TOTAL ANTHOCYANIN LEVELS IN COMMERCIALLY-AVAILABLE PIGMENTED GRAIN PRODUCTS

by

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A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Science
Nutrition

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A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

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ACKNOWLEDGEMENTS

First and foremost, an enormous acknowledgement must go to my advisor Dr. Margaret Slavin. Your expertise, direction, patience, enthusiasm, and commitment to this project were essential. Another big thanks goes to my thesis committee: Dr. Taylor Wallace, Dr. Constance Gewa, and Dr. Lilian de Jonge for providing me with critical advice and suggestions throughout the year, which lead to the completion of my work. Another big thanks goes to Dr. Thomas Huff, and his assistant Zeima, for providing essential equipment and assistance. I am also grateful to the Dr. Lucy Yu, Boyan Gao, and the USDA for allowing access to equipment. Lastly, there is no way I could have finished this project, or degree, without the mountains of support I received from my father, friends, family, and classmates. One last huge thank you especially for Austin, for making sure I always had a smile on my face throughout the entire process. I would not have been able to finish this project without each and every one of you. Thank you!

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LIST OF ABBREVIATIONS

Total Anthocyanin Content ..... TAC
High-Performance Liquid Chromatography ..... HPLC
Gas Chromatography ..... GC
Reverse-Phase High-Performance Liquid Chromatography ..... RP-HPLC
Mobile Phase ..... MP
Stationary Phase ..... SP
Retention Time ..... R_t

ABSTRACT

TOTAL ANTHOCYANIN LEVELS IN COMMERCIALLY-AVAILABLE PIGMENTED GRAIN PRODUCTS

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This thesis reports an observational research project, which analyzed the anthocyanin content of products with black soybeans or blue corn as an ingredient. The objectives of the study were: 1) gather a minimum of 8 commercially available food products with black soybean or blue corn as predominant ingredients, and produce triplicate anthocyanin-rich extractions of each, 2) assess anthocyanin content of each extraction by two methods: HPLC to measure the common anthocyanins as determined by a literature search, and the pH differential colorimetric assay for assessing total anthocyanin content, and 3) compare anthocyanin content of processed pigmented grain products to known values for fruit, and amounts demonstrated to be necessary for achieving health benefits. Total anthocyanin content determined by colorimetric assay ranged from 106.1 mg/kg to 261.5 mg/kg full-fat chip, while yellow corn chips contained a mean of 3.8 mg/kg full-fat chip. All six brands of blue corn tortilla chips had a significantly ($p<0.05$) higher

anthocyanin content than yellow corn chips. HPLC analysis of the individual anthocyanins in blue corn tortilla chips showed the presence of 5 out of 6 of the common anthocyanins. Cyanidin-3-glucoside was the most prominent anthocyanin in blue corn tortilla chips ranging from 13.3 mg/kg full-fat chip to 29.5 mg/kg full-fat chip. Pelargonidin-3-glucoside, delphinidin-3-glucoside, peonidin-3-glucoside, and petunidin-3-glucoside were all present in blue corn chips listed in order of highest quantity. Malvidin-3-glucoside was not found to be present in any of the blue corn chip samples, while no individual anthocyanins were detectable in the yellow corn tortilla chips. The anthocyanins in black soybean milks were unable to be extracted. In a 100 g portion, the amount of anthocyanins found in blue corn tortilla chips is comparable to the amount found in plums.

CHAPTER ONE – LITERATURE REVIEW

With the recommendation to add more pigmented food to one's diet for a better variation of nutrient intake and presence of phytochemicals, food companies are responding by developing new foodstuffs using pigmented grain products to provide convenient, non-perishable, forms that are rich in color ("A More Colorful Diet Is A Healthier Diet.," 2001). However, this puts into question the nutrient density of those new products, particularly the anthocyanin content due to the damage that may occur to the molecules during processing. Anthocyanins are strongly believed to have several health beneficial properties that continue to increase their popularity in fresh and processed foods (Lee et al., 2009).

Pigmented grains are increasingly processed into new forms such as black soybean milks and blue corn tortilla chips, and consumers are unaware of the amount and survival of the anthocyanins in these new products. If the anthocyanin content of these foods is unknown to the public, the health-promoting quality of the food is put into question. Consumers may be deceived if they are purchasing these foods with expectations of certain health-promoting nutrient contents; yet, there is incomplete information on the label. Currently, labels hint at these pigmented grain products being the healthier option using words like "organic" and "simply."

The overall objective of this research was to determine the anthocyanin content of food products containing black soybeans or blue corn as an ingredient. The project did not focus on the distinct impact of processing, but rather observed the composition and quantity of anthocyanins in finished products. The data collected from this research may be used for consumers' and industries' benefit, as well as to inform future dietary intake recommendations. Potentially, this will provide a foundation for future research to understand the effects of processing on black soybean and blue corn products on health.

Chemical Nature

Anthocyanins, a type of phytochemical and subclass of flavonoids, are a natural pigment found in plants, and are responsible for a range of colors occurring in nature, including the red, purple, and blue colors of many fruits, vegetables, cereal grains, and flowers (Bueno et al., 2012; Steimer & Sjöberg, 2011). These phytochemicals compose the largest group of phenolic pigments and are considered to be the most important group of water-soluble pigments in plants (Bueno et al., 2012). The word anthocyanins is derived from the Greek *anthos* meaning flower, and *kianos* meaning blue (Kong, 2003).

Plants produce anthocyanins as a protective mechanism against environmental stress factors, like UV light, cold temperatures, and droughts, but have also been used for human benefit (Wallace, 2011). Foods containing anthocyanins are of interest due to potential health-promoting qualities, their use as natural food colorants, and their antioxidant properties (Papoušková et al., 2011; Steimer & Sjöberg, 2011). Specifically, mounting research suggests that anthocyanins may reduce the risk of cardiovascular disease mortality, and delay cancer development (Damodaran, Parkin, & Fennema, 2008;

Thomasset et al., 2009). Anthocyanins also play a part as pollination attractants and phytoprotective agents (Grotewold, 2006). It is believed that anthocyanins are dissolved in the cell sap found in the vacuole of a plant's epidermis cells and a major function of these compounds is to provide color to most flowers and fruits (Bueno et al., 2012). The color helps to attract birds and bees to pollinate.

