IMPROVED EXTRACTION OF ACYLATED ANTHOCYANINS FROM PURPLE SWEET POTATO (*IPOMOEA BATATA*) FOR ENHANCED ANTI-INFLAMMATORY ACTIVITY AND THEIR METABOLITE PRODUCTION DURING PORCINE FECAL DIGESTION

A Dissertation

by

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ABSTRACT

This study characterized Purple sweet potato (PSP) polyphenolics and heating increments associated to PPO inhibitors (citric acid, oxalic acid, and sodium borate) to inactivate enzyme and enhance pigment recovery. Hydrolyzed fractions were used to evaluate stability using an *in vitro* simulated gastrointestinal digestion for 7 hr, absorption, using a Caco-2 human intestinal cell model, anti-inflammatory and anti-cancer properties via TNF-α induced CCD-18Co fibroblast and HT-29 colon cancer cells. Finally, PSP anthocyanin extracts stability were assessed and compared with other sources using an *in vitro* digestion as well as incubation with pig fecal microflora. Predominant PSP anthocyanins included acylated cyanidin or peonidin derivatives. Non-pigmented cinnamates acted as oxidase substrates and induced co-oxidation reactions with anthocyanins. Pre-heating PSP significantly increased polyphenolic yields in a temperature-dependent manner, consistent with tissue softening and PPO inactivation. Hydrolyzed anthocyanins were degraded in both presence and absence of phenolic acids while non-hydrolyzed anthocyanin fractions presented high recovery after intestinal incubation. Transport from the apical to basolateral side was demonstrated in the Caco-2 cell model where extent of transport was dependent on chemical structure and association complexes. Cancer cells were significantly inhibited (40% survival) whereas no toxicity was observed for non-cancer cells. ROS was suppressed at higher concentrations in non-cancer cells, while an increase was noted in cancerous cells. Fractions containing phenolic acids served to down-regulate mRNA and protein

expressions of inflammatory markers (NF-κB, TNF-α, IL-1β and IL-6) on non-cancer cells, thus showing cancer prevention properties. Non-hydrolyzed fractions modulated these biomarkers on cancer cells, thus acting as cancer inhibitors. Sources presented great stability under gastric digestion, whereas great losses were observed for non-acylated during intestinal digestion. Pig fecal incubation of stable acylated sources (Black carrot and PSP) showed fecal suspension was able to rapidly degrade anthocyanins, with di-acylated degrading faster than mono and non-acylated ones, likely produced as complex anthocyanins were decomposed. Free cinnamic and phenolic acids were formed during incubation with active suspension, with maximum concentration ranging from 1 to 6 hours of exposure, simultaneous to anthocyanin degradation. Anthocyanins from PSP showed great applicability in the food industry and stability during digestion with potential for health benefits.

DEDICATION

To my mom and dad, Rosane and Paulo, for your unconditional love and always believing and being there for me, in the pursuit of my dreams.

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CHAPTER I

INTRODUCTION

Anthocyanins are flavonoids responsible for the bright attractive orange, red, purple and blue colors in flower, fruits and vegetables. There is a great demand for natural food colors such as anthocyanins since they can substitute synthetic dyes such as FD&C Red No. 40, currently of highest consumption in the United States and often associated with health concerns (Weiss, 2012). The use of colorants from natural sources can be challenging: anthocyanins are unstable and may degrade during processing or storage conditions such as pH, temperature and light; they possess low tinctorial power and thus larger doses are required to reproduce synthetic dye hues, which make them expensive ingredients (J. He & Giusti, 2010). Identification of low cost and stable natural sources of anthocyanins is the key to guaranteeing sustainability in the competitive market of natural food colors.

Purple Sweet Potatoes (*Ipoema batatas*, PSP) are rich source of anthocyanins cultivated in Asian and South American countries (Steed & Truong, 2008). In the United States, North Carolina is the main grower of PSP but industry interest over these compounds has driven breeding programs to search for new varieties with improved quality. PSP anthocyanins are an attractive colorant for the food industry due to their high concentrations of stable, acylated anthocyanins that can provide an array of colors depending on their concentration and solution acidity. The acyl substitution on the anthocyanin glycosides enhance the initial extracted color and stabilizes it when exposed

to conditions of food processing such as light, heat, and oxidative conditions (Guiusti & Wrolstad, 2003). As ingredients, they can be applied in several foods applications such as juices, bread, noodles, jams, confectionary, and fermented beverages.

Besides anthocyanins, PSP extracts contain non-pigmented polyphenolics that beneficially contribute to color as copigments and to antioxidant and biological activity of extracts (D.-J. Huang, Chun-Der, Hsien-Jung, & Yaw-Huei, 2004; Yoshimoto, Okuno, Yoshinaga, Yamakawa, Yamaguchi, & Yamada, 1999). However, these polyphenolics also serve as substrates for polyphenol oxidase (PPO; E.C. 1.14.18.1), offering an extra challenge in the process of extracting anthocyanins. Although anthocyanins are not main substrates for PPO, oxidation of polyphenolics to reactive o-quinones leads to co-oxidation of pigment (F Kader, Irmouli, Nicolas, & Metche, 2002) with loss of color, brown pigment formation and a consequent decrease in its sensory and nutritional value. Associated to the difficulties in extracting anthocyanins from a dense tissue structure, there is a great need for optimization extracting processes that help improve the yield and result in quality PSP concentrates that can easily be incorporated to food formulations.

Polyphenolic compounds, like anthocyanins, exert a strong antioxidant activity and are thereby able to protect cells against reactive oxygen species (ROS). ROS act as mitogens to stimulate COX-2 expression via nuclear factor κappa B (NF-κB) activation, thereby inducing inflammatory responses, which are directly related to the incidence of cancer and other chronic diseases (Reuter, Gupta, Chaturvedi, & Aggarwal, 2010; Winrow, Winyard, Morris, & Blake, 1993). Therefore, plant compounds that can

effectively down-regulate NF-κB and scavenge ROS may reduce inflammatory responses and alleviate the related pathological conditions (Aggarwal, Shishodia, Sandur, Pandey, & Sethi, 2006). Research with anthocyanins show its bioavailability and health benefits is highly affected by the different types of substitutions to the aglycone, stability under environment conditions such as due to gastrointestinal digestion and more importantly, due to the breakdown products that are generated under digestive environment. In the human body, these polyphenolic compounds are largely metabolized by liver and kidney enzymes as well as bacteria in the colon, and the question that is raised is whether the real health benefit of consuming anthocyanins is due to the presence of these metabolites. If gastrointestinal metabolites are in fact responsible for health benefits commonly associated with the consumption of anthocyanins, the first step forward is understanding their formation pathways to further comprehend their bioavailability and potential benefits to human health.

The objectives of this study were:

I. To optimize processing extracting conditions for PSP anthocyanins to be used in commercial extraction protocols which will help improve yield and result higher quality PSP concentrates;

II. To determine the role of acylation patterns and naturally occurring copigments in PSP as influencing factors for *in vitro* digestion and absorption using a Caco-2 monolayer cell model;

III. To determine the role of acylation patterns and naturally occurring copigments in PSP anthocyanins as influencing factors for anti-inflammatory properties on cancer and non-cancer colon cells;

IV. To compare *in vitro* stability and anti-cancer properties of PSP anthocyanins and other anthocyanin sources and identify the primary metabolites formed from microbial fermentation of chemically different anthocyanin sources.

CHAPTER II

LITERATURE REVIEW

Purple Sweet Potatoes (*Ipoema batatas*, PSP)

Sweet potatoes are the world's sixth most important food crop with about 100 million metric tons produced globally each year. Developing countries in Asia or Pacific islands are responsible for 95% of crop growth, whereas Africa and Latin America account for the rest (Foundation, 2016). The average annual per capita consumption of sweet potato is estimated to be 18 kg in Asia, 9 kg in Africa, 5 kg in Latin America, and 2.3 kg in the United States (Faostat, 2012).

In the United States, sweet potatoes are better grown in southern states where warmer climates, typically frost free, allow for better adaptation of the crop. In particular, North Caroline presents the appropriate conditions for sweet potato production, and therefore leads the nation in area harvested and total production, responsible for 53% of the countries' production (15.8 million cwt) (Johnson, Wilson, Worosz, Fields, & Bond, 2015). From 2000-2014, states like California and Mississippi have also made significantly increase sweet potato growth, with an increase of 100 and 155%, respectively (Wells, & Thornsbury, 2015). Moreover, new value-added sweet potato products and publishable data on the health benefits associated with sweet potato consumption indicate per capita consumption and trade trends make up for a sustainable demand in the future, domestically and abroad.

Sweet potatoes are rich in vitamins (B1, B2, C and E), minerals (Ca, Mg, K and Zn), dietary fibers and carbohydrates (H. W. Kim, Kim, Cho, Chung, Lee, Chu, et al., 2012) and therefore have great potential for contributing to human diet and reducing hunger around the world. The color of the flesh may vary with cultivar but it does not affect the nutritional value. The main difference is that colored fleshed sweet potatoes may provide several types of health benefits and functional pigments, such as flavones, β-carotene and anthocyanins which correspond to the deep yellow, orange, and purple-fleshed sweet potatoes, respectively (Suda, Oki, Masuda, Kobayashi, Nishiba, & Furuta, 2003).

The purple-fleshed sweet potato (PSP) originated in Asian and South American countries and contains high levels of stable acylated anthocyanins, thus offering a healthier alternative to synthetic food colors such as FD&C red 40. In the United States, North Carolina is the main grower of this cultivar but industry interest over these has driven breeding programs to search for new varieties with improved quality (Montilla, Hillebrand, Butschbach, Baldermann, Watanabe, & Winterhalter, 2010). PSP anthocyanins have been widely used in processed commercial products as natural food colorants in applications such as juices, bread, noodles, jams, confectionary, and fermented beverages (V.-D. Truong, Deighton, Thompson, McFeeters, Dean, Pecota, et al., 2009)

The total monomeric anthocyanin content in PSP ranged from 10 to 96.8mg/100g fw in PSP anthocyanins from different sweet potato cultivars. The anthocyanin content of PSP can be comparable to commodities such as grapes (27-120

mg/100 g fw), plum (19-124 mg/100 g fw), raspberries (92 mg/100 g fw) and red radishes (100 mg/100 g fw) (X. Wu, Pittman, & Prior, 2006).

PSP contains anthocyanins that are predominantly acylated glucosides of cyanidin or peonidin although other anthocyanin glycosides have been reported in low concentrations (V.-D. Truong, et al., 2009). In previous studies, 13 of 15 anthocyanins in *Ipomoea batatas* L. were acylated with ferulic, diferulic, caffeic or hydroxybenzoic acid moieties (Montilla, Hillebrand, Butschbach, Baldermann, Watanabe, & Winterhalter, 2010), whereas acylation with *p*-coumaric acid in the cultivar *Ayamurasaki* has also been identified (Konczak-Islam, Okuno, Yoshimoto, & Yamakawa, 2003). Besides anthocyanins, PSP extracts contain non-pigmented polyphenolics including chlorogenic, caffeic, and ferulic acids which beneficially contribute to color as copigments and to antioxidant and biological activity of extracts (D.-J. Huang, Chun-Der, Hsien-Jung, & Yaw-Huei, 2004).

Anthocyanin Chemistry

Structure and Chemistry

Anthocyanins encompass a class of water soluble natural pigments responsible for the vivid blue, purple and red colors present in many fruits, vegetables and flowers distributed worldwide. They are secondary metabolites synthetized and stored in vacuoles of higher plants in response to strong light, UV-radiation, climate extremes or nutrient deficiency (Gould, Davies, & Winefield, 2008). Anthocyanins also play a role

in attracting animals, leading to seed dispersal and propagation of species (Kong, Chia, Goh, Chia, & Brouillard, 2003).

The food color industry shows great interest in these compounds because they are healthier alternatives to food dyes, which have been associated with adverse behavioral and neurological effects (McCann, Barrett, Cooper, Crumpler, Dalen, Grimshaw, et al., 2007). Anthocyanins are polyphenolic compounds in the class of flavonoids, which share an aglycone structure composed of a C-6 (A ring)-C-3 (C ring)-C-6 (B ring) carbon skeleton (Harborne & Williams, 2000), as shown in Figure1. As of now, 25 different aglycones (anthocyanidins) have been identified (Castañeda-Ovando, de Lourdes Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009; de Rijke, Out, Niessen, Ariese, Gooijer, & Udo, 2006) but only six are commonly found in nature and are cyanidin (Cy), delphinidin (Dp), malvidin (Mv), peonidin (Pn), pelargonidin (Pg) and petunidin (Pt), as listed on Table 1. The differences between aglycones are attributed to the different patterns of hydroxylation and methylations in different positions on the B-ring, which in turn leads to differences in colors as well (Andersen & Markham, 2005).

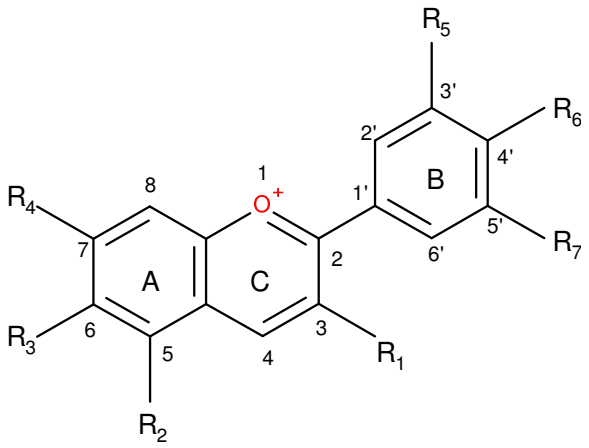


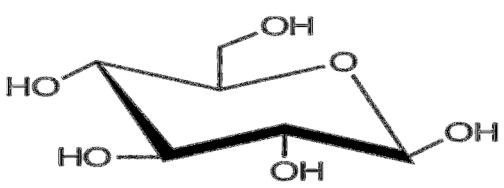
Figure 1: Structural identification of anthocyanidins.

Table 1: Differences on chemical structure of anthocyanidins most commonly found in nature.

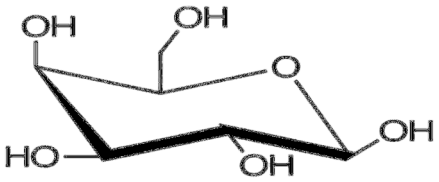
	Substitution pattern at numbered position						
	3	5	6	7	3'	4'	5'
Pelargonidin (Pg)	OH	OH	H	OH	H	OH	H
Cyanidin (Cy)	OH	OH	H	OH	OH	OH	H
Delphinidin (Dp)	OH	OH	H	OH	OH	OH	OH
Peonidin (Pn)	OH	OH	H	OH	OMe	OH	H
Petunidin (Pt)	OH	OH	H	OH	OMe	OH	OH
Malvidin (Mv)	OH	OH	H	OH	OMe	OH	OMe

In nature, aglycones are very unstable being mostly found in their glycosylated form, as anthocyanins. By definition, anthocyanins are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium (flavylium) salts (Brouillard, 1982). The sugar moieties may vary but are commonly mono glucosides such as glucose, rhamnose, galactose or arabinose attached to the C-3, although 3,5- and 3,7-diglycosides may also occur. Combinations of monosaccharides result in di- and trisaccharide

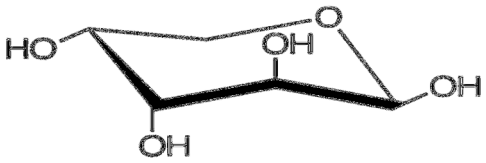
compounds such as rutinose (6-*O*-L-rhamnosyl-D-glucose), sophorose (2-*O*-β-D-glucosyl-D-glucose) or sambubiose (3-O-β-D-xylosyl-D-glucoside), which can also be attached to one of the hydroxyls at C-5, C-7, C-3', C-5' and C-4', but preferably to C3 (Brouillard & Dangles, 1994; Mazza & Miniati, 1993). A list of possible glycosidic substitutions is shown in Figure2. Anthocyanins may also be acylated with an aromatic or aliphatic acid, or a combination of both, creating differentiation among the compounds. The most common acylating agents include derivatives of hydroxycinnamic acids, such as ferulic, sinapic or caffeic or hydroxybenzoic acids, such as gallic or p-hydroxylbeiznoic acids (Clifford, 2000) and are shown on Figure3. With numerous possibilities for combinations of glycosidic and acyl substituents attached to the anthocyanidin molecule, it is no surprise that more than 500 different naturally occurring anthocyanins have been reported (Harborne & Williams, 2000).



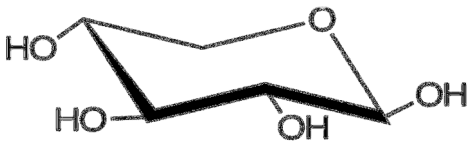
β-D-Glucose



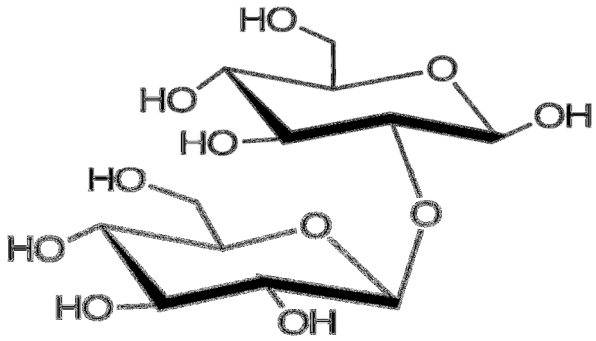
β-D-Galactose



β -D-Arabinose



β -D-Xylose



Sophoroside (2-O-β –D-glucosyl-D-glucoside)

Figure 2: Typical glycosylations in anthocyanins.

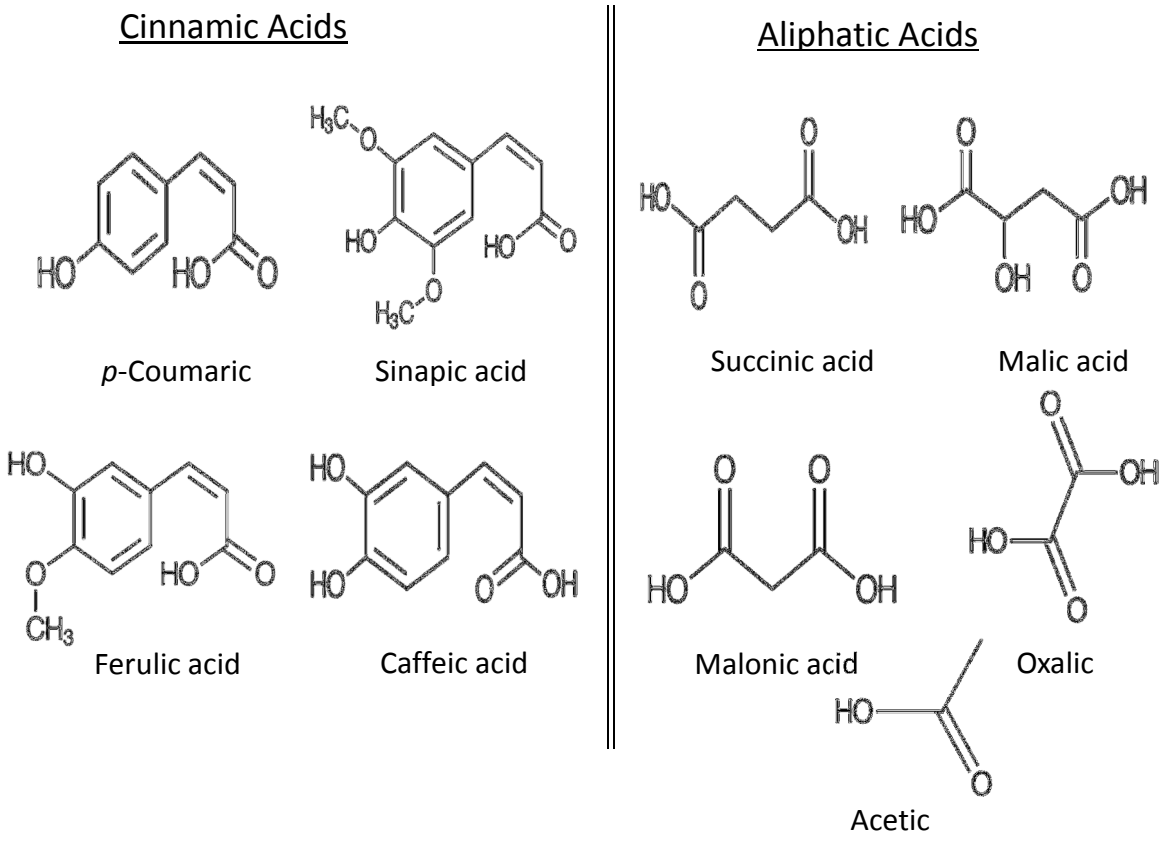


Figure 3: Common acylations in anthocyanin structures.

Anthocyanin Stability

Although anthocyanins are a desirable ingredient for the natural food color industry considering the biological benefits upon consumption, it presents restrictions due to low stability in several processing and storage conditions. A considerate number of factors affect anthocyanin color and stability such as molecular structure and concentration, pH, light, temperature, presence of metallic ions, enzymes, oxygen, copigments and degradation products, amongst others (Cevallos-Casals & Cisneros-

Zevallos, 2004). Also, anthocyanins are highly susceptible to chemical transformations when there is a shift in pH or hydration properties.

The anthocyanin structure plays an important role in their stability and in turn, has an immediate consequence on color. Different ring methylation, hydroxylation, glycosylation and acylation influence the stability of the molecule considering the addition/removal of such groups may hysterically act as a barrier preventing changes in the resonance pattern of the fully conjugated A and B rings, and somewhat to the C-ring, responsible for the reactivity (Jing, 2006). When the resonance is disrupted, color is lost, and can be detrimental to food applications. Increased number of methyl and/ or hydroxyl groups to the B-ring of the aglycone causes bathochromic shifts at low acidic pH's, but addition of a glycosyl group improved the stability at a wider range of pH (1-8) (Welch, Wu, & Simon, 2008).

At pH 2,0 or lower, the flavilium cation is the predominant species and gives purple or red colors (He & Giusti, 2010). Hydration of the flavilium cation in the C2 position leads to the formation of carbinol pseudobase, with pH values ranging from 3-6. A continuous hydration can further equilibrate to form an open ring, a colorless chalcone pseudobase. At the same time, upon acidification, the carbinol pseudobase can be transformed into the flavilium cation. Deprotonation of the flavylium cation into the quinonoidal base occurs at slightly acidic to neutral condition, and the reaction is extremely fast(Brouillard & Dubois, 1977), especially when compared to the hydration reactions. With increases in pH (pH 4-6), the quinoidal base prevails but other structural forms may coexist: flavylium cation, colourless carbinol base and pale yellow chalcone

in which equilibrium between quinoidal base and carbinols occurs via flavilium cation (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). The mechanism is illustrated on Figure 4.

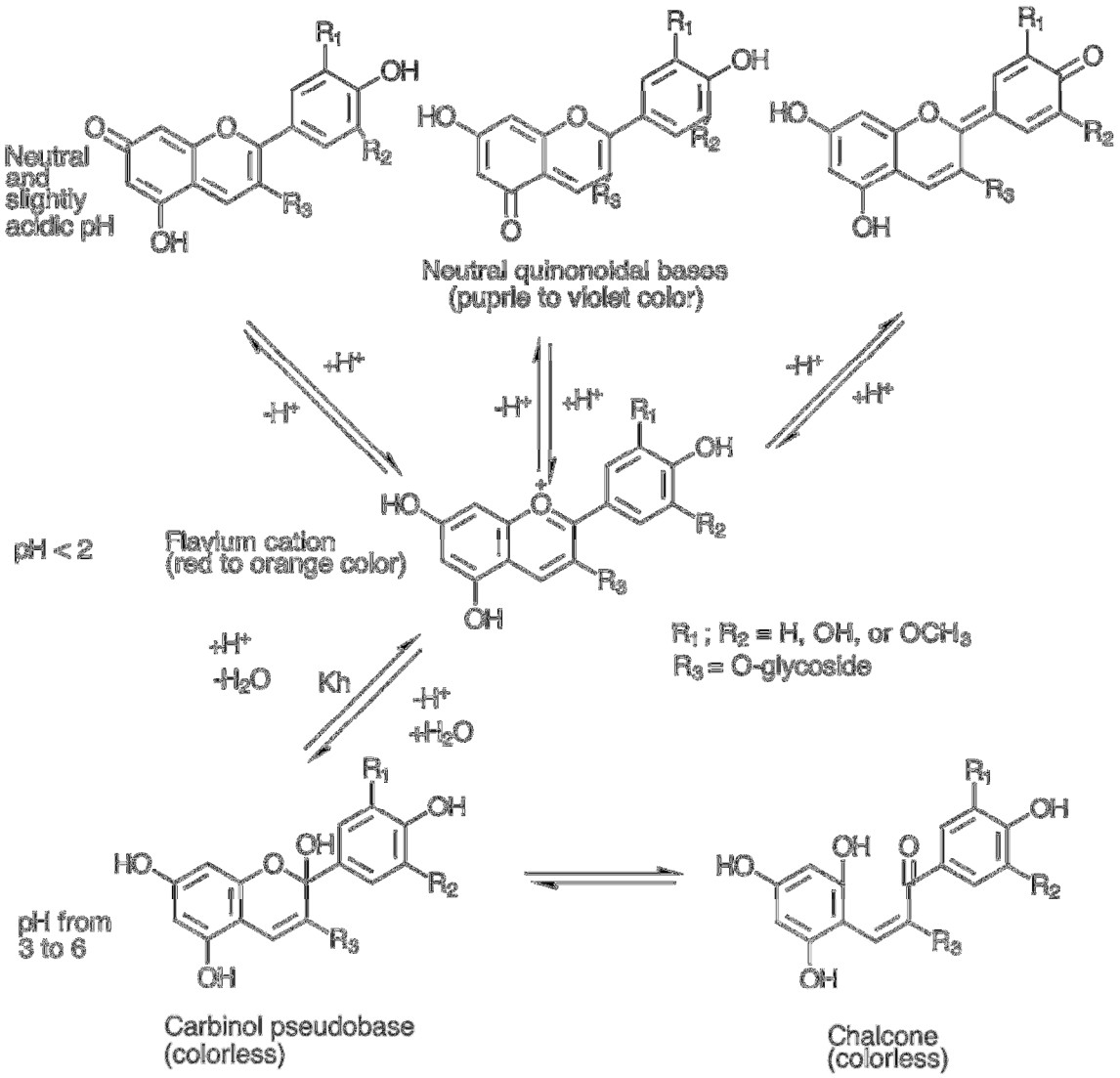


Figure 4: Structural modifications in Anthocyanins at different pH levels.

A mechanism suggested for the degradation of glycosylated forms of anthocyanins (cyanidin-3-glucoside) is shown on Figure5. In the aglycone degradation path, with changes in pH, different structural forms are predominant in the medium. Evaluation of aglycones stability in neutral media at 37°C suggested these compounds may undergo dimerization between the quinoidal moieties, since this is the reactive species at this pH (Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006). More recently, studies have shown that anthocyanin degradation at mildly alkaline conditions go beyond the formation of quinoidal bases and undergo ring-fission with the breakdown of the flavonoid structure and formation of smaller phenolic acids and aldehydes (Figure5) (Anna-Marja Aura, 2008; Dall'Asta, Calani, Tedeschi, Jechiu, Brighenti, & Del Rio, 2012). The characteristics of the new formed products are dictated by the substituents in the 3' and 5' positions in the B-ring (see Figure1), as shown on table 2. Reports have shown that stability of anthocyanins depend on degree of methylation or hydroxylation. Additional of hydroxy or methoxy tend to decrease stability, as observed with increased stability of pelargonidin under neutral pH conditions (Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006). Gut microflora is also responsible for the cleavage of the ring and production of smaller compounds, as will be further discussed in this research.

As for the glycosylated anthocyanin, glycosylation is cleaved at higher pH values, although the aglycone molecule undergoes similar structural changes as in the cases of anthocyanidins. However, it was noted mono, di-glucosides and acylated anthocyanin presented increased stability under neutral pH conditions, as compared to the respective aglycones. A possible explanation could be the fact the glucosides/ acyl

groups provide protection to the aglycone molecule, preventing the rapid formation of the unstable α-diketones intermediates which break down into aldehyde and phenolic acid counterparts (Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006).

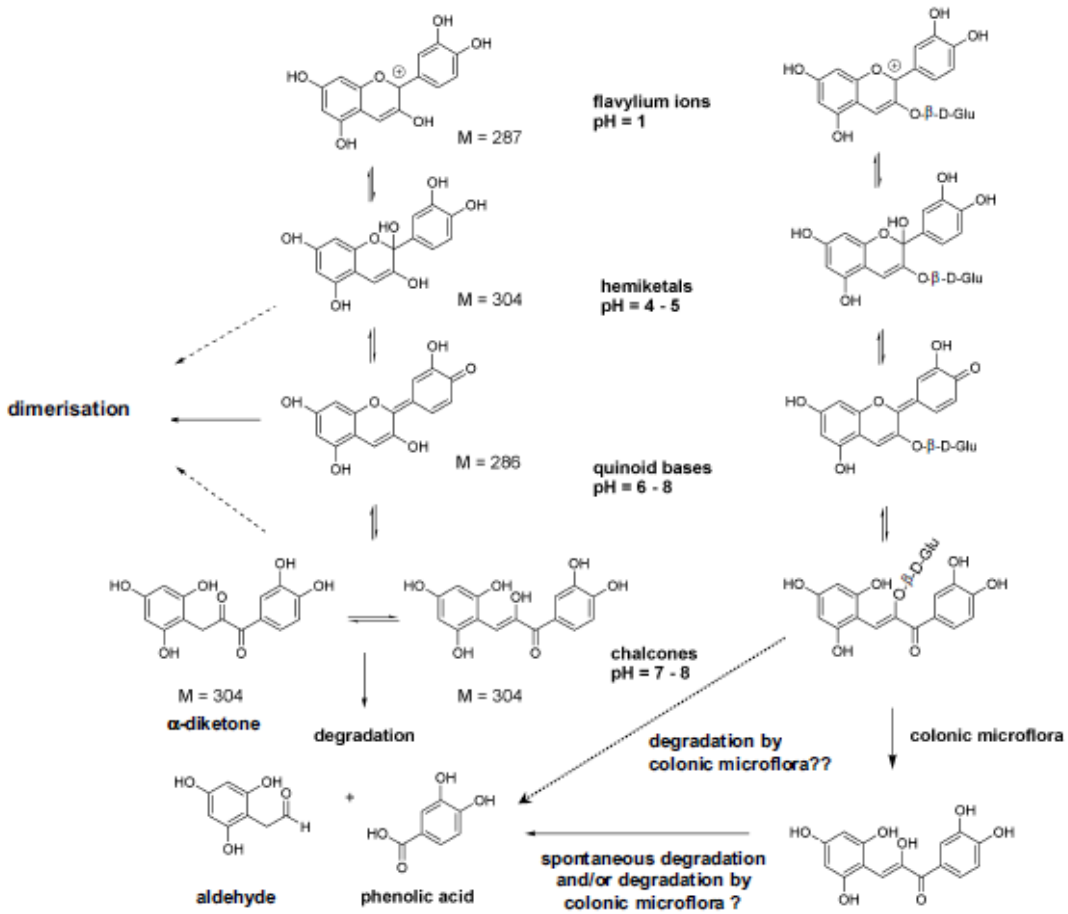


Figure 5: Cyanidin-3-glucoside transformations in pH conditions (Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006).

Table 2: Degradation products of aglycones in neutral pH.

Aglycones	Position in B-ring		Corresponding Phenolic Acid
	3'	5'	
Pelargonidin (Pg)	H	H	4-hydroxybenzoic acid
Cyanidin (Cy)	OH	H	Protocatechuic acid
Delphinidin (Dp)	OH	OH	Gallic acid
Peonidin (Pn)	OMe	H	vanillic acid
Petunidin (Pt)	OMe	OH	3-*O*-methylgallic acid
Malvidin (Mv)	OMe	OMe	Syringic acid

Acylation and Copigmentation

Anthocyanin molecules with complex combinations of glycosylation and acylation patterns are known to present higher stability to processing conditions such as pH shifts, temperature and light exposure (Dangles, Saito, & Brouillard, 1993; M. Giusti & R. Wrolstad, 2003). The bathochromic and hyperchromic effects due to the presence of acylation of sugar substitutions with aromatic or aliphatic organic acids act as both color intensifiers and stabilizers, allowing these pigments to be utilized in a wider range of products when compared to non-acylated anthocyanins (Torskangerpoll & Andersen, 2005). The proposed mechanism for this increased stability is known as the intramolecular/ intermolecular copigmentation, and is well explained by the formation of a sandwich type structure, as previously proposed by Giusti (M. Giusti & R. Wrolstad, 2003). In non-acylated anthocyanins, the charged C ring consists of an electrophilic center which is more susceptible to react with nucleophiles such as water. The presence of acyl groups forms an intermolecular parallel stacking with the anthocyanin nucleus thus protecting the chromophore from the nucleophilic attack (Jing,

2006). As a result, an increase in red color intensity is observed accompanied by the displacement of the equilibrium towards quinoidal base structures, with violet-blue colors existent at neutral pH (F. He, Mu, Yan, Liang, Pan, Wang, et al., 2010).

Similar events occur through intermolecular copigmentations due to the presence of non-anthocyanins polyhenolics or metal ions in the plant matrix, which can bind to anthocyanins via weak hydrophobic forces causing bathochromic and hyperchromic shifts (Mazza & Brouillard, 1990). Therefore, copigment complexes also play a role in the enhancement of the color and chemical stability in non-optimal conditions such as extraction, processing or storage.

Copigment research began with flower colors, based on the theory that the weakly acidic pH conditions in plant vacuoles should shift the equilibrium of the anthocyanin molecule to the colorless chalcone pseudobase. The fact that anthocyanins are extremely colorful in nature led to the understanding that they must be strongly stabilized by other natural components, the copigments, which allow for a shift in the absorbed light (Brouillard, 1982). The first copgiment studies were conducted with grapes, in which it was observed that malvidin-3-glucoside changed hues when added to a solution containing either gallic acid or tannins (Robinson & Robinson, 1931).

Flavonoids (flavones, flavonols, flavanones, and flavanols) and other polyphenols (hydroxycinnamic acids and hydroxybenzoic acids) are the most commonly studied copigments which have shown a significant effect on the enhancement and stabilization of anthocyanin extracts (Markovic, Petranovic, & Baranac, 2000). Berries do not contain high amounts of free phenolic acids, and the existent ones are regarded as

potential copigments (Gao & Mazza, 1994). In Purple sweet potatoes, free chlorogenic acid constitutes 40% to 60% of the total phenolics, followed by caffeic acid derivatives and free caffeic acid (V. D. Truong, McFeeters, Thompson, Dean, & Shofran, 2007). Chlorogenic acid is also the main free phenolic in blueberries, with amounts ranging from 200-1000 mg/kg FW (Gao & Mazza, 1994; Skrede, Wrolstad, & Durst, 2000). In processed berry juices, ferulic and sinapic acids have also been detected as enhancers of colors due to intramolecular copigmentation with anthocyanins (Rein & Heinonen, 2004).

Polyphenol Oxidase and Anthocyanin Degradation

Besides beneficially contributing to color as copigments and to the antioxidant and biological activity of the extracts, the non-pigmented polyphenolics present in PSP including chlorogenic, caffeic, and ferulic acids serve as substrates for polyphenol oxidase (PPO; E.C. 1.14.18.1) (D.-J. Huang, Chun-Der, Hsien-Jung, & Yaw-Huei, 2004; Yoshimoto, Okuno, Yoshinaga, Yamakawa, Yamaguchi, & Yamada, 1999). Processing PSP for commercial anthocyanin recovery offers unique challenges, among which the ability to rapidly inactivate PPO and the inefficient extraction of pigments from the dense tissue structure. PPO catalyzes the O2-dependent oxidation of phenolic acids to o-quinones that can be reactive to anthocyanins leading to loss of color and brown pigment formation (Thipyapong, Stout, & Attajarusit, 2007). It is a limiting factor in the processing of fruits and vegetables as peeled, cut or bruised crops rapidly undergo browning reactions with loss of nutritional and sensory aspects (Yoruk & Marshall,

2003). Commercially edible plant sources highly affected by PPO include apples (Queiroz, da Silva, Lopes, Fialho, & Valente-Mesquita, 2011), bananas (Siyuan Wang, Lin, Man, Li, Zhao, Wu, et al., 2014), mangoes (Cheema & Sommerhalter, 2015) and certain fruits and vegetables containing high levels of anthocyanins such as blueberries (Farid Kader, Rovel, Girardin, & Metche, 1997), eggplants (Barbagallo, Chisarib, & Patanèc, 2012) and Purple sweet potatoes (Jang & Song, 2004). Although not considered substrates for PPO, anthocyanins will rapidly degrade with tissue disruption and co-oxidize from hydrogen peroxide and o-quinone generation (F Kader, Irmouli, Nicolas, & Metche, 2002). Additionally, PSP contain peroxidases (POD; EC 1.11.1.7) that will break down endogenous hydroperoxides, and those formed from PPO action and may serve to exacerbate the degradation of anthocyanins at cut surfaces (Farid Kader, Rovel, Girardin, & Metche, 1997).

The use of enzyme inhibiting agents such as reducing, chelating and complexing agents in addition to acidulants have extensively been employed in order to prevent PPO from acting by eliminating the active elements which participate in the reactions, such as the substrates (phenolics), copper or the reaction intermediates (*O*-quinones) (F Kader, Irmouli, Nicolas, & Metche, 2002). Sulfites, particularly sodium bisulfites or sodium sulfite, were broadly used in the past not only as reducing agents but it was also believed they interacted with quinones preventing further participating. However, due to food safety regulations, such compounds were banned in the food industry by Food and Drug administration (Martinez & Whitaker, 1995). Ascorbic acid then replaced the sulfites but its decreased efficacy led to its combined use with other PPO inhibitors such as citric

acids (Sapers, 1993). Citric and oxalic acid function both as a chelating agent and as lowering the pH below optimal enzyme activity levels, thus making the environment unfavorable for action of (Eidhin, Degn, & O'beirne, 2010). When added to the anthocyanin extracting solution, acidulants and metal chelators like citric, oxalic and sodium borate may effectively inhibit PPO and allow for higher pigment recovery.

Another way to prevent anthocyanin degradation and enhance extraction is through the use of physical agents, such as heat. Thermal processes are conventionally used to inactivate oxidase enzymes and preserve polyphenolics in foods (Steed & Truong, 2008; Terefe, Yang, Knoerzer, Buckow, & Versteeg, 2010). The use of physical processes becomes especially important in PSP because it is plausible that structural changes may take place at the membrane level, disrupting the cell walls which contain the pigment and releasing them into solution. Little information is available regarding the relationship between pre-heating potatoes to temperatures in which gelatinization occurs as well the synergistic effect between heat treatment and the role of chemical additives to inactivate oxidase enzymes and improve anthocyanin and phenolic acid extraction from PSP. In an optimization study to improve anthocyanin extraction (Lu, Zhou, Zhang, Ma, Zhou, Li, et al., 2010) it was demonstrated that the combination of microwave baking and electrolyzed water provided greater pigment extraction yield, indicating the potential for a novel process that is of low cost and is highly effective.

Health Effects of Anthocyanins

An increased interest in dietary polyphenols, which include anthocyanins, is a result of findings correlating an increased consumption of fruits and vegetables and a decreased risk of chronic diseases. The health benefits of anthocyanins have been associated to their antioxidant activity (Kong, Chia, Goh, Chia, & Brouillard, 2003) due to hydroxyl groups in position 3 of the C ring and 3,4 and 5 of the B ring. Therefore their phenolic structure is responsible for their capacity to scavenge oxygen species (ROS) such as superoxide (O2-), singlet oxygen (·O2), peroxide (ROO-) and hydrogen peroxide (H2O2) (L.-S. Wang & G. D. Stoner, 2008; S. Y. Wang & Jiao, 2000) which allows them to present chemoprotective effects. The several chemopreventive mechanisms of anthocyanin action is believed to the through an increased oxygen-radical absorbing capacity of the cells, the stimulation in the expression of Phase II detoxifying enzymes, reduction of oxidative adducts formed in the DNA and lipid peroxidation and reduction of cellular proliferation by modulating signal transduction pathways (L.-S. Wang & G. D. Stoner, 2008). Anthocyanins have been reported to inhibit cancer growth (Hou, 2003), prevent cardiovascular diseases (Matsumoto, Nakamura, Hirayama, Yoshiki, & Okubo, 2002; Wallace, 2011), inflammation (Reddy, Alexander-Lindo, & Nair, 2005; Rossi, Serraino, Dugo, Di Paola, Mondello, Genovese, et al., 2003) and anti-obesity effects (Prior, Wu, Gu, Hager, Hager, & Howard, 2008; Tsuda, 2008), amongst others, widely discussed in the literature (J. He & Giusti, 2010).

Anthocyanins are ubiquitous in the plant kingdom and are consumed as part of a normal diet. Suggested consumption was estimated to be as much as 180-225mg/ person

per day (Keppler & Humpf, 2005), higher than the estimated value for other polyphenols such as quercetin, kaempferol, myricetin, apigenin, and luteolin (23 mg/person totally) (Hertog, Hollman, Katan, & Kromhout, 1993). Currently in the United States, an average consumption of 12.5 mg/day of anthocyanins is suggested per person (X. Wu, Pittman, & Prior, 2006). Consumption of berries will deliver 10s to 100s of milligrams of anthocyanins in a single serving which shows the dietary choice will make an impact on the amount of anthocyanins that will be ingested. Studies have correlated dose response between the concentrations of anthocyanins ingested and health benefits they will exert (McGhie & Walton, 2007).

Anti-inflammatory Effects of Anthocyanins

Inflammation is a natural body response to either internal or external stimuli which counteracts with irritation, injury or infection and is identified by pain, redness and swelling (Aggarwal, Shishodia, Sandur, Pandey, & Sethi, 2006). There are two types of inflammation, acute and chronic. Acute is the initial stage, regulated through an activation of the immune system, and lasts short periods of time (Aggarwal, Vijayalekshmi, & Sung, 2009). Chronic inflammation persists for longer periods of time and may lead to a wide variety of chronic illnesses such as cancer, obesity, diabetes, cardiovascular and neurological disorders (Aggarwal, Shishodia, Sandur, Pandey, & Sethi, 2006; Coussens & Werb, 2001). Chronic inflammation is triggered by multiple factors including stress, bacterial, viral and parasitic infection. Recently it has been suggested that lifestyle factors such as diet can also play a role in promoting chronic inflammation (Aggarwal, Shishodia, Sandur, Pandey, & Sethi, 2006; Shacter &

Weitzman, 2002). Chronic inflammation has been directly related to inducing cellular transformations that may culminate in cancer and metastasis (Balkwill & Mantovani, 2001; Shacter & Weitzman, 2002).

Pro-inflammatory cytokines, chemokines and inflammatory enzymes are associated with chronic inflammation. These genes play an important role in cellular and biological processes such as apoptosis, proliferation, angiogenesis, invasion and metastasis (Aggarwal, Shishodia, Sandur, Pandey, & Sethi, 2006). There are several genes involved in these processes such as TNF-α (tumor necrosis alpha factor) and members of its family such as interlukines (IL-1β, IL-6, IL-8, IL-18) and chemokines (MMP-9, VEGF), all regulated by either the transcription factor nuclear factor kappa B (NF-κB) or cyclooxygenase-2 (COX-2) (L.-S. Wang & G. D. Stoner, 2008). Both proteins are commonly associated with abnormal cellular activity and therefore considered master regulators of inflammation in most tumors (Aggarwal, Shishodia, Sandur, Pandey, & Sethi, 2006; Balkwill & Mantovani, 2001; Coussens & Werb, 2001; Dalgleish & O'Byrne, 2006; Shacter & Weitzman, 2002). Therefore, inhibitors of NF-κB or its downstream genes might exhibit significant chemopreventive properties.

Studies with anthocyanins have proven their ability to inhibit mRNA and/or protein expression levels of COX-2, NF-κB or other interlukines, reaffirming their anti-inflammatory capacities in several cell types *in vitro* (C. Huang, Huang, Li, Hu, Aziz, Tang, et al., 2002; Ramos, 2008; L.-S. Wang & G. D. Stoner, 2008). Anthocyanin rich extracts from black raspberry inhibited tumor development in JB-6 Cl 41 Mouse epidermal cells by regulation of benzoapyrene diol-epoxide (BaPDE)-induced

expression of NF-κB (C. Huang, et al., 2002). Moreover, berry juices containing several different anthocyanins sources (gooseberry, sea buckthorn, cranberry, black currant, white currant, raspberry) significantly inhibited the TNF-α induced activation of COX-2 and nuclear transcription factor NF-κB expression, which emphasizes an inclusion of berries in our daily diets would significantly contribute towards preventing chronic diseases (Boivin, Blanchette, Barrette, Moghrabi, & Beliveau, 2007). When considering anthocyanin bioavailability, it would be more appropriate to evaluate the anti-inflammatory and inhibitory effect on tumorigenesis of anthocyanin-rich commodities in the gastrointestinal tract since current metabolite studies suggests colon is responsible for fate of these compounds. This signifies that the low bioavailability of anthocyanins is actually due to disappearance of the parent compounds and appearance of breakdown metabolites responsible for the biological activities.

Bioavailability of Anthocyanins

Bioavailability can be defined as the proportion of the nutrient that is digested, absorbed and metabolized on normal body pathways (McGhie & Walton, 2007). Numerous mechanisms have been proposed to explain the fate of anthocyanins in the body, although fully understanding the health properties of these compounds will be a constant challenge considering the different molecular structures involved in the process associated with the number of metabolites that can be generated both *in vitro* and in the gastro intestinal tract (GIT).

It is known that the apparent bioavailability of anthocyanins is very low with often less than 0.1% of the ingested dose appearing in the urine (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005) although higher levels of anthocyanin excretion, as up to 5%, has been reported with the consumption of cranberry juice, red wine, and strawberry (Felgines, Talavera, Texier, Gil-Izquierdo, Lamaison, & Remesy, 2005; Lapidot, Harel, Granit, & Kanner, 1998). The absorption of these compounds occurs quickly (tmax in plasma usually 15-60min), with excretion completed within 6-8h, which leads to the suggestion that anthocyanins are absorbed in the stomach (Mazza & Kay, 2008). When comparing the data of 30 published studies involving anthocyanin bioavailability, they observed concentrations of anthocyanins measured in the plasma were consistently low, ranging from 1.4 to 593 nmol/L, after being administered doses ranging from 56 to 3,570 mg anthocyanins in the form of berries, berry extracts, or concentrates. The time to reach the maximum concentration, Cmax, ranged from 0.3 to 2.8 h (mean value 1.4 h) for plasma and from 1.5 to 3.0 h (mean value 1.8 h) for urine (Mazza & Kay, 2008).

A proposed mechanism for anthocyanin absorption and elimination (McGhie & Walton, 2007) indicates that anthocyanin glycosides can be rapidly absorbed from the stomach after ingestion by a process involving the bilitranslocase, enabling these compounds to enter the systemic circulation after passing through the liver and kidney, to be metabolized. Glucuronic acid and methyl groups will be incorporated into the molecule *in vivo*, by the action of UDP- glucuronyl-transferase (Keppler & Humpf, 2005; Talavéra, Felgines, Texier, Besson, Gil-Izquierdo, Lamaison, et al., 2005). In fact,

glucuronidation and/or methylation are the major anthocyanin metabolic pathways, as have been previously reported in several researches (Felgines, Talavera, Texier, Gil-Izquierdo, Lamaison, & Remesy, 2005; Kay, Mazza, Holub, & Wang, 2004; Tian, Giusti, Stoner, & Schwartz, 2006; X. Wu, Cao, & Prior, 2002; X. Wu, Pittman, & Prior, 2006). It is important to consider that intact compounds will also be absorbed and may reach the systemic circulation, organs and tissues. The non-absorbed anthocyanins and metabolites will be directed to the intestine. In exposure to higher pH conditions, structurally different anthocyanin equilibrium forms (hemiketal, chalcone, and quinonoidal) will co-exist, as explained in Figure4. Hydrolases may also act on the intact compounds in the intestine, releasing the aglycone (Cao & Prior, 1999). The anthocyanins that reach the colon will be exposed to substantial microbial population and may be degraded to sugar and phenolic components. The phenolic components will be further degraded with a disruption of the C-ring to yield phenolic acids and aldehydes, as previously discussed in the Stability Section. These products, derived from the ingested anthocyanins, may contribute to the health effect of anthocyanins either directly in the GIT or after absorption from the colon (McGhie & Walton, 2007).

In an attempt to understand the structural relationship of anthocyanins and their pharmacokinetics parameters, purple sweet potato (*Ipomoea batatas* cv Ayamurasaki) extracts containing different substituted anthocyanins were administered to humans and rats (Harada, Kano, Takayanagi, Yamakawa, & Ishikawa, 2004). On both subjects, only two of eight intact acylated anthocyanins (Cy-CafSop-Glc and Pn-CafSop-Glc) were detected in the plasma and urine by LC–MS indicating the moiety CafSop-Glc could be

the reason for better absorbability. These authors also propose that non-acylated anthocyanins are likely methylated to peonidin by catechol-O-methyltransferase in the liver and kidney, and these methylated anthocyanins are excreted from the liver directly into bile, since they are not detected in the blood. The results showed acylated PSP anthocyanins are more difficult to be metabolized than the non-acylated ones, considering they were detected in the blood and urine. It was concluded there must be a function-relationship between the moieties present in the intact absorbed forms of anthocyanins and increased absorbability, indicating acylated anthocyanins can be selectively absorbed after ingesting food.

The bioavailability of acylated and non-acylated anthocyanins from purple corn and the effect of cooking and dose on the comparative bioavailability in a clinical feeding study has also been previously studied (Kurilich, Clevidence, Britz, Simon, & Novotny, 2005). Four of the five carrot anthocyanins were found intact in plasma after consumption. To note, all anthocyanins contained cyanidin based aglycones, one presented a mono-glucoside moiety (galactoside), one presented a di-glucoside moiety (xylose-galactoside), and the other three contained acyl groups: sinapic, ferulic and p-coumaric, attached to a glucose-galactoside unit. Anthocyanins appeared in plasma 30 min after ingestion, rising steadily until 2h, and then slowly decreasing until 8h, in which some were still detected. Cooking increased the recovery of nonacylated anthocyanins but not acylated anthocyanins whereas increasing dose size significantly reduced recovery of both acylated and nonacylated anthocyanins, suggesting saturation of absorption mechanisms, as previously explained. Presence of acylations led to

different bioavailability responses. Although the original dose contained higher amounts of acylated anthocyanins, plasma recovery of non-acylated anthocyanins were 8-10 times higher than for acylated, and urine recovery was 11-14 times higher in non-acylated compounds. On another study, mono- and diacylated intact anthocyanins in rat plasma after consumption of a concentrated extract made from purple fleshed sweet potato were observed (Suda, Oki, Masuda, Kobayashi, Nishiba, & Furuta, 2003). It was suggested the acylation must play a role in the bioavailability, but the mechanism was still unclear. A possible explanation previously proposed for the lower recovery of acylated anthocyanins is that they have to be cleaved to produce more non-acylated compounds, preferably the mono-glucoside form. However, the recoveries of both forms were very similar, which suggested, if cleavage was actually happening, it was at a very low extent (Kurilich, Clevidence, Britz, Simon, & Novotny, 2005).

It can be inferred that the bioavailability and health benefits of anthocyanins will be highly subjected to changes considering the different chemistry involved (type of substitutions to the aglycone), environment (pH, temperature) in which these compounds are exposed to and the breakdown products that are generated after being exposed in the GIT. Considering the compounds are largely metabolized by enzymes and bacteria in the colon, the true health benefit of consuming anthocyanins could be due to the effects of these metabolites and more studies need to be conducted to assay biovailability and absorption parameters of the new compounds.

In vitro Metabolism of Anthocyanins by Gut Microflora

It has recently been suggested that microbiota is a metabolizing organ with functions include that xenobiotic metabolism, vitamin syntheses and macro- and micro-molecule breakdown (Fernandes, Faria, Calhau, de Freitas, & Mateus, 2014). The human intestine is composed of 1013 – 1014 bacterial count, 10 times more than human cells, representing a large genome (Cani & Delzenne, 2009). Colon microflora entails a highly complex ecosystem in which the host supports unlimited metabolic reactions in a symbiotically interaction with the microorganisms (Van Duynhoven, Vaughan, Jacobs, Kemperman, Van Velzen, Gross, et al., 2011) and its dependent on the dietary habits of the host (Cani & Delzenne, 2009). In result, unhealthy microbiota metabolism has been implicated to conditions such as diabetes and obesity (Ley, Bäckhed, Turnbaugh, Lozupone, Knight, & Gordon, 2005).

Studies with anthocyanin bioavailability has demonstrated poor absorbability though the small intestine and excretion in urine, and it is hypothesized the original chemical form of anthocyanins is not the one that reaches colon, but instead these compounds undergo intense metabolism in the body, including methylation, sulphation and glucuronidation (A-M Aura, Martin-Lopez, O'Leary, Williamson, Oksman-Caldentey, Poutanen, et al., 2005; Felgines, Talavera, Texier, Gil-Izquierdo, Lamaison, & Remesy, 2005; X. Wu, Cao, & Prior, 2002). At continuous exposure to alkaline pH conditions, the breaking of glycoside linkage and cleavage of anthocyanin heterocycle has also been observed (Fernandes, Faria, Calhau, de Freitas, & Mateus, 2014; Rechner, Smith, Kuhnle, Gibson, Debnam, Srai, et al., 2004).

In addition to the complexity in flavonoids metabolism, some of the molecules and breakdown products are able to reach the large intestine where, in contact with human microbiome, suffers drastic transformations when compared to those in liver and intestinal enzymes (Selma, Espin, & Tomas-Barberan, 2009). Bacteroides, Clostridium, Eubacterium, Ruminococcus, and Eggertheilla genera (Blaut & Clavel, 2007) are some of the intestinal bacterial responsible for the transformation of compounds (Borges, Roowi, Rouanet, Duthie, Lean, & Crozier, 2007). They present several glycoside deconjugating enzymes such as β-D-glucuronidases, β-D-glucosidases, α-L-rhamnosidases, catechol-O-methyltransferases and aryl-sulfotransferase (A-M Aura, et al., 2005; Bokkenheuser & Winter, 1988; D.-H. Kim, Jung, Sohng, Han, Kim, & Han, 1998; Selma, Espin, & Tomas-Barberan, 2009) as well as the human enzyme lactase phlorizin hydrolase (Day, Cañada, Díaz, Kroon, Mclauchlan, Faulds, et al., 2000).

The aglycones, in contrast, are rapidly consumed by bacteria, with ring fission and production of small phenolic acids skeletons and aldehydes, and can further be absorbed. Moreover, microorganism metabolism and presence of enzymes allow for continuous molecule hydroxylation leading to removal of glucuronides, sulfates, amides, de-glycosylation, and posterior ring-cleavage, reduction, decarboxylation, demethylation and dihydroxylation (Anna-Marja Aura, 2008; A-M Aura, et al., 2005; Dall'Asta, Calani, Tedeschi, Jechiu, Brighenti, & Del Rio, 2012). Such intense metabolism and degradation processes may partially be responsible for the low parental anthocyanin recoveries widely reported in the literature.

On an study to assess whether anthocyanin chemical composition played a role in the gut incubation hydrolysis rate, cyanidin-3-O-glucoside (C3G), malvidin-3-O-glucoside (M3G), cyanidin-3,5-O-diglucoside (C35G), malvidin-3,5-O-diglucoside (M35G), cyanidin-3-O-rutinoside (C3R) and peonidin-3-O-glucoside (P3G) were incubated for 24hours with non-sterilized inoculum filtrate of pig caecum (Keppler & Humpf, 2005). While mono-glycosylated anthocyanins (C3G and M3G) were completely hydrolyzed after 20min incubation, only small amounts of the aglycones (cyanidin, malvidin and peonidin) were detected. At the same time, phenolic degradation products (PCA, syring acid, vanillic acid and phloroglucinol aldehyde) were already detected with 20min of reaction time and the timepoint in which maximum concentration was obtained for the phenolics was directly dependent on the anthocyanin hydrolysis rate. C35G and M35G were hydrolyzed to their monoglycosidic derivative, but it was observed the rate of di-glucoside hydrolysis was slower than the hydrolysis rate of the mono-glucosides, suggesting the di-glucoside bond protected the molecule from degradation. Interestingly, the rate of C3R hydrolysis was much slower compared to other di-glucosides, indicating the increased steric hindrance due to complex sugar moiety rutinoside. They concluded the aglycone had little influence in the hydrolysis rate, in comparison to the sugar substitution. Moreover, concentration of produced phenolics decreased after reaching a maximum, which suggests further microbial metabolism may continue to act upon on these compounds (Keppler & Humpf, 2005). Similar results were observed in this study as well.

Protocatechuic acid (PCA) is the main metabolite formed in cyanidin heterocycle degradation due to fecal suspension. It has been identified as the major metabolite compound in sources such as berries, orange juice and red wine, sources containing cyanidin glucosides as part of their main anthocyanins (Dall'Asta, Calani, Tedeschi, Jechiu, Brighenti, & Del Rio, 2012). It has also been reported that PCA formation could be a result of chlorogenic, caffeic, and ferulic acids conversion in gut microflora, as this was the also the primary metabolite found in coffee and flaxseed (Gonthier, Verny, Besson, Rémésy, & Scalbert, 2003). Interestingly, PSP contain anthocyanins highly acylated with similar cinnamic acids, suggesting the PCA formed in digestion can also be derived from these acylations degradation themselves.

Another product of degradation, 3-O-methylgallic acid, was reported as the main metabolite formed following gut microbiota digestion of petunidin aglycones, and it may also be derived from a demethylation product of malvidin-3-glucoside (syringic acid) or methylation of delphinidin product (gallic acid) (Forester & Waterhouse, 2008). Increases in levels of 3-O-methylgallic acid were positively correlated to fast metabolism of petunidin-3-glycosides after 0.5h of pig fecal incubation (Forester & Waterhouse, 2008). In human clinical study to evaluate the metabolites of red wine, with malvidin as the predominant aglycone in this source, gallic acid, 4-O-methylgallic acid, and 3-O-methylgallic were detected in plasma (Cartron, Fouret, Carbonneau, Lauret, Michel, Monnier, et al., 2003). Other metabolites of malvidin-3-glucoside and of peonidin-3-glucoside were reported to be syringic and vanillic (Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006).

Recent studies demonstrate the metabolites produced by anthocyanin degradation are indeed subjected to further metabolism in the body, and also glucuronidated, sulfated, or methylated *in vivo* with potential to be better absorbed (Forester & Waterhouse, 2008). Other reports have even shown decarboxylation and dehydroxylation products of PCA as metabolites in fecal suspension studies (Hsu, Daniel, Lux, & Drake, 1990). Therefore, more research needs to be conducted to further elucidate anthocaynin metabolism, bioavailability, transport into the cells as well as the characterization and health benefits of the metabolites.

In vitro Models to Measure Anthocyanins Absorption

The common mechanisms involving the absorption of xenobiotic compounds across the intestinal epithelium are passive transcellular, passive paracellular, carrier mediated transport, carrier mediated efflux and transcytosis (Artursson, 1990). Passive transcellular occurs when the xenobiotic penetrates the membrane of epithelial cells and is rapidly distributed into the cell, in the case of lipophilic compounds. Molecules that are slowly absorbed by the membrane are transported through the water-filled pores of the paracellular pathway across the intestinal epithelium, and this transport is called passive paracellular transport (Artursson, 1990; Engman, 2003). Hydrophilic compounds whose chemical structures mimic various nutrients are transported across the intestinal epithelium by an active carrier mediated transport whereas if a compound enters the cytoplasm of mucosal cells and is transported back to the intestinal lumen, it is called carrier-mediated efflux (A. P. Li, 2001). Trancytosis occurs when compounds are

entrapped in vesicles due to invagination of the apical membrane (Artursson, 1990). Figure 6 illustrates the several mechanisms proposed for intestinal absorption.

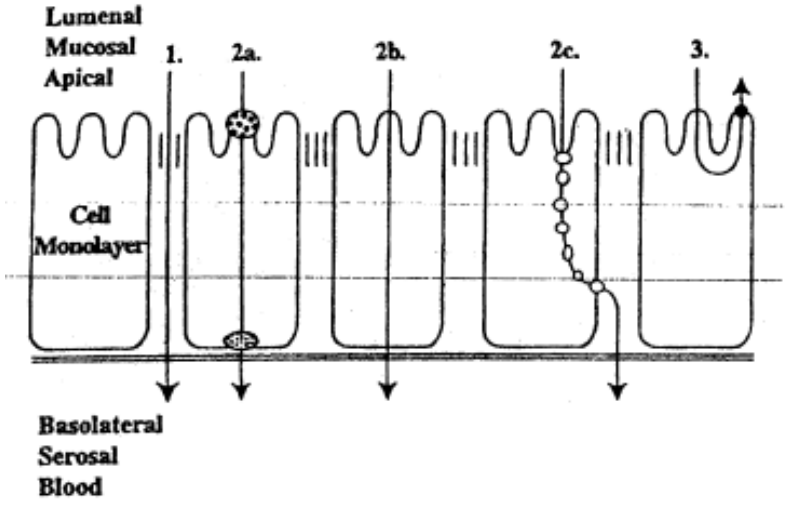


Figure 6: Pathways involved in intestinal absorption.
(1) Paracellular Pathway; (2) transcellular: (2a) Carrier-mediated, (2b) Passive diffusion, (2c) Transcytosis; (3) Carrier-mediated efflux pathways (Balimane, Chong, & Morrison, 2000; Stewart, Chan, Lu, Reyner, Schmid, Hamilton, et al., 1995).

The increased interest in the beneficial health effects of anthocyanins can be explained due to significant increased evidence that these compounds offer a variety of potential biological effects *in vitro*. However, a significant gap exists between what is presented in *in vitro* studies and what can be achieved through *in vivo* experiments. For instance, human pharmacokinetics studies usually identify less than 1% of the non-metabolized compound despite consumption of doses which exceed 500mg (Kay,

Mazza, & Holub, 2005; Kroon, Clifford, Crozier, Day, Donovan, Manach, et al., 2004). This raises the question whether these compounds are biologically active as parent compounds and how absorption plays a role the efficacy of the treatment, making it a fundamental step when understanding the fate of anthocyanins in the body.

Absorption is an important aspect to relate *in vitro* chemoprotective properties to health benefits by polyphenolics (Balimane, Chong, & Morrison, 2000; Yi, Akoh, Fischer, & Krewer, 2006). The Caco-2 cell line is a recognized by the FDA model used in drug permeability and absorption studies and allows for a correlation between absorption in the Caco-2 model and percent of absorption in human. It is a simple, convenient and less expensive method compared to human and animal models that are highly complex and account for a series of variables such as chemical instability and inadequate analytical methodology (Yi, Akoh, Fischer, & Krewer, 2006).

Caco-2 cells from human colon adenocarcinoma undergo a process of spontaneous differentiation when they reach confluence on a porous polymer membrane, leading to the formation of tight cell monolayers that presents a model for a single layer of epithelial cells that covers the inner intestinal wall which is the rate-limiting barrier for the absorption of drugs (Hu, Ling, Lin, & Chen, 2004). Caco-2 cells are commonly cultured on permeable supports that allow free access of nutrients to both sides of the cell monolayer, the apical and basolaterial sides, which resemble the conditions in the intestine *in vivo* (Artursson, 1990). The special feature of this model is that both the both sides of the monolayer are easily accessible, therefore the compounds can be easily

transported from the apical to the basolateral side, making it an excellent model for studying drug excretion or efflux (Hu, Ling, Lin, & Chen, 2004).

Caco-2 cell monolayer models have been used to assess intestinal transport of several sources of anthocyanins including acai (Pacheco-Palencia, Talcott, Safe, & Mertens-Talcott, 2008), Blueberries (Yi, Akoh, Fischer, & Krewer, 2006) and breakdown metabolites of Cabernet Sauvignon anthocyanin extract (Forester & Waterhouse, 2010).

CHAPTER III

PRE-HEATING AND POLYPHENOL OXIDASE INHIBITION IMPACT ON EXTRACTION OF PURPLE SWEET POTATO ANTHOCYANINS

Overview

Purple sweet potatoes (PSP) have been used as a natural food colorant with high acylated anthocyanins concentrations. Commercially extracting pigments from PSP can be challenging due to firm texture and high polyphenol oxidase (PPO) content. These studies evaluated hot water immersions (30, 50, 70, and 90°C for 10 min) as pre-heating treatments and addition of PPO inhibitors (citric acid, oxalic acid, and sodium borate) to aqueous extraction solutions to aid pigment recovery. Predominant PSP anthocyanins included acylated cyanidin or peonidin derivatives. Non-pigmented cinnamates acted as oxidase substrates and induced co-oxidation reactions with anthocyanins. Pre-heating PSP significantly increased polyphenolic yields in a temperature-dependent manner, consistent with tissue softening and PPO inactivation. The use of solvent modifiers in the extraction solution associated with heat helped minimize enzyme action and increased polyphenolic recovery. Minimizing the impact of PPO with heat was critical to the extraction and recovery of PSP anthocyanins, suitable for food use.

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Introduction

Anthocyanins are pigments widely distributed in fruits, cereals, vegetables and are responsible for the intense red, blue and purple colors found in nature. Concentrated anthocyanin isolates from fruits and vegetables have gained popularity as natural color alternatives to synthetic red dyes and even other natural sources such as insect-derived carminic acid. In addition to color contributions, anthocyanins have reported health benefits such as reduction of reactive oxygen species, prevention of cardiovascular diseases, anti-inflammatory and anticarcinogenic activity, as reviewed by others (J. He & Giusti, 2010).

Purple sweet potatoes (PSP; *Ipomoea batatas*) with increased concentration of anthocyanins were originally cultivated in in Japan, Korea, and New Zealand (Steed & Truong, 2008) but are currently grown, processed, and concentrated in the United States and other parts of the world with the intent to meet the growing market for natural colors. PSP anthocyanins are an attractive colorant for the food industry due to their high concentrations of stable, acylated anthocyanins that can provide colors ranging from pink to deep red depending on their concentration and solution acidity. Aromatic or aliphatic organic acids esterified to anthocyanin glycosides are more likely to possess greater initial color and are often more stable when exposed to conditions of food processing such as light, heat, and oxidative conditions (Guiusti & Wrolstad, 2003). PSP possess anthocyanins that are predominantly acylated glucosides of cyanidin or peonidin although other anthocyanin glycosides have been reported in low concentrations (V.-D. Truong, et al., 2009). On previous studies, 13 of 15 anthocyanins

in PSP were acylated with ferulic acid, diferulic acid, caffeic acid, or hydroxybenzoic acid moieties (Montilla, Hillebrand, Butschbach, Baldermann, Watanabe, & Winterhalter, 2010), whereas acylation with *p*-coumaric acid in the cultivar 'Ayamurasaki' has also been identified (Konczak-Islam, Okuno, Yoshimoto, & Yamakawa, 2003; Tian, Giusti, Stoner, & Schwartz, 2006). Besides anthocyanins, PSP extracts contain non-pigmented polyphenolics including chlorogenic, caffeic, and ferulic acids that not only serve as substrates for polyphenol oxidase (PPO; E.C. 1.14.18.1) but beneficially contribute to color as copigments and to antioxidant and biological activity of extracts as well (D.-J. Huang, Chun-Der, Hsien-Jung, & Yaw-Huei, 2004; Yoshimoto, Okuno, Yoshinaga, Yamakawa, Yamaguchi, & Yamada, 1999).

Processing PSP for commercial anthocyanin recovery offers unique challenges, among which are the ability to rapidly inactivate PPO and inefficient extraction of pigments from the dense tissue structure. Although not considered substrates for PPO, anthocyanins will rapidly degrade with tissue disruption and posterior co-oxidation from hydrogen peroxide and o-quinone generation (F Kader, Irmouli, Nicolas, & Metche, 2002). PPO catalyzes the O2-dependent oxidation of phenolic acids to o-quinones that can be reactive to anthocyanins leading to loss of color and brown pigment formation (Thipyapong, Stout, & Attajarusit, 2007). However, some reports have indicated a direct action of PPO on anthocyanins in crushed fresh blueberries, and the addition of chlorogenic acid stimulated browning reactions that led to additional pigment destruction (Farid Kader, Rovel, Girardin, & Metche, 1997). Additionally, PSP contain peroxidase (POD; EC 1.11.1.7) that will break down endogenous hydroperoxides, and

those formed from PPO action may serve to exacerbate the degradation of anthocyanins at cut surfaces (Farid Kader, Rovel, Girardin, & Metche, 1997).

Thermal treatments with temperatures reaching up to 105°C have been used to inactivate oxidase enzymes, considering optimal activity of enzymes such as polyphenol oxidase are near 40°C (Yoruk & Marshall, 2003). Heat can also aid in extraction of pigments from fruits and vegetables including purple and red sweet potatoes (Cevallos-Casals & Cisneros-Zevallos, 2004; Suda, Oki, Masuda, Kobayashi, Nishiba, & Furuta, 2003). In addition to heat, the use of enzyme inhibiting agents added to extraction solvents may enhance extraction efficiency through increased ionic strength (Lu, et al., 2010) and direct action as an oxidase enzyme inhibitor. Chelating acids such as citric or oxalic acid can bind to metal cofactors in enzymes (Yoruk & Marshall, 2003) and lower the pH below optimal activity levels. However, little knowledge exists to link the relationship between heat treatment and the role of chemical additives to inactivate oxidase enzymes to improve anthocyanin and phenolic acid extraction from PSP. Therefore, the aim of these studies was to evaluate the efficacy of pre-heating treatments and PPO-inhibiting chemical agents on the extractability of PSP polyphenolics and evaluate their stability for use as natural food colors.

Material and Methods

Plant Material and Chemicals

Fresh purple sweet potatoes (PSP) from a proprietary development line (25.6 ± 0.6 % dry matter) were obtained from Avoca Farms Inc., Merry Hill, North Carolina

(USA) from the 2012 harvest, and 40 kg were transported the next day to the Department of Nutrition and Food Science at Texas A&M University. PSP were stored dry for 6 months at room temperature and shielded from direct light. Standards of cyanidin-3-glucoside, phenolic acids (caffeic, ferulic and chlorogenic acid) and Trolox were purchased from Sigma Aldrich, Co. (St. Louis, MO, USA). Extraction chemicals such as methanol, ethyl acetate, citric acid, oxalic acid, and sodium borate along with solvents for chromatography were purchased from Fisher Scientific (San Jose, CA, USA).

Effect of Pre-heating

The effects of pre-heating on the polyphenolic recovery from PSP were evaluated. RandomLy selected whole PSP were washed, manually cut into 5 cm cubes, mixed, and divided into five groups for immersion in a deionized water bath (Thermo Scientific SWB25, USA) at 30, 50, 70 and 90°C for 10 min as a pre-heated treatment and compared to a non-heated control group. Following immersion at each temperature, approximate 5 cm PSP cubes were drained of surface water and shredded in a kitchen-scale food processor for subsequent extraction. Pre-heated PSP treatments were extracted in individual vessels (1:6 w/v ratio) containing water acidified to pH 2.0 with citric acid and held for 1 hr in a thermostatic water bath at 70°C with continuous stirring. Following extraction, treatments were cooled in an ice water bath for 5 min, centrifuged at 4,000 x g for 15 min (Eppendorf Centrifuge 5810R, Eppendorf North America, Westbury, NY, USA), and the supernatant filtered through a 1 cm bed (9 cm diameter)

of acid-washed diatomaceous earth under mild vacuum to clarify. Treatments were held frozen at -18°C until analysis.

Effect of PPO Inhibiting Agents

The effect of PPO inhibiting agents and their impact on anthocyanin recovery was evaluated for both pre-heated (90°C for 10 min) and non-heated PSP. To the aqueous citric acid extraction solution at pH 2.0 that served as a control, the addition of 1% w/v sodium borate, 1% oxalic acid, and 1% citric acid were made. Anthocyanins were likewise extracted at 70°C for 1 hr in a thermostatic water bath and clarified as previously described. The effects of PPO, that can act to co-oxidize anthocyanins, were also evaluated on PSP cubes treated with and without pre-heating at 90°C and held for 15 min prior to extraction. Activity of residual PPO was determined by homogenizing PSP cubes in 0.05 M phosphate buffer at pH 6.8 and the homogenate filtered and kept in an ice water bath (Jiang, Pen, & Li, 2004). Extracts were then centrifuged at 19,000 x g for 20 min at 4°C and the supernatant used as the enzyme extract. Chlorogenic acid was used as a substrate and enzyme activities immediately measured at 410 nm and again after 24 hr of holding the extract at 25°C.

Polyphenolic Fractions and Analysis

Total soluble polyphenolics were characterized directly from the clarified PSP extract while sub-fractions were created by partitioning from 5 g pre-conditioned Sep-Pak columns (Waters Corporation, Milford, MA). Partitioning created a non-anthocyanin polyphenolic fraction that was first eluted with 100% ethyl acetate and a

predominantly anthocyanin fraction eluted with 100% methanol containing 0.01% v/v HCl as previously performed (Rodriguez-Saona & Wrolstad, 2001). Solvents were evaporated under vacuum at 35°C and re-dissolved in a known volume of 0.5 M citric acid buffer at pH 3. Analysis of total anthocyanin content was determined using the pH-differential method (Wrolstad, Durst, & Lee, 2005) and results expressed in equivalents of cyanidin-3-glucoside (mg/kg FW). Total soluble polyphenols representing the total metal ion reducing capacity was determined by the Folin-Ciocalteu assay (Singleton & Rossi, 1965) and data expressed in mg/kg chlorogenic acid equivalents (CAE). The antioxidant capacity of PSP extracts was measured by the oxygen radical absorbance capacity method (ORAC) and the results expressed in µM Trolox equivalents per mL (µM TE/mL) (Ou, Hampsch-Woodill, & Prior, 2001). Individual anthocyanin glycosides and non-anthocyanin phenolic acids were tentatively characterized by HPLC-ESI-MSn using a Thermo Finnigan LCQ Deca XP Max ion trap mass spectrometer equipped with an ESI ion source run in positive ionization mode (ThermoFisher, San Jose, CA, USA) according to a previous developed method by Pacheco-Palencia, Hawken and Talcott (2007). Following initial compound characterization, routine quantification of anthocyanins was conducted using a Waters 2695 Alliance HPLC system (Waters Corp., Milford, MA) equipped with a Waters 996 photodiode array detector. Separations were conducted using a 250 x 4.6 mm Acclaim 120 C18 column (Dionex, Sunnyvale, CA) with a C18 guard column. Mobile phases consisted of 10% v/v acetic acid and 5% methanol in water (Phase A) and a 1% acetic and 1% formic acid in methanol (Phase B) run at 0.7 mL/min. A gradient solvent program ran Phase B from 0% to 30% in 3 min,

from 30% to 50% in 2 min, from 50% to 70% in 5 min, from 70% to 80% in 2 min, from 80% to 100% in 3 min prior to re-equilibration. Individual anthocyanins were additionally monitored at 520 nm and quantified in mg/kg equivalents of cyanidin-3-glucoside while individual phenolic acids were monitored at 280 nm and quantified in mg/kg of caffeic, ferulic or chlorogenic acid equivalents. The stability of individual PSP anthocyanins was determined by monitoring changes in total anthocyanin content over 6 hr in a thermostatic water bath at 80°C by removing samples in 1 hr intervals and cooling in an ice bath prior to analysis to determine constant rate (hr^{-1}) and half-life (hr) of anthocyanin color.

Statistical Analyses

The effect of pre-heating temperatures and PSP polyphenolic fractions were evaluated along with the impact of chemical additions to the extraction solvent. All experimental treatment variables were conducted in two independent repetitions on separate days and each analyzed in triplicate. Analysis of variance (ANOVA), linear regression, Pearson correlations and means separation by Tukey's HSD test ($p < 0.05$) were conducted using SAS version 6.11 (SAS Institute, Inc., Cary, NC).

Results and Discussion

Polyphenolic Identifications

Purple sweet potato (PSP) concentrates have attracted considerable interest as a potentially healthy food colorant with a diverse array of anthocyanins that are applicable in many food and beverage applications. Previous studies have identified the

predominant anthocyanin and non-anthocyanin polyphenolics present in PSP, whose concentrations vary depending on the cultivar and method of extraction (Teow, Truong, McFeeters, Thompson, Pecota, & Yencho, 2007; V.-D. Truong, et al., 2009). Using a proprietary PSP variety, the present study identified eight predominant anthocyanins by HPLC-MS that were previously characterized in the literature (Table 3).

The compounds identified were glycosylated and acylated derivatives of cyanidin and peonidin with aglycones at m/z of 287 and 301, respectively; and consisted mostly of mono and diacylated moieties of caffeic, ferulic, or p-hydroxybenzoic acid likely linked to sophorose at the 3-position of an anthocyanin-5-glucoside (Montilla, Hillebrand, Butschbach, Baldermann, Watanabe, & Winterhalter, 2010; Terahara, Konczak, Ono, Yoshimoto, & Yamakawa, 2004; V.-D. Truong, et al., 2009). Only one non-acylated anthocyanin was identified as cyanidin-3-sophoroside-5-glucoside at m/z 773, producing fragment ions at m/z 611, 449 and 287, indicating the loss of 3 glucosyl units.

Table 3: Tentative compound identity and fragmentation patterns of predominant anthocyanin glycosides and phenolic acids present in purple sweet potatoes. Concentrations represent the highest attained under the experimental conditions for each compound.

Tr (Retention Times)	**Tentative Identity**	**MS**	**MS^2**	**MS^3**	**Maximal Concentration (mg/kg)**
9.70	Cyanidin-3-sophoroside-5-glucoside	773	287	611, 449	59.8
12.3	Cyanidin 3-(6”- *p*-hydroxybenzoyl sophoroside)-5-glucoside	893	287	731, 449	255
14.5	Cyanidin 3-(6”-feruloyl sophoroside)-5-glucoside	949	287	787, 449	43.8
15.0	Cyanidin 3-(6”-caffeoyl sophoroside)-5-glucoside	935	287	773, 449	52.6
17.5	Cyanidin 3-(6”-caffeoyl-6”’-p-hydroxybenzoyl sophoroside)-5-glucoside	1055	287	893, 449	318
18.5	Peonidin 3-(6”-caffeoyl sophoroside)-5-glucoside	949	301	787, 463	930
20.5	Cyanidin 3-(6" caffeoyl-6”’-feruloyl sophoroside)-5-glucoside	1111	287	949, 449	120
22.7	Peonidin 3-(6”-caffeoyl-6”’-*p*-hydroxybenzoyl sophoroside)-5-glucoside	1069	301	907, 463	115
8.50	Chlorogenic Acid	353	191	191	1,900
10.5	Feruloylquinic acid	367	193	193	426
11.7	4,5-di-*O*-caffeoylquinic acid	515	353	179, 173	2,330
17.7	3,5-di-*O*-caffeoylquinic acid	515	353	179	5,260
19.5	3,4-di-*O*-caffeoylquinic acid	515	353	203, 173	2,860

Mono acylated compounds generally eluted first from the reversed phase column compared to diacylated anthocyanins with a longer retention time (Figure7). Extracted ion chromatograms at each target mass aided in differentiating compounds due to minor co-elution in the visible spectrum. Among the anthocyanins, three mono-acylated cyanidin based compounds were detected with p-hydroxybenzoyl, feruloyl and caffeoyl moieties with molecular ions of m/z 893, 949 and 935, respectively; with peonidin 3-(6”-caffeoyl sophoroside)-5-glucoside present in the highest concentration. One mono-acylated peonidin base compound consisted of a caffeoyl moiety with a molecular ion at m/z 949. Di-acylated species included cyanidin 3-caffeoyl-p-hydroxybenzoyl sophoroside-5-glucoside (m/z 1055), cyanidin 3-(6" caffeoyl-6"-feruloyl sophoroside) 5-glucoside (m/z 1111), and peonidin 3-caffeoyl-p-hydroxybenzoyl sophoroside 5-glucoside (m/z 1069).

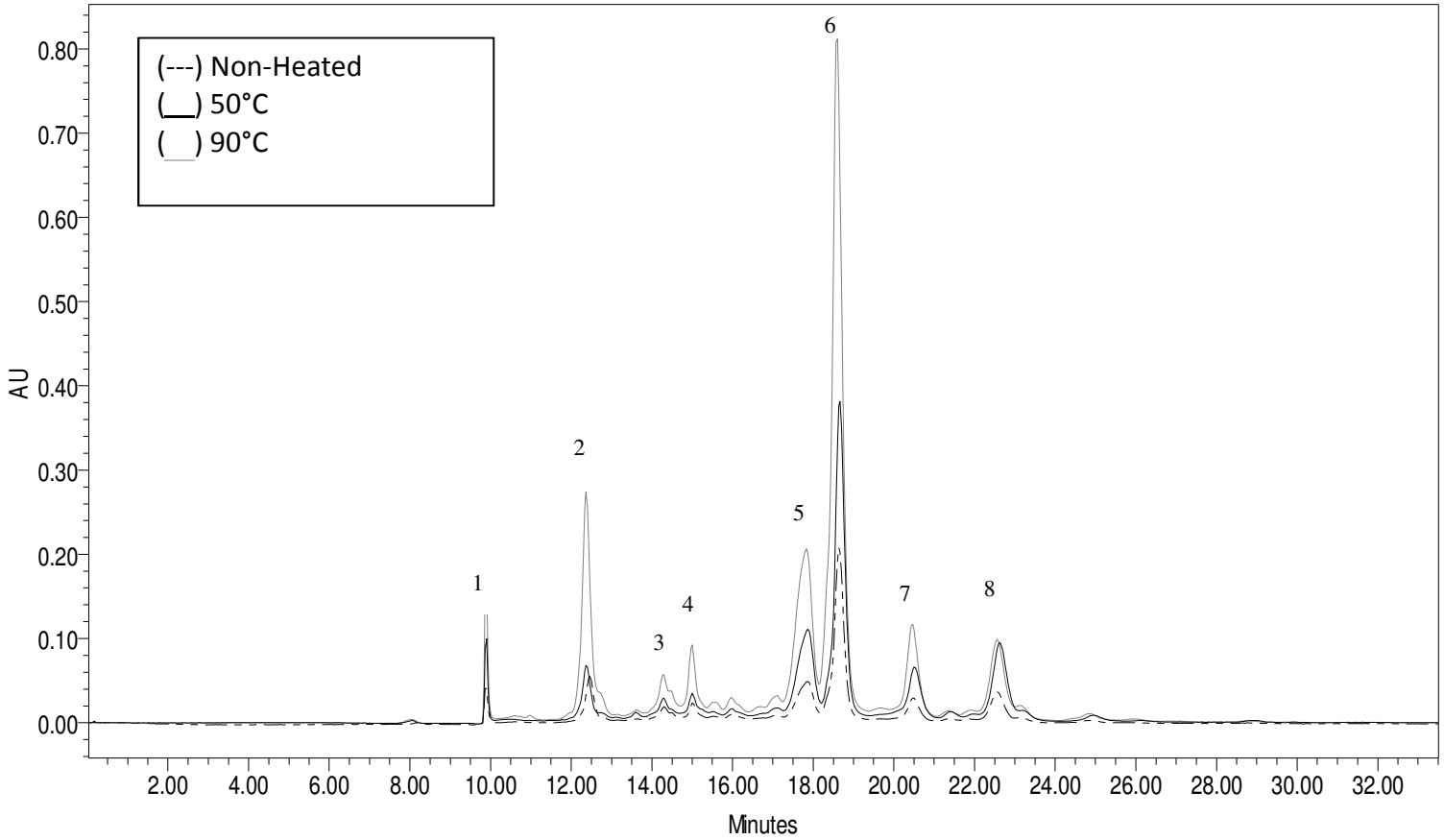


Figure 7: Overlaid HPLC chromatogram (520 nm) of the predominant anthocyanins present in purple sweet potatoes extracted at 70 °C for 1hr min following selected pre-heating temperatures held for 10 min.
Tentative identification with respective to retention times are shown in Table 3.

Non-anthocyanin polyphenolic compounds were also tentatively identified based on the HPLC elution time with standards (caffeic, ferulic and chlorogenic acid), UV absorbance spectra, and MS fragmentation patterns (Table 3). Several studies have also reported non-anthocyanin polyphenolics in PSP, with chlorogenic, caffeic, and di-O-caffeoylquinic acids (3,4-diCQA, 3,5-diCQA, and 4,5-diCQA) among the most commonly reported (Padda & Picha, 2008; V. D. Truong, McFeeters, Thompson, Dean, & Shofran, 2007). These compounds are critical to the quality of PSP extracts not only as potent bioactive compounds (Konczak-Islam, Okuno, Yoshimoto, & Yamakawa, 2003; Steed & Truong, 2008; V.-D. Truong, et al., 2009) and as primary substrates for PPO, but also as important contributors to extract color as anthocyanin cofactors (Pacheco-Palencia, Mertens-Talcott, & Talcott, 2010). Di-O-caffeoylquinic acid was the predominant phenolic acid present in these studies, followed by chlorogenic acid and feruloylquinic acid (V. D. Truong, McFeeters, Thompson, Dean, & Shofran, 2007).

Pre-Heating and Polyphenolic Extraction

Like many fruits and vegetables, the cut surface of PSP can lead to rapid physicochemical changes, with the appearance of visible brown pigments within seconds of air exposure. When quantified, a 15 min air exposure to cut PSP cubes without pre-heating treatment resulted in a 30% loss of anthocyanins compared to cubes pre-heated at 90°C for 10 min. Isolated crude PPO extracted from potato tissue with or without heating at 90°C found no residual enzyme activity under the conditions of the pH 2.0 extraction solution in neither isolates. Holding these isolates for 24 hr post-

extraction at 25°C with and without supplemented chlorogenic acid did not indicate enzyme recovery at this high acidity level.

The impact of pre-heating treatment on anthocyanin and polyphenolic recovery was evaluated after temperature increments of 30, 50, 70 and 90°C for 10 min to soften tissue and inactivate enzymes, such as PPO as compared to a non-heated control (Figure 8). Pre-heating PSP prior to polyphenolic extraction proved to be a fundamental processing step, with a significant increase in individual and total monomeric anthocyanins as well as individual and total phenolics at the highest pre-heating temperature. The non-heated control contained the lowest concentration of total anthocyanins among the extracts at 516 mg/kg cyanidin 3-glucoside equivalents, while the highest pre-heating temperature effectively inactivated PPO, softened tissue, gelatinized starch, and liberated higher total anthocyanin concentrations up to 1,281 mg/kg, a significant 2.5-fold increase in concentration (Figure8A). Tissue softening may also result from physicochemical reactions that take place in the cell wall matrix such as ß-elimination of pectin (Sila, Smout, Elliot, Loey, & Hendrickx, 2006). At the lower temperatures of 30 and 50°C tissue softening, starch gelatinization, and PPO inactivation was minor and resulted in limited to moderate enhancement of anthocyanin extraction. Following the pre-heating treatments, PSP were extracted in acidified water at 70°C, a temperature that would reduce the impact of oxidase enzymes during maceration and holding, and to minimize starch gelatinization in all but the 90°C pre-heating treatment, so that the majority of native starch could easily be removed by precipitation. It is co-incidental that both thermal denaturation of PPO and the lower end of the starch

gelatinization temperature range for PSP are both near 70°C (Almeida & Nogueira, 1995; Noda, Takahata, Sato, Ikoma, & Mochida, 1996). Also, at pre-heating holding temperatures ranging from 50-70°C, the action of pectin methylesterase (PME) in strengthening cell walls may have also contributed to lower pigment yields (Bartolome & Hoff, 1972; Basak & Ramaswamy, 1998). Although pre-heating at 70°C was more effective for anthocyanin recovery compared to lower temperatures, the inherent stability reported for acylated anthocyanins and rapid inactivation of oxidase enzymes made the 90°C pre-heating temperature a better industrial choice based on pigment recovery. The degree of metal ion reducing capacity (total phenolics) in PSP extracts following each pre-heating temperature also indicated either an adverse role of oxidase enzymes such as PPO or an enhancement in extraction, since a 25% higher concentration was found at 90°C in relation the lower pre-heating temperatures (Figure 8B). At 90°C the tissue is likely softened again due to inactivation of PME and ß-elimination reactions in the pectin, which do not allow the formation of calcium bridges between carboxyl groups adjacent to pectin and results in softening of the plant tissue (Abe, Yamamuro, Tau, Takenaga, Suzuki, & Oda, 2011). The highest concentrations of anthocyanins recovered at 90°C were greater than values reported for other PSP cultivars with Truong (V.-D. Truong, et al., 2009) reporting total anthocyanins from 100 to 211 mg/ kg FW for Okinawa and 337 to 968 mg/kg FW for Stokes Purple varieties. In related work to investigate the effects of cooking red colored Solanum tuberosum anthocyanins, it was observed that boiling and steam produced the highest anthocyanin yield at four-fold higher concentrations than their control due to cellular disruption and

ease of extraction(Lachman, Hamouz, Orsák, Pivec, Hejtmánková, Pazderů, et al., 2012). In PSP, microwave heating has been studied and enhanced anthocyanin extraction was attributed to cell wall damage and easy penetration by the extraction solvent (Lu, et al., 2010). Similarly, results of the pre-heating trails with PSP indicated that the combined role of rapid PPO inactivation at higher pre-heating temperatures along with tissue softening and cellular disruption due to starch gelatinization were critical factors for enhanced anthocyanin and polyphenolic recovery.

Polyphenolic Fractions

The partitioning of PSP polyphenolics sub-fractions following pre-heating and extraction provided additional information related to the composition and physicochemical characteristics of the extracts. Removal of phenolic acids from the original extracts revealed their ability to contribute to color of the anthocyanins via hyperchromic shifts as the result of intermolecular associations between phenolic acids and anthocyanins (Pacheco-Palencia, Mertens-Talcott, & Talcott, 2010). The classical intermolecular copigmentation effect, as previously reviewed, (Boulton, 2001) was pronounced in pre-heated PSP samples held at 30 and 50°C for 10 min where the ratio of individual phenolic acids to anthocyanins was approximately 4.5 (Figure 9). The enhancement of visible color at 90°C pre-heated treatment could also be attributed to a copigmentation effect in which the maximum ratio of individual phenolic acids to anthocyanins was achieved at 4.8. The influence of polyphenolic enhancement and stability to visible color has been well documented. Cinnamic acids such as ferulic and sinapic were used as enhancements of juices color, improving color stability through

intramolecular copigmentation during storage especially when compared to commercial color stabilizers (Rein & Heinonen, 2004).

On a similar study, ferulic and caffeic acids were responsible for 260% increases of immediate visible color in malvidin-3-glucoside and 70% in cyanidin-3-glucoside, respectively; while cholorogenic increased color by smaller percentages (Eiro & Heinonen, 2002). It was noted that the copigment/pigment ratio affected the copigmentation effect in a concentration dependent manner, and 100:1 provided the best hyperchromic effect in non-acylated monoglucosides. In diacylated anthocyanins, caffeic also showed 40-50% greater copigmentation than chlorogenic. The differences in increased copigment effect in anthocyanins by simple cinnamic acids (ferulic and caffeic) versus conjugated cinnamics (chlorogenic) could be explained by their different nature in associations with anthocyanins due to different chemical structures (Davies & Mazza, 1993). An increased copigment/pigment ratio was also responsible for an increased color value in grape juice, along with an increased antioxidant activity and greater stability during processing conditions and storage (Pozo-Insfran, Follo-Martinez, Talcott, & Brenes, 2007).

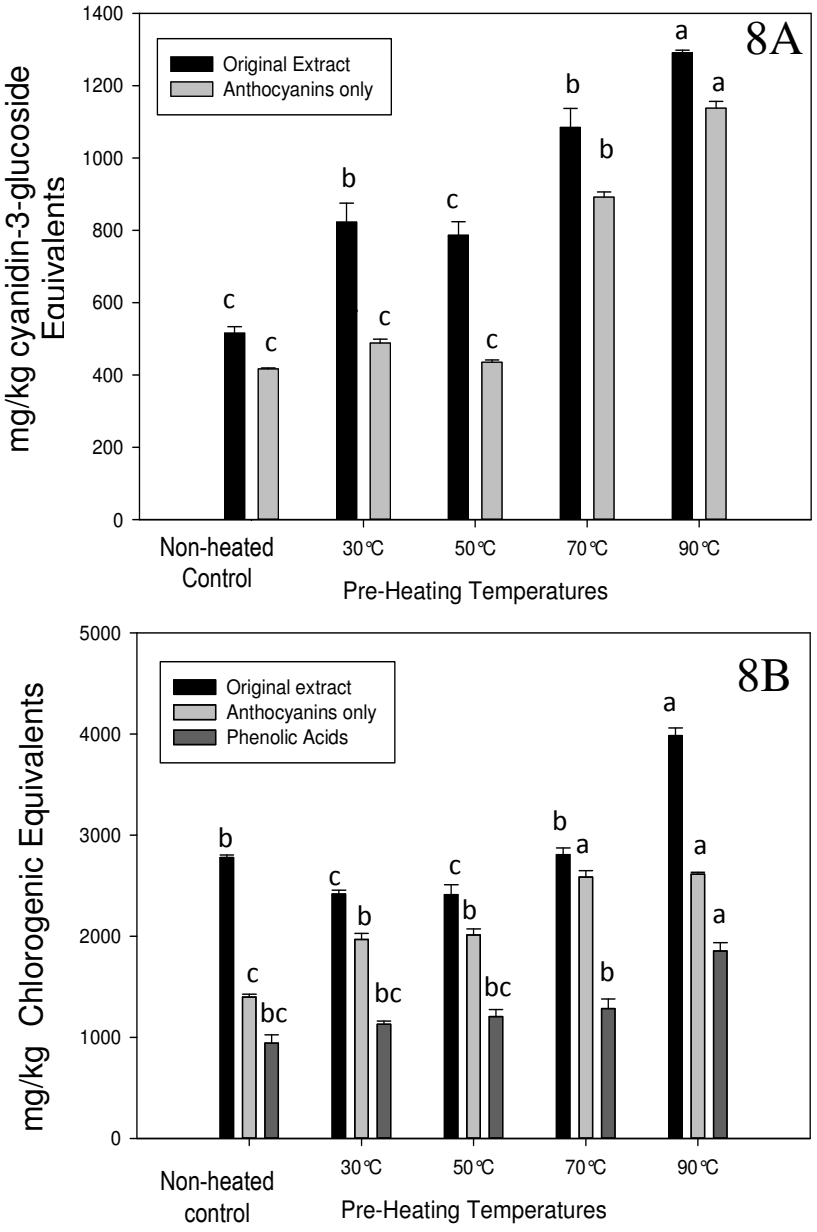


Figure 8: The effect of pre-heating temperatures on the concentration total monomeric anthocyanins measured at 520 nm (8A) or total soluble polyphenolics (8B) of purple sweet potato extracts as compared to a sub-fraction created by removal of phenolic acids with ethyl acetate.