Structures

Anthocyanins are found throughout all plant tissues including leaves, stems, roots, flowers, and fruits. However, the structures of the anthocyanins in the tissues of fruits and vegetables are simpler than those found in flowers (Bueno et al., 2012).

Anthocyanins belong to the flavonoid group due to the unique $C_6C_3C_6$ carbon skeleton (Damodaran et al., 2008). The structure is composed of two aromatic rings linked by three carbons in an oxygenated heterocycle. (**Figure 1**) When a sugar moiety is attached at the 3-position on the C-ring or the 5-position on the A-ring, the structure is deemed an anthocyanin (Bueno et al., 2012). Each anthocyanin has a different chemical structure due to different R-groups (**Figure 1** & **Table 1**). The most widespread anthocyanin in nature is cyanidin-3-glucoside (**Figure 2**) (Kong, 2003; J. Lee, Durst, & Wrolstad, 2005).

In the absence of the sugar component, the molecule is an anthocyanidin (**Figure 3**). The sugar component of anthocyanins is known to increase their chemical stability and solubility compared to anthocyanidins (Navas, Jiménez-Moreno, Bueno, Sáez-Plaza, & Asuero, 2012), thus they are the structures that are typically found and measured within food. There are six anthocyanidins that are most commonly found in food. These

six are pelargonidin (Pg), peonidin (Pn), cyanidin (Cy), malvidin (Mv), petunidin (Pt), and delphinidin (Dp) (Kong, 2003).

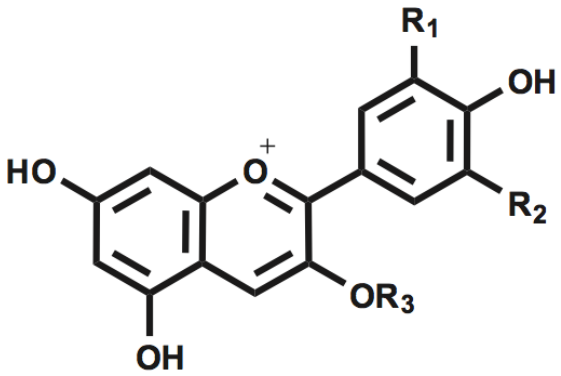

Figure 1. Chemical structure of anthocyanins.

Table 1. Structure and molecular mass (M) of the six most common anthocyanins.

Name	R1	R2	R3	M
Pelargonin-3-glucoside (Pg3glc)	H	H	glucose	271
Cyanidin-3-glucoside (Cy3glc)	OH	H	glucose	287
Peonidin-3-glucoside (Pn3glc)	OCH3	H	glucose	301
Delphinidin-3-glucoside (Dp3glc)	OH	OH	glucose	303
Petunidin-3-glucoside (Pt3glc)	OCH3	OH	glucose	317
Malvidin-3-glucoside (Mv3glc)	OCH3	OCH3	glucose	331

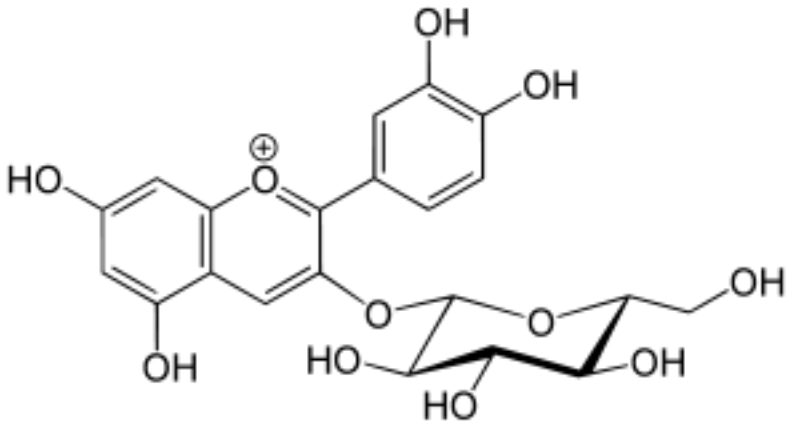

Figure 2. Chemical structure of cyanidin-3-glucoside.

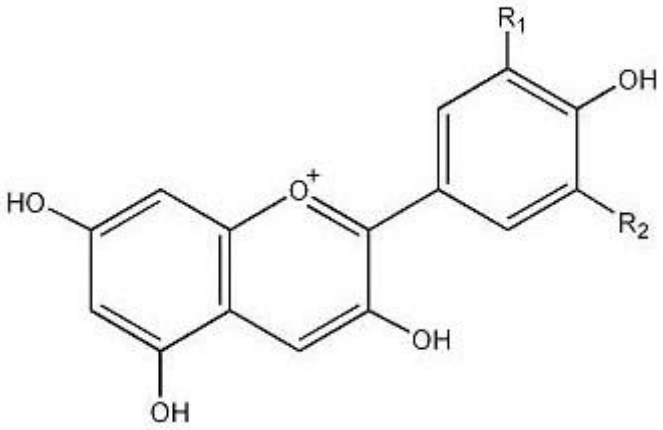

Figure 3. Chemical structure of anthocyanidins.

The color of the anthocyanin within the plant depends upon the presence and number of substituents attached to the molecule (Damodaran et al., 2008). Several identifiers distinguish between specific anthocyanins. These identifiers include the number of hydroxyl and/or methoxy groups present, the types, numbers, sites of

attachment of sugars to the molecule, and the types and numbers of aliphatic or aromatic acids that are attached to the sugars in the molecule (Damodaran et al., 2008).

Anthocyanidins, the sugarless counterpart to an anthocyanin, is produced when the sugar moiety is hydrolyzed and the aglycone, or the non-sugar hydrolysis product, remains. There are 19 naturally occurring anthocyanidins, but only six occur commonly in foods (Damodaran et al., 2008). Anthocyanidins are less water-soluble than their glycoside counterparts (anthocyanins).

The double bonds in the structures are abundant in both anthocyanins and anthocyanidins, and are fundamental for the color (Bueno et al., 2012).These double bonds become excited easily by visible light (Damodaran et al., 2008).

Food Sources

Anthocyanins are found in a variety of foods (**Table 2**). These foods can include fruits, vegetables, and grains (Bueno et al., 2012). Generally, the anthocyanin concentration in most produce ranges from 0.1 to 1% dry weight (Bueno et al., 2012). Anthocyanins are primarily found in the skin of fruits and vegetables, although some types of fruits, strawberries and cherries for example, also contain anthocyanins in the flesh (Bueno et al., 2012).

While the majority of produce contains some level of anthocyanins, each fruit or vegetable contains a different amount of anthocyanins (**Table 3)**. Most species of fruits and vegetables, such as apples, plums, and pears, contain a limited number of anthocyanin pigments, however, red grapes may contain a mixture of more than 20 pigments, provide a richer anthocyanin profile than several other fruits (Clifford, 2000;

McCallum, Yang, Young, Strommer, & Tsao, 2007). However, various fruits, such as berries and black currants, are the fruits richest in anthocyanins (Clifford, 2000). The eggplant is the only common vegetable in the United States that contains a high level of anthocyanins (Horbowicz, Kosson, Grzesiuk, & Dębski, 2008).

Table 2. Six major anthocyanins found in various plant sources.

Anthocyanins	Food Source
Pelargonidin-3-glucoside	Strawberry, banana, red radish, potato
Cyanidin-3-glucoside	Apple, blackberry, elderberry, peach, pear, fig, cherry, red onion, gooseberry, red cabbage, rhubarb, black currant, blood orange, red cabbage, purple carrot, seed coat of soybean, common cranberry, plum, sweet cherry, purple sweet potato
Delphinidin-3-glucoside	Black currant, blood orange, gooseberry, red cabbage, purple carrot, seed coat of soybean, passion fruit, eggplant, green bean, pomegranate
Peonidin-3-glucoside	Common cranberry, plum, sweet cherry, purple sweet potato, mango
Petunidin-3-glucoside	Bilberry, red grape
Malvidin-3-glucoside	Bilberry, red grape

*Adapted from (Bueno et al., 2012).

Table 3. Total anthocyanin content of common fruits and vegetables.

Food Source	Total Anthocyanin Content (mg/kg^{-1})
Apple (peel)	110-2160
Bilberry	4600
Blackberry	820-1800
Blueberry	825-5300
Cabbage, red	250
Cherry	3500-4500
Cranberry	460-2000
Currant, black	119-186
Eggplant	7500

Elderberry	2000-156000
Grape, red	300-7500
Onion, red	Up to 250
Orange, blood	2000
Plum	19-250
Radish, red	110-600
Raspberry, red	100-600
Rhubarb	Up to 2000
Strawberry	127-360

*Adapted from (Horbowicz et al., 2008)

Stability

Anthocyanins are relatively easily damaged in foods because they are highly unstable and very susceptible to degradation (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). The chemical structure of anthocyanins influences stability, and therefore influences which factors will degrade anthocyanins (Jing, 2006). There are several factors that can degrade them including changes in pH, temperature, light, and oxygen concentration (Thomasset et al., 2009). Notably, these are all circumstances that may occur during the processing and storage of food. In other words, anthocyanins are likely to be destroyed to some extent during food processing. However, anthocyanin pigments have the greatest stability occurring under acidic conditions (Damodaran et al., 2008). Anthocyanins that are unsubstituted or mono-substituted anthocyanins are susceptible to nucleophilic attack at the C-2 and/or C-4 positions (Damodaran et al., 2008). To minimize damage when working with anthocyanins, the chemistry, structure, and proper selection processes can be used in conjunction with the appropriate application and each anthocyanin.

pH

Anthocyanins undergo dramatic changes in color when an aqueous environment has a change in pH (Jing, 2006). At pH 4.5 if yellow flavonoids are not present, anthocyanins in fruit juices are nearly colorless (slightly bluish). At pH 1.0, the color of the anthocyanins is strongest. In general, structural features that lead to increased pH stability also lead to increased stability in changing temperatures (Damodaran et al., 2008).

Temperature

The stability of anthocyanins in foods is greatly affected by temperature. Generally, anthocyanins lose stability at temperatures over 100°C (Yue & Xu, 2008). Elevated temperatures will decouple anthocyanins and sugars therefore releasing anthocyanidins (Sadras & Moran, 2012). The mechanism of anthocyanin degradation by temperature has not been completely clarified, but the currently proposed methods indicate that the degradation depends upon the type of anthocyanin involved (Damodaran et al., 2008). In contrast, one study suggests that the thermal degradation of anthocyanins follow first-order reaction kinetics (Wang & Xu, 2007). As previously mentioned, structural features may allow for increased thermal stability (Damodaran et al., 2008).

Light

It is generally recognized that light accelerates degradation of anthocyanins. Anthocyanins substituted at the C-5 hydroxyl groups are more susceptible to photodegradation (Damodaran et al., 2008). Other forms of radiant energy such as ionizing radiation may also result in anthocyanin degradation.

Nutritional Nature

As anthocyanins become increasingly relevant in the food system, it is important to understand the kinetics of these molecules in terms of digestion, absorption, metabolism, transport, and excretion in the body. This is especially important to maximize the health benefits of anthocyanins. With humans consuming a high daily intake of ≥180-215 mg, there is even more reason to further research (Felgines et al., 2003). Currently, a large portion of the literature describing the nutritional nature of these components are animal studies (Bò et al., 2010; Felgines et al., 2006; He, Magnuson, & Giusti, 2005; Ichiyanagi, Shida, Rahman, Hatano, & Konishi, 2006; Walton, Lentle, Reynolds, Kruger, & McGhie, 2006). The majority of human research on the nutritional nature of anthocyanins has used purified aqueous extracts or high concentrations of foods with anthocyanins and few have studied the anthocyanins when consumed in a normal diet consisting of average amounts of food (Walton et al., 2006). Thus, we do not have a full understanding of anthocyanins' actions within the body, but a review of available evidence is presented.