Experiments were performed in three independent repetitions and results expressed as mean ± SE (standard error). Comparisons were made within same compounds and bars with similar letters are not significantly different (Tukey's HSD test, $p < 0.05$).

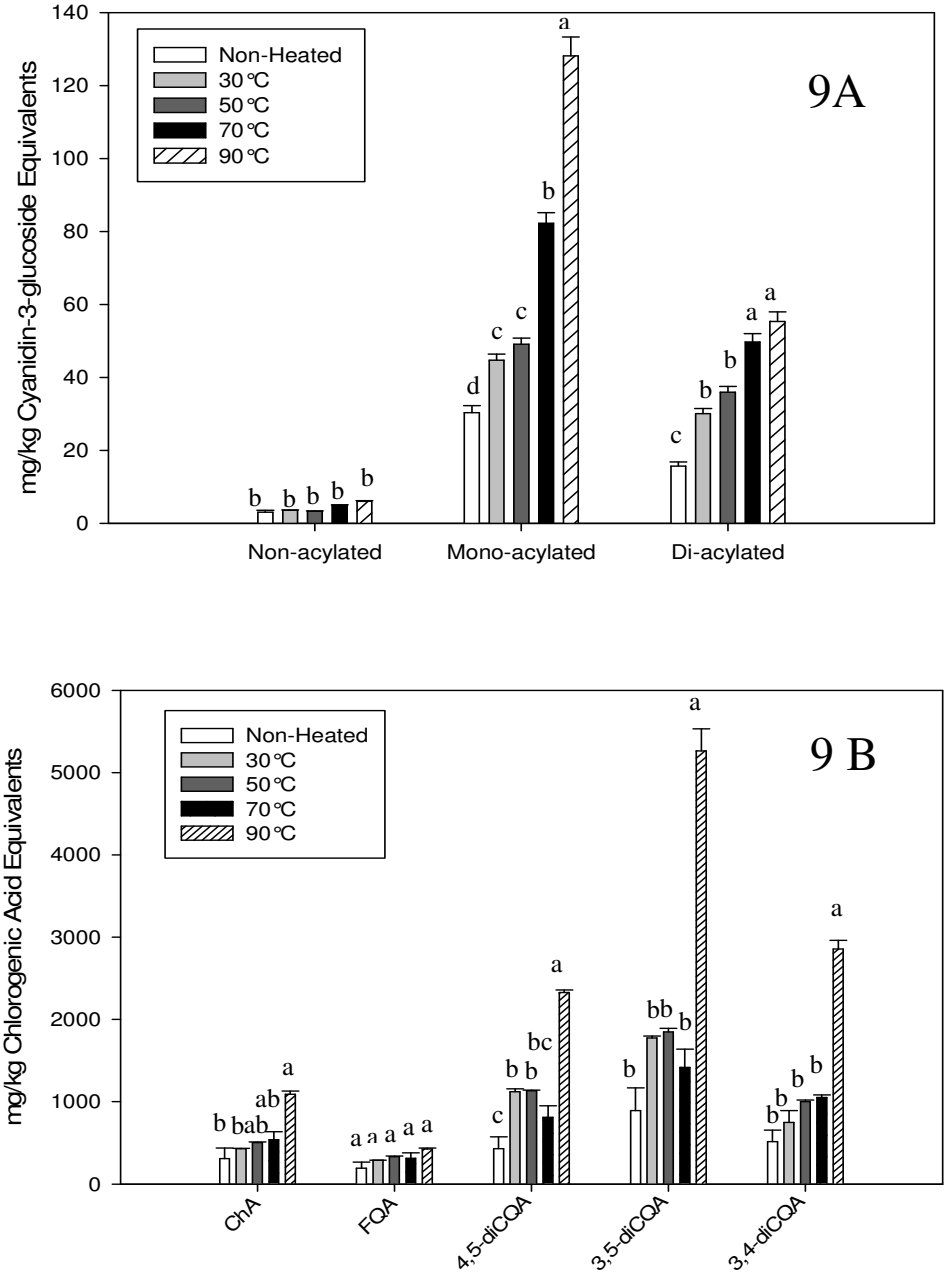


Figure 9: The effect of pre-heating temperatures on type of individual purple sweet potato anthocyanins (9A) and individual phenolic acids (9B).
Tentative compound characterizations are found in Table 1. Results are the average of three independent repetitions and results expressed as mean ± SE (standard error). Bars with similar letters for the same compound are not significantly different (Tukey's HSD test, $p < 0.05$). Compound identification: ChA: Chlorogenic Acid; FQA: feruloylquinic acid; 4,5-di-CQA: 4,5-di-O-caffeoylquinic acids; 3,5-di-CQA: 3,5-di-O-caffeoylquinic acids; 3,4-di-CQA: 3,4-di-O-caffeoylquinic acids.

The importance of the structural characteristics of acylated anthocyanins in both intra- and intermolecular copigmentation phenomenon affords the flavilium cation protection from nucleophilic attack of water and slows the subsequent formation of chalcones that lead to red color loss (M. M. Giusti & R. E. Wrolstad, 2003; Kammerer, Carle, & Schieber, 2004b). The impact of copigmentation can also be realized with a higher antioxidant and biological activity (H. W. Kim, et al., 2012), and may contribute additional benefits to PSP concentrates aside from their color impact. A positive high correlation ($r = 0.87$) with monomeric anthocyanins indicated the antioxidant capacity following pre-heating treatments demonstrated a similar temperature-dependent trend as well with the total reducing capacity ($r = 0.86$), with ORAC values increased from 130 µM TE/mL in the non-heated controls to a high of 240 µM TE/mL after pre-heating at 90°C for 10 min.

Analysis of individual polyphenolics confirmed spectroscopic observations that pre-heating conditions were instrumental in liberating both anthocyanins and phenolic acid from PSP. Anthocyanins were not preferentially extracted from PSP at any pre-heating temperature; rather identical chromatograms that only varied in concentration resulted from each extraction. For individual anthocyanins, pre-heating at 30 or 50°C for 10 min resulted in only minor increases in anthocyanin extraction, but pre-heating to 90°C increased the individual monomeric anthocyanins as up as 4.3-fold, in comparison to the non-heated control groups (Figure 9A). The proportion of mono and di-acylated anthocyanins increased ($p < 0.05$) with the increments in temperature as opposed to non-acylated anthocyanins.

The increase in temperature from 70 to 90°C appreciably increased tissue softening and initiated starch gelatinization and it was also justified by the increase in individual anthocyanin recovery (1.53 to 4.10-fold higher), in particular for mono-acylated anthocyanins, the most abundant anthocyanins in the evaluated PSP variety. For the five individual phenolic acids characterized, appreciable concentration increases were not observed except for the 90°C pre-heating treatment, whereby concentrations were 2.2 to 5.9-fold higher than the non-heated controls (Figure 9B).

Solvent Modifications and Air Effects

Color changes from browning reactions or the loss of anthocyanin color from co-oxidation reactions can adversely impact consumer acceptance of fruits and vegetables. When used in concentrate forms in foods and beverages, discoloration may also prevent their use as natural colors. Many different approaches have been used to minimize the activity of oxidase enzymes such as a PPO in an effort to increase shelf life of fresh foods including modified atmosphere, temperature control, metal chelators, pH control, and the use of chemical inhibitors such as citric and oxalic acid (Jiang, Pen, & Li, 2004). For PSP extracts used as an industrial color additive, the rapid inhibition of PPO was singled out as a critical step to prevent phenolic acid oxidation and subsequent anthocyanin degradation. However, since rapid PPO inactivation is not assured by pre-heating PSP on a commercial scale due to varying tubers sizes and density, the use of chemical inhibitors as extraction aids were evaluated with the addition of citric acid, oxalic acid, and sodium borate to the aqueous extraction solution. In these trials, non-heated and pre-heated at 90ºC for 10 min PSP, the latter with oxidase enzyme denaturing

capabilities, were evaluated with modifications to the aqueous extraction solution for optimal polyphenolic extraction. Extractions were conducted immediately for minimal air exposure and compared to a PSP group held in air for 15 min prior to extraction.

Among the solvent modifications to PSP extracted without air exposure or heat, only sodium borate addition significantly increased the recovery of anthocyanins at 24% over the acidified water control (Figure 10A). When first pre-heated to 90°C, the concentration of anthocyanins was greater with addition of oxalic acid (40%) and citric acid (18%) indicating an enhanced extraction role not attributable to enzyme inhibition. The mechanism of pH-induced oxidase inhibition has been extensively reviewed as attributable to metal chelation and the protonation of catalytic groups essential for enzyme functioning (Tipton & Dixon, 1979; Yoruk & Marshall, 2003). Citric acid has been widely used in the food industry as an anti-browning agent, acting as both an acidulant and metal chelator (Yoruk & Marshall, 2003). However, less is known regarding the effects of sodium borate as a PPO inhibitor, although it can chelate metals and has been previously used as an anti-browning agent in white-fleshed potato cultivars (Ni Eidhin, Degn, & O'beirne, 2010).

As observed with pre-heating at variable temperatures, heat likely induced structural changes to PSP cells that were aided by water-uptake and its composition as starch gelatinized. As a result, vacuole containing anthocyanins and polyphenolics were disrupted thus facilitating the pigment release into solution. In addition to chelation properties, the chemical additives altered ionic strength which helped increase the solubility of polyphenolics from the PSP matrix. In this study, the 1% oxalic acid

solution presented the greatest ionic strength of all, 0.055 M when compared to 1% sodium borate (0.04 M) and 1% citric acid (0.025 M) which explains why oxalic acid and sodium borate aided in the extraction in a more efficient way. A positive correlation between increased ionic strength and polyphenolic extraction have been previously reported (Tabart, Kevers, Sipel, Pincemail, Defraigne, & Dommes, 2007), possibly attributable to the degree of hydrolysis these antioxidant compounds present, considering these redox-active phenolics are found in plants as glycosides and starch polymers (Baugh & Ignelzi, 2000).

By comparison, the exposure of PSP cut surfaces to air for 15 min prior to extraction, representative of an industrial process, resulted in 30% loss of anthocyanins in the absence of a pre-heating treatment (Figure 10 A and B). Addition of sodium borate in non-heated PSP resulted in the greatest recovery of anthocyanins, of 218%, a strong indication of its oxidase-inhibiting properties. However, pre-heating to 90°C for 10 min and holding for 15 min under atmospheric conditions had no effect on pigment recoveries.

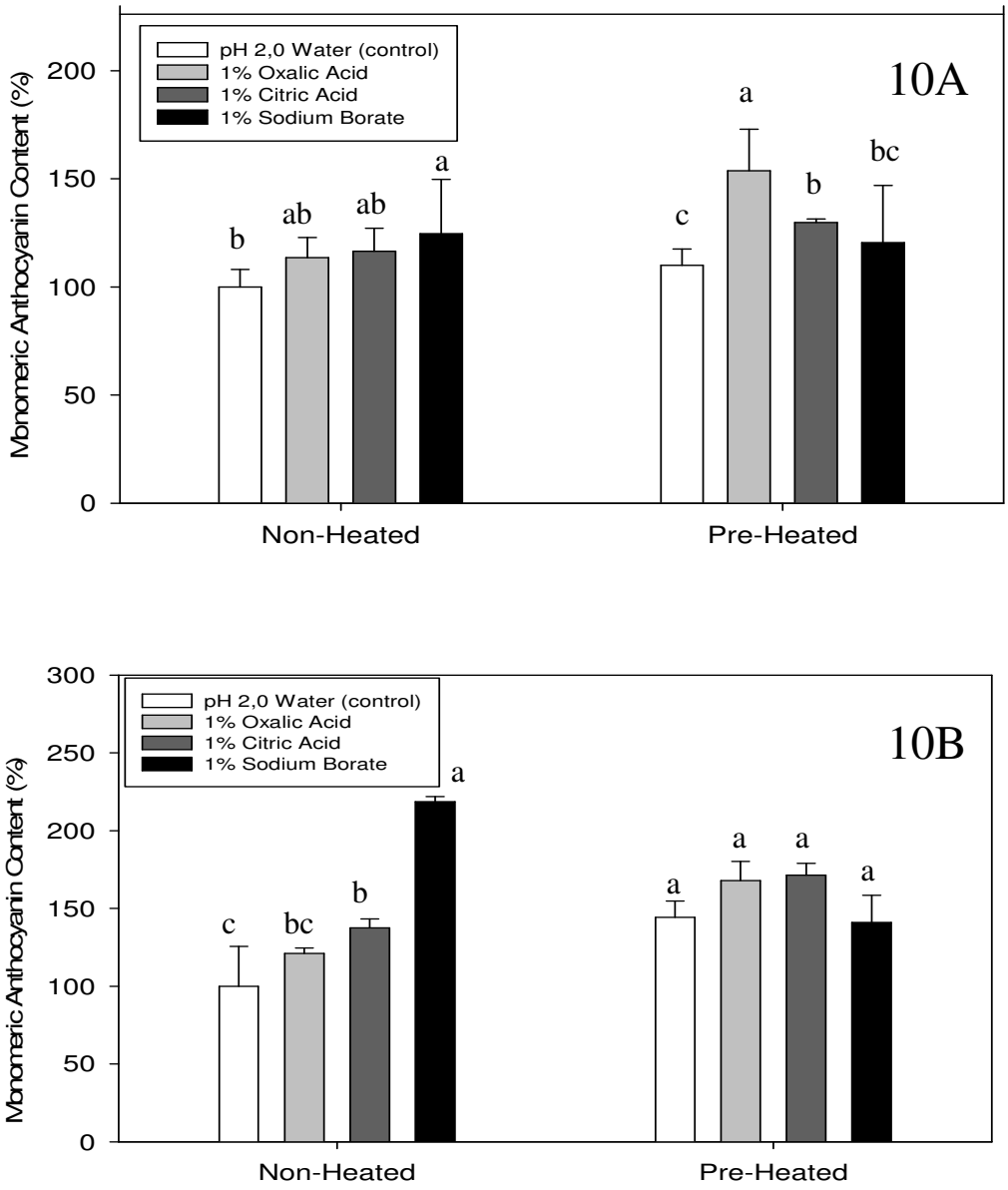


Figure 10: The effect of no-heating and pre-heating at 90°C for 10 min on monomeric anthocyanin concentrations in purple sweet potatoes as influenced by the addition of chemical modifiers to the aqueous extraction solution with limited air exposure (10A) or after 15 minutes of air exposure (10B) prior to extraction.
Results are the average of three independent repetitions and results expressed as mean ± SE (standard error). Bars with similar letters for the same treatment are not significantly different (Tukey's HSD test, $p < 0.05$).

Stability of PSP Anthocyanins

The thermal stability of PSP anthocyanin extracts was determined between non-heated and pre-heated PSP at 90°C for 10 min with and without chemical additions to the extraction solvent and evaluated for 6 hr at 80°C (Table 4). Regression analyses for anthocyanin half-life (t1/2) and rate constants (k) were calculated as first-order degradation kinetics as commonly reported for thermal loss of anthocyanins (J. Li, Li, Zhang, Zheng, Qu, Liu, et al., 2013; Nayak, Berrios, Powers, & Tang, 2011; Reyes & Cisneros-Zevallos, 2007). Despite the observed anthocyanin loss between non-heated and pre-heated PSP, the thermal stability observed at 80°C was higher for non-heated samples containing oxalic and citric acid in the extraction solution. Anthocyanin profiles by HPLC (profiles similar to Figure7) confirmed that these chemical agents were instrumental in increasing the stability of each anthocyanin in the extract without a pre-heating treatment. Results may indicate other physicochemical changes to PSP during pre-heating such as protein denaturation, cell wall degradation, or solute release from starch gelatinization that impacted anthocyanin stability post-extraction. However, no differences in thermal stability were observed among any of the pre-heated extracts, based on half-life parameter. By comparison, the stability of strawberry, elderberry, and black carrot anthocyanin was evaluated under similar heating conditions and t1/2 of 1.95, 1.96, and 2.81 hr, respectively, were observed, when exposed to similar thermal conditions. The higher stability of black carrot anthocyanins was attributed to their acyl moieties (Eva Sadilova, Carle, & Stintzing, 2007), as similarly observed for the PSP anthocyanins. Results indicated that PSP anthocyanins were relatively thermostable

under these extreme heating conditions and compared favorably to other anthocyanin sources.

Conclusions

Anthocyanins and phenolic acid cofactors that enhance the color of PSP anthocyanins were obtained at higher concentrations with use of a pre-heating step sufficient to inactivate PPO and disrupt plant cell structures. The use of chemical modifiers such as oxalic acid and sodium borate to a standard aqueous citric acid solution was additionally beneficial to anthocyanin extraction by serving as a metal chelator and increased ionic strength of the extraction solution. PSP proved to contain relatively stable anthocyanins with the potential for diverse applications in food and beverages.

Table 4: The effect of no-heating and pre-heating at 90°C for 10 min on the kinetic parameters of anthocyanin degradation monitored at 520 nm in purple sweet potato extracts held as 80°C for 6 hr as influenced by the addition of chemical modifiers to the aqueous extraction solvent. Anthocyanin degradation followed first-order kinetics.

	Pre-Heated			**Non Pre-Heated**	
Treatment	$t_{1/2}$ **(hr)**[1]	k **x** 10^3 **(hr**$^{-1}$**)**[2]	**Treatment**	$t_{1/2}$ **(hr)**	k **x** 10^3 **(hr**$^{-1}$**)**
Control	10.94 ± 0.4[a]	6.33	Control	9.06 ± 0.2[d]	7.65
Oxalic Acid	9.69 ± 1.0[a]	7.15	Oxalic Acid	16.45 ± 1.8[a]	4.21
Citric Acid	8.35 ± 2.2[a]	8.30	Citric Acid	13.78 ± 0.2[b]	5.03
Sodium Borate	10.48 ± 0.5[a]	6.61	Sodium Borate	10.41 ± 0.1[c]	6.66

[1]Half life (hours) of initial absorbance value for each treatment. [2]Reaction rate constant (k x 10^3 hr-1). [3]Values with similar letters within columns are not significantly different (Tukey's HSD test, $p < 0.05$) with results expressed as mean ± SE (standard error).

CHAPTER IV

ACYLATED ANTHOCYANINS FROM PURPLE SWEET POTATOES (*IPOMOEA BATATAS*) IN SIMULATED *IN VITRO* DIGESTION AND CACO-2 ABSORPTION MODEL

Overview

The objective of this study was to investigate stability of Purple Sweet Potato (PSP) anthocyanins and phenolic acids in an *in vitro* digestion that simulates gastric and pancreatic conditions, as well as assess their absorption in a Caco-2 human intestinal cell model. PSP compounds were enriched using solid-phase extraction and subjected to stomachal (pH 2.0/ 2 hr) and small intestinal conditions (pH 7.0/ 7 hr) and stability monitored by HPLC-MS. Results indicate that gastric conditions did not cause significant degradation of PSP anthocyanins and phenolic acids, while significant degradation occurred at intestinal conditions. Hydrolyzed anthocyanins were degraded in both conditions presence and absence of phenolic acids (47.45 and 42.2% recovery, respectively). In comparison, the non-hydrolyzed anthocyanin fractions presented a high recovery after intestinal incubation, of 67.92% and 68.16%, in the presence and absence of phenolics, respectively. Ethyl-acetate fractionated phenolic acids were characterized by degradation under intestinal conditions with a recovery of 48.54%. Transport from the apical to the basolateral side was demonstrated in the Caco-2 cell model where the extent of transport was dependent on chemical structure and association complexes. Overall, acylated anthocyanins from PSP are characterized by increased stability

compared to hydrolyzed anthocyanins and therefore may contribute to anthocyanin-associated health benefits.

Introduction

Food coloring is important in the food industry, as it contributes towards the aesthetic appeal of the product. Increased interest in natural colors can be attributed to restrictions on the usage of synthetic dye, which may pose a risk to human health. In 2014, the revenue for natural colors was values at $1,100 millions, with a share estimated to be close to 60% in 2020 (Anonymous, 2016). Additionally, consumer awareness of products' ingredient lists have made simpler, more natural ingredients, such as natural colors, a desirable additive with multiple applications.

Polyphenolic compounds are gaining popularity as consumers become more aware of the health benefits associated with their consumption, such as the decreased incidence of certain, chronic diseases (J. He & Giusti, 2010; Williams, Spencer, & Rice-Evans, 2004). Anthocyanins (polyphenols in the flavonoid class) are a secondary metabolite contributing to intense red and purple coloring in fruits, vegetables, and flowers. Anthocyanins may potentially prevent cardiovascular diseases, diabetes, and obesity (Ghosh & Konishi, 2007; Tsuda, Horio, Uchida, Aoki, & Osawa, 2003; Wallace, 2011); and possess anti-inflammatory and anti-carcinogenic activity (Joseph, Edirisinghe, & Burton-Freeman, 2015). In industry, anthocyanin extracts are used as an inexpensive substitute for synthetic dye red # 40, which is commonly used to color

beverages, confectionary, and snack foods. (Jing, Zhao, Ruan, Xie, Dong, & Yu, 2012; Wrolstad, Durst, & Lee, 2005).

Purple sweet potato (PSP), a nutritionally valuable crop from Asia and South America, contains high levels of anthocyanins in its roots and leaves (Steed & Truong, 2008). Anthocyanins in PSP have long been used in the food industry for presenting highly glycosylated and acylated (caffeoyl, feruloyl, and p-hydroxybenzoyl residues) derivatives of cyanidin and peonidin. The anthocyanins in PSP can easily overcome the usual limitations of natural colors, such as low stability in neutral pH and processing conditions, through inter- and intramolecular copigmentation (M. M. Giusti & R. E. Wrolstad, 2003). In addition, it was characterized several non-anthocyanin phenolic acids including chlorogenic acid and isomers of caffeic acid in sweet potatoes (Islam, Yoshimoto, Yahara, Okuno, Ishiguro, & Yamakawa, 2002; V. D. Truong, McFeeters, Thompson, Dean, & Shofran, 2007), and ferulic acid in PSP (de Aguiar Cipriano, Ekici, Barnes, Gomes, & Talcott, 2015).

Understanding polyphenolic stability in a human digestion system is a key factor in evaluating the health benefits of these compounds in food sources. The low probability of polyphenolic compounds surviving physiological digestive conditions raises the concern of their ability to be posteriorly absorbed and reach tissues and organs (Gil-Izquierdo, Zafrilla, & Tomás-Barberán, 2002; McDougall, Dobson, Smith, Blake, & Stewart, 2005). Therefore, submitting PSP extracts to digestive conditions is necessary to determine the extent that polyphenolic instability in non-optimal conditions affects the low concentration of polyphenols in serum, as determined by *in vivo* studies

(Charron, Kurilich, Clevidence, Simon, Harrison, Britz, et al., 2009; Dupas, Marsset Baglieri, Ordonaud, Tomé, & Maillard, 2006b; Frank, Netzel, Strass, Bitsch, & Bitsch, 2003; X. Wu, Cao, & Prior, 2002).

In addition, anthocyanins are known to possess low bioavailability, less than 1% are observed in plasma and urine in rat and human studies (Charron, et al., 2009; Frank, Netzel, Strass, Bitsch, & Bitsch, 2003). Many human pharmacokinetics studies identify the bioavailability of anthocyanins to range from 0.004 to 1.2% of the non-metabolized compound, regardless of dosage (J. He, Magnuson, Lala, Tian, Schwartz, & Giusti, 2006; Kay, Mazza, & Holub, 2005; Steinert, Ditscheid, Netzel, & Jahreis, 2008; L.-S. Wang & G. D. Stoner, 2008). However, these previous studies solely focused on the overall mechanistic interaction associated with dosage level and how the body metabolizes and excretes the compounds, not accounting for intestinal absorption (Van Duynhoven, et al., 2011). To address this oversight, intestinal absorption studies are needed to better understand how polyphenols interact with intestinal cells and what affects their overall uptake. These studies can show if anthocyanins are biologically active as parent compounds and how absorption plays a role in the efficacy of the treatment. This knowledge is a fundamental piece into understanding the fate of anthocyanins in the body.

Information correlating chemical composition of PSP polyphenols and cellular absorption after ingestion is very limited. Therefore, this study used a simulated gastrointestinal digestion model to assess the stability of PSP extracts and a Caco-2 human intestinal cell monolayer to assess the absorption properties of different chemical

compositions of PSP extracts—obtained through basic hydrolysis and C18 solid phase extraction.

Materials and Methods

Extraction of PSP Polyphenols

As previously described in the Polyphenolic Fractions and Analysis in Material and Methods of Chapter III.

Optimization for Alkaline Hydrolysis of Anthocyanins

Optimization of the alkali conditions tested concentration of base (NaOH) and time of hydrolysis. De-acylation of the anthocyanins (3 mL) was performed via alkaline hydrolysis (Haekkinen, Kaérenlampi, Heinonen, Mykkänen, & Toerroenen, 1998). Anthocyanin-rich extracts were placed in screw-capped test tubes with 12 mL of 1, 2, 3, or 4 N aqueous NaOH for 0, 5, 10 and 15 min at 37°C, then infused with nitrogen to prevent oxidation. At each collecting time, the solution was acidified with concentrated HCl (12 N), cooled in an ice bath for 5min, and purified prior to injection into the HPLC system (Z. Shi, I. Bassa, S. Gabriel, & F. Francis, 1992). The reaction was monitored through the formation of the non-acylated anthocyanins cyanidin-3-glucoside, peonidin-3-glucoside and free acylating acids (Hong & Wrolstad, 1990).

Isolation of Anthocyanin and Non-Anthocyanin Phenolic Acids (NAF)

The PSP whole fraction control (WC) was clarified using 5 g pre-conditioned Sep-Pak columns (Waters Corporation, Milford, MA). Non-anthocyanin phenolic

fraction (NAF) were first eluted with 100% ethyl acetate, then the remaining anthocyanin control (AC) with 100% acidified methanol (0.01% HCl) (Rodriguez-Saona & Wrolstad, 2001). Solvents were evaporated under vacuum at 35°C and re-dissolved in a known volume of 0.5 M citric acid buffer at pH 3. Both WC and AC were hydrolyzed with the prevously chosen method, yielding the de-acylated fractions WH and AH (WH– Whole hydrolyzed and AH- Anthocyanin hydrolyzed).

Simulated Gastrointestinal Digestion

PSP anthocyanin and non-anthocyanin phenolic extracts (1000 mg/kg GAE—gallic acid equivalents) were subjected to two-stages of a simulated human digestion system (Krook & Hagerman, 2012). The initial stage consisted of adding pepsin/HCl to the diluted extracts (1:25 v/v) for 2 hr at 37ºC until pH 2.0 was reached, to simulate gastric conditions. It was followed by a digestion with pancreatic salts (porcile pancreatin and lipase) and bile mixed in $NaHCO_3$ until a final pH of 7.0 ±0.1 was reached, for 7 hr at 37ºC, with shaking, in the absence of light, and under N_2. The control group consisted of the same extracts exposed to a buffered solution with the same final pH values (2.0 and 7.0, respectively), without addition of enzymes. At each collection time, 5% (v/v) 99% formic acid was added to the samples to stop the reaction. Then, the samples were filtered and analyzed via HPLC-MS.

Transepithelial Transport Model

Caco-2 colon carcinoma cells were purchased from American Type Culture Collection (Manassas, VA). These cells were cultured with high glucose Dulbecco's

Modified Eagle Medium (DMEM), supplemented with solutions of 1 % penicillin, 1% non-essential amino acids (10 mM), 1% sodium pyruvate (100 mM), and 20 % fetal bovine serum (Life Technology, Grand Island, NY). Cells were maintained at 37 °C with a humidified 5 % CO_2 atmosphere. Caco-2 colon carcinoma cells (10 x 10^4) between passages 5 and 10 were seeded into 12-mm transparent polyester cell culture well plates (Transwell, Corning Costar Corp., Cambridge, MA) with 0.5 mL of growth medium in the apical side and 1.5 mL in the basolateral side. Cells were cultivated and differentiated to confluent monolayers for 21 days (Hidalgo, Raub, & Borchardt, 1989). Prior to analysis, transepithelial electrical resistance (TEER) values were monitored with an EndOhm Volt ohmmeter equipped with an STX-2 electrode (World Precision Instruments Inc., Sarasota, FL). TEER values of 350 Ωcm^2 and higher had to be obtained at the beginning and at the end of the experiment to insure monolayer integrity. The medium was changed 24 hours prior to the experiment. Prior to transport experiment, growth medium in the apical side was substituted with Hank's balanced salt solution (HBSS, Thermo fisher Scientific, Pittsburgh, PA) containing 10 mM 2-(N- morpholino) ethanesulfonic acid solution (MES, pH 6.0, Life Technology, Grand Island, NY), while HBSS containing 1 M N-[2-hydroxyethyl] piperazine-N0-[2-ethanesulfonic acid] (HEPES, pH 7.4, Life Technology, Grand Island, NY) replaced the media in the basolateral side. Anthocyanin-rich (WC, WH, AC, and AH) and NAF extracts at 50 mg/L GAE—diluted in MES and adjusted to pH 6.0—were then loaded into the apical side of the cell monolayers. Monolayers were incubated for 2 hr at 37°C. Aliquots (200 μL) were taken from the basolateral compartment at 0.5, 1.0, 1.5, and 2.0 hr, then

immediately acidified with 5% (v/v) 99% formic acid and kept frozen at -20°C for further analysis. As sample was removed from the basolateral side, fresh HBSS medium (200 μL) was added to make up for the removed volume and accounted for in the final calculations. Samples were then filtered through 0.45 μm PTFE membranes (Whatman, Florham Park, NJ) and injected directly into the HPLC–ESI-MS system.

Phytochemical Analyses

Total anthocyanin, total soluble phenolics (total reducing capacity), individual and hydrolyzed anthocyanins and phenolic acids were assayed as previously described in the Polyphenolic Fractions and Analysis, in Material and Methods of Chapter III.

Statistical Analyses

The experiment was conducted with 2 triplicates in a randomized complete design. Data were analyzed by SAS version 6.11 (SAS Institute, Inc., Cary, NC). Student t-test and ANOVA were performed, and significance of differences among least square means was determined by Tukey's test ($p < 0.05$).

Results and Discussion

Analysis of the Anthocyanins and Non-Anthocyanins in the PSP

Glycosylated (glucose and sophoroside moieties) and acylated (caffeic, ferulic, or p-hydroxybenzoic acid) derivatives of cyanidin and peonidin, likely linked to sophorose at the 3-position of an anthocyanin-5-glucoside, were detected in the PSP extracts (Figure 11A). Non-anthocyanin phenolic acids fractions (NAF) in PSP consisted of di-

O-caffeoylquinic, chlorogenic, and ferulic acids (Figure 12A). Alkaline hydrolysis was conducted with PSP anthocyanins in order to break the ester bonds of the acyl groups. Since there is no established procedure for alkaline hydrolysis of anthocyanins, an optimization test was performed to determine the best procedure, varying concentration of NaOH and exposure time. The most effective results were obtained when using 3 N NaOH with 5 minutes of exposure, yielding on average 3-4 times higher de-acylated cyanidin and 2-4 times peonidin based compounds compared to the initial conditions (Figure 13A and B, respectively). HPLC chromatograms of PSP after alkaline hydrolysis are shown in Figure 11B. Cyanidin and peonidin-3-glucoside were identified by retention time and spectral characteristics. Contrary to our hypothesis that only cyanidin and peonidin-3-glucoside would remain after alkaline hydrolysis, a peak remained for cyanidin 3-(6”- *p*-hydroxybenzoyl sophoroside)-5-glucoside, suggesting that complex anthocyanins hydrolyze into this form.

The highest concentration of monomeric anthocyanin was observed in WC and AC, with concentrations of 8437.38 ± 270 and 7855.31 ± 284 mg/kg CGE, respectively. Hydrolysis led to loss of total monomeric anthocyanin, as concentrations were measured at 2928.29 ± 69 and 2723 ± 164 mg/kg CGE in WH and AH, respectively. Additionally, the hydrolyzed fractions displayed a loss of color value. Acyl moieties being released into solution during alkaline hydrolysis could explain the loss of color value in the hydrolyzed extracts (Rein & Heinonen, 2004).

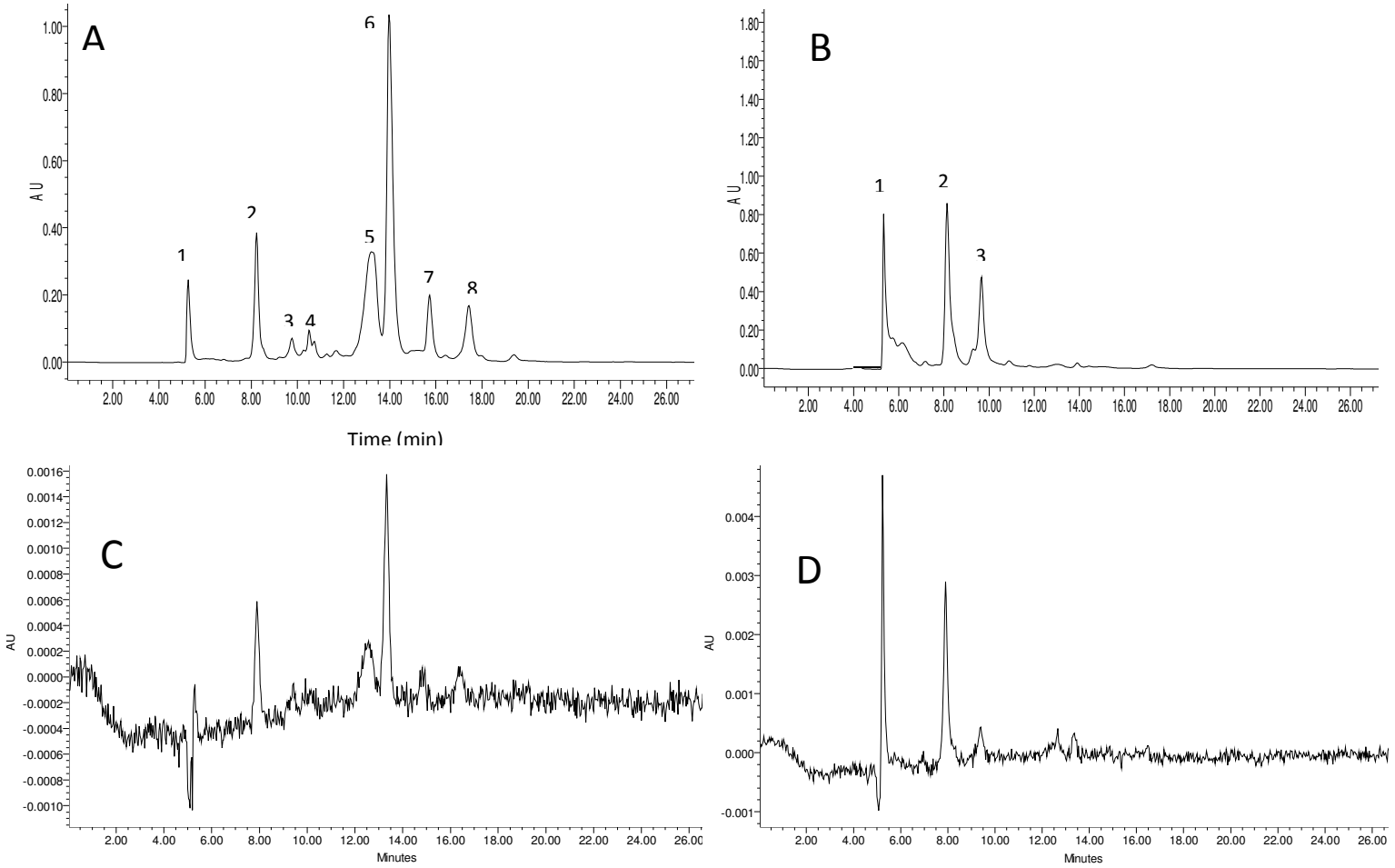


Figure 11: Chromatogram (520 nm) of the anthocyanins present in PSP before (A) and after basic hydrolysis (B).
(C) and (D) represent chromatogram of the anthocyanin fractions in PSP before and after basic hydrolysis, respectively, as recovered in the basolateral compartment of CaCo-2 cell monolayers after 2 hr. Peak assignments: 1, Cyanidin-3-sophoroside-5-glucoside; 2, Peonidin-3-sophoroside-5-glucoside, 3, Cyanidin 3-(6”- p-hydroxybenzoyl sophoroside)-5-glucoside; 4, Cyanidin 3-(6”-feruloyl sophoroside)-5-glucoside; 5, Cyanidin 3-(6”-caffeoyl-6”’-p-hydroxybenzoyl sophoroside)-5-glucoside; 6, Peonidin 3-(6”-caffeoyl sophoroside)-5-glucoside; 7, Cyanidin 3-(6" caffeoyl-6”’-feruloyl sophoroside)-5-glucoside; 8, Peonidin 3-(6”-caffeoyl-6”’-p-hydroxybenzoyl sophoroside)-5-glucoside.

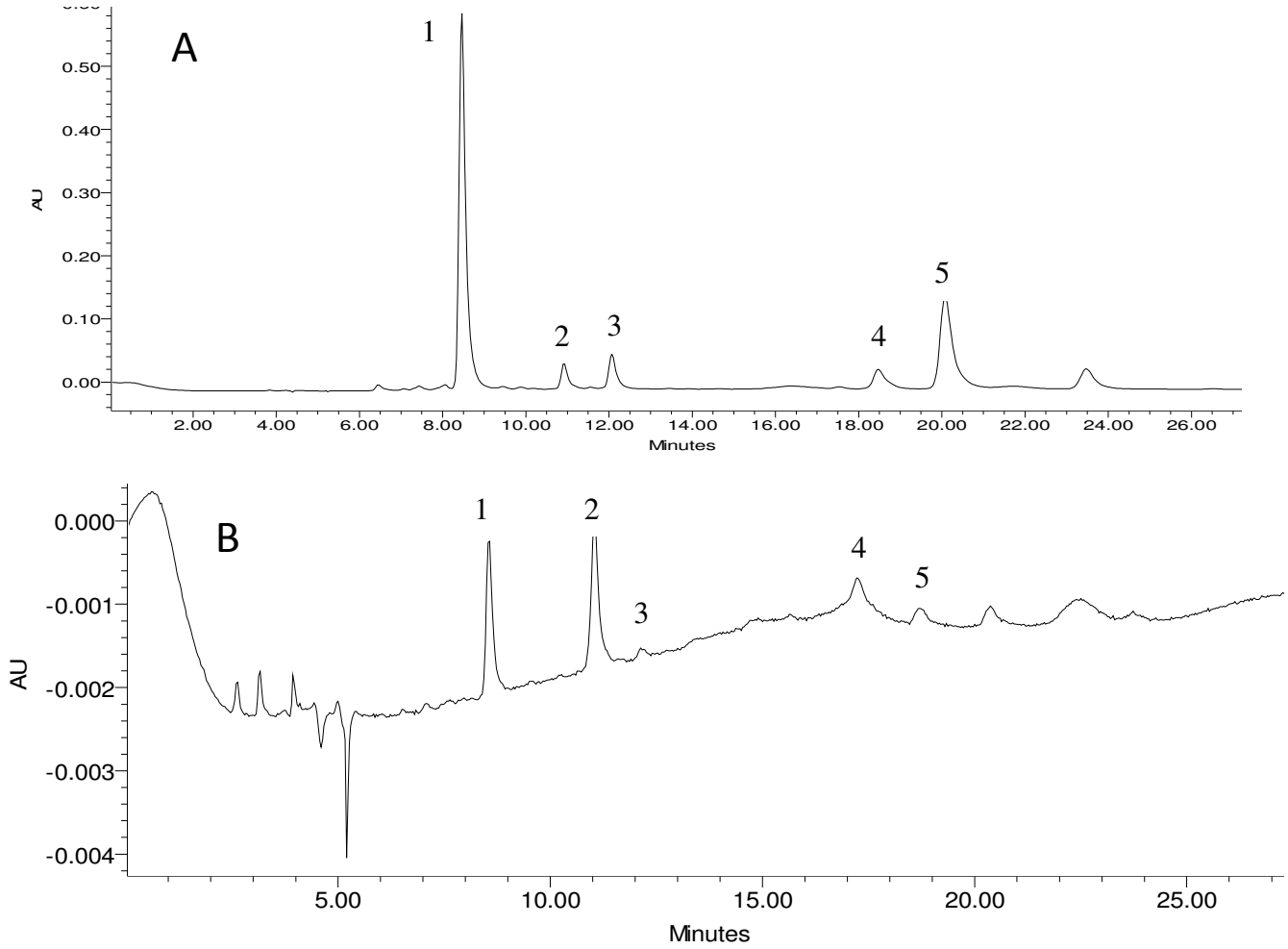


Figure 12: Chromatogram (280 nm) of the non-anthocyanins phenolic acids present in PSP (A) and present in the basolateral compartment of the Caco-2 cell monolayers following incubation for 2 hr.
Peak assignment: 1, Chlorogenic Acid; 2, Feruloylquinic acid; 3, 4,5-di-O-caffeoylquinic acid; 4, 3,5-di-O-caffeoylquinic acid; 5, 3,4-di-O-caffeoylquinic acid.

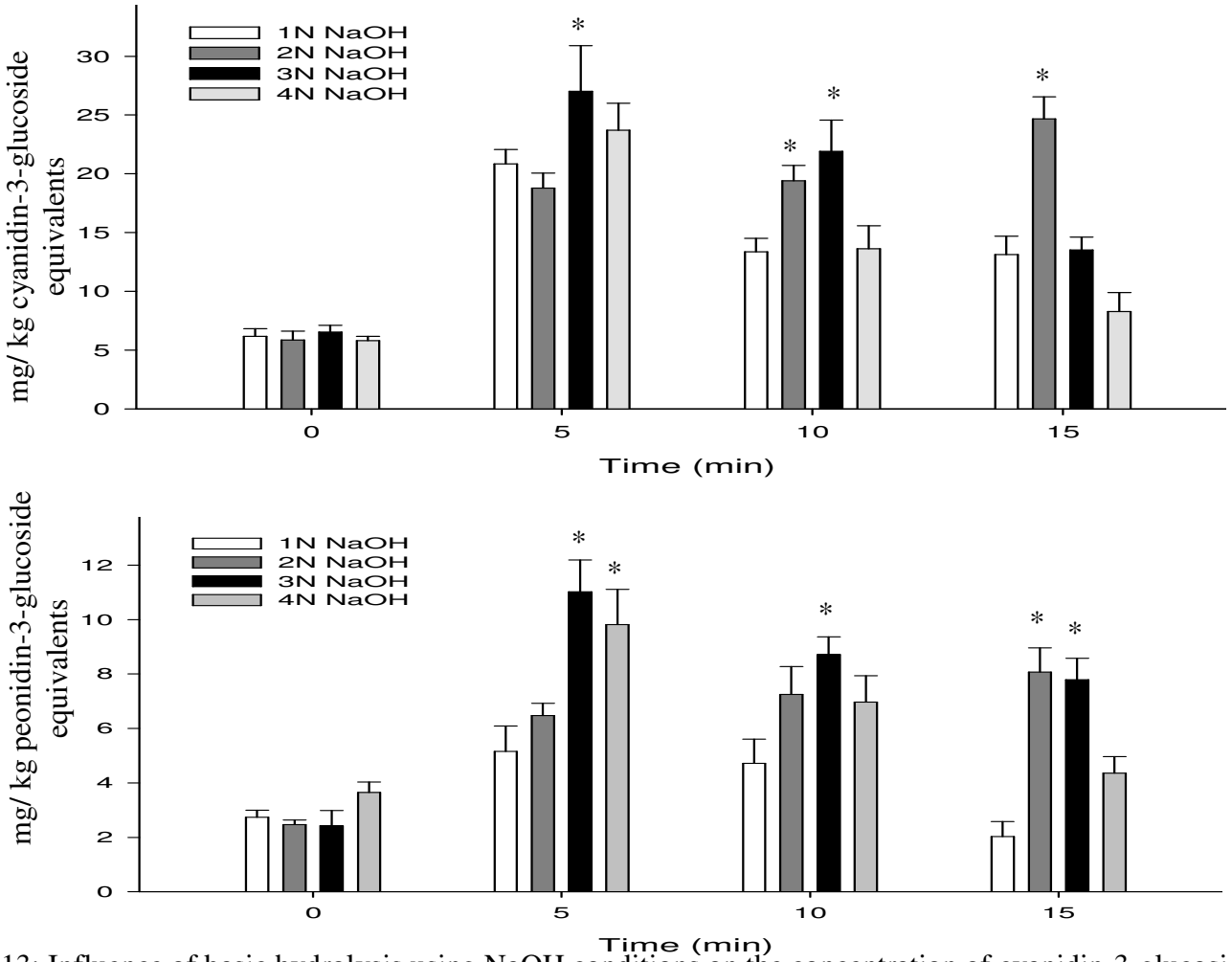


Figure 13: Influence of basic hydrolysis using NaOH conditions on the concentration of cyanidin-3-glucoside (A) and peonidin-3-glucoside (B) in PSP.
Values are average of three replicate injections from three different experiments. *: Indicates significant differences within same time of reaction ($p < 0.05$).