Digestion & Absorption

Anthocyanins are absorbed as glycosides in both humans and rats (Felgines et al., 2003). In humans, absorption occurs in the stomach and small intestine and may involve the use of a specific enzyme such as bilitraslocase (Del Bo' et al., 2012). Additionally, one study described anthocyanins to be more stable in the upper gastrointestinal tract than previously predicted (Crozier, Del Rio, & Clifford, 2010). More recent research explains the anthocyanins being absorbed through the gastric wall (Fernandes, Faria, Calhau, de

Freitas, & Mateus, 2014). Another discovered that blanching increases the absorption of anthocyanins, and found that the process minimally increased absorption about 1.5 to 2 hours after consumption (Del Bo' et al., 2012).

Metabolism & Transport

Anthocyanin metabolism is similar to flavonoid metabolism in that both are metabolized rapidly (Bò et al., 2010; Felgines et al., 2003). Anthocyanins enter the circulatory system after passing through the liver, move freely in the blood, and are then distributed to different tissues. Generally, 15-60 minutes after consumption, about 1% of ingested anthocyanins appear in plasma (Del Bo' et al., 2012; Fernandes et al., 2014). Previously, it was believed that anthocyanins did not appear to undergo extensive metabolism of the parent glycosides to glucurono, sulfo or methyl derivatives (Crozier et al., 2010). Typically, anthocyanins are metabolized by intestinal microbiota in the colon which contributes to the bioavailability of anthocyanins (Del Bo' et al., 2012; Fernandes et al., 2014). However, more recent reviews propose that this previously low observed bioavailability may be mistaken due to high rates of anthocyanin recycling from the liver to the bile and into the small intestine (Lila, Burton-Freeman, Grace, & Kalt, 2016). This recycling leads to the anthocyanins staying in the body for a longer period of time, and suggests that anthocyanins are more bioavailable than previously believed.

Excretion

A portion of the anthocyanins are quickly removed from circulation and are excreted into bile and urine (**Figure 4)** (Fernandes et al., 2014). Typically, less than 0.1% of the ingested amount of anthocyanins is found in urine (Crozier et al., 2010; Walton et

al., 2006). One study demonstrates that the absorption rate of anthocyanins is delayed when simultaneously ingested with other food or other flavonoids (Walton et al., 2006).

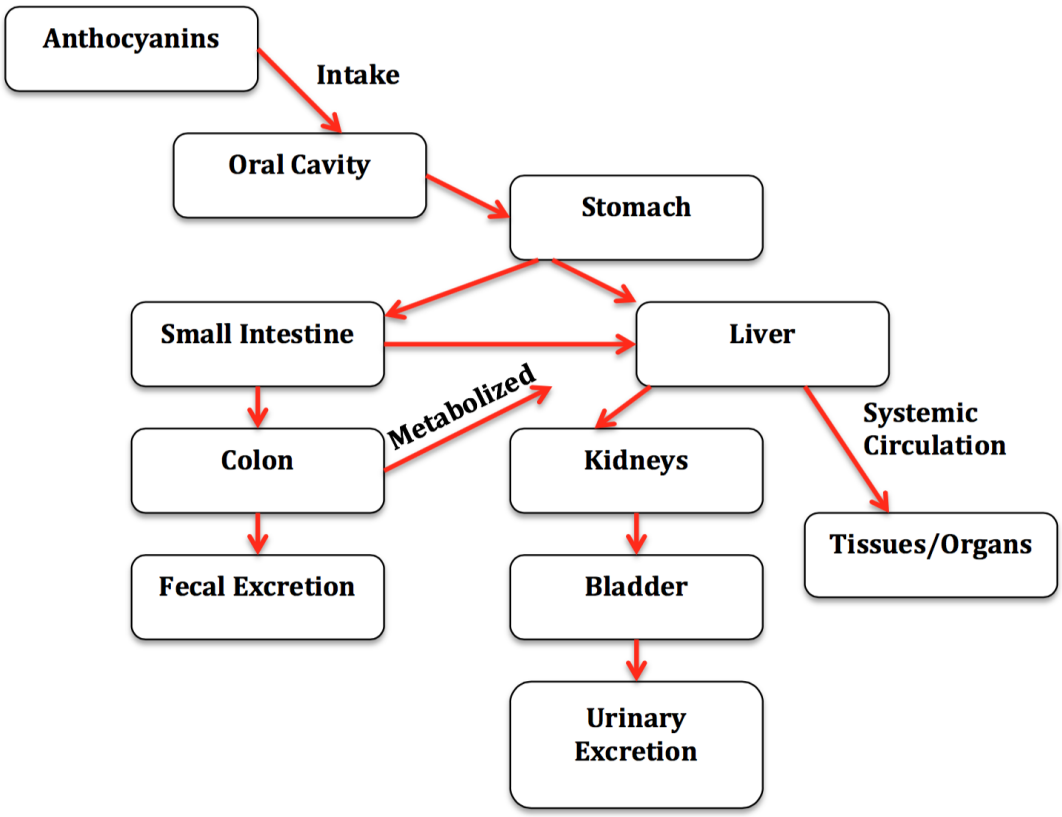

Figure 4. Simplified diagram of hypothetic anthocyanin absorption, metabolism, and excretion pathways.
*Adapted from (Fernandes et al., 2014)

Chosen Foods: Blue Corn and Black Soybeans

This project analyzed the anthocyanin content of processed forms of foods containing black soybeans or blue corn. Both blue corn and black soybeans have been

studied previously for their anthocyanin content, however not for the levels of anthocyanins in commercially available processed foods (**Figures 7 & 8**) (Escalante-Aburto et al., 2013; Jang et al., 2010; Li, 2009; Li, Walker, & Faubion, 2011; Whent, 2009). This project analyzed the content of anthocyanins in processed foods containing blue corn and black soybeans, which are two relevant ingredients on the market. These studies have explored specific processes such as nixtamalization in regards to blue corn, or the health-promoting quality of black soybeans (Escalante-Aburto et al., 2013; Slavin, Kenworthy, & Yu, 2009). The total anthocyanin content for blue corn in its fresh state is 321-620.9 mg/kg (Escalante-Aburto et al., 2013; Li, 2009). Black soybeans have a total anthocyanin content in their fresh state of 12117.9 mg/kg (Jang et al., 2010). Cyanidin-3-glucoside has been found to be the most common anthocyanin in both black soybeans and blue corn (Escalante-Aburto et al., 2013; Jang et al., 2010).

The decision to measure anthocyanins by HPLC was based on the principal components present in previous anthocyanin analyses, as follows. Only the anthocyanins that make up the top 90% of the total anthocyanin content were analyzed. According to Lee et al., cyanidin-3-glucoside makes up 75.8% area of black soybeans, while delphinidin-3-glucoside (8.8%), petunidin-3-glucoside (4.4%), and pelargondidn-3-glucoside (3.4%) follow to make up a total of 92.4% of the total area (Lee et al., 2009). Therefore, these four anthocyanins were analyzed in the black soybean milks. In blue corn, cyanidin-3-glucoside makes up the highest area with 73.1%, while peonidin-3-glucoside (16.1%) and pelargonidin-3-glucoside (10.8%) form the rest of the area of anthocyanins (Moreno et al., 2005). These three anthocyanins total 100% of the

anthocyanin area in blue corn and were measured in the blue corn foods. The chosen HPLC method was able to distinguish amongst these 5 anthocyanins in a dissolved sample.

Blue Corn

Figure 5. Image of blue corn.

Blue corn is an open pollinated flour corn (**Figure 7**) (Li, 2009). Floury corns tend to be popular due to their relatively easy nature to reduce into flour. Blue corn is known to be high in anthocyanins, specifically cyanidin-3-glucoside (Li, 2009). The pigments are found in various locations in different grains, but are found in the highest concentration in the pericarp and aleurone layer of blue corn (Li et al., 2011; Moreno et al., 2005). The pericarp and aleurone layer refers to the outer most layers of the endosperm.

There are many disadvantages for commercial production. First, blue corn only grows well in hot and dry locations, and is prone to diseases and insects (Li, 2009). It has

low grain yields, which causes the cost to be greater than regular dent corn (Li, 2009). However, blue corn grows well in an organic farming setting, which is relevant in today's food system. The health benefits of adding pigments to one's diet, and the sweeter flavor and softness are all advantages that are thought to outnumber the disadvantages under certain circumstances (Cortés, Salinas, Martín-Martinez, & Martínez-Bustos, 2006).

One blue corn product that is relatively popular in the United States is the blue corn tortilla chip. Other products include tortillas, posole, and atole. These foods all use the process of nixtamalization to process the blue corn. Most current research focuses on nixtamalization and the most popular of the blue corn products, tortillas and tortilla chips (Cortés et al., 2006; A. Escalante-Aburto et al., 2013; Gomez, Pérez, Orea, & Martinez, 2006). Unfortunately, this process destroys much of the pigment (50-60%) (Anayansi Escalante-Aburto et al., 2014; Li et al., 2011).

Black Soybean

Figure 6. Image of black soybeans.

Anthocyanins are the primary pigments in black soybeans (**Figure 8**) (Koh, Youn, & Kim, 2014). The distinguishing black pigment is because of the high concentration of anthocyanins found in the epidermis palisade layer of the seed coat (Koh et al., 2014). It has been reported that nine different anthocyanins are found in black soybeans, three major anthocyanins and numerous others (Lee et al., 2009).

Black soybeans are found in several different foods including soymilk, tofu, soy sauce, and soy sprouts (Lee et al., 2009). Black soybeans are becoming increasingly popular over recent years due to consumer awareness of their health benefits (Lee et al., 2009). However, the cooking techniques used with soybeans have the potential to degrade or discard anthocyanins. One particular example is tofu, where soybeans are first boiled in water to produce soymilk, then the proteins are curdled to produce tofu and the

water-soluble anthocyanins may be discarded in the liquid waste. Additional research is needed to determine the amount of anthocyanins remaining after such processing.

Research Question and Objectives

General Research Question

This project aimed to answer one overarching question: what levels of anthocyanins are present in commercially available, shelf-stable, processed foods containing either blue corn or black soybeans?

Objectives

This proposal completed three objectives:

- Gathered a minimum of 8 commercially available food brands in September 2015 with black soybean or blue corn as predominant ingredients, and produce triplicate anthocyanin-rich extractions of each.
- Assessed anthocyanin content of each extraction by two methods: HPLC to measure the five most common anthocyanins as determined by a literature search, and the pH differential colorimetric assay for assessing total anthocyanin content.
- Compared anthocyanin content of processed pigmented grain products to known values for fruit, and amounts demonstrated to be necessary for achieving health benefits.

Eight was decided as the minimum amount of products due to the average number of products in each store only being about 2-3. The date of September 2015 was chosen

because this is close to the time of data collection, and the foods should be as fresh as possible. Pigmented grain products were chosen because of the lack of research currently on these two products, and their prior research has demonstrated these foods contain a high level of anthocyanins in their fresh state.

References

A More Colorful Diet Is A Healthier Diet. (2001, March 19). *The Food Institute Report*, p. 2.

Bò, C. D., Ciappellano, S., Klimis-Zacas, D., Martini, D., Gardana, C., Riso, P., & Porrini, M. (2010). Anthocyanin Absorption, Metabolism, and Distribution from a Wild Blueberry-Enriched Diet (*Vaccinium angustifolium*) Is Affected by Diet Duration in the Sprague−Dawley Rat. *Journal of Agricultural and Food Chemistry*, *58*(4), 2491–2497. http://doi.org/10.1021/jf903472x

Bueno, J. M., Sáez-Plaza, P., Ramos-Escudero, F., Jiménez, A. M., Fett, R., & Asuero, A. G. (2012). Analysis and Antioxidant Capacity of Anthocyanin Pigments. Part II: Chemical Structure, Color, and Intake of Anthocyanins. *Critical Reviews in Analytical Chemistry*, *42*(2), 126–151. http://doi.org/10.1080/10408347.2011.632314

Cassidy, A., Mukamal, K. J., Liu, L., Franz, M., Eliassen, A. H., & Rimm, E. B. (2013). High Anthocyanin Intake Is Associated With a Reduced Risk of Myocardial Infarction in Young and Middle-Aged Women. *Circulation*, *127*(2), 188–196. http://doi.org/10.1161/CIRCULATIONAHA.112.122408

Castañeda-Ovando, A., Pacheco-Hernández, M. de L., Páez-Hernández, M. E., Rodríguez, J. A., & Galán-Vidal, C. A. (2009). Chemical studies of anthocyanins: A review. *Food Chemistry*, *113*(4), 859–871. http://doi.org/10.1016/j.foodchem.2008.09.001

Clifford, M. N. (2000). Anthocyanins–nature, occurrence and dietary burden. *Journal of the Science of Food and Agriculture*, *80*(7), 1063–1072.