Simulated Digestion of Anthocyanin and Non-Anthocyanin Polyphenolic Fractions

As observed with other anthocyanin sources (M. J. Bermúdez-Soto, F. A. Tomás-Barberán, & M. T. García-Conesa, 2007; McDougall, Dobson, Smith, Blake, & Stewart, 2005), gastric simulated digestion presented no significant effect on the total amount of anthocyanins present in PSP fractions (Table 5). In the WC and NAF, a slight increase in the total compounds was observed over the course of 2 hours (~ 2 and 8%, respectively, $p < 0.05$). This is consistent with reports for other anthocyanin sources displaying increased anthocyanin concentrations after gastric incubation, as for chokeberries (M. J. Bermúdez-Soto, F. A. Tomás-Barberán, & M. T. García-Conesa, 2007) and raspberries (McDougall, Dobson, Smith, Blake, & Stewart, 2005). Because the WC was exposed to more acidic conditions than the control and the flavilium cation is favored at low pH conditions, an increased fraction of the cation is expected. A similar trend was observed for NAF, as phenolic acids also possess higher stability under low pH conditions. This phenomena has been previously observed with chokeberry juice (M. J. Bermúdez-Soto, F. A. Tomás-Barberán, & M. T. García-Conesa, 2007).

Table 5: Effect of gastrointestinal digestion on anthocyanin and non-anthocyanin phenolic contents

	% Recovery				
	Whole Control (WC)	Whole Hydrolyzed (WH)	Anthocyanin Control (AC)	Anthocyanin Hydrolyzed (AH)	Non-Anthocyanin Fraction (NAF)
Post-gastric	102.51 ± 4.25^{a}	91.79 ± 6.71^{b}	95.78 ± 4.65^{a}	88.46 ± 4.91^{b}	108.54 ± 2.31^{a}
Pancreatic Digestion (1 hr)	93.06 ± 7.85^{ab}	96.46 ± 3.5^{a}	88.75 ± 3.34^{b}	80.12 ± 5.05^{c}	54.95 ± 1.1^{b*}
Pancreatic Digestion (7 hr)	80.45 ± 13.40^{a*}	53.98 ± 3.02^{b*}	85.78 ± 3.63^{a}	42.20 ± 4.48^{c*}	48.54 ± 8.59^{c*}

Values are averages of three replicate experiments ± standard errors and are compared to values of the post-gastric treatment.
Different letters in the same row indicates statistical differences amongst anthocyanin compounds.
*Significant differences ($p < 0.05$) in comparison to the post-gastric extract, within the same extract.

PSP fractions were submitted to pancreatic digestion conditions for 7 hours, an estimation of the time that simulates complete absorption, metabolization, or excretion of these compounds (Ryan & Prescott, 2010; X. Wu, Pittman, & Prior, 2006). Collection occurred first after 1 hour, to simulate compounds reaching the small intestine (the site of greatest absorption of compounds into the bloodstream), then after 7 hours, to simulate compounds reaching the colon and being subjected to colonic microflora, fermentation, and degradation into smaller phenolic acids (Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006). Despite literature indicating most anthocyanins are unstable under pancreatic conditions, PSP total monomeric anthocyanins were able to withstand drastic pH shifts (from 2.0 to 7.0) with recoveries ranging from 80.12 to 96.46% after 1 hour of exposure (Table 5). After 7 hours of being exposed to pancreatic digestion, recoveries ranged from 42.2 to 85.78% in the order of increasing stability fraction: AH < WH < WC < AC. The recovery rates of the WC and AC were significantly higher than the WH and AH. Inter- and intramolecular copigmentation effects may have been key to protecting these compounds under intestinal conditions (M. M. Giusti & R. E. Wrolstad, 2003). Control groups without addition of enzymes showed similar reduction trends, as expected considering enzymes added in the intestinal digestion (lipase, bile and pancreatic) are not polyphenolic-substrate oriented. The decrease in total anthocyanins is believed to be due to the chemical transformation of the flavilium cation when exposed to neutral pH conditions. The equilibrium shift to colorless chalcones may underrepresent the actual amount of anthocyanins present in the medium. In this study, NAF were affected by pH conditions similarly to anthocyanins.

After 7 hours of exposure to pancreatic enzymes, a significant reduction was observed for these compounds (recovery of 48.59%). In a study with broccoli polyphenolics (Vallejo, Gil-Izquierdo, Pérez-Vicente, & García-Viguera, 2004), a decrease in total polyphenolic content was noted under both gastric and pancreatic conditions for hydroxicinnamic acid derivatives, with losses reaching up to from 92% for ferulic acid. The low stability of these compounds might be due to the low acidity of the medium, as in the case of anthocyanins.

The recoveries for individual anthocyanins and phenolic acids, after submission to pancreatic digestion for 7 hours, have been summarized in Table 6. In the non-hydrolyzed fractions (WC and AC), mono (57-111% for WC and 77-112% for AC) and no-acylated anthocyanins (90-95% for WC and 82-89% for AC) presented higher recovery percentages than di-acylated anthocyanins over time (55-74% for the WC and 62-78% for AC). High recoveries in these fractions could be due to copigmentation with NAF. The high stability of mono-acylated and non-acylated fractions in this study could have been due to degradation of di-acylated compounds yielding mono-acylated which could also be degraded into non-acylated respectives, thus regenerating simpler anthocyanins as digestion proceeded. The type of acylation seemed to influence stability as well.

Table 6: Recovery of individual PSP phenolic compounds determined in fractions after 7 hours *in vitro* gastric digestion.

Ind. Compounds	% Recovery				Ind. Compounds	% Recovery
	Whole Control (WC)	Whole Hydrolyzed (WH)	Anthocyanin Control (AC)	Anthocyanin Hydrolyzed (AH)		Non-Anthocyanin Fraction (NAF)
Cy-3-Sop-5Gluc	95.82 ± 11.91[a]	46.46 ±1.59[c*]	82.91 ± 4.09[b*]	42.84 ± 4.56[c*]	ChA	31.51 ± 1.76[*]
Pn-3-Sop-5Gluc	90.45 ± 12.35[a]	43.12 ± 1.76[b*]	89.35 ± 3.07[a]	38.71 ± 3.45[b*]	FQA	15.73 ± 4.68[*]
Cy-3-*p*hB-Sop-5 Gluc	111.13 ± 4.33[a]	72.37 ± 5.70[b*]	112.16 ± 2.62[a]	45.32 ± 7.15[c*]	4,5-CQA	37.77 ± 3.25[*]
Cy-3-Fer-Sop-5 Gluc	94.28 ± 15.69		107.4 ± 15.60		3,5-CQA	24.87 ± 3.28[*]
Cy-3-(Caf)(*p*hB)-Sop-5 Gluc	54.89 ± 5.23[*]		62.33 ± 0.15[*]		3,4-CQA	21.80 ± 1.87[*]
Pn-3-Caf-Sop-5-Gluc	57.08 ± 8.87[a*]		77.08 ± 1.17[b*]			
Cy-3-(Caf)(Fer)-Sop-5 Gluc	66.46 ± 15.19[*]		78.11 ± 0.57[*]			
Pn-3-(Caf)(*p*hB)-Sop-5 Gluc	73.51 ± 16.87[*]		76.89 ± 1.80[*]			

Recovery was calculated as a comparison to the amount of each compound present in the post-gastric sample. Values are average of three replicate injections from three different experiments ± standard error.
Different letters in the same raw indicates statistical differences amongst anthocyanin compounds.
*Significant differences ($p < 0.05$) in comparison to the post-gastric extract, within the same extract.
Anthocyanins were analysed at 520 nm while NAF at 280 nm.
Cy= Cyanidin, Pn= Peonidin, Sop=Sophoroside, Gluc=Glucose, *p*hB=*p*-hydroxy Benzoic acid,
Fer=Ferulic acid, Caf=Caffeic acid, ChA=Chlorogenic acid, FQA= Ferullyquinic acid, CQA= Caffeoylquinic acid.

Overall, *p*-hydroxybenzoic acid substituted anthocyanins showed the greatest stability. Within the mono-acylated forms, anthocyanins substituted with p-hydroxybenzoic acid and ferulic acid were more stable than those substituted with caffeic acid (Cyanidnin (Cy)-3- p-hydroxy benzoic acid (*p*hB)- sophoroside (Sop)-5 glucose (Gluc) > Cy-3-ferulic acid (Fer)-Sop-5 Gluc > peonidin (Pn)-3-caffeic acid (Caf)-Sop-5 Gluc). The increased stability of hydroxycinnamic acids, present in abundance in PSP was investigated. Their findings showed mono-acylated anthocyanins substituted with *p*-coumaric acid were able to better withstand the neutral pH conditions in digestion, as compared with anthocyanins substituted with ferulic or sinapic acids (McDougall, Fyffe, Dobson, & Stewart, 2007). Another difference in the PSP anthocyanins is the presence of di-glucose sophoroside, which seems to play an important role in the stability of these anthocyanins (possibly through intra-copigmentation). Although poly-acylated anthocyanins are known for conferring stability to the overall molecule, the results presented here support a more intensified effect with acylations and sugar molecules or even the interaction between them.

In the hydrolyzed fractions (WH and AH), removal of acylation led to significant reduction in the remaining compounds. WH presented recoveries ranging from 43-72% in comparison to AH with ranging from 38-45%. These results indicate that the presence of acylation leads to more protective effects than inter-copigmentation with NAF in the WH. There was also a significant decrease in the NAF alone, with recoveries ranging from 15-37% (Table 6). Previous studies suggest that this could be due to esterase

activity of pancreatin used during digestion, with consequent loss of chlorogenic acid (M. J. Bermúdez-Soto, F. A. Tomás-Barberán, & M. T. García-Conesa, 2007).

Our results suggest PSP anthocyanins and phenolic acids are highly stable compounds which can undergo drastic pH shifts in the body. As a result, they are likely to be absorbed by the intestines or reach the colon intact, thus permitting them to exert health benefits. Understanding the polyphenol breakdown compound pharmacokinetics and physiological stability would be the next step into further elucidating the fate of these compounds in the body as well as their potential health properties.

Transepithelial Transport Study

Caco-2 cell monolayer models have been used to assess intestinal transport of several sources of anthocyanins (Pacheco-Palencia, Mertens-Talcott, & Talcott, 2010) and were therefore used in this study to evaluate the intestinal absorption of different fractions of PSP anthocyanins. There are few conclusive studies with highly acylated anthocyanins in similar absorption models to Caco-2. In addition, most previous studies that involve animal or human bioavailability are unable to accurately model the complex mechanisms involved once polyphenolics reach the digestive system, making the comparison difficult. Using Caco-2 cells will allow us to better model and understand these mechanisms.

In this study, all individual anthocyanins from PSP fractions were transported from the apical side to the basolateral side of the Caco-2 monolayer on the basis of retention time and spectrum characteristics (Fig 11C and D). It has been suggested that anthocyanin absorption may be mediated by sodium-dependent glucose transporter

(SGLT1) (Wolffram, Blöck, & Ader, 2002) or hexose pathway receptors (Steinert, Ditscheid, Netzel, & Jahreis, 2008). Although more research is needed in order to elucidate the actual mechanisms of anthocyanin absorption, the efficiency in anthocyanin transport is likely related to their structure and conjugated forms, which affect metabolism and stability at time of exposure.

Individual anthocyanin transport efficiency across Caco-2 monolayers are summarized in Table 7. Transport efficiency ranged from 0.72% to 10.44% on all analyzed fractions, and the highest transport percentages were in the WC and AC, with 10.44% and 8.33% for the mono-acylated anthocyanin, Pn-3-(Caf)-Sop-5 Gluc, respectively. Within each fraction, lowest transport percentages were associated with di-acylated anthocyanins, especially for those with higher molecular weight (Cy3-(Caf)(Fer)-Sop-5 Gluc and Pn-3-(Caf)(phB)-Sop-5 Gluc), ranging from 0.91% - 1.50%. Results from this study suggest that the presence of NAF may aid in absorption. In the presence of NAF in the WC, the transport efficiencies of non-acylated anthocyanins (Cy-3-Sop-5Gluc and Pn-3-Sop-5 Gluc) were increased by 15% and 224% compared to those in the AC. The transport efficiency of the Pn-3-Sop-5 Gluc in the WH was increased by 75% compared to that in the AH in the hydrolyzed fraction.

Table 7: Transport efficiency (%) and transport rates (µg/h/cm^2) of PSP anthocyanins from apical to basolateral side of Caco-2 monolayers after 2h of incubation.

Ind. Compounds	% Polyphenolic transport				Ind. Compounds	% Polyphenolic transport
	Whole Control (WC)	Whole Hydrolyzed (AC)	Anthocyanin Control (AC)	Anthocyanin Hydrolyzed (AH)		Non-Anthocyanin Fraction (NAF)
Cy-3-Sop-5Gluc	2.25 ± 0.08^{c}	1.37 ± 0.25^{b}	1.95 ± 0.10^{c}	2.54 ± 0.77^{b}	ChA	0.47 ± 0.01^{c}
Pn-3-Sop-5Gluc	7.59 ± 0.14^{b}	6.83 ± 0.22^{a}	2.34 ± 0.33^{bc}	3.89 ± 0.27^{a}	FQA	5.27 ± 0.58^{a}
Cy-3-*p*hB-Sop-5 Gluc	2.06 ± 0.32^{d}	0.72 ± 0.09^{c}	3.35 ± 0.14^{b}	1.67 ± 0.15^{c}	4,5-CQA	5.25 ± 0.26^{a}
Cy-3-Fer-Sop-5 Gluc	2.25 ± 0.01^{c}		2.39 ± 0.11^{bc}		3,5-CQA	1.38 ± 0.05^{b}
Cy3-(Caf)(*p*hB)-Sop-5 Gluc	2.95 ± 0.22^{c}		2.45 ± 0.34^{bc}		3,4-CQA	0.47 ± 0.01^{c}
Pn-3-Caf-Sop-5-Gluc	10.44 ± 0.37^{a}		8.33 ± 0.68^{a}			
Cy-3-(Caf)(Fer)-Sop-5 Gluc	1.07 ± 0.03^{d}		0.91 ± 0.10^{d}			
Pn-3-(Caf)(*p*hB)-Sop-5 Gluc	1.46 ± 0.03^{d}		1.50 ± 0.11^{c}			
Ind. Compounds	Polyphenolic transport rate (µg/h/cm^2)				Ind. Compounds	Polyphenolic transport rate (µg/h/cm^2)
	Whole Control (WC)	Whole Hydrolyzed (AC)	Anthocyanin Control (AC)	Anthocyanin Hydrolyzed (AH)		Non-Anthocyanin Fraction (NAF)
Cy-3-Sop-5Gluc	15.11 ± 0.08^{c}	20.32 ± 0.25^{b}	8.50 ± 0.10^{d}	46.68 ± 0.77^{a}	ChA	0.72 ± 0.03^{c}
Pn-3-Sop-5Gluc	25.43 ± 0.14^{b}	11.17 ± 0.22^{c}	27.33 ± 0.33^{b}	35.95 ± 0.27^{b}	FQA	0.89 ± 0.09^{c}
Cy-3-*p*hB-Sop-5 Gluc	7.87 ± 0.32^{d}	33.16 ± 0.09^{a}	11.03 ± 0.14^{c}	23.89 ± 0.15^{c}	4,5-CQA	3.94 ± 0.26^{a}
Cy-3-Fer-Sop-5 Gluc	6.10 ± 0.01^{d}		9.06 ± 0.11^{cd}		3,5-CQA	1.03 ± 0.03^{bc}
Cy3-(Caf)(*p*hB)-Sop-5 Gluc	37.74 ± 0.22^{b}		29.70 ± 0.34^{b}		3,4-CQA	1.71 ± 0.05^{b}
Pn-3-Caf-Sop-5-Gluc	63.19 ± 0.37^{a}		56.48 ± 0.68^{a}			
Cy-3-(Caf)(Fer)-Sop-5 Gluc	8.67 ± 0.03^{d}		8.74 ± 0.10^{d}			
Pn-3-(Caf)(*p*hB)-Sop-5 Gluc	15.62 ± 0.03^{c}		10.53 ± 0.11^{c}			

Values are average of three triplicates ± standard error.Different letters in the same column indicate significant differences ($p < 0.05$).Anthocyanins were analysed at 520nm while NAF at 280nm.Cy= Cyanidin, Pn= Peonidin, Sop=Sophoroside,Gluc=Glucose, *p*hB=*p*-hydroxy Benzoic acid, Fer=Ferulic acid, Caf=Caffeic acid, ChA=Chlorogenic acid, FQA= Ferullyquinic acid, CQA= Caffeoylquinic acid.

The chemical structure of aglycones, acyl substitutions, and sugar substituents are known for affecting absorption parameters. Methylated forms of aglycones, such as peonidin-based anthocyanins, appear to have increased absorption, due to lower hydrophobicity and facilitated portioning across the membrane (Yi, Akoh, Fischer, & Krewer, 2006). Results from this study suggest peonidin-based anthocyanins present improved absorption on all analyzed fractions, especially non- and mono-acylated fractions (2.34% - 10.44%). Di-acylated anthocyanins appear to have lower absorption rates than non- and mono-acylated anthocyanins. This result is similar to previous studies involving red cabbage, which possesses highly acylated anthocyanins (Charron, Clevidence, Britz, & Novotny, 2007). One such group of anthocyanins, those with a cyanidin-3-diglucoside-5-glucoside skeleton, have a similar structure to anthocyanins in PSP. Complex sugar substituents, such as rutinosides, attached to anthocyanin molecules were found to be more stable when compared to simple sugars, such as glucose, therefore presenting better absorption properties (X. Wu, Pittman, & Prior, 2006). In this study, sugar substituents of PSP anthocyanins (mainly 3-sophoroside-5-glucoside, which has been reported to possess high absorption efficiencies) helped increase absorption efficiencies. Overall, PSP anthocyanins showed higher absorption efficiencies compared to anthocyanins from other sources (e.g. acai, black currant, blueberry) studied using Caco-2 monolayer cell models (Pacheco-Palencia, Mertens-Talcott, & Talcott, 2010; Steinert, Ditscheid, Netzel, & Jahreis, 2008; Yi, Akoh, Fischer, & Krewer, 2006).

Average transport rates (μg/h/cm^2) of individual anthocyanins were calculated after incubation of chemically modified PSP anthocyanin extracts (50 μg/mL) for 2 hours. Transport rates ranged from 6.10 to 63.19 μg/h/cm^2 (Table 7). Pn-3-Caf-Sop-5 Gluc presented the highest transport rates in WC and AC fractions (63.19 and 56.48 μg/h/cm^2, respectively), followed by Cy-3-(Caf)(*p*hB)-Sop-5 Gluc (37.74 and 29.70 μg/h/cm^2, WC and AC respectively). Non-acylated Cy-3-Sop-5 Gluc, which could be a degradation product of more complex anthocyanins, presented higher transport rates in the hydrolyzed fractions of the extracts, WH (20.32 μg/h/cm^2) and AH (46.68 μg/h/cm^2) compared to WC (15.11 μg/h/cm^2) and AC (8.50 μg/h/cm^2).

A high correlation (r = 0.84) was found between the transport efficiencies (%) and transport rates (μg/h/cm^2) in WC and AC fractions, while WH and AH poorly correlated (r = -0.41), indicating that hydrolyzed anthocyanins may have different factors affecting transport rates than their non-hydrolyzed counterparts. One possible mechanism affecting transport rates for hydrolyzed anthocyanins is the concentration load. Although previous studies have shown increased concentration loads do not influence transport efficiency (Pacheco-Palencia, Mertens-Talcott, & Talcott, 2010), transport rate is driven by concentration. When hydrolyzed anthocyanins are exposed to physiological pH conditions, complex anthocyanins may degrade into simpler anthocyanins, increasing the concentration load for the simpler polyphenols over time, thereby decreasing their transport rates.

Because the health benefits of polyphenols are partially related to their bioavailability, the absorption efficiencies of the NAF in PSP were also investigated in

this study. Although extensive research has been done to show complex metabolic pathways (Van Duynhoven, et al., 2011), understanding their transport across epithelial cells (Caco-2) is the first limiting step in the cycle. Although the precise mechanism for absorption of phenolic acid is still unclear, paracellular diffusion, solute carriers (SGLT-1), and monocarboxylic acid transporters (MCT) are the most commonly accepted theories (Konishi & Kobayashi, 2004; Williamson, Meskin, Bidlack, Davies, Lewis, & Randolph, 2004).

Representative chromatograms of NAF in PSP before and after 2 hr incubation with 50 μg/mL GAE of the extracts are shown in Figs. 2A and B. The major polyphenolics detected in PSP were identified as chlorogenic, ferulic, and isomers of caffeic acid. Transport efficiencies and rates of the NAF in the PSP are shown in Table 7. 4,5 – Caffeic and ferulic acid showed the highest efficiencies (5.27 and 5.25%, respectively), while chlorogenic and 3,4- caffeic acid showed the lowest (0.47% each) Individual polyphenolic transport rates were significantly higher for 4,5 – caffeic acid (3.94 μg/h/cm^2). Cholorogenic acid presented the lowest transport rate (0.72 μg/h/cm^2), agreeing with previous studies (Dupas, Marsset Baglieri, Ordonaud, Tomé, & Maillard, 2006a; Konishi & Kobayashi, 2004). Previous study also showed that absorption efficiency of caffeic acid was greater than that of chlorogenic acid (Konishi & Kobayashi, 2004). The low absorption of chlorogenic acid could also be explained due to intestinal mucosal esterases (A-M Aura, et al., 2005) or deglycosylation by β-glucosidases (Van Duynhoven, et al., 2011; Walle, Walle, & Halushka, 2001), especially the cytosolic ones (Williamson, Meskin, Bidlack, Davies, Lewis, & Randolph, 2004)

cleaving the molecule into caffeic and free quinic acids, which can further be better absorbed.

Conclusions

In conclusion, we have demonstrated that polyphenolics in Purple Sweet Potato, especially acylated copigmented compounds, have the capability to withstand *in vitro* physiological conditions of the body and remain bioavailable after digestion, a prerequisite for providing protective health benefits upon consumption. Currently, studies target the cometabolome interactions of colonic microbiota and the human superorganism in order to better understand the complex metabolic fate of polyphenols (Van Duynhoven, et al., 2011). Further studies of polyphenols with microbiota incubation are warranted to apply our observations and theories to more complex and realistic biological systems in order to fully understand what the breakdown products of metabolism are. Other human and animal studies should be carried out to understand the metabolic pathways involved in the digestion, absorption, and excretion of polyphenols. In addition, the study of protective properties of PSP polyphenols in chronic disease will bring trust to consumers, who rely on scientific research to make their buying choices.

CHAPTER V

ANTIPROLIFERATIVE AND ANTI-INFLAMMATORY EFFECTS OF PURPLE SWEET POTATO POLYPHENOL EXTRACTS IN TNF-α INDUCED NON-MALIGNANT INTESTINAL MYOFIBROBLAST CCD-18 COLON AND COLON CANCER CELL LINES HT-29

Overview

Acylated anthocyanins from purple sweet potatoes are used as natural food colors. Digestion impacts their stability and composition due to enzymatic hydrolysis and thus requires additional study for biological activity. However, the signaling pathways in which these compounds can exert health benefits such as anti-inflammatory and anti-cancer is not fully understood. This study evaluated the anti-inflammtory properties of different fractions of PSP polyphenolics in inhibiting TNF-α induced CCD-18Co fibroblast and HT-29 colon cancer cells via NF-κB or its downstream genes. Anthocyanins were alkali hydrolyzed and fractionated to create distinctive polyphenolic isolates to simulate digestive processes and evaluate anti-inflammatory effects in CCD-18Co colon cells and selective cell proliferation in HT-29 cancer cells. A 40% cell inhibition was observed for HT-29 cells for all anthocyanin-containing fractions, while no toxicity was observed for non-cancer cells (CCD-18Co cells). Anthocyanins with and without hydrolysis suppressed TNF-α induced ROS at 25 CAE mg/L, while phenolic acids alone promoted it on HT-29 cells. While fractions containing phenolic acids (WC, WH and NAF) served to down-regulate mRNA, protein expressions of inflammatory

markers (NF-κB, TNF-α, IL-1β and IL-6) on non-cancer cells, thus showing cancer prevention properties, non-hydrolyzed fractions (WC and AC) better modulated these biomarkers on cancer cells, thus acting as cancer inhibitors. Results indicated the importance of polyphenolic composition and hydrolysis effects on physiological relevant anthocyanin moietes, and serves to provide valuable knowledge to the natural color industry for healthier alternatives for food colors.

Introduction

Over a million cases of colorectal cancer are diagnosed per year, making it the 3rd most common cause of cancer mortality around the globe (Terzić, Grivennikov, Karin, & Karin, 2010). More recently, studies have revealed a connection between inflammation and carcinogenesis, through pathways that involve primary inflammatory cytokines (IL-1, TNF-α), IL-6, and the nuclear factor (NF-κB) family members (Greten, Eckmann, Greten, Park, Li, Egan, et al., 2004; Karin, 2006; Soly Wang, Liu, Wang, & Zhang, 2009) and generation of ROS (Waris & Ahsan, 2006; Winrow, Winyard, Morris, & Blake, 1993). ROS can activate the nuclear factor (NF-κB), a master switch that triggers inflammatory responses. Moreover, under increased oxidative stress conditions, produced ROS may lead to structurally cell damages, induced DNA mutations and cellular transformation that link to genetic instability and cancer cell proliferation (Reuter, Gupta, Chaturvedi, & Aggarwal, 2010; Visconti & Grieco, 2009). The involvement of ROS, chronic inflammation and cancer has thus been extensively studied

(Reuter, Gupta, Chaturvedi, & Aggarwal, 2010; Winrow, Winyard, Morris, & Blake, 1993).

Fruits and vegetables containing phytochemicals such as phenolic acids and flavonoids such as anthocyanins, have been widely researched for providing strong antioxidant capacity *in vitro* (Moyer, Hummer, Finn, Frei, & Wrolstad, 2002; H. Wang, Cao, & Prior, 1996). Therefore, great interest exists to link these compounds to presenting strong chemoprotective properties as being inhibitors of cancer cell proliferation, preventers of cell activity transformation and anti-inflammatory agents (Boivin, Blanchette, Barrette, Moghrabi, & Beliveau, 2007; Terzić, Grivennikov, Karin, & Karin, 2010; Visconti & Grieco, 2009).

Anthocyanins are flavonoids responsible for the intense red, purple and blue color in fruits and vegetables, and its consumption has been highly associated to cancer prevention due to their anti-inflammatory properties (Boivin, Blanchette, Barrette, Moghrabi, & Beliveau, 2007; J. He & Giusti, 2010; Lee, Kim, Yang, Pham, Park, Manatou, et al., 2014; L.-S. Wang & G. D. Stoner, 2008; Zhu, Ling, Guo, Song, Ye, Zou, et al., 2013). Anthocyanin rich extracts from black raspberry inhibited tumor development in JB-6 Cl 41 Mouse epidermal cells by regulation of benzoapyrene diol-epoxide (BaPDE)-induced expression of NF-κB (C. Huang, et al., 2002). On another study, blackberries, Korean raspberries and black raspberries anthocyanins showed anti-inflammatory activity on LPS-stimulated colon cells (Jung, Lee, Cho, & Hwang, 2015), significantly decreasing the expression of iNOS (inducible nitric oxide synthase) and COX-2 (Cyclooxygenase-2), both distinguished biomarkers of inflammation and

colorectal tumorigenesis (Terzić, Grivennikov, Karin, & Karin, 2010), suggesting these compounds play an important role in the regulation of NF-κB activation and subsequent cell events, essential in inflammatory body processes.

Recently, new sources of highly acylated and stable anthocyanins have received attention in health care. That is the case of Purple Sweet Potatoes, a nutritionally valuable crop originated from Asia and South America which contains high levels of complex anthocyanins and non-anthocyanin phenolic acids (de Aguiar Cipriano, Ekici, Barnes, Gomes, & Talcott, 2015; Islam, Yoshimoto, Yahara, Okuno, Ishiguro, & Yamakawa, 2002; V. D. Truong, McFeeters, Thompson, Dean, & Shofran, 2007) responsible for increased stability under non-optimal conditions. Such advantage favors not only the food industry, which can now use these pigments to easily overcome the usual limitations of natural colors, such as low stability in neutral pH and processing conditions, but also the pharmaceutical community, which can now focus on extracting and purifying compounds that are likely to survive gastric and intestinal conditions *in vitro* and exert the proposed health benefits. Reports have shown anthocyanins to possess numerous biological functions including ROS-scavenging, antimutagenic, anti-carcinogenic, and anti-hypertensive effects (Ahmed, Akter, & Eun, 2010; Hwang, Choi, Yun, Han, Kim, Kim, et al., 2011)

Considering the lack of studies utilizing purple sweet potato anthocyanins on inflammation and carcinogenesis and the importance of identifying new suitable natural food colors with enhanced stability and health benefits, this study aimed to evaluate PSP polyphenols-rich extracts on cell viability, ROS generation, and pro-inflammatory

biomarkers (NF-κB, TNF-α, IL-6 and IL-1β) in inflamed CCD-18Co non-malignant and HT-29 colon cancer cells.

Material and Methods

Chemicals, Antibodies and Primers

Standards of cyanidin-3-Glucoside, 2'7'-dichlorofluorescein diacetate (DCFH-DA), dimethyl sulfoxide (DMSO), Tumor Necrosis Factor alpha (TNF-α) and solutions for chromatographic analysis were purchased from Sigma-Aldrich (St Louis, MI). Methanol, formic acid, ethyl acetate citric acid and Hank's balanced salt solution (HBSS) were purchased from Fisher Scientific (Pittsburgh, PA). 2-(N- morpholino) ethanesulfonic acid solution (MES) and N-[2-hydroxyethyl] piperazine-N0-[2-ethanesulfonic acid] (HEPES) were obtained from Gibco BRL Life Technology (Grand Island, NY). The Folin-Ciocalteu reagent was purchased from MP biochemical, LLC (Solon, Ohio). Bradford reagent was obtained from BioRad (Hercules, CA). Antibodies against NF-kB p65 and phospho-NF-kB p65 were obtained from Cell Signaling Technology (Beverly, MA). All primers were purchased from Integrated DNA Technologies (San Diego, CA). MirVana TM extraction kit, reverse transcription (RT) and real-time PCR amplification kit were purchased from Applied Biosciences (Foster City, CA).

Plant Materials

Purple sweet potatoes were obtained as previously described in Material and Methods section of Chapter III.

Cell Lines

HT-29 human colon adenocarcinoma cells and non-malignant CCD-18Co colon fibroblast cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). HT-29 human colon adenocarcinoma cells were cultured in 1 McCoy's-5a modified medium, supplemented with a 1% penicillin solution and a 10% solution of fetal bovine serum (SBF, Invitrogen, Carlsbad, CA). CCD-18Co cells and Caco-2 colon carcinoma cells were cultured with high glucose Dulbecco's Modified Eagle Medium (DMEM), supplemented with solutions of 1% penicillin, 1 % non-essential amino acids (10 mM), 1% sodium pyruvate (100 mM), 1% Glutamine (100 mM) and 20 % SBF. Cells were maintained at 37 °C with a humidified 5 % CO_2 atmosphere.

Extraction of PSP Anthocyanins

Purple sweet potatoes were extracted according to the methodology described in Material and Methods of Chapter III.

Alkali Hydrolysis of Anthocyanins

De-acylation of the anthocyanins (3 mL) was obtained by alkaline hydrolysis, according to a previous method (Z. Shi, I. A. Bassa, S. Gabriel, & F. J. Francis, 1992). Anthocyanin-rich extracts were placed in screw-caped test tubes with 12 mL of 1, 2, 3 or 4N aqueous NaOH for 0, 5, 10 and 15 min at 37°C, and infused with nitrogen for prevention of oxidation. At each collecting time, the solution was acidified with 5% (v/v) 99% formic acid, cooled in an ice bath for 5 min and purified prior to injection into the HPLC system.

Isolation of Phenolics and Phytochemical Analysis

Isolation of phenolics, total anthocyanin, total soluble phenolics and identification of individual anthocyanins followed the same methodology as previously described, in the Material and Methods of Chapter III.

Cell Proliferation

CCD18 and HT29 cells were seeded at a density of 3000 and 5000 cells per well, respectively, in a 96-well plate and incubated for 24 h to allow cell attachment prior to PSP extract treatment. Different PSP fractions (WC, WH, AC, AH and NAF) dissolved in DMSO at different concentrations (0 – 100 mg GAE/L) replaced medium, whereas the negative control group received medium containing DMSO. Cell proliferation was determined after 48 h incubation with extracts using a cell counter (Z2 Series Beckman Coulter, Fullerton, CA), and the results were expressed as a percentage of the control cells, as previously described (Noratto, Bertoldi, Krenek, Talcott, Stringheta, & Mertens-Talcott, 2010).

Generation of Reactive Oxygen Species

The 2', 7'-dichlorofluorescein (DCFH-DA) assay was used to determine the intracellular generation of ROS (H. Wang & Joseph, 1999). HT-29 human colon adenocarcinoma and CCD-18Co non cancer colon fibroblast cells (3.0×10^3) were seeded in a black bottom 96 well plate 24 hours prior to incubation with PSP extracts in 2 concentrations: 25 and 50 μg/mL. The generation of ROS was accomplished by exposure of cells to TNF-α (20 ng/mL) for 3h at 37 °C. HT-29 cells were not stimulated

with TNF-α because they normally present higher oxidative stress than normal cells (Pathi, Jutooru, Chadalapaka, Sreevalsan, Anand, Thatcher, et al., 2011). Later, cells were washed with colorless DMEM and replaced with DCFH-DA (10 μM) for 30 min at 37 °C. After incubation, cells were washed twice with DMEM and the fluorescence intensity was measured using a fluorescent microplate reader (BMG Labtech Inc., Durham, NC) at 485 nm excitation and 520 nm emission.

Real Time PCR Analysis of mRNA

CCD-18 cells ($3x10^5$ cells/well) were seeded into 6-well plates and incubated for 24 h to allow cell attachment before treatment with PSP anthocyanin extracts (50 μg GAE/mL) for 24 h. mRNA and miRNA were isolated using the mirVanaTMmiRNA Isolation Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's recommended protocol. The quality and quantity of RNA samples were assessed using the NanoDrop ® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) at 260 and 280 nm. The isolated RNA was used to synthetize cDNA using a Reverse Transcription Kit (Invitrogen Corp., Grand Island, NY) following the manufacturer's recommended protocol. Each primer tested was designed using Primer Express software (Applied Biosystems, Foster City, CA); homology searched by an NCBI BLAST and the specificity was examined by a dissociation curve analysis. Real time PCR reactions were conducted using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on a 7900HT Fast Real Time PCR System (Applied Biosystems, Foster City, CA). Primers were purchased from Integrated DNA

Technologies, Inc. (San Diego, CA) and the sequences of the primers used were as follows:

TBP F: 5'- TGCACAGGAGCCAAGAGTGAA-3'

TBP R: 5'- CACATCACAGCTCCCCACCA-3'

NFKB F: 5'- TGGGAATGGTGAGGTCACTCT-3'

NFKB R: 5'- TCCTGAACTCCAGCACTCTCTTC-3'

IL-1B F: 5'- CTTCAGGCAGGCCGCGTCAG-3'

IL-1B R: 5'- TGCTGTGAGTCCCGGAGCGT-3'

IL-6 F: 5' - AGGGCTCTTCGGCAAATGTA-3'

IL-6 R: 5'-GAAGGAATGCCCATTAACAACAA-3'

TNF-α F: 5'-TGTGTGGCTGCAGGAAGAAC-3'

TNF-α R: 5'-GCAATTGAAGCACTGGAAAAGG-3'

Quantification and analysis of miRNA-126 and RNU6B (endogenous control) were assessed using the Taqman® MicroRNA reverse transcription kit (Applied Biosystems, Foster City, CA) as previously described (Del Follo, Noratto, & Mertens-Talcott, 2009). Briefly, the reverse transcription samples were diluted in a 1:15 ratio and amplified with Taqman ® Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, Foster City, CA).

Protein Expression by Luminex®

Protein expression by Luminex was assessed using an 11-plex AKT/mTOR phosphoprotein magnetic bead kit (Millipore, Billerica, MA) following the manufacturer's protocol. Data was analyzed using Luminex xPonent 3.0 software.

Statistical Analyses

The experiment was conducted with 3 triplicates in a randomized complete design. Data were analyzed by SAS version 6.11 (SAS Institute, Inc., Cary, NC). Analysis of variance was performed, and significance of differences among least square means was determined by Tukey's test ($P < 0.05$).

Results and Discussion

Polyphenols in Purple Sweet Potatoes

Purple sweet potatoes (PSP) are of great interest to the natural color industry as this vegetable contains high concentration of anthocyanins, a natural colorant with capabilities to substituting synthetic FD&C red#40 in several food applications (Suda, Oki, Masuda, Kobayashi, Nishiba, & Furuta, 2003). Moreover, non-anthocyanin phenolics have been reported to be present in PSP as well (V. D. Truong, McFeeters, Thompson, Dean, & Shofran, 2007). Acylated anthocyanins, as the majority of PSP anthocyanins, are reported to present great stability under non-optimal conditions due to copigmentation associations. They would be likely to survive physiological conditions of the digestions system, thus reaching endemic circulation or tissues where they may exert health benefits. However, upon digestion, these compounds may go through hydrolysis and it is questioned whether parental compounds or product of hydrolysis would be responsible for health properties. With that, this research aimed to evaluate different forms of PSP anthocyanins and how effective different fractions can be in mediating inflammatory processes in the body.

The PSP whole fraction control (WC) and anthocyanin control (AC) were obtained as previously described in the material and methods of Chapter III. Both WC and AC were submitted to basic hydrolysis using 3 N NaOH for 5 min, which yielded the highest concentration of the de-acylated fractions (WH– Whole hydrolyzed and AH-Anthocyanin hydrolyzed), which were used for comparison with the non-anthocyanin phenolic fractions (NAF).

The highest concentration of monomeric anthocyanin was observed for the non-hydrolyzed anthocyanin fractions with and without the non-anthocyanin phenolics (WC and AC, respectively), with concentrations of 8437.38 ± 270 and 7855.31 ± 284 mg/kg cianidin-3-glucoside equivalents, as shown on Figure 14. It was reported PSP contain free chlorogenic, ferulic and caffeic acid (Cipriano, Ekici, Barnes, Gomes, & Talcott, 2015) and PSP anthocyanins can be acylated at R1, R2 or R3 position by caffeic, ferulic and/ or *p*-hydroxybenzoic acid (Xu, Su, Lim, Griffin, Carey, Katz, et al., 2015), conferring stability to molecule. While it has been reported most berry anthocyanins will greatly degrade upon heat, as 50% less anthocyanins were recovered in elderberries after 95°C for 3 hours (E Sadilova, Stintzing, & Carle, 2006), or 43% recovered in blueberries after blanching at 95°C for 3 min (Brownmiller, Howard, & Prior, 2008), PSP anthocyanins only degraded 8-16% upon steaming, microwaving or frying (Xu, et al., 2015). These authors attributed such heat resistance to the highly acylated anthocyanins found in this vegetable, especially mono-acylated ones containing *p*-hydroxybenzoic acids.

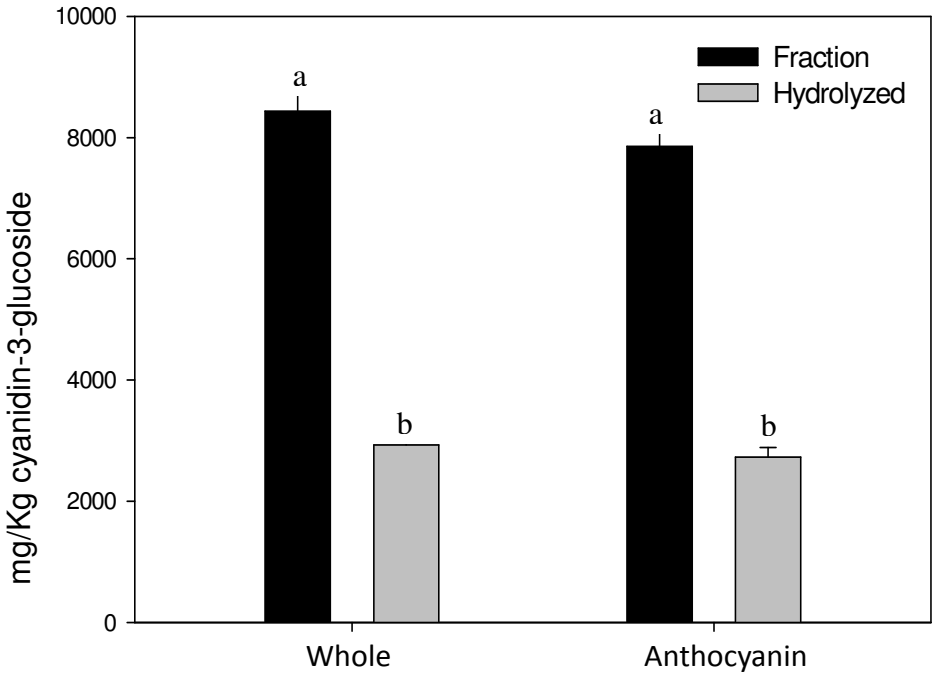


Figure 14: Anthocyanin content in different fractions of PSP after C18 solid phase extraction and basic hydrolysis (3N NaOH for 5min).
Experiments were performed in three independent repetitions and results expressed as mean ± SE (standard error). Common letters are not statistically different (Tukey's HSD test, $p < 0.05$).

Effects of Purple Sweet Potato Polyphenol Extracts on TNF-α Induced Non-Malignant CCD-18 Colon and HT-29 Colon Cancer Cell Growth

Cell proliferation is an important indication of initiation stages of colon cancer development and the usage of nutraceutical extracts, such as anthocyanins, may play an important role in preventing growth and multiplication of damaged cells (Coates, Popa, Gill, McCann, McDougall, Stewart, et al., 2007). Therefore, great interest exists in the

use of polyphenols in disease prevention as they are ubiquitous in nature and their regular consumption is highly recommended as part of a healthy diet. However, in order for polyphenolic extracts to show potential chemo-preventive effects on cancer cells and be indicated for usage in preventive treatment, they must not present any cytotoxicity towards non-cancer cells (Noratto, Kim, Talcott, & Mertens-Talcott, 2011). Therefore, the cell inhibition of PSP anthocyanin and non-anthocyanin extracts (5.0 – 100 mg GAE/L) on CCD-18 non-malignant and HT-29 cancer colon cells after 48 hr treatment was tested.

Results show the net growth of CCD-18Co cells was not significantly inhibited by PSP Polyphenolic extracts in the tested range (5.0-100 mg GAE/L) after 48 hr of incubation (Figure 15A) and therefore, 50 mg GAE/L of the extracts was used in the subsequent experiments (> 85% surviving cells). HT-29Co cancer cells were significantly reduced in a dose dependent manner, reaching less than 60% at higher concentrations (12.5-100 mg GAE/L). The extracts were found to have different efficacies in reducing HT-29Co cancer cells, as can be seen on Figure 15B. PSP anthocyanin fraction showed increased anticancer activity in comparison to non-anthocyanin fraction. At 100 mg GAE/L, all anthocyanin fractions (WC, AC, WH and AH) were able to reduce cancer cells to nearly 60% (in comparison to control), while the non-anthocyanin extract reduced the growth only by 10%. These results are important to confirm the potential of PSP polyphenolic extracts to inhibit cancer initiation or prevent progression without having cytotoxic effects on normal cells (Russo, 2007).

Anthocyanins have been previously reported for possessing anticancer properties with low cytotoxicity on non-malignant cells. Human colon SW480 cells have been treated with extracts from P40 purple sweet potatoes, which contained complex anthocyanins highly acylated with caffeic, ferulic and p-hydroxybenzoic and concentrations of 0-40 μM of Peonidin-3-glucoside decreased malignant cells in a dose-dependent manner (Lim, Xu, Kim, Chen, Su, Standard, et al., 2013). On a similar trend, viability of non-malignant cells did not differ statistically from the control group, thus showing potato anthocyanin extract presented little cytotoxicity amongst normal cells. A dose-dependent effect was observed when anthocyanin extracts from raspberry (CARE - colon-available raspberry extract) was applied to HT29 H_2O_2 challenged cells, with reduction of approximately 50% when the highest concentration was used (Coates, et al., 2007). They concluded this was an indication the anthocyanin extracts provided increased cellular capabilities to protect DNA against damage.

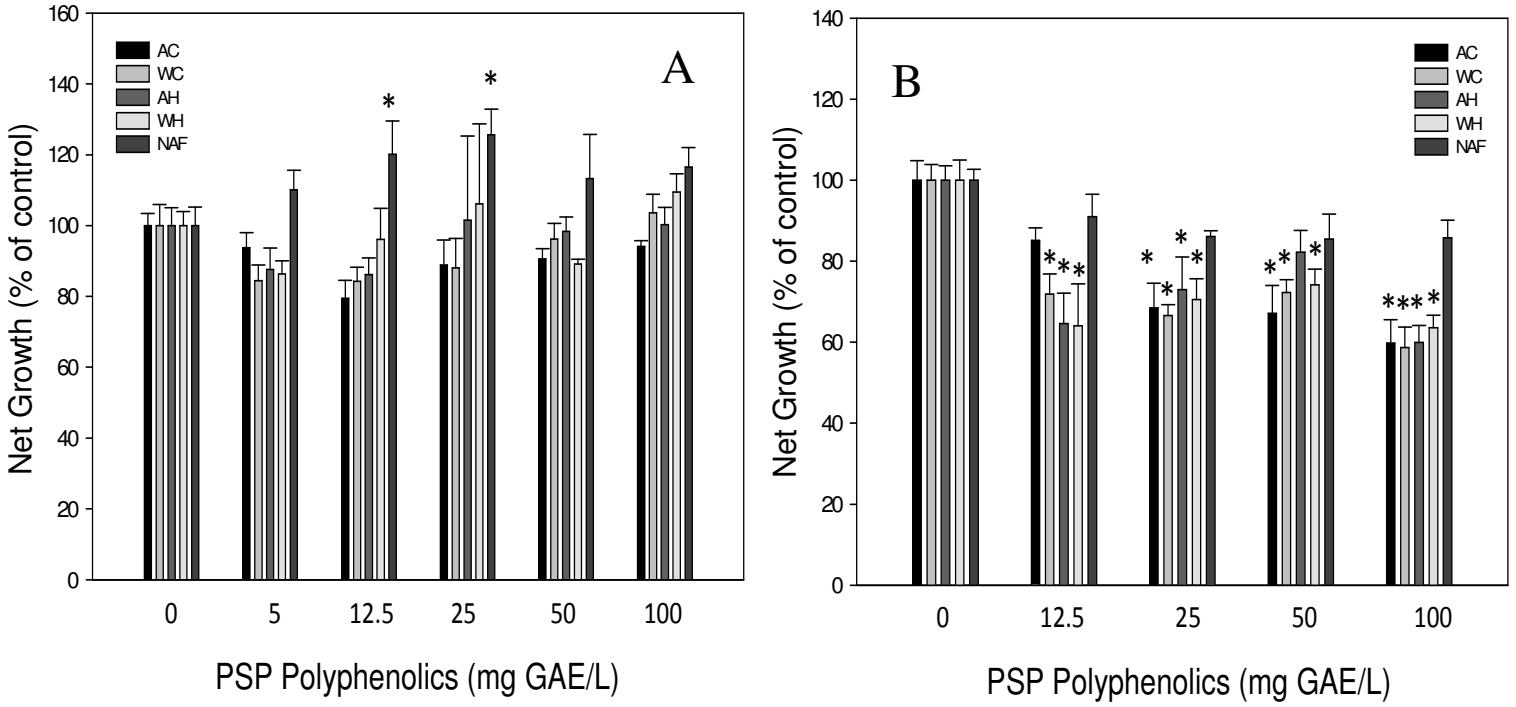


Figure 15: Cell proliferation of CCD-18 non-malignant and HT-29 colon cancer cells treated with chemically modified PSP Polyphenolic extracts.
Cells were treated with different concentrations of PSP extracts (5.0-100 mg/L GAE) and cell growth was assessed after 48 h of incubation. Values are means SE (n=3). *: Statistically different from TNF-α positive control group (ANOVA-Dunnet, $p < 0.05$).

Original extract of chokeberry showed similar growth inhibition capabilities as anthocyanin fraction alone, confirming the anthocyanin fractions was most effective contributor for the chemopreventive properties of this fruit at 25 μg/mL GAE (Jing, Bomser, Schwartz, He, Magnuson, & Giusti, 2008). When separated by solid phase extraction, the phenolic fraction presented lowered cancer cell inhibition, and these authors attribute the strong inhibition of the original extract through a synergistic effect within the fractions. On another study with bilberry, it was discovered the anthocyanin fraction suppressed the growth of HT-60 colon cancer cells to a greater extent than the flavonols present in the extract (myricetin, quercetin, and kaempferol) (Katsube, Iwashita, Tsushida, Yamaki, & Kobori, 2003). In blueberries, the phenolic fraction also showed lowest antiproliferation properties, on both HT-29 and Caco-2 colon cancer cells, compared to their anthocyanin fraction (Yi, Fischer, Krewer, & Akoh, 2005) and they concluded anthocyanins were the major contributors to cancer anti-proliferation and inducers of apoptosis.

Substitutions in the aglycone may also lead to different antiproliferation properties, as previously reported. The role of anthocyanin chemical structure and the ability to inhibit cancer cells was previously researched, noting that Chokeberry, which contained monoglycosylated cyanidin derivatives, inhibited cancer cells at a greater extent than grapes or bilberries, if compared for similar concentrations of monomeric anthocyanins (Zhao, Giusti, Malik, Moyer, & Magnuson, 2004). When growth inhibition was compared based on phenolic acid content (200 μM GAE), grape extracts presented higher growth inhibition after 24 hr, of 12%, compared to 7% for both bilberry and

chockeberry, respectively. These authors attribute the efficacy of the grape extracts to being less polymerized, in comparison to the others. Purple corn, which contains mostly monoglucosylated cyanidin, peonidin and pelargonidin, demonstrated to have the highest inhibitory effect on HT-29Co cancer cells, when compared to other anthocyanin sources containing more complex acylations and sugar substitutions (Jing, Bomser, Schwartz, He, Magnuson, & Giusti, 2008). These authors found that grapes, radish and elderberry possessed the least effect, and attributed them to type of aglycone, type of acylation substitution and glycosylation patterns.

Therefore, it can be concluded the chemistry of anthocyanins is directly linked with the health properties it will promote. The PSP extracts containing phenolic acids (WC and AC) may have presented higher cancer antiproliferative activity due to presence of copigments, the free phenolic acids, which are widely discussed for their anticancer properties (Zhao, Giusti, Malik, Moyer, & Magnuson, 2004) but also due to increased chemoprotection effects owned to anthocyanin interactions (Brownmiller, Howard, & Prior, 2008). Moreover, considering the extracts are exposed to neutral pH media and hydrolysis of the acylations may occur, which in result may release free phenolic acids, it becomes difficult to attribute efficacy to specific compounds at this point and therefore more studies are needed to understand the effects of copigments versus acylation, on the chemopreventive properties of anthocyanins.

Protection Against Production of Reactive Oxygen Species (ROS) on Colon HT-29 Malignant and CCD-18Co Myofribroblast Cells

Oxidative stress in the cell is caused by presence of reactive oxygen species, or ROS, originated during the metabolism of oxygen on all aerobic cells, and are counterbalanced by the action of antioxidant enzymes under homeostatic conditions (Waris & Ahsan, 2006). When excess ROS cannot be overcompensated by the low level of natural antioxidants in the body, oxidative stress occurs with potential damage to DNA, to tissues by disruption of the lipid membrane (Waris & Ahsan, 2006) as well as proteins, by modifying their ability to participate in the nuclear signal transduction pathways and interfering with production of DNA repair enzymes (Wiseman & Halliwell, 1996). Consequently, ROS plays an important role in stimulating several clinical conditions as chronic inflammation and other malignant transformations that could lead to cancer (Waris & Ahsan, 2006; Wiseman & Halliwell, 1996). PSP polyphenolics, composed mainly of mono- and di-acylated anthocyanins as well as free phenolic acids, are highly electron deficient and thus have the ability to react and control ROS production by interrupting radical chain reactions (Shahidi, Janitha, & Wanasundara, 1992). Therefore, intracellular ROS DCF assay was performed in order to evaluate efficacy and chemo-preventive properties of chemically modified PSP polyphenolic extracts in protecting non-cancerous CCD-18Co and cancer HT-29Co cells from generation of ROS.

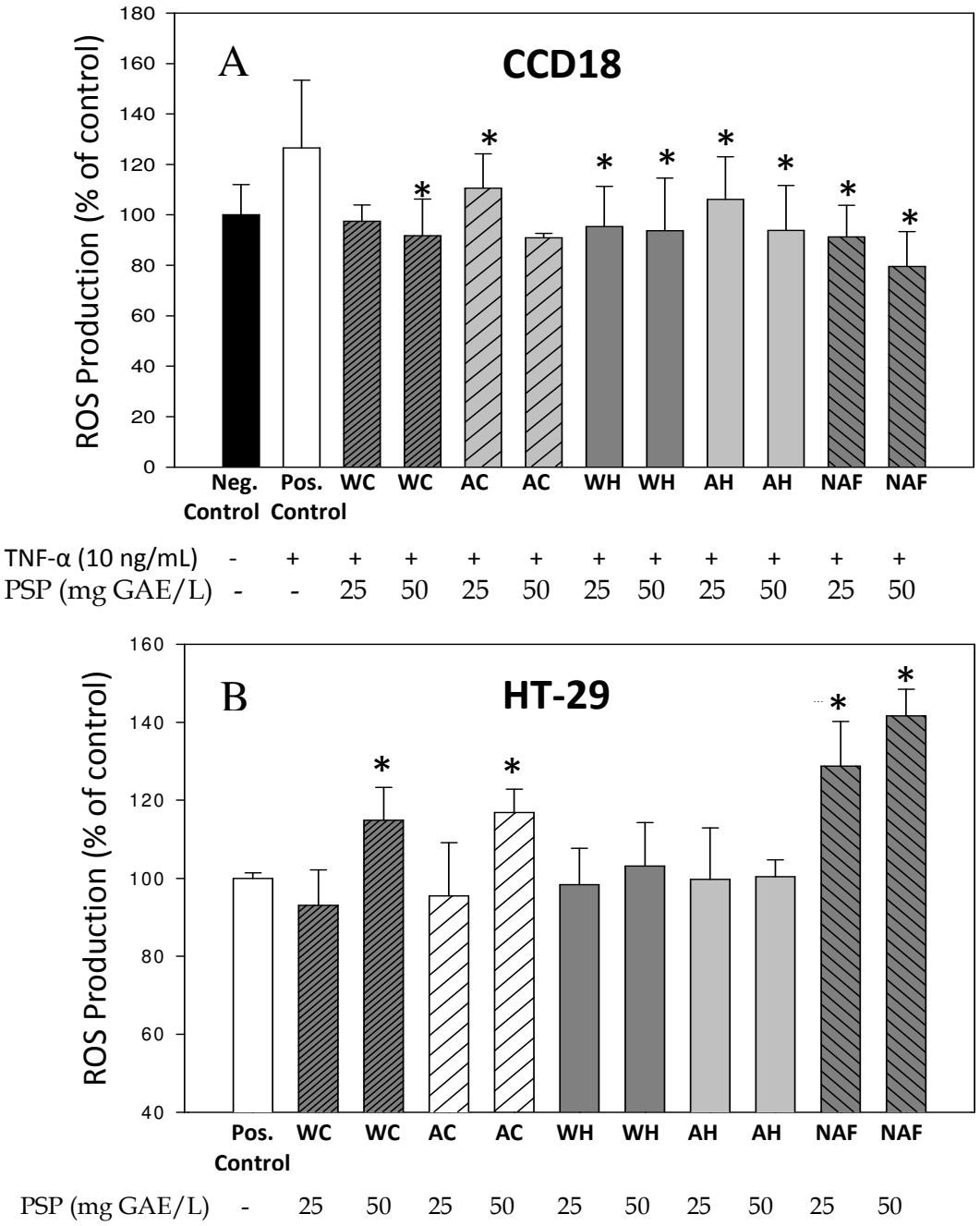


Figure 16:TNF-α induced generation of reactive oxygen species (ROS) in CCD-18 non-malignant colon cells treated with chemically modified PSP extracts (A) and HT-29 non treated cells (B) after incubation with chemically modified PSP polyphenolic extracts for 24 hr.

Each experiment was performed at least three times and results are expressed as means ± SE. *: Statistically different from positive control group (ANOVA-Dunnet, $p < 0.05$).

Results indicate positive control TNF-α challenged cells had their ROS levels increased by 1.3-fold in non-cancerous colon CCD-18 cells in comparison to negative control. The enhancement of ROS generation by TNF- α was inhibited, in a dose-dependent manner, after pre-treatment with PSP anthocyanin and non-anthocyanin extracts (Figure 16A). At the highest concentration (50 mg GAE/L), levels of anthocyanin fractions (WC, AC, WH and AH) reduced production of ROS to 0.69- to 0.76- fold of TNF-α challenged cells, while non-anthocyanin reduced ROS to 0.61-fold of the positive control. These results are important to show the potential chemoprotective effects of PSP polyphenols in CCD-18Co non-cancer cells. The increased intracellular levels of oxidative stress, as measured by the oxidative stress-sensitive dye DCF-DA, was significantly reduced by PSP polyphenolic compounds to levels nearly equal to the negative control (Figure 16A), providing evidence that PSP antioxidant properties may be able to protect TNF-α induced cytotoxic cells (Ye, Meng, Yan, & Wang, 2010). In a study to assess the oxidative state in kidneys of HFD-treated mice, it was also noted oral administration of Purple Sweet Potato Color (PSPC) significantly reduced the levels of ROS to levels compared to control group, indicating promising usage of these extracts against ROS induced inflammatory conditions (Shan, Zheng, Lu, Zhang, Wu, Fan, et al., 2014).

In contrast, higher concentrations (50 mg GAE/L) of PSP polyphenolic compounds induced a significant increase in the generation of ROS in HT-29Co malignant cells (Figure 16B), as up to 1.18-fold for both WC and AC, respectively; and a 1.3- and 1.40-fold increase for NAF, at both concentrations (25 and 50 mg GAE/L)

(Figure 16B). Induced ROS generation in HT-29Co cancer have been previously reported for acai anthocyanins (Pacheco-Palencia, Talcott, Safe, & Mertens-Talcott, 2008) and mango polyphenols using SW-480 breast cancer cells (Noratto, Bertoldi, Krenek, Talcott, Stringheta, & Mertens-Talcott, 2010). Considering ROS is used as signaling to cell proliferation and further events involved in cancer progression, its inhibition is assumed to be in the direction of cancer retardation. However, cancer cells are known for having increased basal oxidative stress, required for the increased rate of growth (Brownmiller, Howard, & Prior, 2008; Schumacker, 2006). Therefore, the induction in ROS in HT-29 cancer cells, as noted with PSP polyphenols, especially the ones containing the free phenolic acids, could have been due to an amplified oxidative stress pushing the cells beyond the threshold for survival, with consequences in lipid and protein oxidation, DNA damage and eventually leading to cell death (Noratto, Bertoldi, Krenek, Talcott, Stringheta, & Mertens-Talcott, 2010; Schumacker, 2006). Moreover, these results are important to show that concentrations that induces ROS in malignant cells (50 mg GAE/L), PSP polyphenolics were still able to inhibit ROS in non-malignant CCD-18Co cells.

Anti-inflammatory Activity of Polyphenolics from Purple Sweet Potato Polyphenolic Extracts in Intestinal Myofibroblasts CCD-18 Cells and HT-29 Malignant Cells

Inflammation is a natural body response to either internal or external stimuli which counteracts with irritation, injury or infection and is identified by pain, redness and swelling (Aggarwal, Shishodia, Sandur, Pandey, & Sethi, 2006). While acute inflammation lasts shorted periods and can be through an activation of the immune system, chronic inflammation persists for longer periods, is triggered by multiple factors including stress, bacterial, viral and parasitic infection and may lead to a wide variety of chronic illnesses such as cancer, obesity, diabetes, cardiovascular and neurological disorders (Aggarwal, Shishodia, Sandur, Pandey, & Sethi, 2006; Aggarwal, Vijayalekshmi, & Sung, 2009).

Pro-inflammatory cytokines, chemokines and inflammatory enzymes are associated with chronic inflammation. These genes play an important role in cellular and biological processes such as apoptosis, proliferation, angiogenesis, invasion and metastasis (Aggarwal, Shishodia, Sandur, Pandey, & Sethi, 2006). There are several genes involved in these processes such as TNF-α (tumor necrosis alpha factor) and members of its family such as interlukines (IL-1β, IL-6, IL-8, IL-1β) and chemokines (MMP-9, VEGF), all regulated by either the transcription factor nuclear factor kappa B (NF-κB) or cyclooxygenase-2 (COX-2) (L.-S. Wang & G. D. Stoner, 2008). Both proteins are commonly associated with abnormal cellular activity and therefore considered master regulators of inflammation in most tumors. Inhibitors of NF-κB or its

downstream genes might exhibit significant chemopreventive or therapeutic properties and therefore this study aimed to evaluate the potential anti-inflammatory properties of PSP polyphenolic extracts on HT-29 cancer and TNF-α challenged CCD-18Co cells.

Pro-inflammatory cytokine tumor necrosis factor alfa (TNF-α) is known to induce inflammation on CCD-18 non-cancerous colon cells as previously tested for significantly up-regulating mRNA and protein expression of cytokines, in comparison to estrogen, glucose and LPS (Arbizu Berrocal, 2013). The mRNA expressions of inflammatory cytokines were significantly up-regulated upon TNF-α stimulation (Figure 17A). Inflammation was not induced on HT-29 cell lines (Figure 17B) as these cells already contain high levels of ROS and are thus found in an inflamed stage. Results shown on Figure 17A indicate that at the mRNA levels on CCD-18co cells, TNF-α induced expression of NF-κB, TNF-α, IL-1β, and IL-6 was significantly down-regulated by PSP polyphenolic fractions at 50 mg/L GAE, to as low as 24.74% on the NAF. Similar results were observed on HT-29 cancer cells at the mRNA levels, where all PSP fractions presented efficacy towards down-regulating TNF-α, IL-1β, and IL-6. It was observed a reduction in the biomarker IL-1β to as low as 31.62, 33.36 and 34.43% for NAF, AH and WC, respectively, on cancer cells.

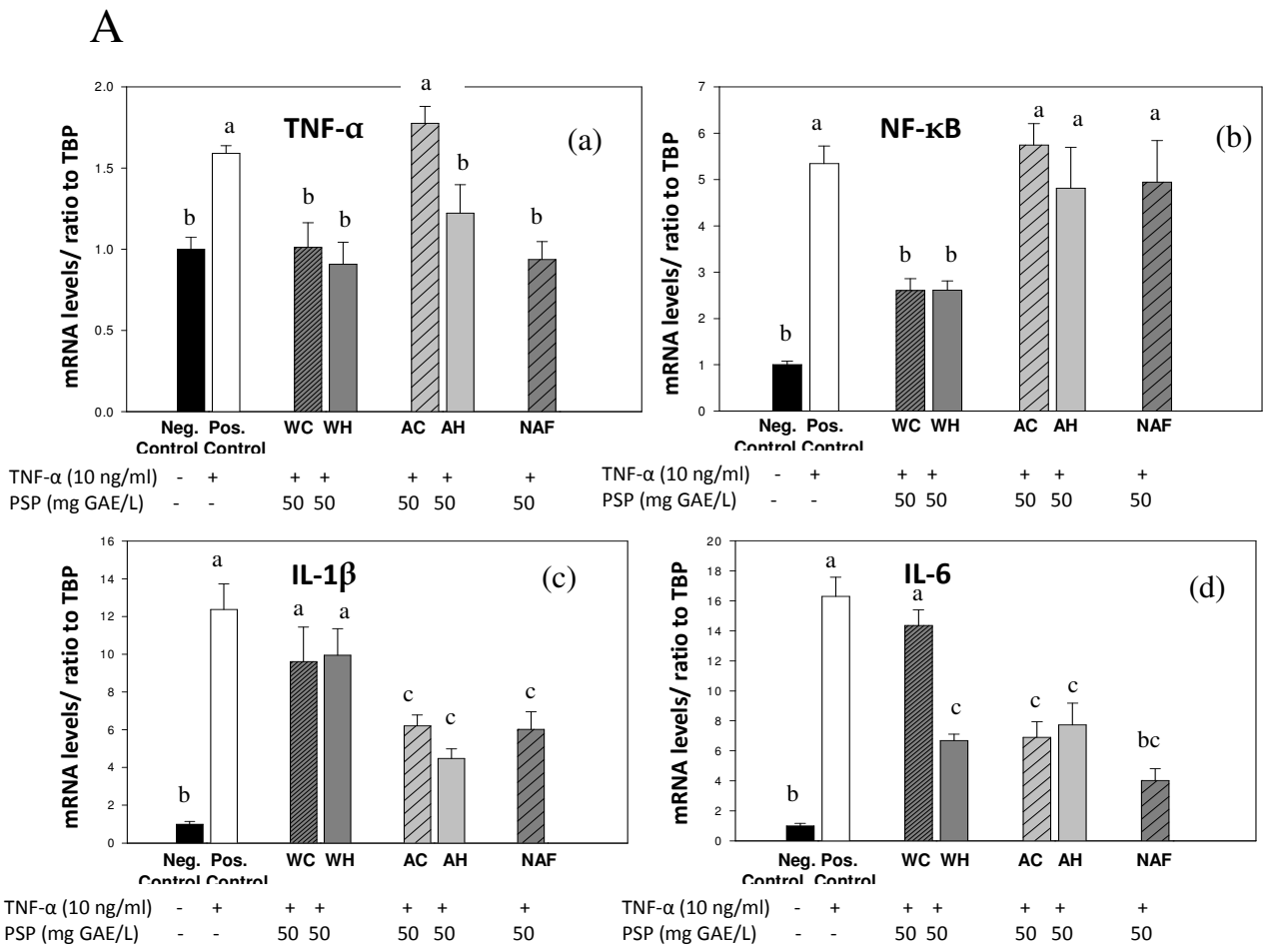


Figure 17: Differential mRNA expression of (a) NF-κB, (b) TNF-α, (c) IL-1β and (d) IL-6 in TNF-α-challenged CCD-18Co (A) and (e) TNF-α, (f) IL-1β and (g) IL-6 in HT-29 cells (B). Gene expression was analyzed by qRT-PCR as a ratio to TATA binding protein (TBP) mRNA. Each experiment was performed at least three times and results are expressed as means ± SE. Different letters indicate significance at $P < 0.05$.

B

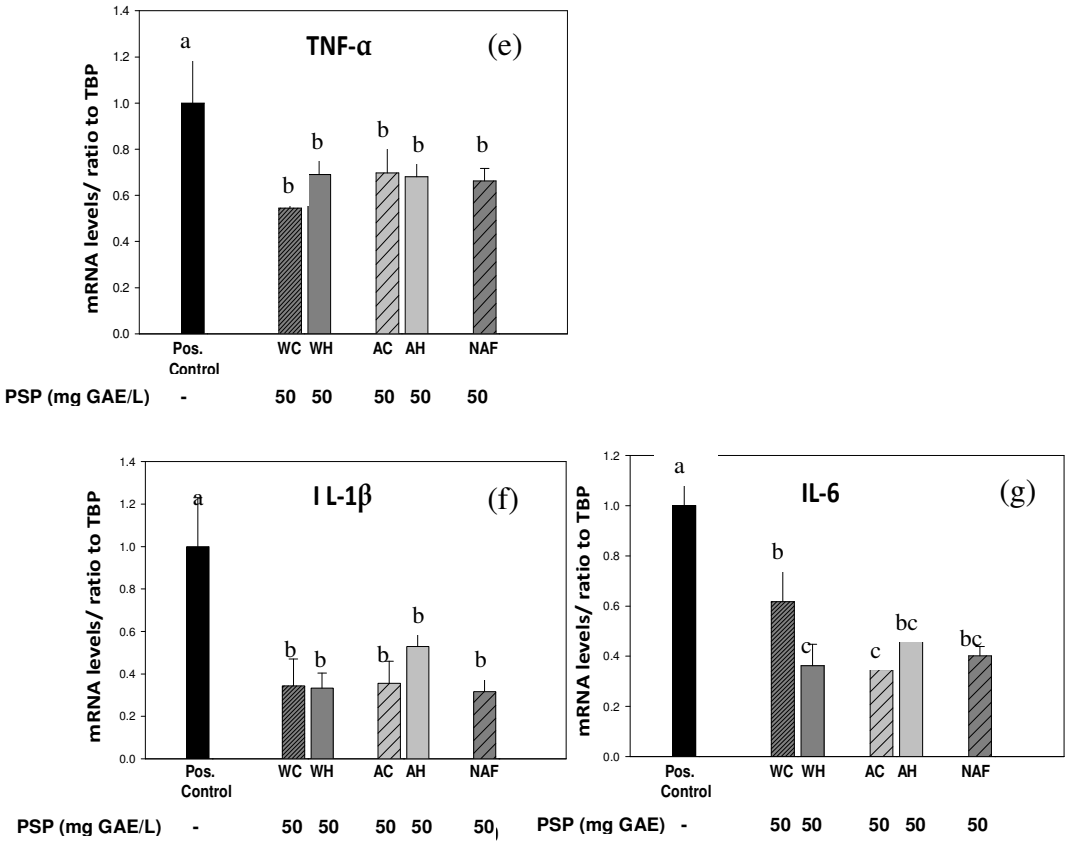


Figure 17: Continued

Protein levels of NF-κB, TNF-α, IL-1β, and IL-6 were also down-regulated by PSP polyphenolic extracts, on both CCD-18Co and HT-29Co cancer cells (Figure 18A and B), but PSP fractions showed different efficacies on the tested cell lines. As a trend, on non-cancer cells (Figure 17A), TNF-α induced expression of NF-κB, TNF-α, IL-1β was significantly down-regulated after treatment with WC, WH and NAF extracts, which indicates presence of NAF in extracts may have a synergistic effect with the anthocyanins in preventing inflammation. In contrast, on cancer cells (Figure 18B), expression of TNF-α, IL-1β and IL-6 were significantly reduced following treatment with WC and AC, indicating presence of acylation on the anthocyanin structure may play a significant role as anti-inflammatory agents and therefore could be used in cancer treatment.

The nuclear factor- κB (NF-κB) has been associated with the development of inflammation and carcinogenesis, as a major inducer of the expression of several pro-inflammatory cytokines genes, contributing to inflammation-related tissue damage (Rathee, Chaudhary, Rathee, Rathee, Kumar, & Kohli, 2009; Soly Wang, Liu, Wang, & Zhang, 2009). Risk factors such as endotoxins, carcinogens, tumor developers and inflammatory cytokines (TNF-α) or interleukins (IL-1 or IL-8) (Aggarwal, 2004; Aggarwal, Vijayalekshmi, & Sung, 2009) stimulate the activation of IKK complex, which in turn ignites the phosphorylated stage of NF-κB, leading to subsequent DNA modifications and initiation of transcription for specific inflammatory genes such as primary inflammatory cytokines (TNF-α, IL-1β), IL-6 and chemokines (Aggarwal, Vijayalekshmi, & Sung, 2009; Terzić, Grivennikov, Karin, & Karin, 2010).

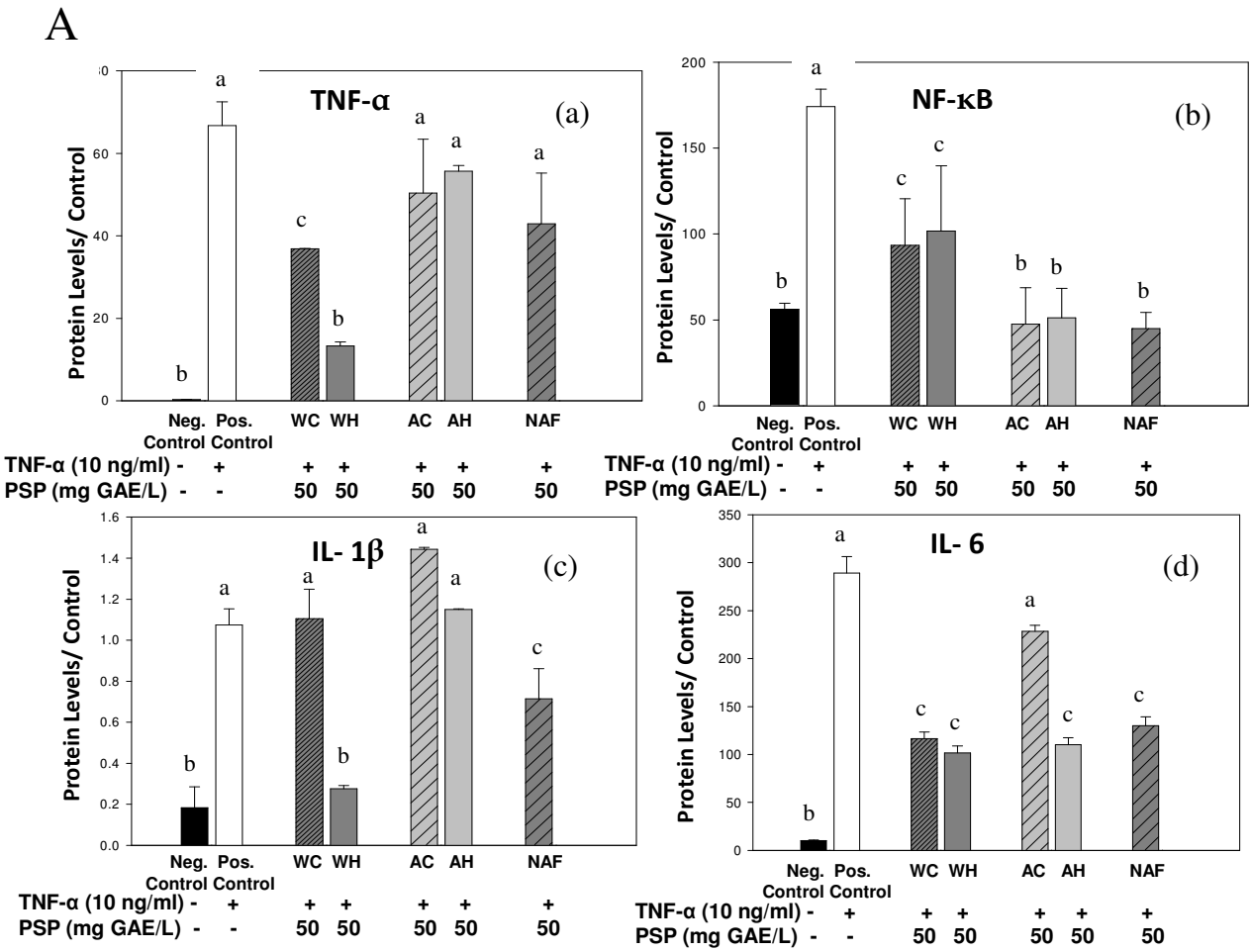


Figure 18: Expression of pro-inflammatory proteins NF-κB (a), TNF-α (b), IL-1β (c), and IL-6 (d) expression on CCD-18Co (A) and TNF-α (e), IL-1β (f), and IL-6 (g) on HT-29Co cancer cells (B).
Each experiment was performed at least three times and results are expressed as means ± SE. Different letters indicate significance at $P < 0.05$.

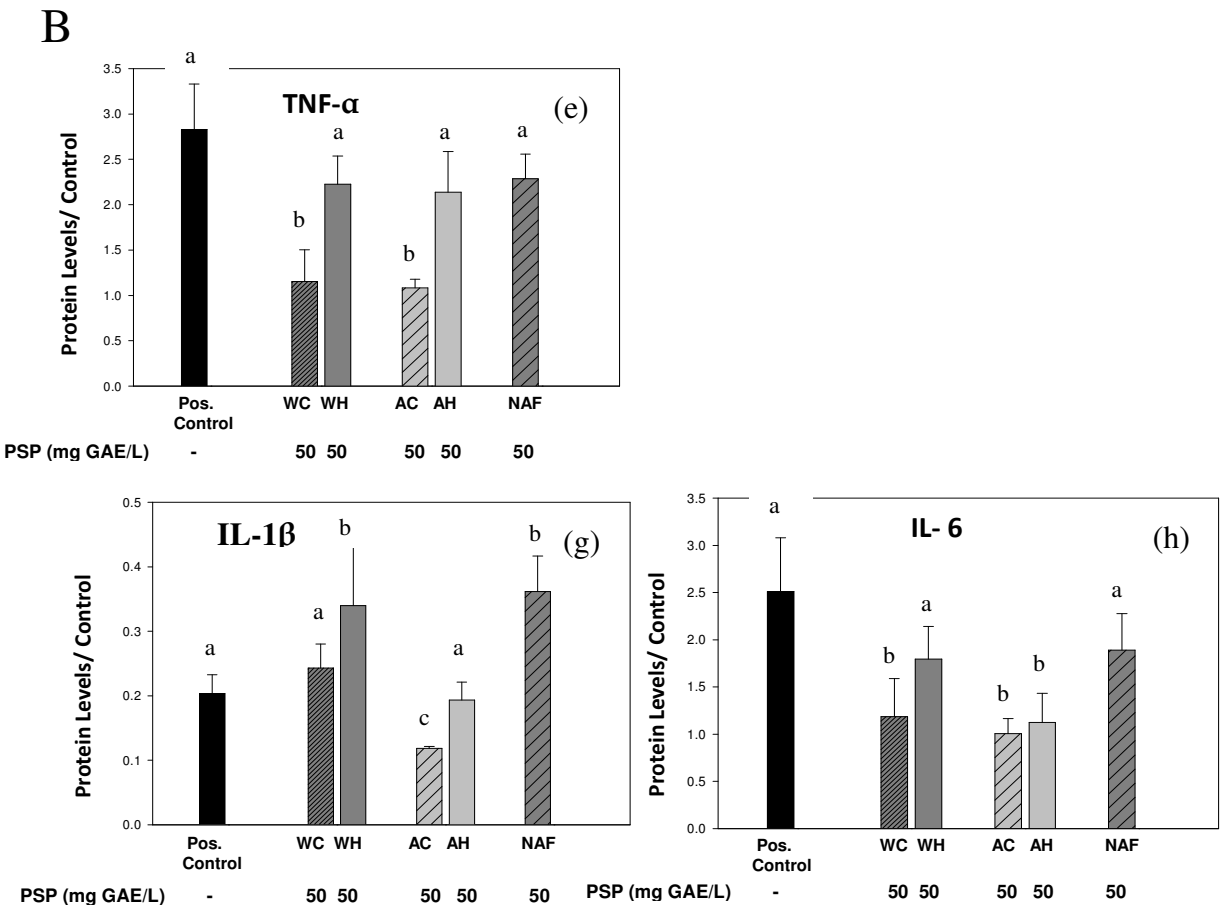


Figure 18: Continued

The overexpression of these markers may lead to upregulation of tumorigenic, adhesion proteins and inhibitors of malignant cell death, increasing the chances of several inflammatory conditions such as bowel diseases (IBDs) and colorectal cancer, as consequence (S. Kim, Keku, Martin, Galanko, Woosley, Schroeder, et al., 2008; Popivanova, Kitamura, Wu, Kondo, Kagaya, Kaneko, et al., 2008; Soly Wang, Liu, Wang, & Zhang, 2009).

Studies with anthocyanins have proven their ability to inhibit mRNA and/or protein expression levels of COX-2, NF-κB or other interlukines, reaffirming their anti-inflammatory capacities in several cell types *in vitro* (C. Huang, et al., 2002; Ramos, 2008; Terzić, Grivennikov, Karin, & Karin, 2010; L. Wang & G. D. Stoner, 2008). Berry juices containing several different anthocyanins sources (gooseberry, sea buckthorn, cranberry, black currant, white currant, raspberry) significantly inhibited the TNF-α induced activation of cyclooxygenase 2 (COX-2) and NF-κB expression (Boivin, Blanchette, Barrette, Moghrabi, & Beliveau, 2007) both distinguished biomarkers of inflammation and colorectal tumorigenesis (Terzić, Grivennikov, Karin, & Karin, 2010). Anthocyanin rich extracts from black raspberry also inhibited tumor development in JB-6 Cl 41 Mouse epidermal cells by regulation of benzoapyrene diol-epoxide (BaPDE)-induced expression of NF-κB (C. Huang, et al., 2002). Anthocyanins from blackberries, Korean raspberries and black raspberries were also tested for anti-inflammatory activity on LPS-stimulated colon cells (Jung, Lee, Cho, & Hwang, 2015). The expression of iNOS and COX-2 significantly inhibited after treatment with 20 μg/mL of the respective purified anthocyanins, suggesting these compounds may play an important role in the

regulation of NF-κB activation and subsequent cell events, essential in inflammatory body processes.

Purple sweet potato anthocyanins have been evaluated for anti-inflammatory properties. After inducing liver injury in rats dimethylnitrosamine (DMN), a carcinogen found in processed meats, protein expression of COX-2 and iNOS were significantly reduced after administration of anthocyanin fractions (AC) from PSP, in comparison to the injury group (Hwang, et al., 2011). AC was also able to significantly reduce NF-κB in the injury group, in a dose dependent manner. COX-2 and iNOS (inducible nitric oxide synthase), both enzymes involved in inflammatory processes and tumor progression (Chung, Park, Kim, Kim, Hwang, Lee, et al., 2007; Terzić, Grivennikov, Karin, & Karin, 2010), have recently been associated as activators of NF-κB and therefore modulation of NF-κB by phytochemical treatment seems to be a viable alternative in chemoprevention (Chung, et al., 2007; Hwang, et al., 2011; Surh, 2003; Terzić, Grivennikov, Karin, & Karin, 2010).

Enough evidence exists relating the incidence of colitis-associated cancers and NF-κB (Ardite, Panes, Miranda, Salas, Elizalde, Sans, et al., 1998; Eaden, Abrams, & Mayberry, 2001; Rogler, Brand, Vogl, Page, Hofmeister, Andus, et al., 1998), TNF-α (Terzić, Grivennikov, Karin, & Karin, 2010), IL-6 (Becker, Fantini, Wirtz, Nikolaev, Lehr, Galle, et al., 2005) and IL-1β (Popivanova, et al., 2008) presence and therefore the enhanced effect of PSP polyphenols in down-regulating the expression of NF-κB and downstream genes suggests their potential for use in preventive (CCD-18) and treatment (HT-29) of colorectal cancer.

Conclusions

We demonstrated that PSP polyphenols have the ability to inhibit cancer cell growth HT-29, with little toxicity towards the non-malignant cells CCD-18Co colon fibroblast cells. More importantly, different chemical structures of PSP polyphenols presented anti-inflammatory and anti-cancer properties in colon cells, and the mechanisms may involve modulation of ROS generation as well as down-regulation of pro-inflammatory markers NF-κB, TNF-α, IL-1β, and IL-6. However, follow-up studies in animals and humans are needed to support these beneficial effects and to determine clinical relevance of different chemical structures of anthocyanins, as they are abundant in different forms, which is dependent on its original source and processing treatment they are subjected to. Inflammation and colorectal cancer are life-threating complications and diet can play a major role in preventing and overcoming these conditions. Understanding anthocyanin stability under physiological conditions and how the chemical structure affects uptake in the body and their pharmacometrics will allow scientists to formulate and optimize their production for enhanced protection and prevention of colon cancer.

CHAPTER VI

IN VITRO DIGESTIVE STABILITY AND BIODEGRADATION OF VARIOUS ANTHOCYANINS FROM DIFFERENT SOURCES

Overview

Bioavailability studies with anthocyanins commonly support the idea of low absorption. Most anthocyanins are inherently unstable in low-acid conditions as found in the body as food transitions from stomach to the small intestine and the intestine into circulating blood. These studies assessed the stability of anthocyanins during *in vitro* digestion and incubation with pig fecal microflora. Both acylated (purple sweet potato - PSP, black carrot- BC and blackberry- BB) and non-acylated (jaboticaba, pomegranate and raspberry) anthocyanins were isolated from C18 cartridges and subjected to stomachal (pH 2.0 for 2 hr) and intestinal/blood conditions (pH 7.0 for up to 7 hr) under a blanket of nitrogen and stability of individual anthocyanins was monitored by HPLC-MS. Active and inactive pig fecal suspension were prepared, and anthocyanins incubated for up to 6 hr, when stability was assessed. At 1000 mg/L, each anthocyanin source exhibited excellent stability during gastric digestion. However, significant degradation occurred with pH increases to simulate the transition to the intestines or absorption into the bloodstream. Sources containing acylated anthocyanins were more stable to low-acid conditions at 1hr of exposure with 5-51% losses, when reaching the small intestine, site of greatest absorption. After 7 hr of exposure in such harsh conditions, degradation reached up to 80%. Individual compounds significantly

decreased in non-acylated sources, especially after 1 hr, with losses ranging from 67 to 100%, in the case of pomegranate. After 7 hr, losses reached 88-100%. Pig fecal incubation of acylated sources (BC and PSP) showed these were rapidly degraded upon exposure, with di-acylated compounds degrading faster than mono and non-acylated ones, the latter being produced as more complex structures were decomposed. Free hydroxycinnamic and phenolic acids were formed as both sources were incubated with active suspension, with maximum concentration ranging from 1 to 6 hours of exposure, simultaneous to anthocyanin degradation. Digestion leads to rapid loss of anthocyanins, partly explaining the low bioavailability reported for the intact compounds. Evidence shows degradation compounds and equilibrium forms are greatly responsible for health attributes and should be better understood. Therefore, when the stability of individual anthocyanins during digestion and absorption is considered over time, the consumption of stable acylated anthocyanins has a greater chance of exerting health benefits generally associated with the consumption of anthocyanin-rich foods.

Introduction

Polyphenols are secondary metabolites in plants that have received much attention due to potential antioxidant properties, thus appearing as a healthy diet alternative to treatment of chronic diseases. Amongst the flavonoid class, anthocyanins are especially important as they are widely used in the food industry as synthetic color replacement, with desirable health effects towards inflammation and certain cancer

types, such as colon (Aggarwal, Vijayalekshmi, & Sung, 2009; Dalgleish & O'Byrne, 2006; J. He & Giusti, 2010).

However, anthocyanin bioavailability often shows these compounds are poorly absorbed, with low recovery in urine and feces (Felgines, Talavera, Texier, Gil-Izquierdo, Lamaison, & Remesy, 2005; J. He, Magnuson, & Giusti, 2005; Keppler & Humpf, 2005; Mazza, Kay, Cottrell, & Holub, 2002). These results suggest anthocyanins must undergo extensive biotransformation in the body after oral ingestion. Conjugated metabolites have been previously reported (Felgines, Talavera, Texier, Gil-Izquierdo, Lamaison, & Remesy, 2005; X. Wu, Cao, & Prior, 2002), but recently, it has been hypothesized bacterial metabolism in the large intestine to be the primary route of degradation and subsequent absorption. Studies have shown de-glycosylation followed by ring-fission, with appearance of small phenolic acids and aldehydes, were the primary products after incubation with pig cecum contents (A-M Aura, et al., 2005; Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006; Forester & Waterhouse, 2008). It may be the protective health effects anthocyanins have in the colon is due to the new formed metabolites in direct contact with cells (Forester & Waterhouse, 2008). Therefore, besides the degradation at neutral pH in the small and large intestine, metabolism in the gut microflora strongly contributes to the low bioavailability of anthocyanins and needs to be evaluated

Identification of stable anthocyanin sources, that can withstand acidic-gastric and pancreatic pH conditions during digestion, reaching the colon to be metabolized and bioavailable, has become a challenge. Therefore, the aim of this research was to identify

stable anthocyanin sources likely to survive gastrointestinal digestion and incubation with pig fecal suspension, as well as monitor the microbial metabolism over a period of 24 hr and quantify the metabolites.

Material and Methods

Materials

Standards of cyanidin-3-glucoside, phenolic acids (caffeic, chlorogenic, ferulic, gallic, protocatechuic, syringic, vanillic acid and 2,4,6-trihydroxybenzaldehyde) were purchased from Sigma Aldrich, Co. (St. Louis, MO, USA). Extraction chemicals such as methanol, ethyl acetate, citric acid, formic acid along with solvents for chromatography were purchased from Fisher Scientific (San Jose, CA, USA). The Folin-Ciocalteu reagent was purchased from MP biochemical, LLC (Solon, Ohio).

Plant Materials

Fresh purple sweet potatoes (PSP) from a proprietary genotype were obtained from Avoca Farms Inc., Merry Hill, North Carolina (USA) from the 2013 harvest. PSP were transported to the Department of Nutrition and Food Science at Texas A&M University (ca. 40 kg) and stored dry at room temperature room shielded from direct light, until use. Raspberry, Jaboticaba, blackberry, pomegranate and black carrot extracts were obtained from Fruitsmart (Houston, TX).

Anthocyanin Sources and Preparation

A total of six anthocyanin extracts were used with the objective of assessing stability under gastrointestinal conditions and fecal incubation: different aglycone groups, with different simple sugars substitutions: Jaboticaba – JAB (*Myrciaria cauliflora*); different aglycones with different simple sugars substitutions: Pomegranate-POM (*Punica granatum*); a single aglycone group, with different glucose substitutions: Raspberry- RBY (*Rubus idaeus* L.); a single aglycone with different sugar substitutions and one acylating group: Blackberry - BBY (*Rubus* sp.); a single aglycone with different sugar substitutions and acylating groups: Black carrot – BC (*Daucus carota* L.); different aglycones with a high number of substitutions, including different sugars and acylating groups: Purple Sweet Potatoes - PSP (*Ipomoea batatas* L.). PSP extracts were obtained as described in the Material and Methods of chapter III. All other extracts were commercially available.

In Vitro Gastrointestinal Digestion

Selected anthocyanin sources (800 mg/kg GAE gallic acid equivalents) were subjected to two-stages of a simulated human digestion system (Krook & Hagerman, 2012). The initial stage, to simulate gastric conditions, consisted of adding pepsin/HCl to the diluted extracts (1:25 v/v) until pH 2.0 was reached. The falcon tubes were then incubated for 1 hr at 37ºC, under shaking (200 rpm). To simulate intestinal conditions, $NaHCO_3$ (0.5 N), pancreatin, porcile and lipase were added to the gastric digesta until a final pH of 7.0 ± 0.1 was reached, for 7 hr at 37ºC. The intestinal conditions were

simulated with shaking, absence of light and under N_2. Control group consisted of the same extracts exposed to a buffered solution with the same final pH values (2.0 and 7.0, respectively), without addition of enzymes. At each collection time, 5% (v/v) 99% formic acid was added to the samples, which were then filtered and analyzed via HPLC-MS. To evaluate their stability during digestion, a percentage degradation was calculated for each individual compound within anthocyanin source, in comparison to the initial loaded sample.

Preparation of Inoculum and Anthocyanin Incubation

The large intestine material of three different pig caeca was used in this experiment, as it contains similar microflora as humans (W. Pond & Houpt, 1978; W. G. Pond & Mersmann, 2001). The pigs were not taking antibiotics and were excluded of any polyphenol content in their diet. The preparation of the inoculum and incubation of anthocyanins followed a previous published method (Keppler & Humpf, 2005), with some modifications. The caeca were isolated from freshly slaughtered pigs and immediately stored in an anaerobic jar containing the same volume of reducing solution, prepared as follow: 0.15 M PBS (pH 6.4) containing 0.0125% (13.2 g/100 mL $CaCl_2.2H_2O$, 10.0 g/100 mL $MnCl_2.4H_2O$, 1.0 g/100 mL $CoCl_2.6\ H_2O$ and 8.0 g/100 mL $FeCl_3.\ 6H_2O$) and a Na_2S-solution 11.11% (575.9 mg/100 mL of 0.037 M NaOH). All solutions were flushed with N_2 prior to being in contact with the caeci material. The fecal solution was then blended and homogenized for 10 minutes, followed by filtration with a 1 mm sieve, divided into 15 mL falcon tubes and stored at -80°C for posterior use. All experiments with the caeci were conducted in a glove bag flushed with CO_2, in order to

maintain the anaerobiose (no available O_2). An aliquot of the fecal suspension material was inactivated at 121°C for 20 min at 1.1 bar in a small scale laboratory autoclave (Hirayama HVE-50 Autoclave). The inactivated samples were used as control, in order to differentiate chemical degradation from bacterial metabolism (active in the non-sterilized fecal inoculum).

The anthocyanin incubation was performed in different days, as triplicate. At each experiment day, triplicates of both active and inactive fecal suspension were thawed and immediately transported to the anaerobic chamber, where they were homogenized. The incubation experiment was also performed inside the anaerobic chamber. Moreover, in the chamber, all flasks and solutions were gassed with a mixture of N_2/CO_2 (4:1 v/v, 4 L/min), in order to maintain anaerobiose. With triplicates, anthocyanins (1 mL) were added of either active or inactive fecal suspension (9 mL) and the falcon tubes (15 mL) were taken into a water bath at 37°C under shaking (200 rpm). A control group (also in triplicates) contained water (1 mL), instead of anthocyanins. Samples, including control, were collected at 0, 0.5, 3 and 6 hr of experiment. After removed from the incubation period, the samples were immediately acidified with 1mL of 5% (v/v) 99% formic acid, sonicated for 10 min, centrifuged for 20 min at 4000 rpm at 4°C and filtered, using 1 mm sieve. The phenolic fraction was then obtained from partitioning using a 5 g pre-conditioned Sep-Pak columns (Waters Corporation, Milford, MA). The non-anthocyanin polyphenolic fraction that was first eluted with 100% ethyl acetate and a predominantly anthocyanin fraction was eluted with 100% methanol containing 0.01% v/v HCl as previously performed (Rodriguez-Saona & Wrolstad,

2001). Solvents were evaporated under vacuum at 35°C and re-dissolved in a known volume of 0.5 M citric acid buffer at pH 3. Samples were filtered using a 0.45 μm PTFE membrane filter and the stability was analyzed by HPLC-ESI-MS^n using a Thermo Finnigan LCQ Deca XP Max ion trap mass spectrometer equipped with an ESI ion source run in negative and positive ionization mode (ThermoFisher, San Jose, CA, USA) according to a previous developed method (Pacheco-palencia, Hawken, & Talcott, 2007). Separations were conducted using a 250 x 4.6 mm Acclaim 120 C18 column (Dionex, Sunnyvale,CA) with a C18 guard column.