Cortés, G. A., Salinas, M. Y., Martín-Martinez, E. S., & Martínez-Bustos, F. (2006). Stability of anthocyanins of blue maize (Zea mays L.) after nixtamalization of seperated pericarp-germ tip cap and endosperm fractions. *Journal of Cereal Science*, *43*(1), 57–62. http://doi.org/10.1016/j.jcs.2005.05.003

Crozier, A., Del Rio, D., & Clifford, M. N. (2010). Bioavailability of dietary flavonoids and phenolic compounds. *Molecular Aspects of Medicine*, *31*(6), 446–467. http://doi.org/10.1016/j.mam.2010.09.007

Damodaran, S., Parkin, K. L., & Fennema, O. R. (Eds.). (2008). *Fennema's Food Chemistry* (4th ed.). Taylor & Francis Group, LLC.

Del Bo', C., Riso, P., Brambilla, A., Gardana, C., Rizzolo, A., Simonetti, P., … Porrini, M. (2012). Blanching Improves Anthocyanin Absorption from Highbush Blueberry (Vaccinium corymbosum L.) Purée in Healthy Human Volunteers: A Pilot Study. *Journal of Agricultural and Food Chemistry*, *60*(36), 9298–9304. http://doi.org/10.1021/jf3021333

Escalante-Aburto, A., Ramírez-Wong, B., Torres-Chávez, P. I., Figueroa-Cárdenas, J. D., López-Cervantes, J., Barrón-Hoyos, J. M., & Morales-Rosas, I. (2013). Effect of extrusion processing parameters on anthocyanin content and physicochemical properties of nixtamalized blue corn expanded extrudates. *CyTA - Journal of Food*, *11*(sup1), 29–37. http://doi.org/10.1080/19476337.2013.764929

Escalante-Aburto, A., Ramírez-Wong, B., Torres-Chávez, P., López-Cervantes, J., de Dios Figueroa-Cárdenas, J., Barrón-Hoyos, J., … Gutiérrez-Dorado, R. (2014). Obtaining Ready-to-Eat Blue Corn Expanded Snacks with Anthocyanins Using an Extrusion Process and Response Surface Methodology. *Molecules*, *19*(12), 21066–21084. http://doi.org/10.3390/molecules191221066

Felgines, C., Talavéra, S., Gonthier, M.-P., Texier, O., Scalbert, A., Lamaison, J.-L., & Rémésy, C. (2003). Strawberry anthoycanins are recovered in urine as glucuro- and sulfoconjugates in humans. *The Journal of Nutrition*, *133*(5), 1296–1301.

Felgines, C., Talavéra, S., Texier, O., Besson, C., Fogliano, V., Lamaison, J.-L., … Galvano, F. (2006). Absorption and metabolism of red orange juice anthocyanins in rats. *British Journal of Nutrition*, *95*(5), 898–904. http://doi.org/10.1079/BJN20061728

Fernandes, I., Faria, A., Calhau, C., de Freitas, V., & Mateus, N. (2014). Bioavailability of anthocyanins and derivatives. *Journal of Functional Foods*, *7*, 54–66. http://doi.org/10.1016/j.jff.2013.05.010

Gomez, A. C., Pérez, J. L. J., Orea, A. C., & Martinez, E. S. M. (2006). Photoacoustic Analysis of Blue Corn Pigments in Nixtamalized Flours. *International Journal of Thermophysics*, *27*(4), 1274–1280. http://doi.org/10.1007/s10765-006-0085-7

Gössinger, M., Moritz, S., Hermes, M., Wendelin, S., Scherbichler, H., Halbwirth, H., … Berghofer, E. (2009). Effects of processing parameters on colour stability of strawberry nectar from puree. *Journal of Food Engineering*, *90*(2), 171–178. http://doi.org/10.1016/j.jfoodeng.2008.06.018

Grotewold, E. (2006). The genetics and biochemistry of floral pigments. *Annu. Rev. Plant Biol.*, *57*, 761–780.

He, J., Magnuson, B. A., & Giusti, M. M. (2005). Analysis of Anthocyanins in Rat Intestinal ContentsImpact of Anthocyanin Chemical Structure on Fecal Excretion. *Journal of Agricultural and Food Chemistry*, *53*(8), 2859–2866. http://doi.org/10.1021/jf0479923

Horbowicz, M., Kosson, R., Grzesiuk, A., & Dębski, H. (2008). Anthocyanins of Fruits and Vegetables - Their Occurrence, Analysis and Role in Human Nutrition. *Vegetable Crops Research Bulletin*, *68*(-1). http://doi.org/10.2478/v10032-008-0001-8

Ichiyanagi, T., Shida, Y., Rahman, M. M., Hatano, Y., & Konishi, T. (2006). Bioavailability and Tissue Distribution of Anthocyanins in Bilberry (*Vaccinium myrtillus* L.) Extract in Rats. *Journal of Agricultural and Food Chemistry*, *54*(18), 6578–6587. http://doi.org/10.1021/jf0602370

Jang, H., Ha, U.-S., Kim, S.-J., Yoon, B.-I., Han, D.-S., Yuk, S.-M., & Kim, S.-W. (2010). Anthocyanin Extracted from Black Soybean Reduces Prostate Weight and Promotes Apoptosis in the Prostatic Hyperplasia-Induced Rat Model. *Journal of Agricultural and Food Chemistry*, *58*(24), 12686–12691. http://doi.org/10.1021/jf102688g

Jing, P. (2006). *Purple corn anthocyanins: chemical structure, chemoprotective sctivity and structure/function relationships*. The Ohio State University. Retrieved from http://rave.ohiolink.edu/etdc/view?acc_num=osu1155738398

Koh, K., Youn, J. E., & Kim, H.-S. (2014). Identification of anthocyanins in black soybean (Glycine max (L.) Merr.) varieties. *Journal of Food Science and Technology*, *51*(2), 377–381. http://doi.org/10.1007/s13197-011-0493-y

Kong, J. (2003). Analysis and biological activities of anthocyanins. *Phytochemistry*, *64*(5), 923–933. http://doi.org/10.1016/S0031-9422(03)00438-2

Lee, J., Durst, R. W., & Wrolstad, R. E. (2005). Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study. *Journal of AOAC International*, *88*(5), 1269–1278.