Phytochemical Analyses

Following fecal incubations, analyzed anthocyanin sources (PSP, Black carrot, Raspberry, Blueberry, Jaboticaba and Pomegranate) were separated from the non-anthocyanin fractions according to the methodology proposed in the Material and Methods section of Chapter III, as well as the total anthocyanin content, total soluble phenolics (total reducing capacity) and individual anthocyanins and phenolic acids.

Statistical Analyses

The experiment was conducted with 3 triplicates in a randomized complete design. Data were analyzed by SAS version 6.11 (SAS Institute, Inc., Cary, NC). Analysis of variance was performed, and significance of differences among least square means was determined by Tukey's test ($P < 0.05$).

Results and Discussion

Individual Anthocyanin Characterization and Quantification

Identification of individual anthocyanins was based on comparison of retention time (t_R), spectral characteristics and mass spectrometry data with that of standards and references. Individual anthocyanins, retention times, MS characteristics and concentrations found in selected fruits and vegetables are summarized on Table 8.

Jaboticaba (*Myrciaria cauliflora*) is a tropical fruit which grows in the southeastern of Brazil, and contains high levels of anthocyanins (314 mg/ 100 g of fruit) (Castro, Silva, Oliveira, Desobry, & Humeau, 2014; Leite, Malta, Riccio, Eberlin, Pastore, & Marostica Junior, 2011). Jaboticaba extract was found to contain 2 major anthocyanins, simple glucosides of delphinidin and cyanidin, in agreement with previous research (Castro, Silva, Oliveira, Desobry, & Humeau, 2014; Reynertson, Yang, Jiang, Basile, & Kennelly, 2008).

Pomegranate (*Punica granatum*) is a semiarid climate fruit native of the Middle East, which contains high levels of polyphenols such as ellagic acid, ellagitannins, punicalagin, flavonoids (Banerjee, Talcott, Safe, & Mertens-Talcott, 2012; Jurenka, 2008). Previous identification of anthocyanins shows these fruit contain 3-glucosides/3,5-diglucosides of the delphinidin, cyanidin and pelargonidin aglycones (Banerjee, Talcott, Safe, & Mertens-Talcott, 2012; Santiago, Gouvêa, Godoy, Borguini, Pacheco, Nogueira, et al., 2014) , and are in accordance with the findings of this research.

The genus *Rubus* comprise a large class of fruits, naturally grown in the northern hemisphere (Mertz, Cheynier, Günata, & Brat, 2007), include raspberries as well as blackberries. Raspberries (*Rubus idaeus* L.) are also fruits which contain high levels of polyphenols, including ellagitannins and anthocyanins (Ludwig, Mena, Calani, Borges, Pereira-Caro, Bresciani, et al., 2015). Our findings suggest raspberries contain two di- and tri- glucosides of cyanidin (cyanidin-3-sophoroside-5-glucoside and cyanidin-3-glucosyl-rutinoside), while other reports have accounted for the presence of di- and tri-glucosides of pelargonidin, as well as the simpler derivatives of both aglycones (McDougall, Dobson, Smith, Blake, & Stewart, 2005). On another study, cyanidin-3-sophoroside, cyanidin-3- glucosyl- rutinoside, cyanidin-3-glucoside and cyanidin-3-rutinoside were the most predominant anthocyanins, whereas only traces of pelargonidin anthocyanins were quantified (Ludwig, et al., 2015).

Blackberries (*Rubus* sp.) are currently endorsed as rich sources of polyphenols, which include hydrolysable tannins and anthocyanins, as well as hydroxycinnamic acids, flavonols, flavan-3-ols and proanthocyanidins in smaller amounts (Mertz, Cheynier, Günata, & Brat, 2007; Siriwoharn & Wrostald, 2004). On Table 8, major anthocyanins of Blackberries consisted of cyanidin-based compounds, containing one acylation and rutinoside as a di-glucoside. These findings were in agreement with previous reports (Fan-Chiang & Wrolstad, 2005; Mertz, Cheynier, Günata, & Brat, 2007).

Table 8: Identification of anthocyanins in Jaboticaba, Pomegranate, Raspberry, Blackberry, PSP and BC.

Peak	tR (min)	$[M]^+$ (m/z)	MS/MS (m/z)	Individual Anthocyanin	Concentration (mg/Kg)
				Jaboticaba	
1	20.71	465	303	Delphinidin-3-glucoside	10.91 ± 0.03
2	22.2	449	287	Cyanidin-3-glucoside	174.44 ± 1.33
				Pomegranate	
1	18.83	627	465, 303	Delphinidin-3,5-glucoside	2.04 ± 0.45
2	20.23	611	449, 287	Cyanidin-3,5-diglucoside	3.67 ± 0.36
3	22.25	465	303	Delphinidin-3-glucoside	0.52 ± 0.11
4	23.68	449	287	Cyanidin-3-glucoside	0.73 ± 0.24
5	24.76	433	271	Pelargonidin-3-glucoside	0.27 ± 0.10
				Raspberry	
1	20.76	773	611, 449, 287	Cyanidin-3-sophoroside-5-glucoside	136.65 ± 1.15
2	22.21	773	611, 449, 287	Cyanidin-3-glucosylrutinoside	19.98 ± 0.35
				Blackberry	
1	8.7	449	287	Cyanidin-3-glucoside	144.83 ± 2.36
2	11.46	595	449, 287	Cyanidin-3-rutinoside	3.65 ± 0.02
3	12.92	535	287	Cyanidin-3 malonylglucoside	4.09 ± 0.04

Table 8: Continued

Peak	tR (min)	$[M]^+$ (m/z)	MS/MS (m/z)	Individual Anthocyanin	Concentration (mg/Kg)
				Purple Sweet Potato	
1	9.7	773	611, 449, 287	Cyanidin-3-sophoroside-5-glucoside	22.12 ± 2.98
2	10.1	787	625, 463, 301	Peonidin-3-sophoroside-5-glucoside	50.58 ± 7.46
3	12.3	893	731, 449, 287	Cyanidin-3-(6"-*p*-hydroxbenzoyl sophoroside)-5-glucoside	19.54 ± 2.36
4	13.7	907	745, 463, 301	Peonidin-3-(6"-*p*-hydroxbenzoyl sophoroside)-5-glucoside	9.45 ± 1.10
5	14.5	949	787, 449, 287	Cyanidin-3-(6"-feruloyl sophoroside)-5-glucoside	33.74 ± 5.01
6	17.5	1055	893, 449, 287	Cyanidin-3-(6"-caffeoyl -6'"-*p*-hydroxybenz sophoroside)-5-glucoside	33.83 ± 5.73
7	20.5	1111	949, 449, 287	Cyanidin-3-(6"-caffeoyl-6'"- feruloyl sophoroside)-5-glucoside	5.25 ± 1.38
8	22.7	1069	907, 463, 301	Ponidin-3-(6"-caffeoyl -6'"-*p*-hydroxybenz sophoroside)-5-glucoside	8.81 ± 2.05
				Black Carrot	
1	2.041	743	449, 287	Cyanidin-3-xylosyl-glucosyl-galactoside	11.00 ± 0.04
2	2.469	581	449, 287	Cyanidin-3-xyloyl-galactoside	11.88 ± 0.21
3	3.11	595	463, 301	Peonidin-3-xyloyl-galactoside	19.50 ± 1.72
4	4.839	863	743, 449, 287	Cyanidin-3-xylosyl-*p*-hydroxybenzoyl-glucosyl-galactoside	17.76 ± 0.23
5	6.347	949	743, 449, 287	Cyanidin-3-sinapoyl-xylosyl-glucosyl-galactoside	20.41 ± 1.26
6	7.119	919	743, 449, 287	Cyanidin-3-feruloyl-xylosyl-glucosyl-galactoside	45.82 ± 0.39
7	7.372	889	743, 449, 287	Cyanidin-3-coumaroyl-xylosyl-glucosyl-galactoside	30.60 ± 3.82
8	7.932	903	727, 433, 271	Pelargonidin-3-feruloyl-xylosyl-glucosyl-galactoside	42.14 ± 3.27
9	8.159	963	757, 463, 301	Peonidin-3-sinapoyl-xylosyl-glucosyl-galactoside	27.45 ± 0.23
10	8.965	933	757, 463, 301	Peonidin-3-feruloyl-xylosyl-glucosyl-galactoside	52.81 ± 2.76

Currently, the food industry has shown great interest in using vegetables such as black carrot, red cabbage and purple sweet potatoes as sources of anthocyanins, considering these pigments are highly acylated and therefore may be a viable alternative to overcome the low stability barrier commonly associated with use of natural colors. Purple sweet potatoes (*Ipomoea batatas*) are vegetables commonly grown in Asia and western countries such as New Zealand, and are rich in mineral, vitamins, besides offering health benefits due to presence of yellow or purple flesh (V.-D. Truong, et al., 2009). Our previous work reported 6 major anthocyanins in Purple sweet potatoes, containing highly acylated and complex sugar substitutions of peonidin and cyanidin (de Aguiar Cipriano, Ekici, Barnes, Gomes, & Talcott, 2015).

Carrots are widely consumed vegetable, as they are a source of health promoting benefits such as carotene (orange) or anthocyanins (Black). Black carrot (*Daucus carota* L.) are traditionally grown in western countries such as Turkey, Afghanistan, Egypt, Pakistan and India (Kammerer, Carle, & Schieber, 2004b). Black carrots anthocyanins are commonly reported to contain mostly cyanidin and peonidin based aglycones, substituted with xylosyl galactoside sacharides or xylosylglucosylgalactoside sacharides, that can be further acylated with sinapic, *p*-coumaric or ferulic acid as well (Kammerer, Carle, & Schieber, 2004b). Similar findings are reported on Table 8. Other research have reported up to 10 different anthocyanins, which included peonidin and pelargonidin based anthocyanins substituted and similar sugar and cinnamic acid substitutions (Montilla, Arzaba, Hillebrand, & Winterhalter, 2011).

Stability of Individual Anthocyanins from Different Sources During *In Vitro* Digestion of Different Anthocyanin Sources

The effect of *in vitro* gastrointestinal digestion on individual anthocyanins estimated by HPLC method of the previously selected sources is presented on Table 9. The digestion consisted of two steps. The gastric step consisted of the addition of HCl/ pepsin at pH 2.0 for 2 hr, and subsequent pancreatic/ bile salts at pH 7,0 for 7 hr, to simulate intestinal conditions and results expressed as a percentage variation between the initial load submitted to the digestion and the recovered amounts after digestion. Moreover, the *in vitro* gastric and intestinal digestive simulation is a powerful tool which enables to identify the compounds that would likely survive GI conditions and reach colon, where they can exert their antioxidant properties as well as be metabolized by colonic bacteria, be absorbed and promote health benefits (A-M Aura, et al., 2005; McDougall, Fyffe, Dobson, & Stewart, 2007).

Gastric simulated digestion had a small effect on individual anthocyanins of all sources, except the jaboticaba extract, in which reduction of 39 and 66% were observed. Anthocyanins from POM, RBY, BBY, BC and PSP remained stable during the first digestion, with slight increases in concentration, as it has been reported for other sources as well (M.-J. Bermúdez-Soto, F.-A. Tomás-Barberán, & M.-T. García-Conesa, 2007; Podsędek, Redzynia, Klewicka, & Koziołkiewicz, 2014).

Table 9: Individual quantities (μg/mL) of the main anthocyanins of selected sources and percentage recovered after gastric and intestinal *in vitro* digestion.

Individual Anthocyanin	Initial Concentration (mg/Kg)	Gastric Digestion	Pancreatic Digestion (1hr)	Pancreatic Digestion (7hr)
Jaboticaba				
Delphinidin-3-glucoside	10.91 ± 0.03	44.00%	12%	0%
Cyanidin-3-glucoside	174.44 ± 1.33	61.00%	43%	2%
Pomegranate				
Delphinidin-3,5-glucoside	2.04 ± 0.45	127%	0%	0%
Cyanidin-3,5-diglucoside	3.67 ± 0.36	124%	0%	0%
Delphinidin-3-glucoside	0.52 ± 0.11	135%	0%	0%
Cyanidin-3-glucoside	0.73 ± 0.24	145%	0%	0%
Pelargonidin-3-glucoside	0.27 ± 0.10	148%	0%	0%
Raspberry				
Cyanidin-3-sophoroside-5-glucoside	136.65 ± 1.15	117%	43%	5%
Cyanidin-3-glucosylrutinoside	19.98 ± 0.35	107%	60%	12%
Blackberry				
Cyanidin-3-glucoside	144.83 ± 2.36	111%	96%	3%
Cyanidin-3-rutinoside	3.65 ± 0.02	113%	117%	74%
Cyanidin-3 malonylglucoside	4.09 ± 0.04	122%	110%	63%
Purple Sweet Potato				
Cyanidin-3-sophoroside-5-glucoside	22.12 ± 2.98	99%	95%	53%
Peonidin-3-sophoroside-5-glucoside	30.58 ± 7.46	104%	100%	45%
Cyanidin-3-(6"-*p*-hydroxbenzoyl sophoroside)-5-glucoside	19.54 ± 2.36	102%	129%	58%
Peonidin-3-(6"-*p*-hydroxbenzoyl sophoroside)-5-glucoside	9.45 ± 1.10	104%	109%	75%

Table 9: Continued.

Individual Anthocyanin	Initial Concentration (mg/Kg)	Gastric Digestion	Pancreatic Digestion (1hr)	Pancreatic Digestion (7hr)
Cyanidin-3-(6"-feruloyl sophoroside)-5-glucoside	33.74 ± 5.01	101%	95%	17%
Cyanidin-3-(6"-caffeoyl -6'"-*p*-hydroxybenz sophoroside)-5-glucoside	33.83 ± 5.73	104%	114%	19%
Cyanidin-3-(6"-caffeoyl-6'"- feruloyl sophoroside)-5-glucoside	5.25 ± 1.38	115%	135%	45%
Peonidin-3-(6"-caffeoyl -6'"-*p*-hydroxybenz sophoroside)-5-glucoside	8.81 ± 2.05	106%	111%	38%
Black Carrot				
Cyanidin-3-xylosyl-glucosyl-galactoside	11.00 ± 0.04	125%	129%	81%
Cyanidin-3-xyloyl-galactoside	11.88 ± 0.21	123%	92%	71%
Peonidin-3-xyloyl-galactoside	42.14 ± 3.27	99%	57%	42%
Cyanidin-3--xylosyl-*p*-hydroxybenzoyl-glucosyl-galactoside	19.50 ± 1.72	65%	51%	39%
Cyanidin-3-sinapoyl-xylosyl-glucosyl-galactoside	17.76 ± 0.23	75%	45%	37%
Cyanidin-3-feruloyl-xylosyl-glucosyl-galactoside	20.41 ± 1.26	85%	58%	35%
Cyanidin-3-coumaroyl-xylosyl-glucosyl-galactoside	45.82 ± 0.39	69%	55%	34%
Pelargonidin-3-feruloyl-xylosyl-glucosyl-galactoside	30.60 ± 3.82	83%	88%	34%
Peonidin-3-sinapoyl-xylosyl-glucosyl-galactoside	27.45 ± 0.23	88%	66%	47%
Peonidin-3-feruloyl-xylosyl-glucosyl-galactoside	52.81 ± 2.76	102%	66%	46%

A possible explanation for the increased anthocyanin content could be that extracts are exposed to pH 2.0 for digestion, a lower pH compared to the original extract they are formulated with (pH 3.0). With that, more flavilium cation may be generated and can be detected with the HPLC method, at 520 nm (Bouayed, Hoffmann, & Bohn, 2011; Mosele, Macià, Romero, Motilva, & Rubió, 2015). Jaboticaba anthocyanins were evaluated for stability and compared to those of grapes and blueberries in acidic medium, showing less stability than the other anthocyanin sources. These authors suggested anthocyanins linked to simple glucosides showed less stability than those linked to galactosides or arabinosides (S.-B. Wu, Dastmalchi, Long, & Kennelly, 2012). The absence of more complex sugar substitutions and acylations may have led to such increased anthocyanin degradation, even at lower pH when these compounds are commonly stable at.

Lower anthocyanin recoveries were observed in the intestinal digestion step, as has been previously reported in wine (McDougall, Dobson, Smith, Blake, & Stewart, 2005), red cabbage (McDougall, Fyffe, Dobson, & Stewart, 2007), chockeberry (M.-J. Bermúdez-Soto, F.-A. Tomás-Barberán, & M.-T. García-Conesa, 2007), blueberries (Liu, Zhang, Wu, Wang, Wei, Wu, et al., 2014), amongst others. To the best of our knowledge, no previous studies have reported findings for an *in vitro* digestion of anthocyanins from Purple sweet potatoes and Jaboticaba fruit.

This study evaluated compounds after 1 hr, which would simulate compounds reaching the small intestine which is the site of greatest absorption of compounds into the bloodstream, and 7 hr of exposure to intestinal digestion, to simulate compounds

reaching the colon and being subjected to colonic microflora, fermentation, and degradation into smaller phenolic acids (Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006). After 1 hr of exposure, PSP and BBY individual anthocyanins presented highest stability, with recoveries ranging from 95 to 135%. BC and RBY were also stable during the exposure to neutral conditions for 1 hr, with recoveries varying from 46 to 76%. POM and JAB individual anthocyanins presented the least stability, ranging from 43% to complete degradation, as in the case of POM. After 7 hr of exposure, JAB anthocyanins were reduced to only 2% (Cyanidin-3-glucoside), while RBY presented a maximum of 12% (cyanidin-3-glycosil-rutinoside). BC, BBY and PSP individual anthocyanins remained at high levels of anthocyanins (See Table 9).

Anthocyanins stability is directly related to its chemistry composition. Acylated compounds tend to be more stable than non-acylated; for instance, 3,5-dyglicosides confer more stability than 3 mono glucosides; and increased number of hydroxyl metoxylation to the aglycone molecule also increases stability, according to previous studies (Liu, et al., 2014; McDougall, Dobson, Smith, Blake, & Stewart, 2005). An *in vitro* study with pomegranate juice showed a slight increase during gastric digestion, followed by a significant reduction of individual compounds after 2.5 hr under intestinal conditions at 37°C (Pérez-Vicente, Gil-Izquierdo, & García-Viguera, 2002). In comparison with other anthocyanin sources, pomegranate extract contains non-acylated mono or di-glucosides of cyanidin and delphinidin at low concentrations (0.11 μmol/ g) (Mosele, Macià, Romero, Motilva, & Rubió, 2015), making them susceptible to degradation. The authors suggest the explanation for such loss of compounds is the

transformation that occurs from the flavilium cation (stable at pH 2.0) to the colorless chalcone (prevalent at pH 7.0) and consequently into anydrobases, with ring fission and productions of aldehydes and small phenolic acids (Brouillard, 1982; Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006; Keppler & Humpf, 2005).

In an attempt to understand blueberry anthocyanin stability in an *in vitro* simulated digestion, it was observed that the delphinidin glycosides showed the least stability, followed by petunidin-3-glucoside. The high degradation rate was attributed to the mono-glycosidic substitution associated to increased number of hydroxyls in the aglycone molecule, making them more susceptible to oxidation reactions (Liu, et al., 2014). Malvidin, which contains more metoxy groups, presented higher stability than petunidin and delphinidin-3-glucosides. The most stable anthocyanidin was cyanidin-3-galactoside, which according to previous reports, presents the least substituents in the B-ring, leading to increased stability (Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006). Our results followed similar trends: in JAB, Pelargonidin aglycones, which contains the least of substituents to the B-ring, presented increased stability in comparison to cyanidin, which was also more stable than delphinidin anthocyanins; and in POM, cyanidin aglycones also presented higher stability than delphinidin ones.

In vitro studies with more complex anthocyanin sources have shown increased stability for these compounds (McDougall, Fyffe, Dobson, & Stewart, 2007). Red cabbage non-acylated anthocyanins showed less stability than those containing acyl substitutions, especially when the compounds contained hydroxycinnamic acids. This study suggested mono acylated anthocyanins containing *p*-coumaric was more stable

than those which were substituted with ferulic or sinapic (McDougall, Fyffe, Dobson, & Stewart, 2007). These published results are in much agreement with the ones found here. PSP, BC and BBY, selected sources containing acylated anthocyanins, were less degraded after 7 hr of exposure to pancreatic conditions, in comparison to RBY, JAB or POM. In fact, PSP contain mono and di-acylated anthocyanins which presented greatly stability during the *in vitro* study. However, an increased amount was still observed for the non-acylated anthocyanins in PSP and BC, which could have been formed as the di and mono-derivatives were degraded. It has been reported that glucose and acyl moieties could be the first to hydrolyze under neutral pH conditions, which could potentially generate more mono and non-acylated compounds (Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006).

The precise fate of anthocyanins upon digestion is still an object of future studies, considering in the human body, these compounds are subjected to metabolism, oxidation reactions and degradation to other chemical species (Pérez-Vicente, Gil-Izquierdo, & García-Viguera, 2002), making detection a difficult task to accomplish. Therefore, evaluating different sources is the first step into better understanding and predicting how the chemical structure will be affected under non-optimal conditions. Our findings suggest vegetable sources containing highly acylated and complex sugar moieties would most likely survive the GI conditions in which the combination of intact molecules, degradation products and/ or metabolites may exert beneficial effects in the stomach, intestine and as absorbed into circulatory system, acting as effective antioxidant and

promoters of health, as it has been widely proposed (A-M Aura, et al., 2005; Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006; J. He & Giusti, 2010).

Stability of Individual Anthocyanins from Acylated Sources after Incubation with Pig Fecal Microflora

Acylated individual anthocyanins from purple sweet potato and black carrots showed great stability during *in vitro* digestion, and therefore, were selected for the incubation with pig microflora. The selected anthocyanins were exposed to an active and inactive microflora, as previously described. Following incubation, C18 cartridges were used to separate and isolate the non-degraded anthocyanins from the phenolic acids, assumed to be the degradation products.

On both evaluated sources, compounds exposed to the inactivated medium were degraded, but intact structure was still detected even after 6 hours of exposure (Figure 19). For black carrots, in the first 30 min of analysis, a rapid decrease in the acylated compounds was concomitantly observed with an increase of non-acylated derivatives. Recoveries of anthocyanins after 6 hr ranged from 34 to 81% (Figure 19A). Purple sweet potato anthocyanins showed increased stability under inactivated fecal suspension, as can be seen in Figure 19B. A slight decrease was observed in the beginning of the exposure time, followed by an increase in the non-acylated cyanidin individual compound. Recovery ranged from 60% to complete recovery.

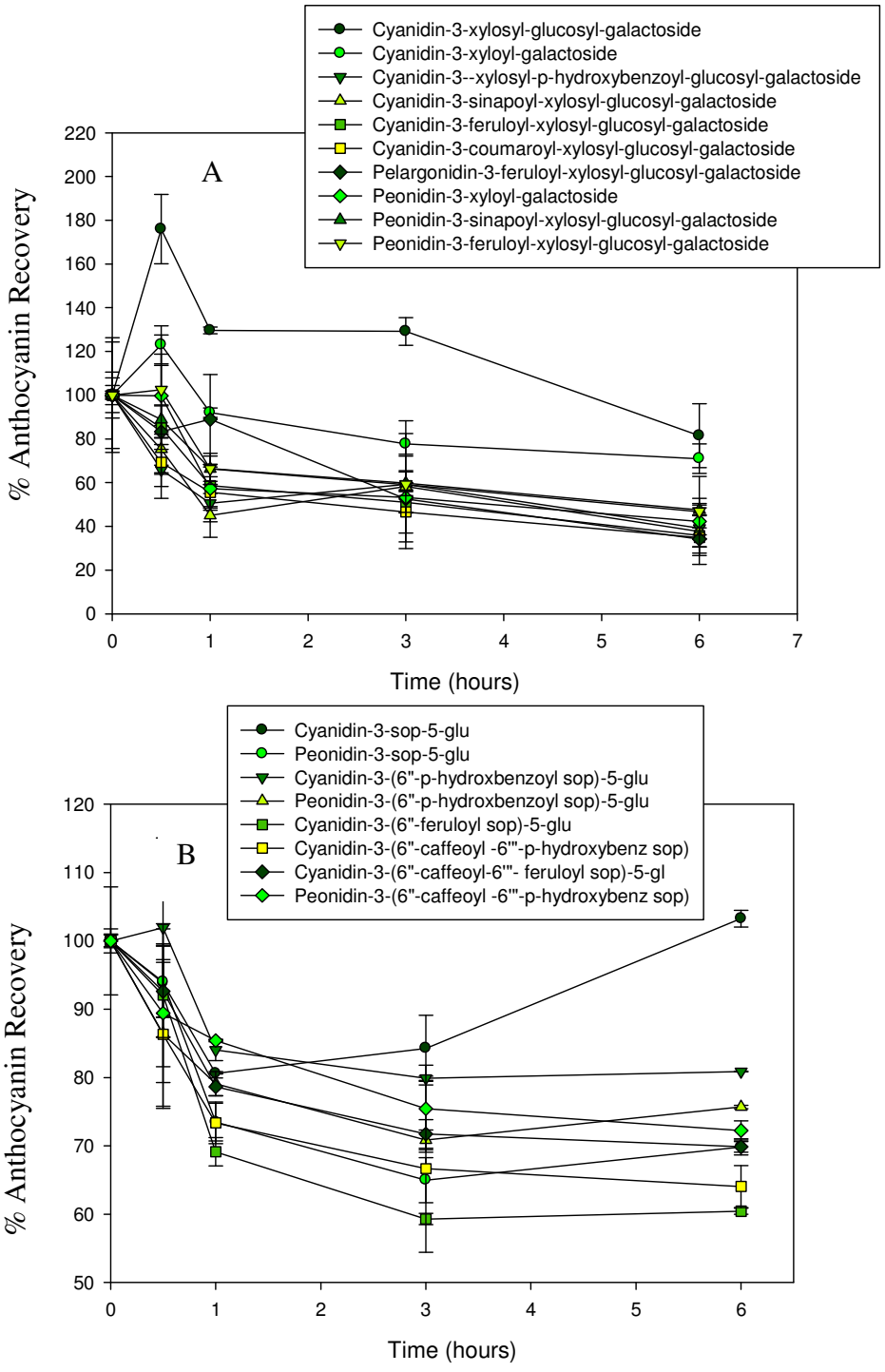


Figure 19: Degradation of Black carrot (A) and Purple sweet potato (B) individual anthocyanins upon incubation with inactive pig microflora suspension for 6hrs. Initial concentration of extract was 1000 mg/ Kg gallic acid equivalent.

Figure 20 shows the degradation of JAB (A), PSP (B) and Black carrot (C) anthocyanins exposed to the active pig fecal suspension. Anthocyanins from acylated souces (BC and PSP) showed great stability after exposure to microflora, after 24h of exposure to active and inactive pig fecal suspension, when compared to non-acylated sources (JAB) (data not shown).

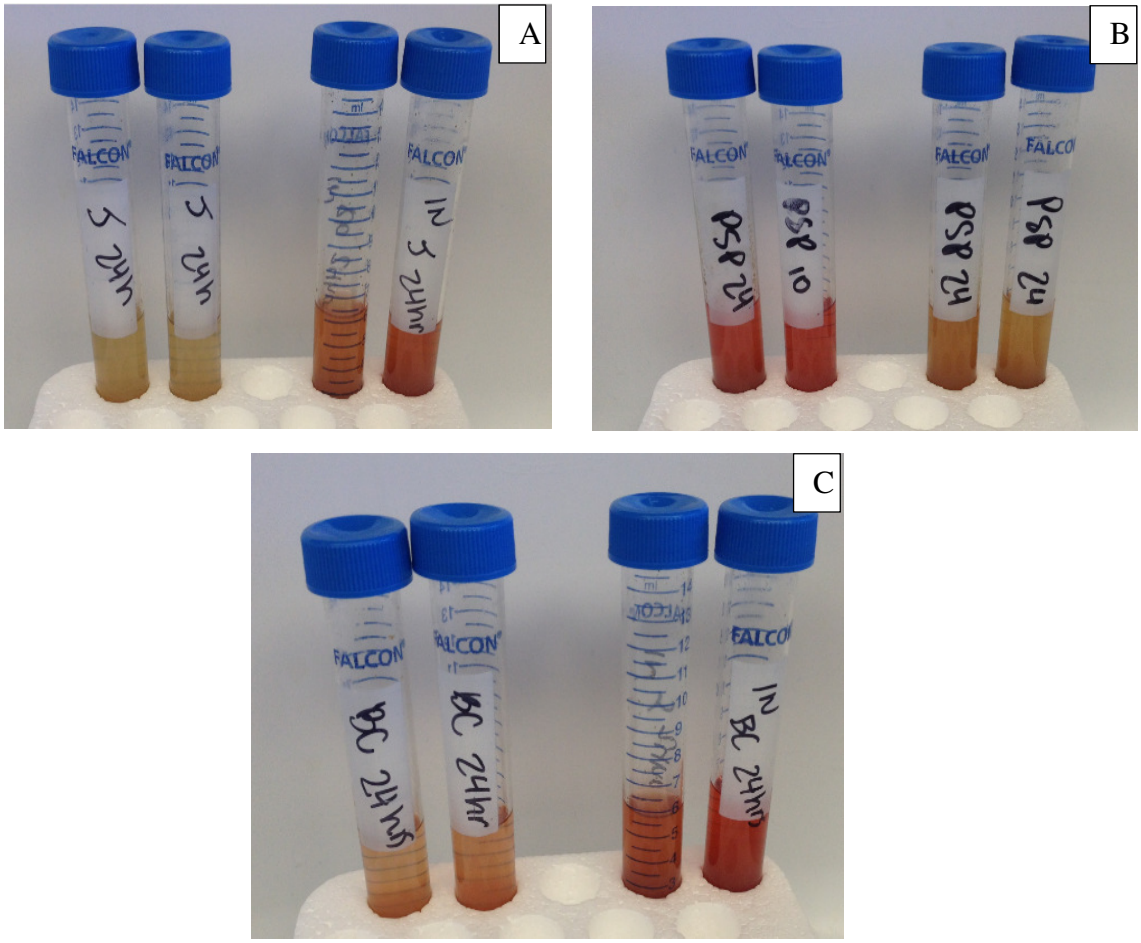


Figure 20: Jaboticaba - JAB (A), Purple sweet potato - PSP (B) and Black carrot - BC (C) anthocyanin extracts after exposure to 24h of active fecal microflora.

In the black carrot extract, after 1 hr of incubation, formation of non-acylated anthocyanins (2.95, 1.45 and 1.44-fold increase in cyanidin-3-xylosil-galactoside, cyanidin-3-xylosil-glucosil-galactoside and peonidin-3-xylosil-galactoside, respectively) was observed as an indication of de-acylation of more complex anthocyanins. Maximum concentration of cyanidin-3-xylosil-galactoside and cyanidin-3-xylosil-glucosil-galactoside were achieved after 12 hours of exposure, whereas peonidin-3-xylosil-galactoside reached its maximum at 1 hr, and decreased after it (Figure 21A).

Although vast literature supports acylated compounds present high stability under non-optimum pH conditions (Castañeda-Ovando, de Lourdes Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009; M. Giusti & R. Wrolstad, 2003), these compounds were rapidly degraded by active fecal suspension (Figure 21B). With 30 min of exposure, sinapic, *p*-coumaric and ferulic acid substituted anthocyanins degraded up to 60%. Cyanidin based anthocyanins showed increased stability in comparison to pelargonidin or peonidin anthocyanins. Compounds reached their lowest concentrations after 24h, varying from 2 to 6% (Figure 21A). Concomitantly, increases in free hydroxycinnamic acids were also noted as acylated anthocyanins decreased (Figure 21C, detection at 320 nm), identified during incubation with the active suspension based on mass to charge ratio and retention time of standards (Kammerer, Carle, & Schieber, 2004a). Samples treated in inactive fecal suspension presented slight increase in free hydroxycinnamic acids, indicating most of the de-acylation occurred due to presence of microflora, and not pH effect (data not shown).

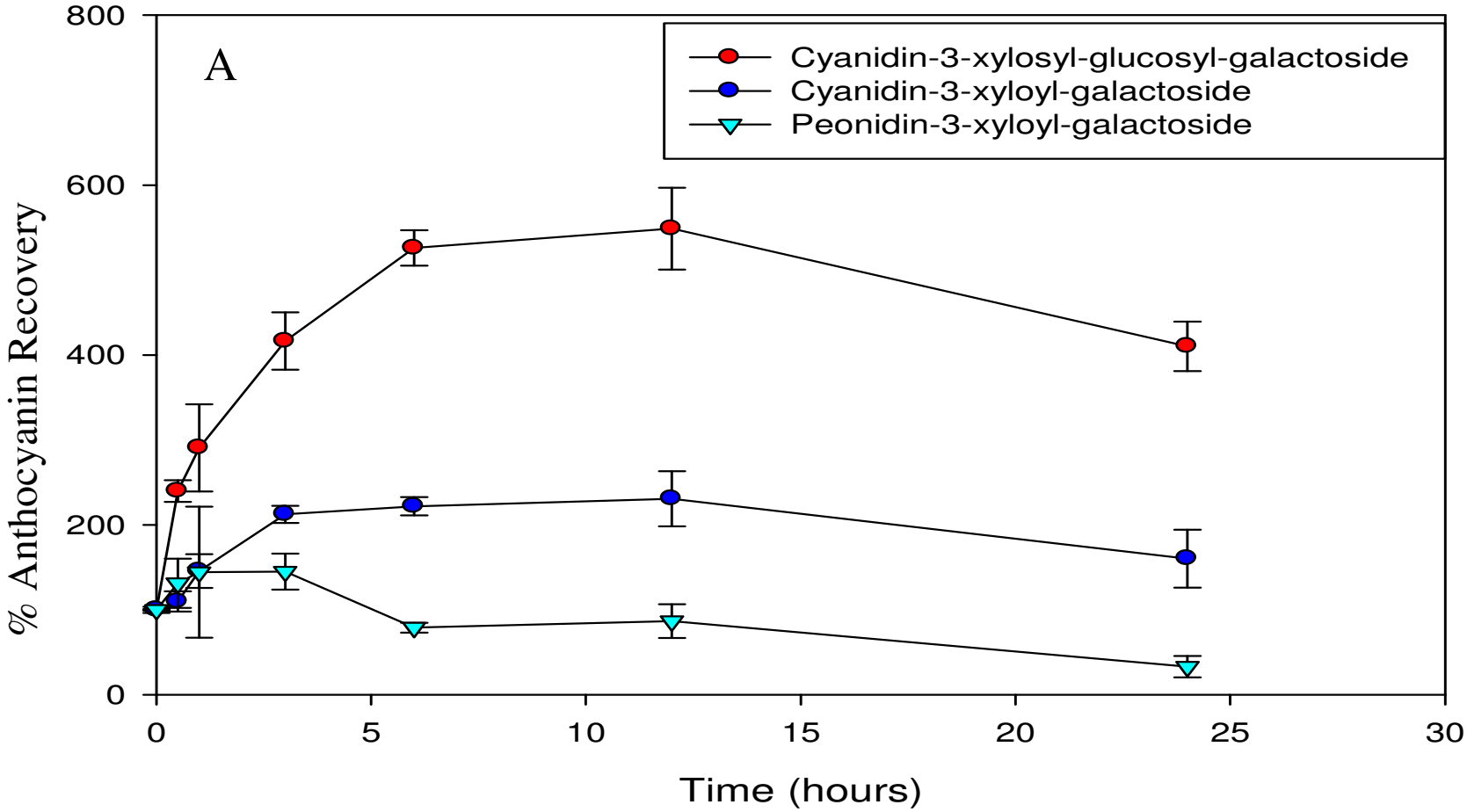
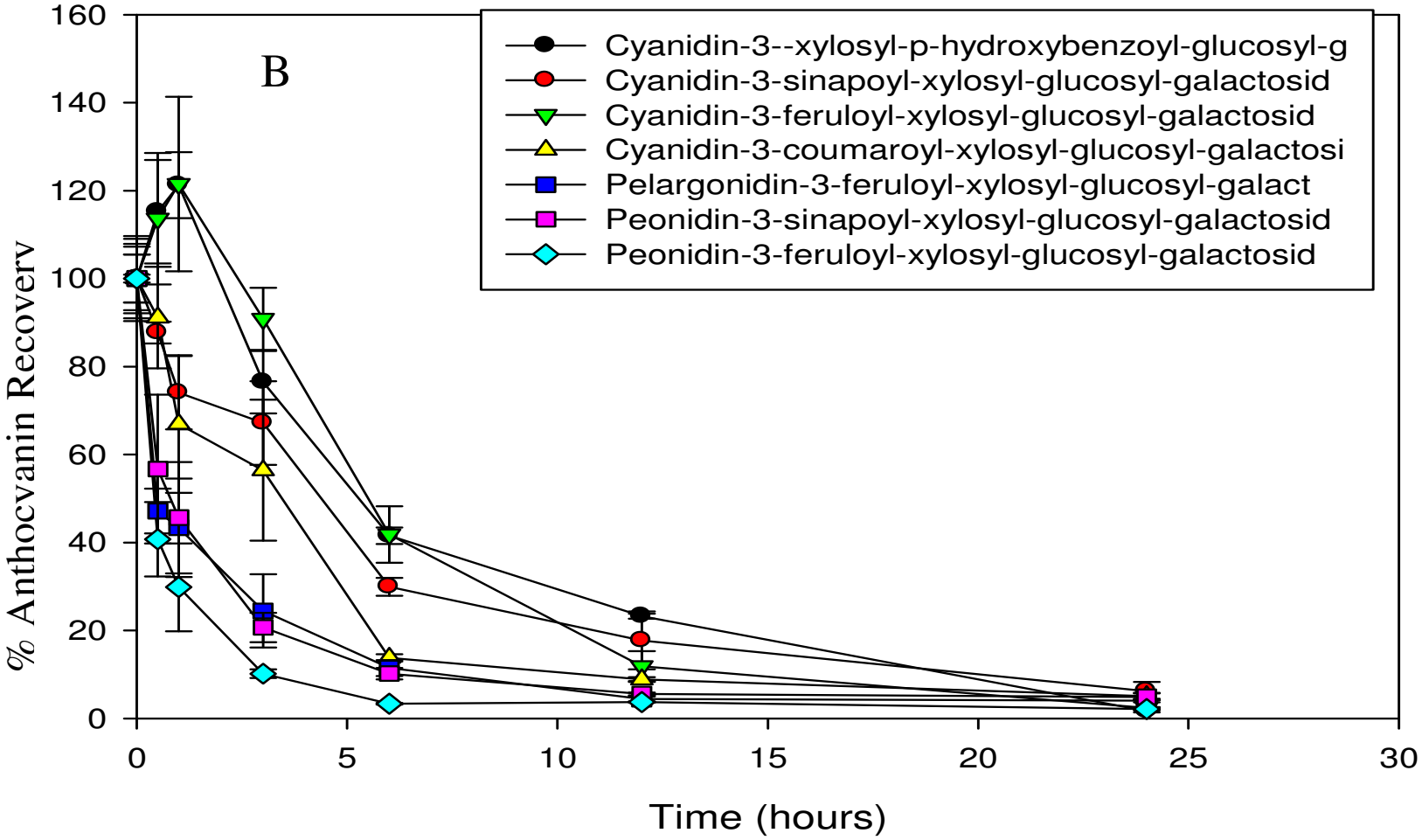


Figure 21: Degradation of Black carrot non-acylated (A) and acylated (B) individual anthocyanins upon incubation with active pig microflora suspension for 24 hours, at 520 nm;(C) shows the formation of hydroxycinnamic acid from black carrot, measured at 320 nm.
Initial concentration of extract was 1000 mg/ kg.



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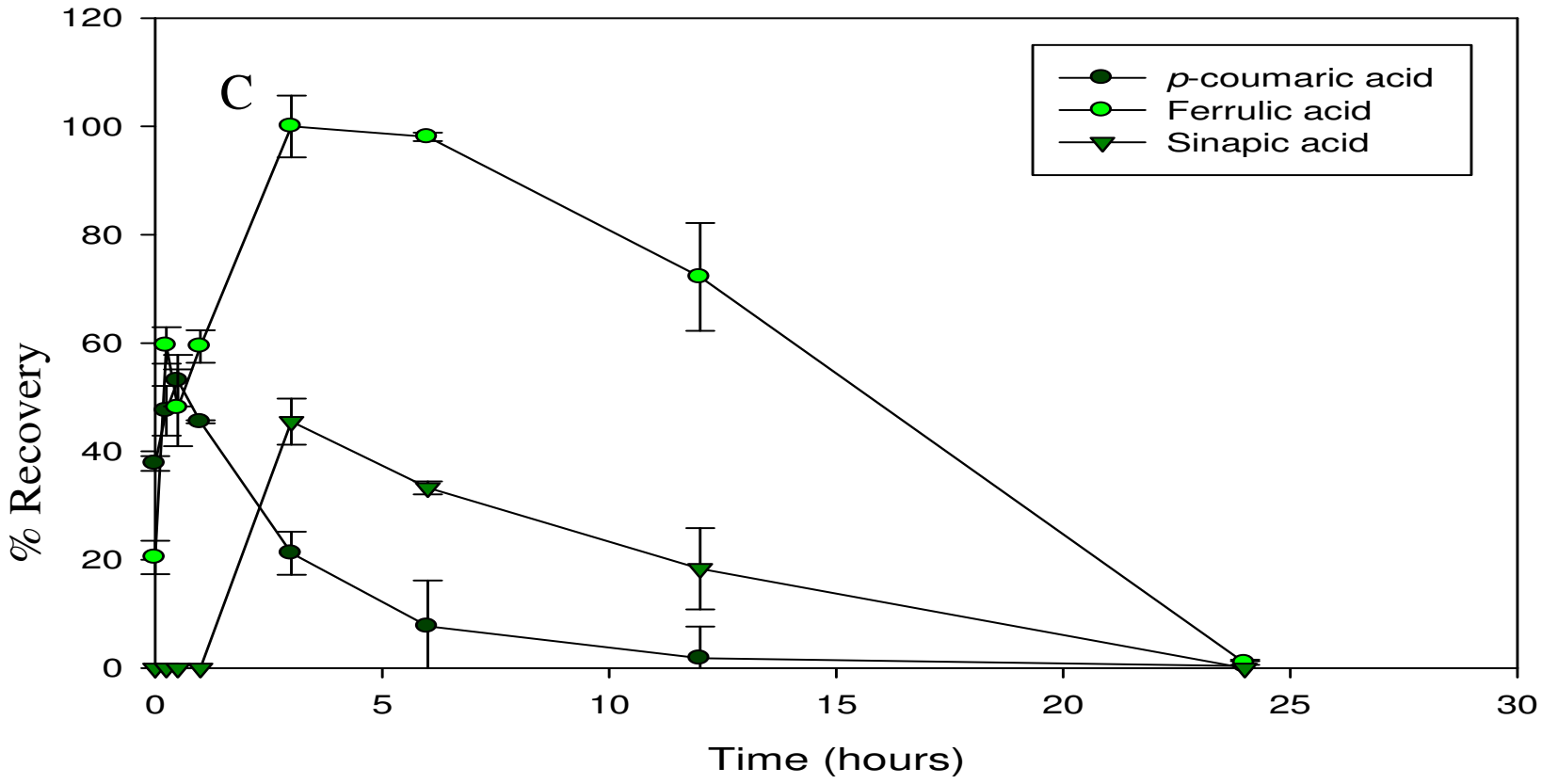


Figure 21: Continued.

In PSP, non-acylated anthocyanins increased, specially the cyanidin based ones, reaching a maximum after 3 hr of exposure (Figure 22A). Peonidin non-acylated anthocyanin remained stable during the 6 hours of incubation. The majority of PSP anthocyanins comprise cyanidin based compounds, which explains the reason for the sudden increase of cyanidin based compounds in regards to peonidin. After 6 hr of incubation, only the non-acylated anthocyanins were still detected in the suspension (226 and 80%, respectively)

Deacylated anthocyanins in purple sweet potato anthocyanins were also rapidly degraded, in comparison to mono- and non-acylated compounds, which could also have been formed as complex compounds that were de-acylated and de-glycosylated. Di-acylated anthocyanins were reduced in the first hour of exposure, with recoveries ranging from 6.55 to 11%; mono-acylated recoveries ranged from 69 to 113%; non-acylated anthocyanins greatly increased in the first part of the experiment, reaching its peaks at 3 hr (450 and 8% for cyanidin-3-sophoroside-5-glucoside and peonidin3-sophoroside-5-glucoside, respectively). After 6h of incubation, mono and di-acylated compounds reached concentrations below our detection capabilities (Figure 22B).

At 320 nm, increases in the concentration of free ferulic and caffeic acid were also observed in purple sweet potato extracts (Figure 22C), while no *p*-hydroxybenzoic acid was detected. A maximum concentration of hydroxycinnamic acids was observed after 30 minutes of incubation.