Lee, J. H., Kang, N. S., Shin, S.-O., Shin, S.-H., Lim, S.-G., Suh, D.-Y., … Ha, T. J. (2009). Characterisation of anthocyanins in the black soybean (Glycine max L.) by HPLC-DAD-ESI/MS analysis. *Food Chemistry*, *112*(1), 226–231. http://doi.org/10.1016/j.foodchem.2008.05.056

Li, J. (2009). *Total anthocyanin content in blue corn cookies as affected by ingredients and oven types*. Kansas State University. Retrieved from http://krex.k-state.edu/dspace/handle/2097/1673

Li, J., Walker, C. E., & Faubion, J. M. (2011). Acidulant and oven type affect total anthocyanin content of blue corn cookies. *Journal of the Science of Food and Agriculture*, *91*(1), 38–43. http://doi.org/10.1002/jsfa.4173

Lila, M. A., Burton-Freeman, B., Grace, M., & Kalt, W. (2016). Unraveling Anthocyanin Bioavailability for Human Health. *Annual Review of Food Science and Technology*, *7*(1), 375–393. http://doi.org/10.1146/annurev-food-041715-033346

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*(26), 7731–7737. http://doi.org/10.1021/jf020690l

McCallum, J. L., Yang, R., Young, J. C., Strommer, J. N., & Tsao, R. (2007). Improved high performance liquid chromatographic separation of anthocyanin compounds from grapes using a novel mixed-mode ion-exchange reversed-phase column. *Journal of Chromatography A*, *1148*(1), 38–45. http://doi.org/10.1016/j.chroma.2007.02.088

Moreno, Y. S., Sánchez, G. S., Hernández, D. R., & Lobato, N. R. (2005). Characterization of anthocyanin extracts from maize kernels. *Journal of Chromatographic Science*, *43*(9), 483–487.

Navas, M. J., Jiménez-Moreno, A. M., Bueno, J. M., Sáez-Plaza, P., & Asuero, A. G. (2012). Analysis and Antioxidant Capacity of Anthocyanin Pigments. Part IV: Extraction of Anthocyanins. *Critical Reviews in Analytical Chemistry*, *42*(4), 313–342. http://doi.org/10.1080/10408347.2012.680343

Papoušková, B., Bednář, P., Hron, K., Stávek, J., Balík, J., Myjavcová, R., … Lemr, K. (2011). Advanced liquid chromatography/mass spectrometry profiling of anthocyanins in relation to set of red wine varieties certified in Czech Republic. *Journal of Chromatography A*, *1218*(42), 7581–7591. http://doi.org/10.1016/j.chroma.2011.07.027

Patras, A., Brunton, N. P., O'Donnell, C., & Tiwari, B. K. (2010). Effect of thermal processing on anthocyanin stability in foods; mechanisms and kinetics of degradation. *Trends in Food Science & Technology*, *21*(1), 3–11. http://doi.org/10.1016/j.tifs.2009.07.004

Prior, R. L. (2004). Absorption and metabolism of anthocyanins: potential health effects. *Phytochemicals: Mechanisms of Action*, 1–19.

Rodriguez-Saona, L. E., & Wrolstad, R. E. (2001). Extraction, Isolation, and Purification of Anthocyanins. *Current Protocols in Food Analytical Chemistry*, F1.1.1–F1.1.11.

Sadras, V. O., & Moran, M. A. (2012). Elevated temperature decouples anthocyanins and sugars in berries of Shiraz and Cabernet Franc: Thermal decoupling of anthocyanins and sugars. *Australian Journal of Grape and Wine Research*, *18*(2), 115–122. http://doi.org/10.1111/j.1755-0238.2012.00180.x

Slavin, M., Kenworthy, W., & Yu, L. (Lucy). (2009). Antioxidant Properties, Phytochemical Composition, and Antiproliferative Activity of Maryland-Grown Soybeans with Colored Seed Coats. *Journal of Agricultural and Food Chemistry*, *57*(23), 11174–11185. http://doi.org/10.1021/jf902609n

Steimer, S., & Sjöberg, P. J. R. (2011). Anthocyanin Characterization Utilizing Liquid Chromatography Combined with Advanced Mass Spectrometric Detection. *Journal of Agricultural and Food Chemistry*, *59*(7), 2988–2996. http://doi.org/10.1021/jf104840v

Thomasset, S., Teller, N., Cai, H., Marko, D., Berry, D. P., Steward, W. P., & Gescher, A. J. (2009). Do anthocyanins and anthocyanidins, cancer chemopreventive pigments in the diet, merit development as potential drugs? *Cancer Chemotherapy and Pharmacology*, *64*(1), 201–211. http://doi.org/10.1007/s00280-009-0976-y

Wallace, T. C. (2011). Anthocyanins in Cardiovascular Disease. *Advances in Nutrition: An International Review Journal*, *2*(1), 1–7. http://doi.org/10.3945/an.110.000042

Wallace, T., Slavin, M., & Frankenfeld, C. (2016). Systematic Review of Anthocyanins and Markers of Cardiovascular Disease. *Nutrients*, *8*(1), 32. http://doi.org/10.3390/nu8010032

Walton, M. C., Lentle, R. G., Reynolds, G. W., Kruger, M. C., & McGhie, T. K. (2006). Anthocyanin Absorption and Antioxidant Status in Pigs. *Journal of Agricultural and Food Chemistry*, *54*(20), 7940–7946. http://doi.org/10.1021/jf061527j