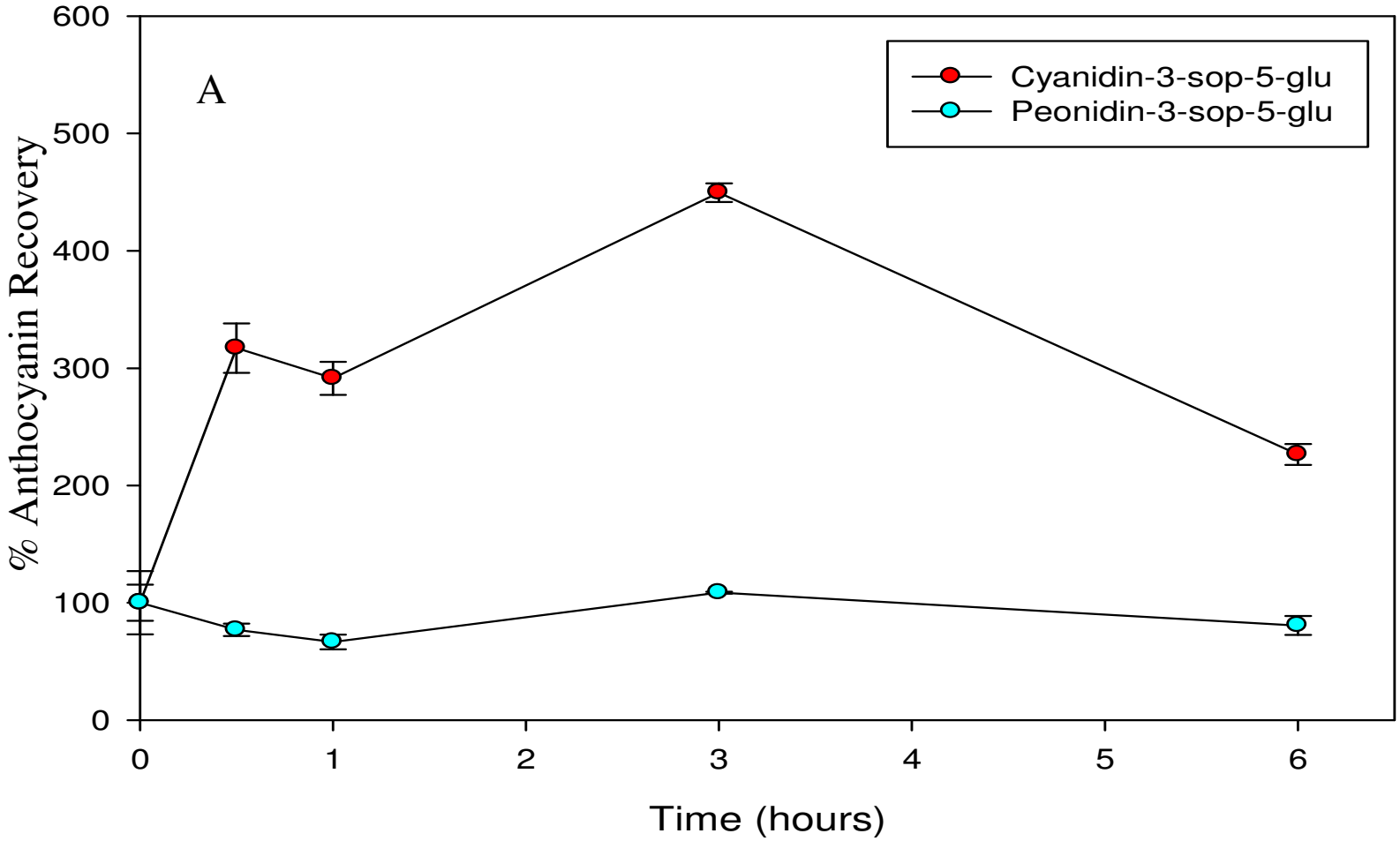
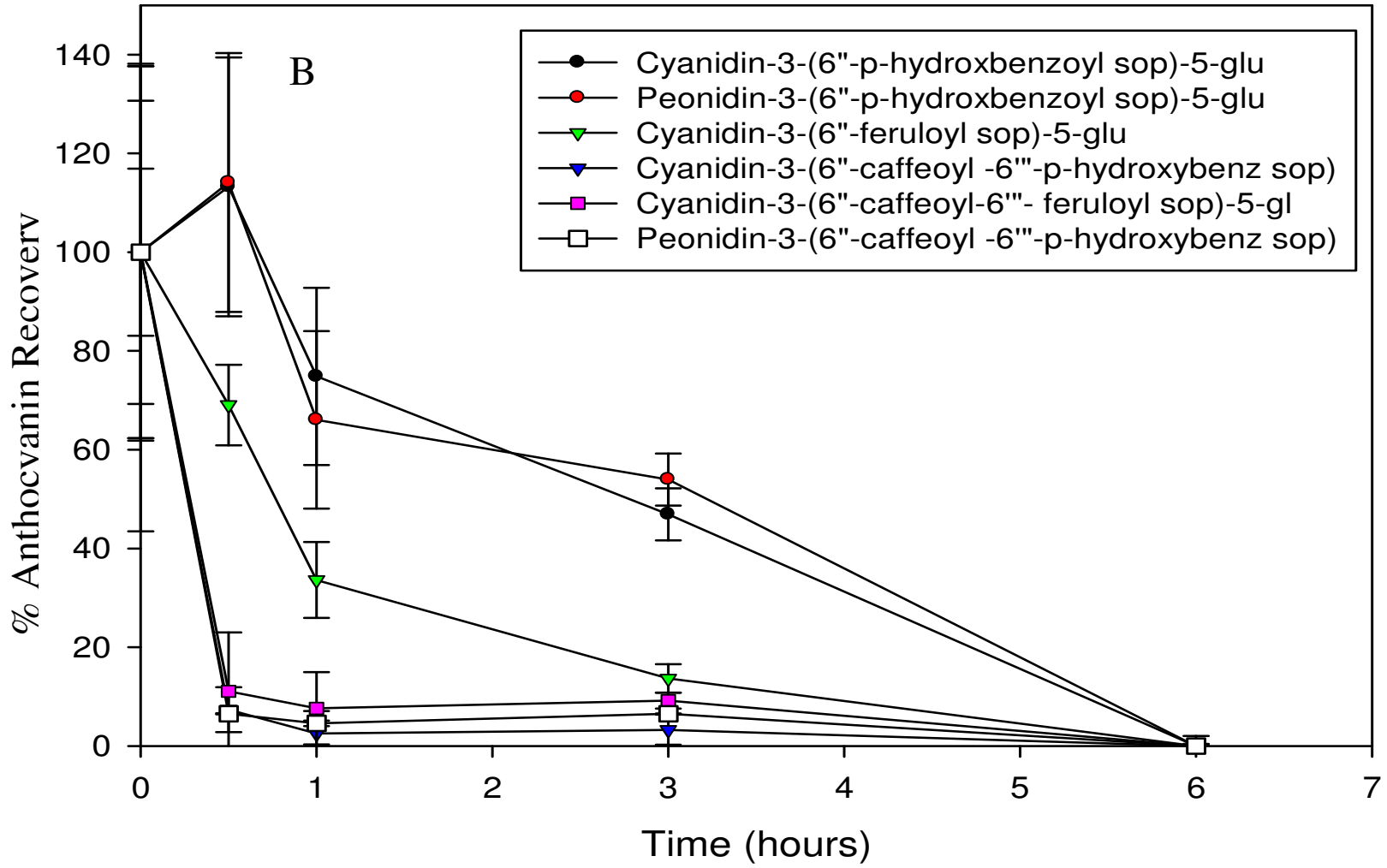


Figure 22: Degradation of Purple sweet potato non-acylated (A) and acylated (B) individual anthocyanins upon incubation with active ig microflora suspension for 24 hours. (C) Free hydroxycinnamic acids kinetics from Purple sweet potato after incubation with active pig microflora suspension for 24 hours. Initial concentration of extract was 1000 mg/ kg.



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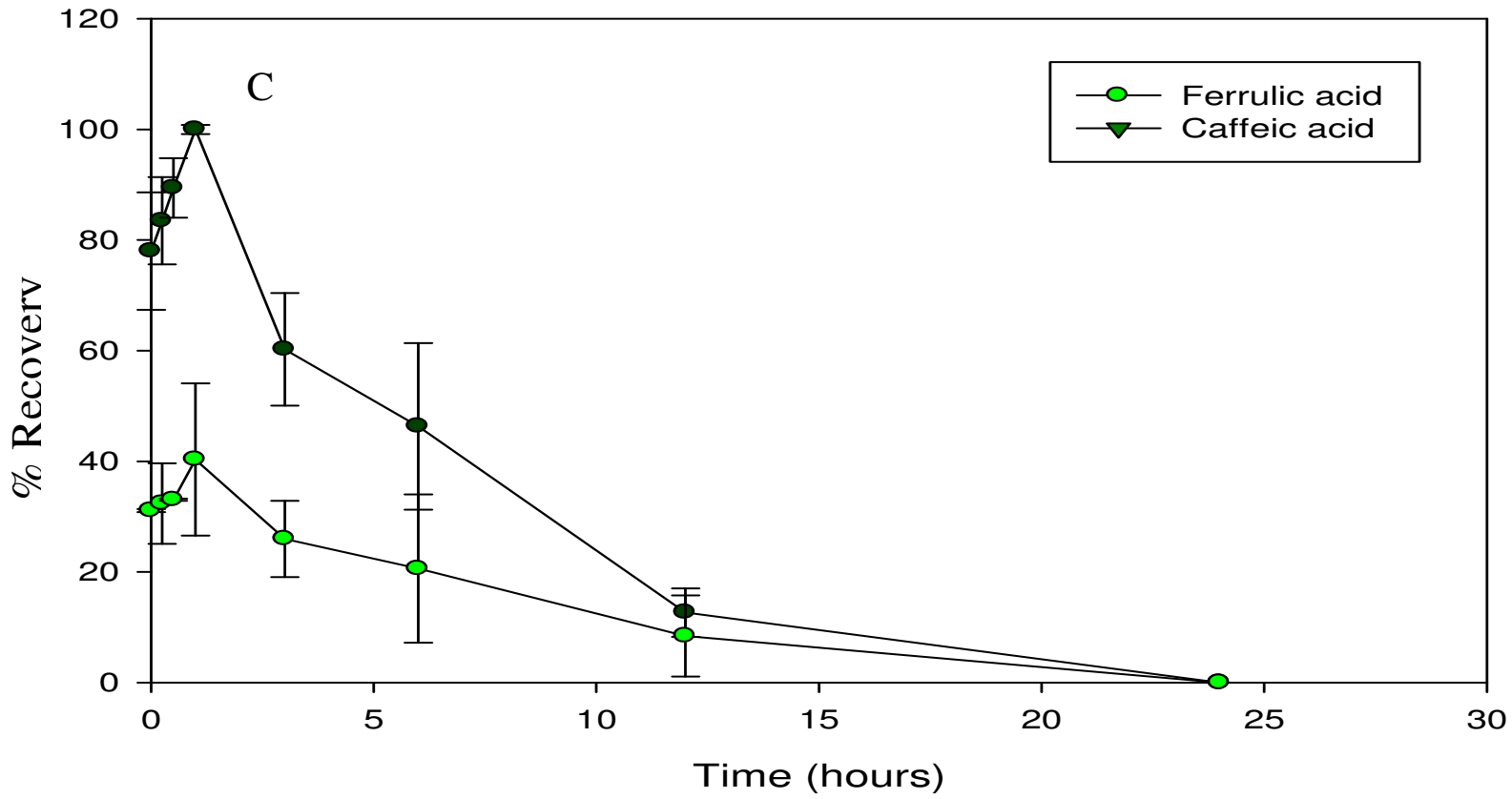

Figure 22: Continued.

The representative HPLC chromatograms (at 520 and 320 nm) of anthocyanins and hydrocinanmic acids isolated from Black Carrot and Purple Sweet Potato after incubation with active fecal microflora are shown on Figure 23 (1 and 2, respectively).

Similar findings were observed when chemically complex anthocyanins, such as acylated ones from radish extracts, were evaluated in *in vitro* human fecal digestion, with the objective of determining rate of hydrolysis and degradation products (Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006). Acylated anthocyanins were incubated with fecal microflora and a loss of the acyl groups was detected at 320 nm, along with the further degradation of the di-glucosides to their mono-glucoside derivatives. When di-glycoside anthocyanins standards were exposed to the fecal suspension, an increase in the mono-glucoside derivatives was observed, following a decrease after 30 minutes of exposure and a concomitant increase in phenolic acid content. Mono-glucosides were rapidly decreased after exposure to fecal flora, while the heat-inactivated suspension did not degrade the compounds (Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006).

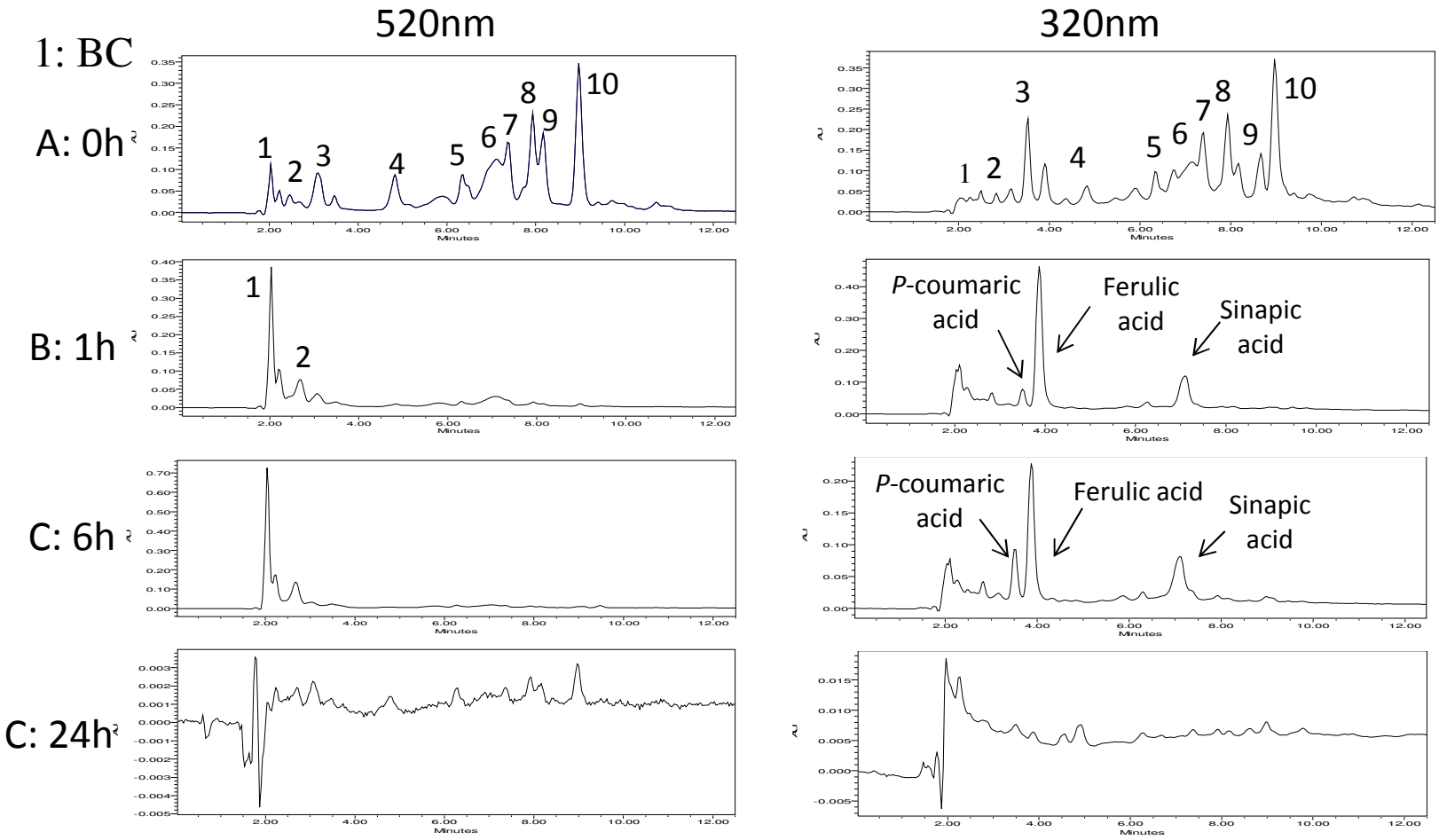

Figure 23: representative HPLC chromatograms (at 520 and 320 nm) of anthocyanins and hydrocinanmic acids isolated from Black Carrot (1:BC) and Purple Sweet Potato (2:PSP) after incubation with active fecal microflora for 24 hr. Refer to Table 1 for identification.

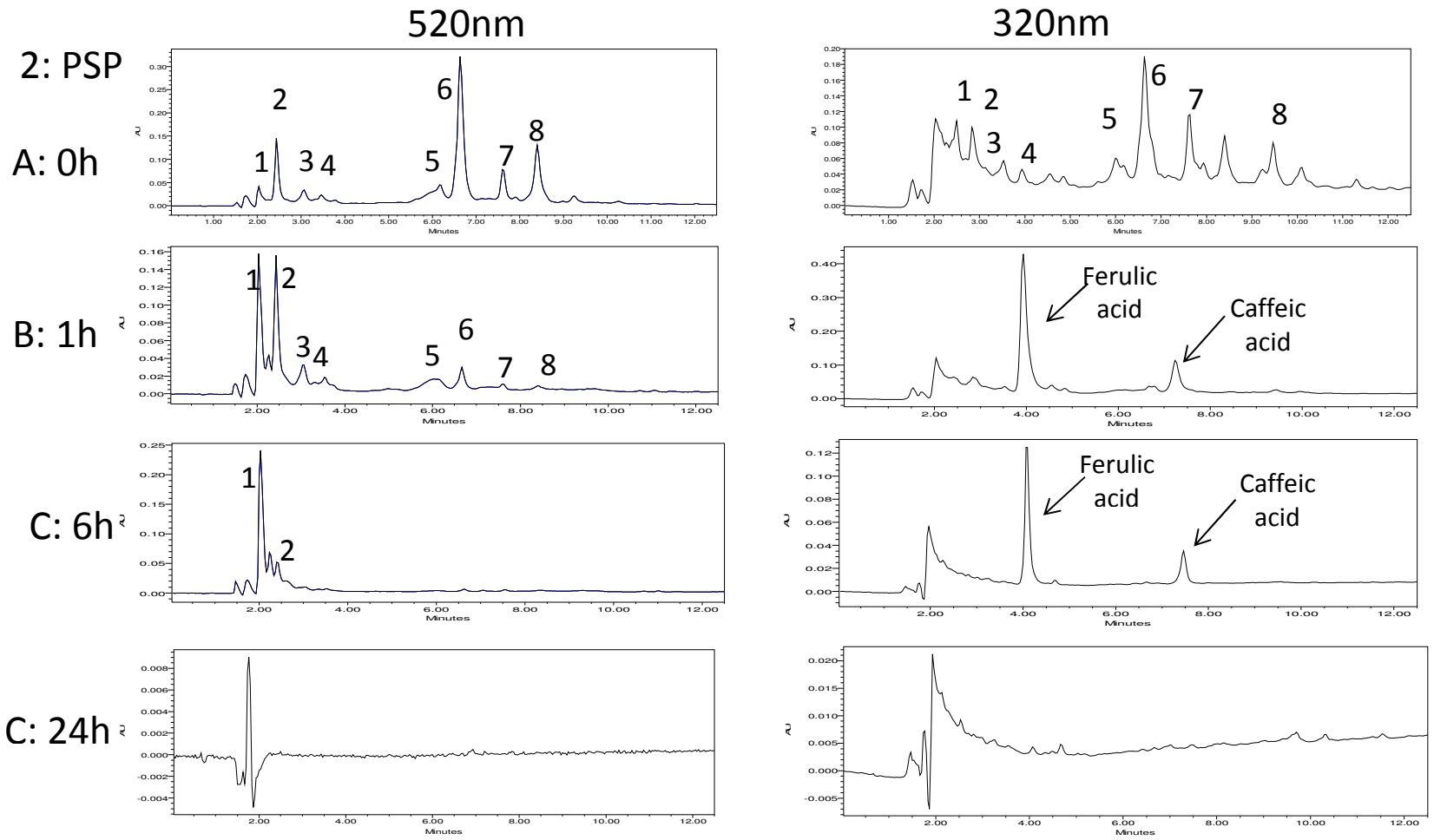

Figure 23: Continued.

In a study to evaluate anthocyanin metabolites using fecal suspension as an in vitro gut microflora model, cyanidin-3-glucoside and cyanidin aglycone were formed as a degradation product of cyanidin-3-rutinoside and cyanidin-3-glucoside, respectively, confirming the deglycosylation of anthocyanins bythe gut flora and deconjugating enzymes in the suspension (A-M Aura, et al., 2005). In contrast, small decreases were observed when anthocyanins are exposed to inactivated fecal suspension. Similar results were also observed in this research. A mechanism suggested for the gut microflora degradation of glycosylated forms of anthocyanins assumes the cleavage of the ring and production of smaller compounds. In the aglycone degradation path, with changes in pH, different structural forms are predominant in the medium. Evaluation of aglycones stability in neutral media at 37°C suggested these compounds may undergo dimerization between the quinoidal moieties, since this is the reactive species at this pH (Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006). More recently, studies have shown that anthocyanin degradation at mildly alkaline conditions go beyond the formation of quinoidal bases and undergo ring-fission with the breakdown of the flavonoid structure and formation of smaller phenolic acids and aldehydes (Anna-Marja Aura, 2008; Dall'Asta, Calani, Tedeschi, Jechiu, Brighenti, & Del Rio, 2012). The characteristics of the new formed products are dictated by the substituents in the 3' and 5' positions in the B-ring, with protocatechuic and vanillic acid as the main degradation products of cyanidin and peonidin aglycones, respectively (Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006).

In this research, a clear increase in metabolites detected at 280nm (characteristic of phenolic acids) was observed simultaneously to the degradation of anthocyanins from Black carrot (Figure 25A and B) and Purple sweet potato (Figure 26A and B). As the

majority of anthocyanins on both sources consisted of cyanidin and peonidin, protocatechuic (PCA) and vanillic acid (VA) were identified as metabolites. Maximum concentration of PCA and VA were approximately 20 and 40 μM for Black carrot while in Purple sweet potato, concentrations of PCA and VA reached 30 and 220 μM, respectively. Other metabolites were also formed, but not identified (data not shown). In all incubations with the heat-inactived suspension, the anthocyanins were stable and phenolic acids were produced at a much lesser rate.

Although low levels of metabolites were encountered, protocatechuic acid was found as the major breakdown metabolite from cyanidin, and has been reported a transient compound as it is likely converted into other metabolites with continuous exposure in the fecal suspension (Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006). In the same study VA was also reported as the main degradation compound of peonidin type anthocyanidins.

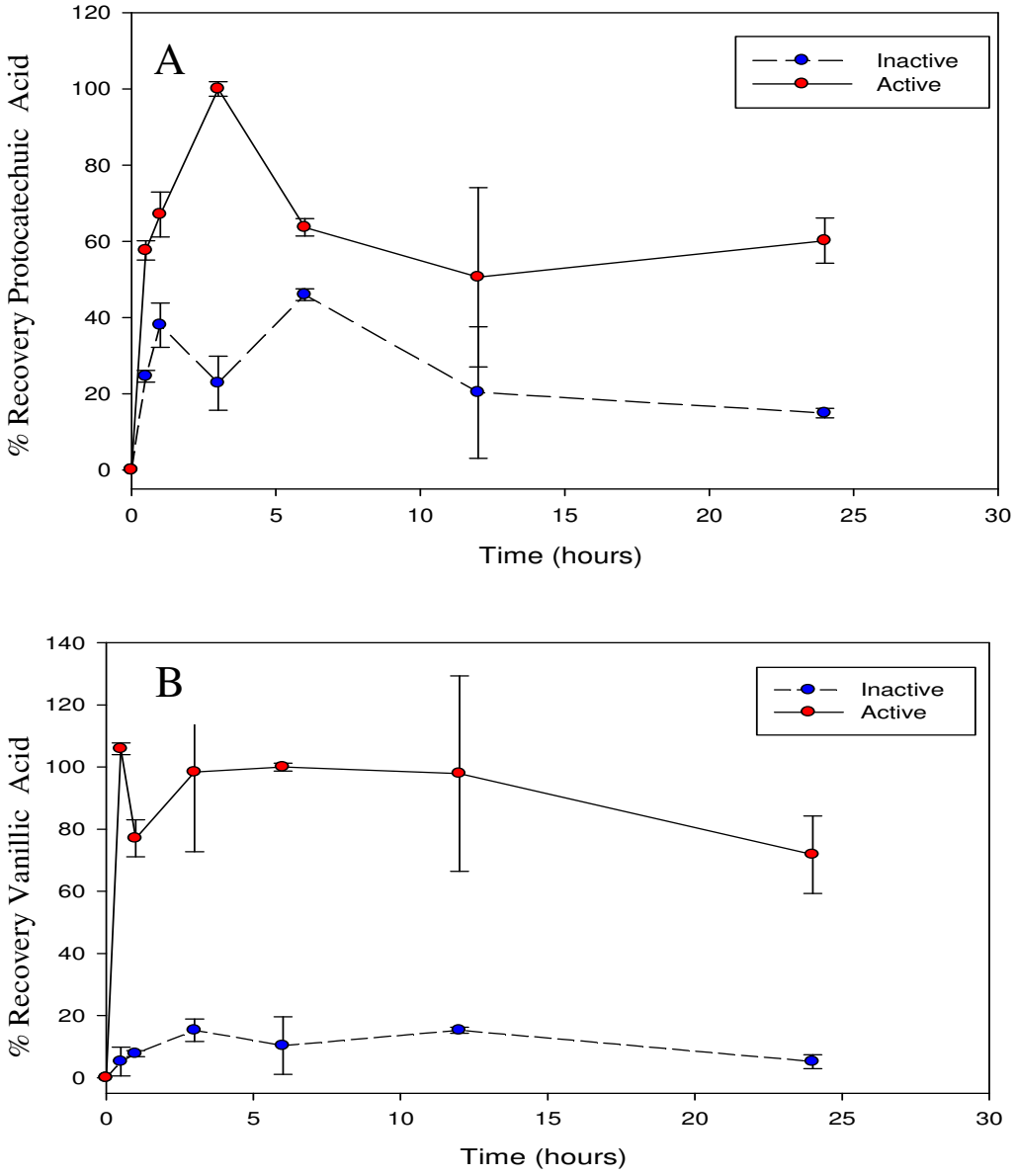

Figure 24: Time dependent formation of Protocatechuic acid (PCA- A) and Vanillic acid (VA- B) during incubation of Black carrot anthocyanins with pig fecal active and inactive microflora suspension. Values are means ± SD, n=2.

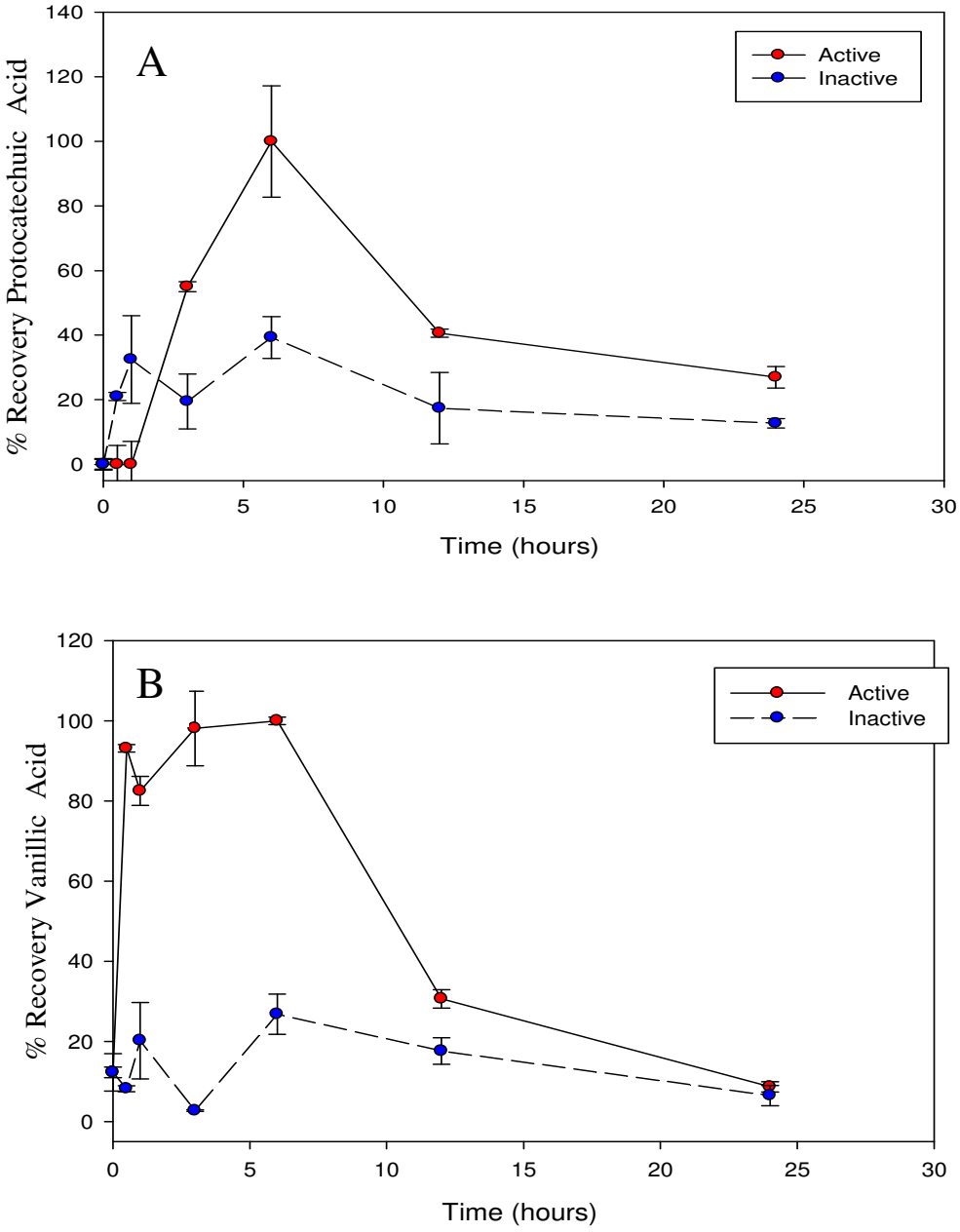

Figure 25: Time dependent formation of Protocatechuic acid (PCA- A) and Vanillic acid (VA- B) during incubation of Purple sweet potato with pig fecal active and inactive microflora suspension. Values are means ± SD, n=2.

When assessing microbiota metabolism of phenolic acids, an observed decreased in the produced acids after incubation time may be an indication that further metabolism continues to occur on these compounds, as they can also be utilized as source of energy by the intestinal bacteria (Keppler & Humpf, 2005). It was reported, only after 2 to 4 hours of incubation, a decrease of syringic and vanillic acid accompanied by an increase in gallic acid and protochatechuic acid (PCA), by O-demethylation and demethylation, respectively. This additional metabolic pathway of PCA suggests this phenolic acid presents lower degradation rate in comparison to other produced phenolic acids. Moreover, the demethylation processes were not detected in the sterilized control samples (prepared in a similar fashion as the non-sterilized fecal suspension, which is believed to contain the live bacteria), a sign that active gut microorganisms are responsible for the intense metabolism occurring to phenolic acids after anthocyanin ingestion (Keppler & Humpf, 2005).

In our study, PCA was found at a much lower concentration than vanillic acid on both sources. BC anthocyanins, which are mostly composed of mono-acylated anthocyanins, presented increased stability over time in comparison to PSP anthocyanins, an reasonable explanation to decreased recovered degradation compounds.

Non-identified compounds were formed for different anthocyanins after incubation with active fecal suspension (Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006), as observed in this research. These authors suggested the new compounds presented phenoxy acid, aldehyde or flavonoid like structures, due to UV spectrum characteristics, but were not ionized in the conditions of the experiment and thus not well identified. All metabolites were found at very low levels (less than 5%) when compared to the original dose, making it difficult for identification. Moreover, affinity to protein-

binding in the fecal matrix, anthocyanin low stability in mildly alkaline pH conditions and formation of unknown metabolites such as nitrogenous (or sulphur) based or even gases (Walle, Walle, & Halushka, 2001) were also addressed as possible causes to the low recoveries found for both parent compounds and metabolites measured in their study. Neither gallic acid nor benzaldehydes, as the opening of the aglycone would suggest, were identified in this study. The difficulty in identifying other compounds in our study such as gallic acid or aldehydes could have partially been attributed to the aforementioned reasons.

Moreover, in trying to understand the real fate of anthocyanins in the human body, it was hypothesized the low apparent bioavailability is not only caused by the bacterial biotransformation which cleaves sugars and degrades the aglycone into phenolic acids, but also by the co-existence of anthocyanin oxidation forms, which are formed due to pH shift and hydration (Castañeda-Ovando, de Lourdes Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009; Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006). Both mechanisms lead to the underestimated amounts of anthocyanins detected through the flavilium cation assays. In addition, it has been reported anthocyanins may bind to other molecules in solution such as free thiols or amino groups of protein, leading to low recovery.

The importance of exposing anthocyanins to an *in vitro* digestion prior to analyzing the metabolites formed after a fecal incubation is that it allows for a more realistic pathway of these compounds in the human body. Anthocyanins from vegetable sources presented increased stability under gastric and even intestinal digestion, differentiating them from non-acylated sources, such as fruits. However, on both cases, when exposed to neutral pH for longer periods of time, anthocyanins could dimerise via

quinoid anydrobases and form different equilibrium forms in solution (flavylium cation, hemiketal quinoid base and α-diketone); or degrade into phenolic acids and aldehyde, via the α-diketone intermediate (Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006). Both mechanisms lead to a decreased amount of parent compound, a false target when assessing potential degradation compounds in anthocyanin metabolism. Therefore, targeting the degradation compounds as well as oxidation products should be the focus on cell culture, *in vitro* and *in vivo* studies assessing health attributes of anthocyanins.

Conclusions

The anthocyanin in vitro digestion followed by fecal incubation has shown that anthocyanins stability is entirely related to its chemical structure. Non-acylated sources are easily degraded upon exposure to neutral pH conditions and would likely be fastly degraded as it reaches colon, during digestion. Hence, vegetable anthocyanin sources, such as Black carrot and Purple sweet potatoes, present a great advantage, which is the capability to withstand unfavorable conditions and be likely available to present health benefits, if not in intact form, by the degradation compounds that are formed.

Poor bioavailability of anthocyanins has raised the questions whether these compounds are indeed capable of health attributes. This research comes as evidence to show anthocyanins undergo a series of transformations in the body, due to colonic bacteria as well as increased pH conditions, forming smaller phenolic acids or aglycone conjugates which need to be better characterized and evaluated for health benefits.

CHAPTER VII

CONCLUSIONS AND FUTURE RESEARCH

In conclusion, we have demonstrated that polyphenolics in Purple Sweet Potato were obtained at higher concentrations with use of a pre-heating step sufficient to inactivate PPO and disrupt plant cell structures. The use of chemical modifiers to a standard aqueous citric acid solution was additionally beneficial to anthocyanin extraction by serving as a metal chelator and increased ionic strength of the extraction solution.

We observed PSP polyphenolics fractions, especially those containning acylated copigmented compounds, that have the capability to withstand physiological conditions of the human body and remain bioavailable after digestion, a prerequisite for providing protective health benefits upon consumption. Compounds in PSP were fully absorbed, as assessed using the the Caco2 cell model. Polyphenolic fractions in PSP also presented the ability to inhibit cancer cell growth HT-29, with little toxicity towards the non-malignant cells CCD-18Co colon fibroblast cells. More importantly, they possess strong anti-inflammatory and anti-cancer properties in colon cells, as seen by down regulations in mRNA and protein levels of important pro-inflammatory markers (NF-κB, TNF-α, IL-1β, and IL-6).

In assessing stable anthocyanin sources that could be used in food formulations and likely survive gastrointestinal tract and metabolism in colon, vegetable acylated alternatives (PSP and BC) seemed to present advantageous stability due to presence of complex anthocyanin structures which can withstand unfavorable pH conditions and be likely available during digestion to present health benefits to the human body. Moreover,

differences in anthocyanin degradation due to incubation with pig microbiota was assessed and metabolites were identified and quantified.

Overall, this research targeted to prove PSP polyphenolics contain a relatively stable source of compounds with the potential for diverse applications in food and beverages. Poor bioavailability of anthocyanins has raised questions whether these compounds are indeed capable of health benefits. This research comes as evidence to show anthocyanins undergo a series of transformations upon consumption, due to colonic bacteria as well as increased pH conditions during digestion, forming smaller phenolic acids or aglycone conjugates which need to be better characterized and evaluated for health benefits.

Further studies are still needed to better understand the bioavailability and potential health benefits of anthocyanins, metabolites and degradation compounds. Follow up studies could focus on:

How interaction in food matrices will affect absorption and stability of PSP polyphenolic compounds using in vitro and in vivo models;

Further studies of polyphenols with microbiota incubation, in order to apply our observations and theories to more complex and realistic biological systems and fully understand what the breakdown products of metabolism are;

Animal and human studies with the scope of the metabolic pathways involved in the digestion, absorption, and excretion of polyphenols and determining clinical relevance of different chemical structures of anthocyanin, as they are abundant in different forms, depending their source and the processing treatment they are subjected to.

Assessing health benefits of metabolites and degradation compounds in place of intact anthocyanins. Understanding the protective properties of PSP polyphenols in chronic disease will bring trust to consumers, who rely on scientific research to make their buying choices.

REFERENCES

Abe, S., Yamamuro, Y., Tau, K., Takenaga, F., Suzuki, K., & Oda, M. (2011). High-pressure and heat pretreatment effects on rehydration and quality of sweet potato. *Am J Food Technol, 6*, 63-71.

Aggarwal, B. B. (2004). Nuclear factor-κB: the enemy within. *Cancer cell, 6*, 203-208.

Aggarwal, B. B., Shishodia, S., Sandur, S. K., Pandey, M. K., & Sethi, G. (2006). Inflammation and cancer: how hot is the link? *Biochemical pharmacology, 72*, 1605-1621.

Aggarwal, B. B., Vijayalekshmi, R., & Sung, B. (2009). Targeting inflammatory pathways for prevention and therapy of cancer: short-term friend, long-term foe. *Clinical Cancer Research, 15*, 425-430.

Ahmed, M., Akter, M. S., & Eun, J. B. (2010). Impact of α-amylase and maltodextrin on physicochemical, functional and antioxidant capacity of spray-dried purple sweet potato flour. *Journal of the Science of Food and Agriculture, 90*, 494-502.

Almeida, M., & Nogueira, J. (1995). The control of polyphenol oxidase activity in fruits and vegetables. *Plant Foods for Human Nutrition, 47*, 245-256.

Andersen, O. M., & Markham, K. R. (2005). *Flavonoids: chemistry, biochemistry and applications*: CRC Press.

Anonymous. (2016). Natural Food Colors Market - Global Analysis, Market Size, Application Analysis, Regional Outlook, Competitive Strategies And Forecasts, 2014 To 2020 : Hexa Research. In).

Arbizu Berrocal, S. (2013). *Anti-inflammatory and Cytotoxic Activities of Mango (Mangifera indica L. var Keitt) Polyphenols in Cancer and Non-cancer Breast Fibroblasts in Vitro.*

Ardite, E., Panes, J., Miranda, M., Salas, A., Elizalde, J., Sans, M., Arce, Y., Bordas, J., Fernández-Checa, J., & Pique, J. (1998). Effects of steroid treatment on activation of nuclear factor κB in patients with inflammatory bowel disease. *British journal of pharmacology, 124*, 431-433.

Artursson, P. (1990). Epithelial transport of drugs in cell culture. I: A model for studying the passive diffusion of drugs over intestinal absorbtive (Caco-2) cells. *Journal of pharmaceutical sciences, 79*, 476-482.

Aura, A.-M. (2008). Microbial metabolism of dietary phenolic compounds in the colon. *Phytochemistry Reviews, 7*, 407-429.

Aura, A.-M., Martin-Lopez, P., O'Leary, K. A., Williamson, G., Oksman-Caldentey, K.-M., Poutanen, K., & Santos-Buelga, C. (2005). In vitro metabolism of anthocyanins by human gut microflora. *European journal of nutrition, 44*, 133-142.

Balimane, P. V., Chong, S., & Morrison, R. A. (2000). Current methodologies used for evaluation of intestinal permeability and absorption. *Journal of pharmacological and toxicological methods, 44*, 301-312.

Balkwill, F., & Mantovani, A. (2001). Inflammation and cancer: back to Virchow? *The Lancet, 357*, 539-545.

Banerjee, N., Talcott, S., Safe, S., & Mertens-Talcott, S. U. (2012). Cytotoxicity of pomegranate polyphenolics in breast cancer cells in vitro and vivo: potential role of miRNA-27a and miRNA-155 in cell survival and inflammation. *Breast cancer research and treatment, 136*, 21-34.

Barbagallo, R. N., Chisarib, M., & Patanèc, C. (2012). Use in vivo of natural anti-browning agents against polyphenol oxidase activity in minimally processed eggplant. *CHEMICAL ENGINEERING, 27*, 49-54.

Bartolome, L. G., & Hoff, J. E. (1972). Firming of potatoes. Biochemical effects of preheating. *Journal of agricultural and food chemistry, 20*, 266-270.

Basak, S., & Ramaswamy, H. (1998). Effect of high pressure processing on the texture of selected fruits and vegetables. *Journal of Texture Studies, 29*, 587-601.

Baugh, S., & Ignelzi, S. (2000). Hydrolysis and redox factors affecting analysis of common phenolic marker compounds in botanical extracts and finished products. *Journal of AOAC International, 83*, 1135-1140.

Becker, C., Fantini, M., Wirtz, S., Nikolaev, A., Lehr, H., Galle, P., John, R., & Neurath, M. (2005). IL-6 signaling promotes tumor growth in colorectal cancer. *Cell Cycle, 4*, 220-223.

Bermúdez-Soto, M.-J., Tomás-Barberán, F.-A., & García-Conesa, M.-T. (2007). Stability of polyphenols in chokeberry (*Aronia melanocarpa*) subjected to in vitro gastric and pancreatic digestion. *Food chemistry, 102*, 865-874.

Bermúdez-Soto, M. J., Tomás-Barberán, F. A., & García-Conesa, M. T. (2007). Stability of polyphenols in chokeberry (*Aronia melanocarpa*) subjected to *in vitro* gastric and pancreatic digestion. *Food chemistry, 102*, 865-874.

Blaut, M., & Clavel, T. (2007). Metabolic diversity of the intestinal microbiota: implications for health and disease. *The Journal of nutrition, 137*, 751S-755S.

Boivin, D., Blanchette, M., Barrette, S., Moghrabi, A., & Beliveau, R. (2007). Inhibition of cancer cell proliferation and suppression of TNF-induced activation of NFκB by edible berry juice. *Anticancer research, 27*, 937-948.

Bokkenheuser, V., & Winter, J. (1988). Hydrolysis of flavonoids by human intestinal bacteria. *Progress in clinical and biological research, 280*, 143.

Borges, G., Roowi, S., Rouanet, J. M., Duthie, G. G., Lean, M. E., & Crozier, A. (2007). The bioavailability of raspberry anthocyanins and ellagitannins in rats. *Molecular nutrition & food research, 51*, 714-725.

Bouayed, J., Hoffmann, L., & Bohn, T. (2011). Total phenolics, flavonoids, anthocyanins and antioxidant activity following simulated gastro-intestinal digestion and dialysis of apple varieties: Bioaccessibility and potential uptake. *Food chemistry, 128*, 14-21.

Boulton, R. (2001). The copigmentation of anthocyanins and its role in the color of red wine: a critical review. *American journal of Enology and Viticulture, 52*, 67-87.

Brouillard, R. (1982). *Chemical structure of anthocyanins* (Vol. 1): Academic Press: New York.

Brouillard, R., & Dangles, O. (1994). Anthocyanin molecular interactions: the first step in the formation of new pigments during wine aging? *Food chemistry, 51*, 365-371.

Brouillard, R., & Dubois, J.-E. (1977). Mechanism of the structural transformations of anthocyanins in acidic media. *Journal of the American Chemical Society, 99*, 1359-1364.

Brownmiller, C., Howard, L., & Prior, R. (2008). Processing and storage effects on monomeric anthocyanins, percent polymeric color, and antioxidant capacity of processed blueberry products. *Journal of food science, 73*, H72-H79.

Cani, P. D., & Delzenne, N. M. (2009). The role of the gut microbiota in energy metabolism and metabolic disease. *Current pharmaceutical design, 15*, 1546-1558.

Cao, G., & Prior, R. L. (1999). Anthocyanins are detected in human plasma after oral administration of an elderberry extract. *Clinical Chemistry, 45*, 574-576.

Cartron, E., Fouret, G., Carbonneau, M.-A., Lauret, C., Michel, F., Monnier, L., Descomps, B., & Léger, C. L. (2003). Red-wine Beneficial Long-term Effect on

Lipids but not on Antioxidant Characteristics in Plasma in a Study Comparing Three Types of Wine--Description of two methylated Derivatives of Gallic Acid in Humans. *Free radical research, 37*, 1021-1035.

Castañeda-Ovando, A., de Lourdes Pacheco-Hernández, M., Páez-Hernández, M. E., Rodríguez, J. A., & Galán-Vidal, C. A. (2009). Chemical studies of anthocyanins: A review. *Food chemistry, 113*, 859-871.

Castañeda-Ovando, A., Pacheco-Hernández, M. d. L., Páez-Hernández, M. E., Rodríguez, J. A., & Galán-Vidal, C. A. (2009). Chemical studies of anthocyanins: A review. *Food chemistry, 113*, 859-871.

Castro, V. C., Silva, P. H. A., Oliveira, E. B., Desobry, S., & Humeau, C. (2014). Extraction, identification and enzymatic synthesis of acylated derivatives of anthocyanins from jaboticaba (*Myrciaria cauliflora*) fruits. *International journal of food science & technology, 49*, 196-204.

Cevallos-Casals, B. A., & Cisneros-Zevallos, L. (2004). Stability of anthocyanin-based aqueous extracts of Andean purple corn and red-fleshed sweet potato compared to synthetic and natural colorants. *Food chemistry, 86*, 69-77.

Charron, C. S., Clevidence, B. A., Britz, S. J., & Novotny, J. A. (2007). Effect of dose size on bioavailability of acylated and nonacylated anthocyanins from red cabbage (*Brassica oleracea* L. Var. capitata). *Journal of agricultural and food chemistry, 55*, 5354-5362.

Charron, C. S., Kurilich, A. C., Clevidence, B. A., Simon, P. W., Harrison, D. J., Britz, S. J., Baer, D. J., & Novotny, J. A. (2009). Bioavailability of anthocyanins from purple carrot juice: effects of acylation and plant matrix. *Journal of agricultural and food chemistry, 57*, 1226-1230.

Cheema, S., & Sommerhalter, M. (2015). Characterization of polyphenol oxidase activity in Ataulfo mango. *Food chemistry, 171*, 382-387.

Chung, W. Y., Park, J. H., Kim, M. J., Kim, H. O., Hwang, J. K., Lee, S. K., & Park, K. K. (2007). Xanthorrhizol inhibits 12-O-tetradecanoylphorbol-13-acetate-induced acute inflammation and two-stage mouse skin carcinogenesis by blocking the expression of ornithine decarboxylase, cyclooxygenase-2 and inducible nitric oxide synthase through mitogen-activated protein kinases and/or the nuclear factor-κB. *Carcinogenesis, 28*, 1224-1231.

Cipriano, P. A., Ekici, L., Barnes, R. C., Gomes, C., & Talcott, S. T. (2015). Pre-heating and polyphenol oxidase inhibition impact on extraction of purple sweet potato anthocyanins. *Food chemistry, 180*, 227-234.

Clifford, M. N. (2000). Anthocyanins–nature, occurrence and dietary burden. *Journal of the Science of Food and Agriculture, 80*, 1063-1072.

Coates, E. M., Popa, G., Gill, C. I., McCann, M. J., McDougall, G. J., Stewart, D., & Rowland, I. (2007). Colon-available raspberry polyphenols exhibit anti-cancer effects on in vitro models of colon cancer. *Journal of carcinogenesis, 6*, 4.

Coussens, L. M., & Werb, Z. (2001). Inflammatory Cells and Cancer Think Different! *The Journal of experimental medicine, 193*, F23-F26.

Dalgleish, A. G., & O'Byrne, K. (2006). Inflammation and Cancer. In *The Link Between Inflammation and Cancer*, (pp. 1-38): Springer.

Dall'Asta, M., Calani, L., Tedeschi, M., Jechiu, L., Brighenti, F., & Del Rio, D. (2012). Identification of microbial metabolites derived from in vitro fecal fermentation of different polyphenolic food sources. *Nutrition, 28*, 197-203.

Dangles, O., Saito, N., & Brouillard, R. (1993). Anthocyanin intramolecular copigment effect. *Phytochemistry, 34*, 119-124.

Davies, A., & Mazza, G. (1993). Copigmentation of simple and acylated anthocyanins with colorless phenolic compounds. *Journal of agricultural and food chemistry, 41*, 716-720.

Day, A. J., Cañada, F. J., Díaz, J. C., Kroon, P. A., Mclauchlan, R., Faulds, C. B., Plumb, G. W., Morgan, M. R., & Williamson, G. (2000). Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. *FEBS letters, 468*, 166-170.

de Aguiar Cipriano, P., Ekici, L., Barnes, R. C., Gomes, C., & Talcott, S. T. (2015). Pre-heating and polyphenol oxidase inhibition impact on extraction of purple sweet potato anthocyanins. *Food chemistry, 180*, 227-234.

de Rijke, E., Out, P., Niessen, W. M., Ariese, F., Gooijer, C., & Udo, A. T. (2006). Analytical separation and detection methods for flavonoids. *Journal of Chromatography A, 1112*, 31-63.

Del Follo, A., Noratto, G. D., & Mertens-Talcott, S. (2009). Resveratrol and quercetin target microRNA 27a in their anti-cancer effects in colon cancer cells. *The FASEB Journal, 23*, 71-74.

Dupas, C., Marsset Baglieri, A., Ordonaud, C., Tomé, D., & Maillard, M. N. (2006a). Chlorogenic acid is poorly absorbed, independently of the food matrix: a Caco-2 cells and rat chronic absorption study. *Molecular nutrition & food research, 50*(11), 1053-1060.

Dupas, C., Marsset Baglieri, A., Ordonaud, C., Tomé, D., & Maillard, M. N. (2006b). Chlorogenic acid is poorly absorbed, independently of the food matrix: a Caco-2 cells

and rat chronic absorption study. *Molecular nutrition & food research, 50*, 1053-1060.

Eaden, J., Abrams, K., & Mayberry, J. (2001). The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut, 48*, 526-535.

Eidhin, N., Degn, P., & O'beirne, D. (2010). Characterization of polyphenol oxidase from rooster potato (*solanum tuberosum* cv rooster). *Journal of Food Biochemistry, 34*, 13-30.

Eiro, M. J., & Heinonen, M. (2002). Anthocyanin color behavior and stability during storage: effect of intermolecular copigmentation. *Journal of agricultural and food chemistry, 50*, 7461-7466.

Engman, H. (2003). Intestinal barriers to oral drug absorption: Cytochrome P450 3A and ABC-transport proteins. 61p.

Fan-Chiang, H. J., & Wrolstad, R. E. (2005). Anthocyanin pigment composition of blackberries. *Journal of food science, 70*, C198-C202.

Felgines, C., Talavera, S., Texier, O., Gil-Izquierdo, A., Lamaison, J.-L., & Remesy, C. (2005). Blackberry anthocyanins are mainly recovered from urine as methylated and glucuronidated conjugates in humans. *Journal of agricultural and food chemistry, 53*, 7721-7727.

Fernandes, I., Faria, A., Calhau, C., de Freitas, V., & Mateus, N. (2014). Bioavailability of anthocyanins and derivatives. *Journal of functional foods, 7*, 54-66.

Fleschhut, J., Kratzer, F., Rechkemmer, G., & Kulling, S. E. (2006). Stability and biotransformation of various dietary anthocyanins in vitro. *European journal of nutrition, 45*, 7-18.

Forester, S. C., & Waterhouse, A. L. (2008). Identification of Cabernet Sauvignon anthocyanin gut microflora metabolites. *Journal of agricultural and food chemistry, 56*, 9299-9304.

Forester, S. C., & Waterhouse, A. L. (2010). Gut metabolites of anthocyanins, gallic acid, 3-O-methylgallic acid, and 2, 4, 6-trihydroxybenzaldehyde, inhibit cell proliferation of Caco-2 cells. *Journal of agricultural and food chemistry, 58*, 5320-5327.

Frank, T., Netzel, M., Strass, G., Bitsch, R., & Bitsch, I. (2003). Bioavailability of anthocyanidin-3-glucosides following consumption of red wine and red grape juice. *Canadian journal of physiology and pharmacology, 81*, 423-435.

Gao, L., & Mazza, G. (1994). Quantitation and distribution of simple and acylated anthocyanins and other phenolics in blueberries. *Journal of food science, 59*, 1057-1059.

Ghosh, D., & Konishi, T. (2007). Anthocyanins and anthocyanin-rich extracts: role in diabetes and eye function. *Asia Pacific journal of clinical nutrition, 16*, 200.

Gil-Izquierdo, A., Zafrilla, P., & Tomás-Barberán, F. A. (2002). An *in vitro* method to simulate phenolic compound release from the food matrix in the gastrointestinal tract. *European Food Research and Technology, 214*, 155-159.

Giusti, M., & Wrolstad, R. (2003). Acylated anthocyanins from edible sources and their application in food systems. *Biochem. Eng. J, 14*, 217-225.

Giusti, M. M., & Wrolstad, R. E. (2003). Acylated anthocyanins from edible sources and their applications in food systems. *Biochemical Engineering Journal, 14*, 217-225.

Gonthier, M.-P., Verny, M.-A., Besson, C., Rémésy, C., & Scalbert, A. (2003). Chlorogenic acid bioavailability largely depends on its metabolism by the gut microflora in rats. *The Journal of nutrition, 133*, 1853-1859.

Gould, K., Davies, K. M., & Winefield, C. (2008). *Anthocyanins: biosynthesis, functions, and applications*: Springer Science & Business Media.

Greten, F. R., Eckmann, L., Greten, T. F., Park, J. M., Li, Z.-W., Egan, L. J., Kagnoff, M. F., & Karin, M. (2004). IKKβ links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell, 118*, 285-296.

Guiusti, M., & Wrolstad, R. (2003). Acylated anthocyanins from edible sources and their application in food systems. *Biochemical Engineering Journal, 14*, 217-225.

Haekkinen, S. H., Kaérenlampi, S. O., Heinonen, I. M., Mykkänen, H. M., & Toerroenen, A. R. (1998). HPLC method for screening of flavonoids and phenolic acids in berries. *Journal of the Science of Food and Agriculture, 77*, 543-551.

Harada, K., Kano, M., Takayanagi, T., Yamakawa, O., & Ishikawa, F. (2004). Absorption of acylated anthocyanins in rats and humans after ingesting an extract of Ipomoea batatas purple sweet potato tuber. *Bioscience, biotechnology, and biochemistry, 68*, 1500-1507.

Harborne, J. B., & Williams, C. A. (2000). Advances in flavonoid research since 1992. *Phytochemistry, 55*, 481-504.

He, F., Mu, L., Yan, G.-L., Liang, N.-N., Pan, Q.-H., Wang, J., Reeves, M. J., & Duan, C.-Q. (2010). Biosynthesis of anthocyanins and their regulation in colored grapes. *Molecules, 15*, 9057-9091.

He, J., & Giusti, M. M. (2010). Anthocyanins: natural colorants with health-promoting properties. *Annual review of food science and technology, 1*, 163-187.

He, J., Magnuson, B. A., & Giusti, M. M. (2005). Analysis of anthocyanins in rat intestinal contents impact of anthocyanin chemical structure on fecal excretion. *Journal of agricultural and food chemistry, 53*, 2859-2866.

He, J., Magnuson, B. A., Lala, G., Tian, Q., Schwartz, S. J., & Giusti, M. M. (2006). Intact anthocyanins and metabolites in rat urine and plasma after 3 months of anthocyanin supplementation. *Nutrition and Cancer, 54*, 3-12.

Hertog, M. G., Hollman, P. C., Katan, M. B., & Kromhout, D. (1993). Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. *Nutrition and Cancer, 20*, 21-29.

Hidalgo, I. J., Raub, T. J., & Borchardt, R. T. (1989). Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology, 96*, 736-749.

Hong, V., & Wrolstad, R. E. (1990). Use of HPLC separation/photodiode array detection for characterization of anthocyanins. *Journal of agricultural and food chemistry, 38*, 708-715.

Hou, D.-X. (2003). Potential mechanisms of cancer chemoprevention by anthocyanins. *Current molecular medicine, 3*, 149-159.

Hsu, T., Daniel, S. L., Lux, M. F., & Drake, H. L. (1990). Biotransformations of carboxylated aromatic compounds by the acetogen Clostridium thermoaceticum: generation of growth-supportive CO2 equivalents under CO2-limited conditions. *Journal of bacteriology, 172*, 212-217.

Hu, M., Ling, J., Lin, H., & Chen, J. (2004). Use of Caco-2 cell monolayers to study drug absorption and metabolism. *Optimization in drug discovery: in vitro methods*, 19-35.

Huang, C., Huang, Y., Li, J., Hu, W., Aziz, R., Tang, M.-s., Sun, N., Cassady, J., & Stoner, G. D. (2002). Inhibition of benzo (a) pyrene diol-epoxide-induced transactivation of activated protein 1 and nuclear factor κB by black raspberry extracts. *Cancer research, 62*, 6857-6863.

Huang, D.-J., Chun-Der, L., Hsien-Jung, C., & Yaw-Huei, L. (2004). Antioxidant and antiproliferative activities of sweet potato (*Ipomoea batatas* [L.] 'LamTainong 57') constituents. *Botanical Bulletin of Academia Sinica, 45*.

Hwang, Y. P., Choi, J. H., Yun, H. J., Han, E. H., Kim, H. G., Kim, J. Y., Park, B. H., Khanal, T., Choi, J. M., & Chung, Y. C. (2011). Anthocyanins from purple sweet potato attenuate dimethylnitrosamine-induced liver injury in rats by inducing Nrf2-mediated antioxidant enzymes and reducing COX-2 and iNOS expression. *Food and chemical toxicology, 49*, 93-99.

Islam, M. S., Yoshimoto, M., Yahara, S., Okuno, S., Ishiguro, K., & Yamakawa, O. (2002). Identification and characterization of foliar polyphenolic composition in sweetpotato (*Ipomoea batatas* L.) genotypes. *Journal of agricultural and food chemistry, 50*, 3718-3722.

Jang, J., & Song, K. (2004). Purification of Polyphenoloxidase from the Purple-fleshed Potato (*Solanum tuberosum* Jasim) and its Secondary Structure. *Journal of food science, 69*, C648-C651.

Jiang, Y., Pen, L., & Li, J. (2004). Use of citric acid for shelf life and quality maintenance of fresh-cut Chinese water chestnut. *Journal of Food Engineering, 63*, 325-328.

Jing, P. (2006). *Purple corn anthocyanins: chemical structure, chemoprotective sctivity and structure/function relationships.* The Ohio State University.

Jing, P., Bomser, J. A., Schwartz, S. J., He, J., Magnuson, B. A., & Giusti, M. M. n. (2008). Structure– function relationships of anthocyanins from various anthocyanin-rich extracts on the inhibition of colon cancer cell growth. *Journal of agricultural and food chemistry, 56*(20), 9391-9398.

Jing, P., Zhao, S.-J., Ruan, S.-Y., Xie, Z.-H., Dong, Y., & Yu, L. L. (2012). Anthocyanin and glucosinolate occurrences in the roots of Chinese red radish (*Raphanus sativus* L.), and their stability to heat and pH. *Food chemistry, 133*, 1569-1576.

Joseph, S. V., Edirisinghe, I., & Burton-Freeman, B. M. (2015). Fruit polyphenols: a review of anti-inflammatory effects in humans. *Critical reviews in food science and nutrition*(just-accepted), 00-00.

Jung, H., Lee, H. J., Cho, H., & Hwang, K. T. (2015). Anti-Inflammatory Activities of Rubus Fruit Anthocyanins in Inflamed Human Intestinal Epithelial Cells. *Journal of Food Biochemistry, 39*, 300-309.

Jurenka, J. (2008). Therapeutic applications of pomegranate (*Punica granatum* L.): a review. *Alternative medicine review, 13*, 128.

Kader, F., Irmouli, M., Nicolas, J., & Metche, M. (2002). Involvement of blueberry peroxidase in the mechanisms of anthocyanin degradation in blueberry juice. *Journal of food science, 67*, 910-915.

Kader, F., Rovel, B., Girardin, M., & Metche, M. (1997). Mechanism of browning in fresh highbush blueberry fruit (*Vaccinium corymbosum* L). Role of blueberry polyphenol oxidase, chlorogenic acid and anthocyanins. *Journal of the Science of Food and Agriculture, 74*, 31-34.

Kammerer, D., Carle, R., & Schieber, A. (2004a). Characterization of phenolic acids in black carrots (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) by high-performance liquid chromatography/electrospray ionization mass spectrometry. *Rapid Communications in Mass Spectrometry, 18*, 1331-1340.

Kammerer, D., Carle, R., & Schieber, A. (2004b). Quantification of anthocyanins in black carrot extracts (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) and

evaluation of their color properties. *European Food Research and Technology, 219*, 479-486.

Karin, M. (2006). Nuclear factor-κB in cancer development and progression. *Nature, 441*, 431-436.

Katsube, N., Iwashita, K., Tsushida, T., Yamaki, K., & Kobori, M. (2003). Induction of apoptosis in cancer cells by bilberry (Vaccinium myrtillus) and the anthocyanins. *Journal of agricultural and food chemistry, 51*(1), 68-75.

Kay, C. D., Mazza, G., Holub, B. J., & Wang, J. (2004). Anthocyanin metabolites in human urine and serum. *British Journal of Nutrition, 91*, 933-942.

Kay, C. D., Mazza, G. J., & Holub, B. J. (2005). Anthocyanins exist in the circulation primarily as metabolites in adult men. *The Journal of nutrition, 135*, 2582-2588.

Keppler, K., & Humpf, H.-U. (2005). Metabolism of anthocyanins and their phenolic degradation products by the intestinal microflora. *Bioorganic & medicinal chemistry, 13*, 5195-5205.

Kim, D.-H., Jung, E.-A., Sohng, I.-S., Han, J.-A., Kim, T.-H., & Han, M. J. (1998). Intestinal bacterial metabolism of flavonoids and its relation to some biological activities. *Archives of pharmacal research, 21*, 17-23.

Kim, H. W., Kim, J. B., Cho, S. M., Chung, M. N., Lee, Y. M., Chu, S. M., Che, J. H., Kim, S. N., Kim, S. Y., & Cho, Y. S. (2012). Anthocyanin changes in the Korean purple-fleshed sweet potato, Shinzami, as affected by steaming and baking. *Food chemistry, 130*, 966-972.

Kim, S., Keku, T. O., Martin, C., Galanko, J., Woosley, J. T., Schroeder, J. C., Satia, J. A., Halabi, S., & Sandler, R. S. (2008). Circulating levels of inflammatory cytokines and risk of colorectal adenomas. *Cancer research, 68*, 323-328.

Konczak-Islam, I., Okuno, S., Yoshimoto, M., & Yamakawa, O. (2003). Composition of phenolics and anthocyanins in a sweet potato cell suspension culture. *Biochemical Engineering Journal, 14*, 155-161.

Kong, J.-M., Chia, L.-S., Goh, N.-K., Chia, T.-F., & Brouillard, R. (2003). Analysis and biological activities of anthocyanins. *Phytochemistry, 64*, 923-933.

Konishi, Y., & Kobayashi, S. (2004). Transepithelial transport of chlorogenic acid, caffeic acid, and their colonic metabolites in intestinal Caco-2 cell monolayers. *Journal of agricultural and food chemistry, 52*(9), 2518-2526.

Krook, M. A., & Hagerman, A. E. (2012). Stability of polyphenols epigallocatechin gallate and pentagalloyl glucose in a simulated digestive system. *Food Research International, 49*, 112-116.

Kroon, P. A., Clifford, M. N., Crozier, A., Day, A. J., Donovan, J. L., Manach, C., & Williamson, G. (2004). How should we assess the effects of exposure to dietary polyphenols *in vitro*? *The American journal of clinical nutrition, 80*, 15-21.

Kurilich, A. C., Clevidence, B. A., Britz, S. J., Simon, P. W., & Novotny, J. A. (2005). Plasma and urine responses are lower for acylated vs nonacylated anthocyanins from raw and cooked purple carrots. *Journal of agricultural and food chemistry, 53*, 6537-6542.

Lachman, J., Hamouz, K., Orsák, M., Pivec, V., Hejtmánková, K., Pazderů, K., Dvořák, P., & Čepl, J. (2012). Impact of selected factors–cultivar, storage, cooking and baking on the content of anthocyanins in coloured-flesh potatoes. *Food chemistry, 133*, 1107-1116.

Lapidot, T., Harel, S., Granit, R., & Kanner, J. (1998). Bioavailability of red wine anthocyanins as detected in human urine. *Journal of agricultural and food chemistry, 46*, 4297-4302.

Lee, S. G., Kim, B., Yang, Y., Pham, T. X., Park, Y.-K., Manatou, J., Koo, S. I., Chun, O. K., & Lee, J.-Y. (2014). Berry anthocyanins suppress the expression and secretion of proinflammatory mediators in macrophages by inhibiting nuclear translocation of NF-κB independent of NRF2-mediated mechanism. *The Journal of nutritional biochemistry, 25*, 404-411.

Leite, A. V., Malta, L. G., Riccio, M. F., Eberlin, M. N., Pastore, G. M., & Marostica Junior, M. R. (2011). Antioxidant potential of rat plasma by administration of freeze-dried jaboticaba peel (Myrciaria jaboticaba Vell Berg). *Journal of agricultural and food chemistry, 59*, 2277-2283.

Ley, R. E., Bäckhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D., & Gordon, J. I. (2005). Obesity alters gut microbial ecology. *Proceedings of the National Academy of Sciences of the United States of America, 102*, 11070-11075.

Li, A. P. (2001). Screening for human ADME/Tox drug properties in drug discovery. *Drug discovery today, 6*, 357-366.

Li, J., Li, X.-d., Zhang, Y., Zheng, Z.-d., Qu, Z.-y., Liu, M., Zhu, S.-h., Liu, S., Wang, M., & Qu, L. (2013). Identification and thermal stability of purple-fleshed sweet potato anthocyanins in aqueous solutions with various pH values and fruit juices. *Food chemistry, 136*, 1429-1434.

Lim, S., Xu, J., Kim, J., Chen, T. Y., Su, X., Standard, J., Carey, E., Griffin, J., Herndon, B., & Katz, B. (2013). Role of anthocyanin-enriched purple-fleshed sweet potato p40 in colorectal cancer prevention. *Molecular nutrition & food research, 57*, 1908-1917.

Liu, Y., Zhang, D., Wu, Y., Wang, D., Wei, Y., Wu, J., & Ji, B. (2014). Stability and absorption of anthocyanins from blueberries subjected to a simulated digestion process. *International journal of food sciences and nutrition, 65*, 440-448.

Lu, L. Z., Zhou, Y. Z., Zhang, Y. Q., Ma, Y. L., Zhou, L. X., Li, L., Zhou, Z. Z., & He, T. Z. (2010). Anthocyanin extracts from purple sweet potato by means of microwave baking and acidified electrolysed water and their antioxidation in vitro. *International journal of food science & technology, 45*, 1378-1385.

Ludwig, I. A., Mena, P., Calani, L., Borges, G., Pereira-Caro, G., Bresciani, L., Del Rio, D., Lean, M. E., & Crozier, A. (2015). New insights into the bioavailability of red raspberry anthocyanins and ellagitannins. *Free Radical Biology and Medicine, 89*, 758-769.

Manach, C., Williamson, G., Morand, C., Scalbert, A., & Rémésy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *The American journal of clinical nutrition, 81*, 230S-242S.

Markovic, D., Petranovic, N. A., & Baranac, J. M. (2000). A spectrophotometric study of the copigmentation of malvin with caffeic and ferulic acids. *Journal of agricultural and food chemistry, 48*, 5530-5536.

Martinez, M. V., & Whitaker, J. R. (1995). The biochemistry and control of enzymatic browning. *Trends in Food Science & Technology, 6*, 195-200.

Matsumoto, H., Nakamura, Y., Hirayama, M., Yoshiki, Y., & Okubo, K. (2002). Antioxidant activity of black currant anthocyanin aglycons and their glycosides measured by chemiluminescence in a neutral pH region and in human plasma. *Journal of agricultural and food chemistry, 50*, 5034-5037.

Mazza, G., & Brouillard, R. (1990). The mechanism of co-pigmentation of anthocyanins in aqueous solutions. *Phytochemistry, 29*, 1097-1102.

Mazza, G., & Kay, C. D. (2008). Bioactivity, absorption, and metabolism of anthocyanins. *Recent advances in polyphenols research, 1*, 228-262.

Mazza, G., Kay, C. D., Cottrell, T., & Holub, B. J. (2002). Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. *Journal of agricultural and food chemistry, 50*, 7731-7737.

Mazza, G., & Miniati, E. (1993). *Anthocyanins in fruits, vegetables, and grains*: CRC press.

McCann, D., Barrett, A., Cooper, A., Crumpler, D., Dalen, L., Grimshaw, K., Kitchin, E., Lok, K., Porteous, L., & Prince, E. (2007). Food additives and hyperactive behaviour in 3-year-old and 8/9-year-old children in the community: a randomised, double-blinded, placebo-controlled trial. *The Lancet, 370*, 1560-1567.

McDougall, G. J., Dobson, P., Smith, P., Blake, A., & Stewart, D. (2005). Assessing potential bioavailability of raspberry anthocyanins using an in vitro digestion system. *Journal of agricultural and food chemistry, 53*, 5896-5904.

McDougall, G. J., Fyffe, S., Dobson, P., & Stewart, D. (2007). Anthocyanins from red cabbage–stability to simulated gastrointestinal digestion. *Phytochemistry, 68*, 1285-1294.

McGhie, T. K., & Walton, M. C. (2007). The bioavailability and absorption of anthocyanins: towards a better understanding. *Molecular nutrition & food research, 51*, 702-713.

Mertz, C., Cheynier, V., Günata, Z., & Brat, P. (2007). Analysis of phenolic compounds in two blackberry species (*Rubus glaucus* and *Rubus adenotrichus*) by high-

performance liquid chromatography with diode array detection and electrospray ion trap mass spectrometry. *Journal of agricultural and food chemistry, 55*, 8616-8624.

Montilla, E. C., Arzaba, M. R., Hillebrand, S., & Winterhalter, P. (2011). Anthocyanin composition of black carrot (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) cultivars antonina, beta sweet, deep purple, and purple haze. *Journal of agricultural and food chemistry, 59*, 3385-3390.

Montilla, E. C., Hillebrand, S., Butschbach, D., Baldermann, S., Watanabe, N., & Winterhalter, P. (2010). Preparative isolation of anthocyanins from Japanese purple sweet potato (*Ipomoea batatas* L.) varieties by high-speed countercurrent chromatography. *Journal of agricultural and food chemistry, 58*, 9899-9904.

Mosele, J. I., Macià, A., Romero, M.-P., Motilva, M.-J., & Rubió, L. (2015). Application of in vitro gastrointestinal digestion and colonic fermentation models to pomegranate products (juice, pulp and peel extract) to study the stability and catabolism of phenolic compounds. *Journal of functional foods, 14*, 529-540.

Moyer, R. A., Hummer, K. E., Finn, C. E., Frei, B., & Wrolstad, R. E. (2002). Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: Vaccinium, Rubus, and Ribes. *Journal of agricultural and food chemistry, 50*, 519-525.

Nayak, B., Berrios, J. D. J., Powers, J. R., & Tang, J. (2011). Thermal degradation of anthocyanins from purple potato (Cv. *Purple Majesty*) and impact on antioxidant capacity. *Journal of agricultural and food chemistry, 59*, 11040-11049.

Ni Eidhin, D., Degn, P., & O'beirne, D. (2010). Characterization of polyphenol oxidase from rooster potato (*solanum tuberosum* cv rooster). *Journal of Food Biochemistry, 34*, 13-30.

Noda, T., Takahata, Y., Sato, T., Ikoma, H., & Mochida, H. (1996). Physicochemical properties of starches from purple and orange fleshed sweet potato roots at two levels of fertilizer. *Starch-Stärke, 48*, 395-399.

Noratto, G. D., Bertoldi, M. C., Krenek, K., Talcott, S. T., Stringheta, P. C., & Mertens-Talcott, S. U. (2010). Anticarcinogenic effects of polyphenolics from mango (*Mangifera indica*) varieties. *Journal of agricultural and food chemistry, 58*, 4104-4112.

Noratto, G. D., Kim, Y., Talcott, S. T., & Mertens-Talcott, S. U. (2011). Flavonol-rich fractions of yaupon holly leaves (*Ilex vomitoria,* Aquifoliaceae) induce microRNA-146a and have anti-inflammatory and chemopreventive effects in intestinal myofribroblast CCD-18Co cells. *Fitoterapia, 82*, 557-569.

Pacheco-palencia, L. A., Hawken, P., & Talcott, S. T. (2007). Juice matrix composition and ascorbic acid fortification effects on the phytochemical, antioxidant and pigment stability of acai (Euterpe oleracea Mart.). *Food chemistry, 105*, 28-35.

Pacheco-Palencia, L. A., Mertens-Talcott, S. U., & Talcott, S. T. (2010). In vitro absorption and antiproliferative activities of monomeric and polymeric anthocyanin fractions from açai fruit (*Euterpe oleracea* Mart.). *Food chemistry, 119*, 1071-1078.

Pacheco-Palencia, L. A., Talcott, S. T., Safe, S., & Mertens-Talcott, S. (2008). Absorption and biological activity of phytochemical-rich extracts from acai (*Euterpe oleracea* Mart.) pulp and oil *in vitro*. *Journal of agricultural and food chemistry, 56*, 3593-3600.

Padda, M. S., & Picha, D. H. (2008). Quantification of phenolic acids and antioxidant activity in sweetpotato genotypes. *Scientia Horticulturae, 119*, 17-20.

Pathi, S. S., Jutooru, I., Chadalapaka, G., Sreevalsan, S., Anand, S., Thatcher, G. R., & Safe, S. (2011). GT-094, a NO-NSAID, inhibits colon cancer cell growth by activation of a reactive oxygen species-microRNA-27a: ZBTB10-specificity protein pathway. *Molecular Cancer Research, 9*, 195-202.

Pérez-Vicente, A., Gil-Izquierdo, A., & García-Viguera, C. (2002). *In vitro* gastrointestinal digestion study of pomegranate juice phenolic compounds, anthocyanins, and vitamin C. *Journal of agricultural and food chemistry, 50*, 2308-2312.

Podsędek, A., Redzynia, M., Klewicka, E., & Koziołkiewicz, M. (2014). Matrix effects on the stability and antioxidant activity of red cabbage anthocyanins under simulated gastrointestinal digestion. *BioMed Research International, 2014*, 1-11.

Pond, W., & Houpt, K. (1978). The biology of the pig, Comstock Pub. *Associates, Ithaca, NY*.

Pond, W. G., & Mersmann, H. J. (2001). *Biology of the domestic pig*: Comstock Pub. Associates, Cornell University Press.

Popivanova, B. K., Kitamura, K., Wu, Y., Kondo, T., Kagaya, T., Kaneko, S., Oshima, M., Fujii, C., & Mukaida, N. (2008). Blocking TNF-α in mice reduces colorectal carcinogenesis associated with chronic colitis. *The Journal of clinical investigation, 118*, 560-570.

Pozo-Insfran, D., Follo-Martinez, D., Talcott, S., & Brenes, C. (2007). Stability of copigmented anthocyanins and ascorbic acid in muscadine grape juice processed by high hydrostatic pressure. *Journal of food science, 72*, S247-S253.

Prior, R. L., Wu, X., Gu, L., Hager, T. J., Hager, A., & Howard, L. R. (2008). Whole berries versus berry anthocyanins: interactions with dietary fat levels in the C57BL/6J mouse model of obesity. *Journal of agricultural and food chemistry, 56*, 647-653.

Queiroz, C., da Silva, A. J. R., Lopes, M. L. M., Fialho, E., & Valente-Mesquita, V. L. (2011). Polyphenol oxidase activity, phenolic acid composition and browning in cashew apple (*Anacardium occidentale*, L.) after processing. *Food chemistry, 125*, 128-132.

Ramos, S. (2008). Cancer chemoprevention and chemotherapy: dietary polyphenols and signalling pathways. *Molecular nutrition & food research, 52*, 507-526.

Rathee, P., Chaudhary, H., Rathee, S., Rathee, D., Kumar, V., & Kohli, K. (2009). Mechanism of action of flavonoids as anti-inflammatory agents: a review. *Inflammation & Allergy-Drug Targets (Formerly Current Drug Targets-Inflammation & Allergy), 8*, 229-235.

Rechner, A. R., Smith, M. A., Kuhnle, G., Gibson, G. R., Debnam, E. S., Srai, S. K. S., Moore, K. P., & Rice-Evans, C. A. (2004). Colonic metabolism of dietary polyphenols: influence of structure on microbial fermentation products. *Free Radical Biology and Medicine, 36*, 212-225.

Reddy, M. K., Alexander-Lindo, R. L., & Nair, M. G. (2005). Relative inhibition of lipid peroxidation, cyclooxygenase enzymes, and human tumor cell proliferation by natural food colors. *Journal of agricultural and food chemistry, 53*, 9268-9273.

Rein, M. J., & Heinonen, M. (2004). Stability and enhancement of berry juice color. *Journal of agricultural and food chemistry, 52*, 3106-3114.

Reuter, S., Gupta, S. C., Chaturvedi, M. M., & Aggarwal, B. B. (2010). Oxidative stress, inflammation, and cancer: how are they linked? *Free Radical Biology and Medicine, 49*, 1603-1616.

Reyes, L. F., & Cisneros-Zevallos, L. (2007). Degradation kinetics and colour of anthocyanins in aqueous extracts of purple-and red-flesh potatoes (< i> Solanum tuberosum</i> L.). *Food chemistry, 100*, 885-894.

Reynertson, K. A., Yang, H., Jiang, B., Basile, M. J., & Kennelly, E. J. (2008). Quantitative analysis of antiradical phenolic constituents from fourteen edible Myrtaceae fruits. *Food chemistry, 109*, 883-890.

Robinson, G. M., & Robinson, R. (1931). A survey of anthocyanins. I. *Biochemical Journal, 25*, 1687.

Rodriguez-Saona, L. E., & Wrolstad, R. E. (2001). Extraction, isolation, and purification of anthocyanins. In R. E. Wrolstad (Ed.), *Current protocols in food analytical chemistry*, (pp. Unit F1.1.1-11). New York: John Wiley & Sons.

Rogler, G., Brand, K., Vogl, D., Page, S., Hofmeister, R., Andus, T., Knuechel, R., Baeuerle, P. A., Schölmerich, J., & Gross, V. (1998). Nuclear factor κB is activated in macrophages and epithelial cells of inflamed intestinal mucosa. *Gastroenterology, 115*, 357-369.

Rossi, A., Serraino, I., Dugo, P., Di Paola, R., Mondello, L., Genovese, T., Morabito, D., Dugo, G., Sautebin, L., & Caputi, A. P. (2003). Protective effects of anthocyanins from blackberry in a rat model of acute lung inflammation. *Free radical research, 37*, 891-900.

Russo, G. L. (2007). Ins and outs of dietary phytochemicals in cancer chemoprevention. *Biochemical pharmacology, 74*, 533-544.

Ryan, L., & Prescott, S. L. (2010). Stability of the antioxidant capacity of twenty-five commercially available fruit juices subjected to an in vitro digestion. *International journal of food science & technology, 45*, 1191-1197.

Sadilova, E., Carle, R., & Stintzing, F. C. (2007). Thermal degradation of anthocyanins and its impact on color and in vitro antioxidant capacity. *Molecular nutrition & food research, 51*, 1461-1471.

Sadilova, E., Stintzing, F., & Carle, R. (2006). Thermal degradation of acylated and nonacylated anthocyanins. *Journal of food science, 71*, C504-C512.

Santiago, M. C. P. d. A., Gouvêa, A. C. M. S., Godoy, R. L. d. O., Borguini, R. G., Pacheco, S., Nogueira, R. I., Nascimento, L. d. S. d. M., & Freitas, S. P. (2014). Analytical standards production for the analysis of pomegranate anthocyanins by HPLC. *Brazilian Journal of Food Technology, 17*, 51-57.

Sapers, G. (1993). Browning of foods: control by sulfites, antioxidants, and other means. *Food Technology, 47*, 75-84.

Schumacker, P. T. (2006). Reactive oxygen species in cancer cells: live by the sword, die by the sword. *Cancer cell, 10*, 175-176.

Selma, M. V., Espin, J. C., & Tomas-Barberan, F. A. (2009). Interaction between phenolics and gut microbiota: role in human health. *Journal of agricultural and food chemistry, 57*, 6485-6501.

Shacter, E., & Weitzman, S. A. (2002). Chronic inflammation and cancer. *Oncology, 16*, 217-226.

Shahidi, F., Janitha, P., & Wanasundara, P. (1992). Phenolic antioxidants. *Critical reviews in food science & nutrition, 32*, 67-103.

Shan, Q., Zheng, Y., Lu, J., Zhang, Z., Wu, D., Fan, S., Hu, B., Cai, X., Cai, H., & Liu, P. (2014). Purple sweet potato color ameliorates kidney damage via inhibiting oxidative stress mediated NLRP3 inflammasome activation in high fat diet mice. *Food and chemical toxicology, 69*, 339-346.

Shi, Z., Bassa, I., Gabriel, S., & Francis, F. (1992). Anthocyanin pigments of sweet potatoes–*Ipomoea batatas*. *Journal of food science, 57*, 755-757.

Shi, Z., Bassa, I. A., Gabriel, S., & Francis, F. J. (1992). Anthocyanin pigments of sweet potatoes–*Ipomoea batatas*. *Journal of food science, 57*, 755-757.

Sila, D. N., Smout, C., Elliot, F., Loey, A. V., & Hendrickx, M. (2006). Non-enzymatic Depolymerization of Carrot Pectin: Toward a Better Understanding of Carrot Texture During Thermal Processing. *Journal of food science, 71*, E1-E9.

Singleton, V., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American journal of Enology and Viticulture, 16*, 144-158.

Siriwoharn, T., & Wrostald, R. E. (2004). Characterization of phenolics in Marion and Evergreen blackberries. *Journal Food Science, 69*, 233-240.

Skrede, G., Wrolstad, R., & Durst, R. (2000). Changes in anthocyanins and polyphenolics during juice processing of highbush blueberries (*Vaccinium corymbosum* L.). *Journal of food science, 65*, 357-364.

Steed, L., & Truong, V. D. (2008). Anthocyanin Content, Antioxidant Activity, and Selected Physical Properties of Flowable Purple-Fleshed Sweetpotato Purees. *Journal of food science, 73*, S215-S221.

Steinert, R. E., Ditscheid, B., Netzel, M., & Jahreis, G. (2008). Absorption of black currant anthocyanins by monolayers of human intestinal epithelial Caco-2 cells

mounted in ussing type chambers. *Journal of agricultural and food chemistry, 56*, 4995-5001.

Stewart, B. H., Chan, O. H., Lu, R. H., Reyner, E. L., Schmid, H. L., Hamilton, H. W., Steinbaugh, B. A., & Taylor, M. D. (1995). Comparison of intestinal permeabilities determined in multiple *in vitro* and *in situ* models: relationship to absorption in humans. *Pharmaceutical research, 12*, 693-699.

Suda, I., Oki, T., Masuda, M., Kobayashi, M., Nishiba, Y., & Furuta, S. (2003). Physiological functionality of purple-fleshed sweet potatoes containing anthocyanins and their utilization in foods. *Japan Agricultural Research Quarterly: JARQ, 37*, 167-173.

Surh, Y.-J. (2003). Cancer chemoprevention with dietary phytochemicals. *Nature Reviews Cancer, 3*(10), 768-780.

Tabart, J., Kevers, C., Sipel, A., Pincemail, J., Defraigne, J.-O., & Dommes, J. (2007). Optimisation of extraction of phenolics and antioxidants from black currant leaves and buds and of stability during storage. *Food chemistry, 105*, 1268-1275.

Talavéra, S., Felgines, C., Texier, O., Besson, C., Gil-Izquierdo, A., Lamaison, J.-L., & Rémésy, C. (2005). Anthocyanin metabolism in rats and their distribution to digestive area, kidney, and brain. *Journal of agricultural and food chemistry, 53*, 3902-3908.

Teow, C. C., Truong, V.-D., McFeeters, R. F., Thompson, R. L., Pecota, K. V., & Yencho, G. C. (2007). Antioxidant activities, phenolic and β-carotene contents of sweet potato genotypes with varying flesh colours. *Food chemistry, 103*, 829-838.

Terahara, N., Konczak, I., Ono, H., Yoshimoto, M., & Yamakawa, O. (2004). Characterization of acylated anthocyanins in callus induced from storage root of

purple-fleshed sweet potato, *Ipomoea batatas* L. *BioMed Research International, 2004*, 279-286.

Terefe, N. S., Yang, Y. H., Knoerzer, K., Buckow, R., & Versteeg, C. (2010). High pressure and thermal inactivation kinetics of polyphenol oxidase and peroxidase in strawberry puree. *Innovative Food Science & Emerging Technologies, 11*, 52-60.

Terzić, J., Grivennikov, S., Karin, E., & Karin, M. (2010). Inflammation and colon cancer. *Gastroenterology, 138*, 2101-2114. e2105.

Thipyapong, P., Stout, M. J., & Attajarusit, J. (2007). Functional analysis of polyphenol oxidases by antisense/sense technology. *Molecules, 12*, 1569-1595.

Tian, Q., Giusti, M. M., Stoner, G. D., & Schwartz, S. J. (2006). Urinary excretion of black raspberry (Rubus occidentalis) anthocyanins and their metabolites. *Journal of agricultural and food chemistry, 54*, 1467-1472.

Tipton, K. F., & Dixon, H. B. (1979). Effects of pH on enzymes. *Methods in enzymology, 63*, 183-234.

Torskangerpoll, K., & Andersen, Ø. M. (2005). Colour stability of anthocyanins in aqueous solutions at various pH values. *Food chemistry, 89*, 427-440.

Truong, V.-D., Deighton, N., Thompson, R. T., McFeeters, R. F., Dean, L. O., Pecota, K. V., & Yencho, G. C. (2009). Characterization of anthocyanins and anthocyanidins in purple-fleshed sweetpotatoes by HPLC-DAD/ESI-MS/MS. *Journal of agricultural and food chemistry, 58*, 404-410.

Truong, V. D., McFeeters, R., Thompson, R., Dean, L., & Shofran, B. (2007). Phenolic acid content and composition in leaves and roots of common commercial sweetpotato (*Ipomea batatas* L.) cultivars in the United States. *Journal of food science, 72*, C343-C349.

Tsuda, T. (2008). Regulation of adipocyte function by anthocyanins; possibility of preventing the metabolic syndrome. *Journal of agricultural and food chemistry, 56*, 642-646.

Tsuda, T., Horio, F., Uchida, K., Aoki, H., & Osawa, T. (2003). Dietary cyanidin 3-O-β-D-glucoside-rich purple corn color prevents obesity and ameliorates hyperglycemia in mice. *The Journal of nutrition, 133*, 2125-2130.

Vallejo, F., Gil-Izquierdo, A., Pérez-Vicente, A., & García-Viguera, C. (2004). In vitro gastrointestinal digestion study of broccoli inflorescence phenolic compounds, glucosinolates, and vitamin C. *Journal of agricultural and food chemistry, 52*(1), 135-138.

Van Duynhoven, J., Vaughan, E. E., Jacobs, D. M., Kemperman, R. A., Van Velzen, E. J., Gross, G., Roger, L. C., Possemiers, S., Smilde, A. K., & Doré, J. (2011). Metabolic fate of polyphenols in the human superorganism. *Proceedings of the national academy of sciences, 108*, 4531-4538.

Visconti, R., & Grieco, D. (2009). New insights on oxidative stress in cancer. *Current opinion in drug discovery & development, 12*, 240-245.

Wallace, T. C. (2011). Anthocyanins in cardiovascular disease. *Advances in Nutrition: An International Review Journal, 2*, 1-7.

Walle, T., Walle, U. K., & Halushka, P. V. (2001). Carbon dioxide is the major metabolite of quercetin in humans. *The Journal of nutrition, 131*, 2648-2652.

Wang, H., Cao, G., & Prior, R. L. (1996). Total antioxidant capacity of fruits. *Journal of agricultural and food chemistry, 44*, 701-705.

Wang, H., & Joseph, J. A. (1999). Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radical Biology and Medicine, 27*, 612-616.

Wang, L.-S., & Stoner, G. D. (2008). Anthocyanins and their role in cancer prevention. *Cancer letters, 269*, 281-290.

Wang, L., & Stoner, G. D. (2008). Anthocyanins and their role in cancer prevention. *Cancer letters, 269*, 281-290.

Wang, S., Lin, T., Man, G., Li, H., Zhao, L., Wu, J., & Liao, X. (2014). Effects of anti-browning combinations of ascorbic acid, citric acid, nitrogen and carbon dioxide on the quality of banana smoothies. *Food and Bioprocess Technology, 7*, 161-173.

Wang, S., Liu, Z., Wang, L., & Zhang, X. (2009). NF-κB signaling pathway, inflammation and colorectal cancer. *Cellular and molecular immunology, 6*, 327.

Wang, S. Y., & Jiao, H. (2000). Scavenging capacity of berry crops on superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. *Journal of agricultural and food chemistry, 48*, 5677-5684.

Waris, G., & Ahsan, H. (2006). Reactive oxygen species: role in the development of cancer and various chronic conditions. *Journal of carcinogenesis, 5*, 14.

Weiss, B. (2012). Synthetic food colors and neurobehavioral hazards: the view from environmental health research. *Environmental health perspectives, 120*, 1.

Welch, C. R., Wu, Q., & Simon, J. E. (2008). Recent advances in anthocyanin analysis and characterization. *Current analytical chemistry, 4*, 75.

Williams, R. J., Spencer, J. P., & Rice-Evans, C. (2004). Flavonoids: antioxidants or signalling molecules? *Free Radical Biology and Medicine, 36*, 838-849.

Williamson, G., Meskin, M., Bidlack, W., Davies, A., Lewis, D., & Randolph, R. (2004). Common features in the pathways of absorption and metabolism of flavonoids. *Phytochemicals: mechanisms of action*, 21-33.

Winrow, V., Winyard, P., Morris, C., & Blake, D. (1993). Free radicals in inflammation: second messengers and mediators of tissue destruction. *British Medical Bulletin, 49*, 506-522.

Wiseman, H., & Halliwell, B. (1996). Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochemical Journal, 313*, 17.

Wolffram, S., Blöck, M., & Ader, P. (2002). Quercetin-3-glucoside is transported by the glucose carrier SGLT1 across the brush border membrane of rat small intestine. *The Journal of nutrition, 132*(4), 630-635.

Wrolstad, R. E., Durst, R. W., & Lee, J. (2005). Tracking color and pigment changes in anthocyanin products. *Trends in Food Science & Technology, 16*, 423-428.

Wu, S.-B., Dastmalchi, K., Long, C., & Kennelly, E. J. (2012). Metabolite profiling of jaboticaba (*Myrciaria cauliflora*) and other dark-colored fruit juices. *Journal of agricultural and food chemistry, 60*, 7513-7525.

Wu, X., Cao, G., & Prior, R. L. (2002). Absorption and metabolism of anthocyanins in elderly women after consumption of elderberry or blueberry. *The Journal of nutrition, 132*, 1865-1871.

Wu, X., Pittman, H. E., & Prior, R. L. (2006). Fate of anthocyanins and antioxidant capacity in contents of the gastrointestinal tract of weanling pigs following black raspberry consumption. *Journal of agricultural and food chemistry, 54*, 583-589.

Xu, J., Su, X., Lim, S., Griffin, J., Carey, E., Katz, B., Tomich, J., Smith, J. S., & Wang, W. (2015). Characterisation and stability of anthocyanins in purple-fleshed sweet potato P40. *Food chemistry, 186*, 90-96.

Ye, J., Meng, X., Yan, C., & Wang, C. (2010). Effect of purple sweet potato anthocyanins on β-amyloid-mediated PC-12 cells death by inhibition of oxidative stress. *Neurochemical research, 35*(3), 357-365.

Yi, W., Akoh, C. C., Fischer, J., & Krewer, G. (2006). Absorption of anthocyanins from blueberry extracts by caco-2 human intestinal cell monolayers. *Journal of agricultural and food chemistry, 54*, 5651-5658.

Yi, W., Fischer, J., Krewer, G., & Akoh, C. C. (2005). Phenolic compounds from blueberries can inhibit colon cancer cell proliferation and induce apoptosis. *Journal of agricultural and food chemistry, 53*(18), 7320-7329.

Yoruk, R., & Marshall, M. R. (2003). Physicochemical properties and function of plant polyphenol oxidase: a review. *Journal of Food Biochemistry, 27*, 361-422.

Yoshimoto, M., Okuno, S., Yoshinaga, M., Yamakawa, O., Yamaguchi, M., & Yamada, J. (1999). Antimutagenicity of sweetpotato (*Ipomoea batatas*) roots. *Bioscience, biotechnology, and biochemistry, 63*, 537-541.

Zhao, C., Giusti, M. M., Malik, M., Moyer, M. P., & Magnuson, B. A. (2004). Effects of commercial anthocyanin-rich extracts on colonic cancer and nontumorigenic colonic cell growth. *Journal of agricultural and food chemistry, 52*(20), 6122-6128.

Zhu, Y., Ling, W., Guo, H., Song, F., Ye, Q., Zou, T., Li, D., Zhang, Y., Li, G., & Xiao, Y. (2013). Anti-inflammatory effect of purified dietary anthocyanin in adults with hypercholesterolemia: a randomized controlled trial. *Nutrition, Metabolism and Cardiovascular Diseases, 23*, 843-849.