Wang, W.-D., & Xu, S.-Y. (2007). Degradation kinetics of anthocyanins in blackberry juice and concentrate. *Journal of Food Engineering*, *82*(3), 271–275. http://doi.org/10.1016/j.jfoodeng.2007.01.018

Whent, M. M. (2009). *Nutraceutical Properties of Low Alpha-linolenic Soybeans Grown in Maryland*. ProQuest. Retrieved from http://books.google.com/books?hl=en&lr=&id=0tdGVzbAwwYC&oi=fnd&pg=PR2&dq=%22Table+1:+Correlation+between+18:3n-3+and+other+soybean%22+%221:+Desired+fatty+acid+profile+for+oil+used+in

+commercial+food%22+%223:+Isoflavone,+lutein,+and+tocopherol+content+of%22+&ots=ARezyc0P2N&sig=Lk5Ge5q1KuN0Q7FITb0_VL6e57w

Wu, Y., Wang, Y., Zhang, W., Han, J., Liu, Y., Hu, Y., & Ni, L. (2014). Extraction and preliminary purification of anthocyanins from grape juice in aqueous two-phase system. *Separation and Purification Technology*, *124*, 170–178. http://doi.org/10.1016/j.seppur.2014.01.025

Yue, X., & Xu, Z. (2008). Changes of Anthocyanins, Anthocyanidins, and Antioxidant Activity in Bilberry Extract during Dry Heating. *Journal of Food Science*, *73*(6), C494–C499. http://doi.org/10.1111/j.1750-3841.2008.00845.x

Zhu, Y., Xia, M., Yang, Y., Liu, F., Li, Z., Hao, Y., … Ling, W. (2011). Purified Anthocyanin Supplementation Improves Endothelial Function via NO-cGMP Activation in Hypercholesterolemic Individuals. *Clinical Chemistry*, *57*(11), 1524–1533. http://doi.org/10.1373/clinchem.2011.167361

CHAPTER TWO – ANTHOCYANIN CONTENT IN BLUE CORN TORTILLA CHIPS

Introduction

Anthocyanins, a type of phytochemical and subclass of flavonoids, are a natural pigment found in plants, and are responsible for a range of colors occurring in nature, including the red, purple, and blue colors of many fruits, vegetables, cereal grains, and flowers (Bueno et al., 2012; Steimer & Sjöberg, 2011). Plants produce anthocyanins as a protective mechanism against environmental stress factors, like UV light, cold temperatures, and droughts, but have also been used for human benefit (Wallace, 2011). Due to potential health-promoting qualities, their use as natural food colorants, and their antioxidant properties foods containing anthocyanins are of interest (Papoušková et al., 2011; Steimer & Sjöberg, 2011). Specifically, mounting research suggests that anthocyanins may reduce the risk of cardiovascular disease mortality, and delay cancer development (Thomasset et al., 2009; Wallace, 2011; Wallace, Slavin, & Frankenfeld, 2016).

However, anthocyanins are not stable compounds. The chemical structure of anthocyanins influences stability, and therefore influences which factors will degrade anthocyanins (Jing, 2006). There are several factors that can degrade them including changes in pH, temperature, light, and oxygen concentration (Thomasset et al., 2009).

Notably, these are all circumstances that may occur during the processing and storage of food. In other words, some proportion of anthocyanins from raw ingredients is likely to be destroyed to some extent during food processing.

Blue corn is increasingly popular as an alternative to white or yellow corn in the mainstream food supply of the United States. Several different products are currently made using this ingredient, one product being blue corn tortilla chips. Other products include tortillas, posole, and atole. These foods all use the process of nixtamalization to process the blue corn. Unfortunately, this process destroys much of the pigment (50-60%) in blue corn due to the changes in pH when the food is soaked and cooked in an alkaline solution, usually limewater (Escalante-Aburto et al., 2014; Li et al., 2011). Most current research on blue corn focuses on the process of nixtamalization (Cortés et al., 2006; Escalante-Aburto et al., 2013; Gomez et al., 2006). However, there is a lack of research specifically focusing on anthocyanins in processed foods.

The overall objective of this research was to determine the anthocyanin content of several different blue corn tortilla chips. The project did not focus on the distinct impact of processing, but rather observed the composition and quantity of anthocyanins in finished processed products currently available in the food supply. Ultimately, the blue corn chip anthocyanin content results are contextualized with regard to current scientific understanding of levels needed to impact health outcomes and in comparison to levels found in other anthocyanin-containing foods.

Materials & Methods

Chemicals

Certified ACS grade acetone, ACS grade hexane, Certified ACS grade potassium chloride, and granular USP/FCC sodium chloride were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Certified ACS grade methanol, HPLC grade water, and Certified ACS grade chloroform were purchased from Fisher Chemical (Waltham, MA, USA). ACS grade hydrochloric acid and 99.95% sodium carbonate were purchased from VWR (Radnor, PA, USA). Kuromanin chloride (Cyanidin-3-glucoside) standard, lot number BCBQ9084V, and sodium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium phosphate, dibasic, anhydrous was purchased from EMD Chemicals, Inc. (Gibbstown, NJ, USA).

Samples

Four grocery stores in northern Virginia were visited between December 2015 and January 2016. Every unique brand of blue corn chip or unique product encountered was purchased and analyzed. Only products prior to the expiration date (if listed) at time of collection were collected to assure all samples were commercially-available and fresh. Six blue corn tortilla chip brands and one yellow corn tortilla chip brand were collected. Bags of chips were opened at time of extraction to maintain freshness. Samples were labeled for ease of identification as follows: each blue corn chip brand was labeled A-F and sample G was the yellow corn chip sample. Chip samples were ground using a kitchen grade coffee grinder to a consistent particle size fitting through 40-mesh sieve.

Fat Extraction

Ten grams of ground corn chips were weighed and placed in 125mL bottle with 40mL hexane (1:4 (w/v)) for fat extraction. Samples were left to sit overnight at room temperature (22°C) in the dark. Samples were filtered by Buchner filtration to separate hexane and fat from ground chips on a pre-weighed filter paper. Extra hexane was used to wash all chip residue from the bottle onto the filter paper. Chip residue was allowed to dry on the filter paper under vacuum until a constant weight, approximately 2 hours. Weight of chips was measured and subtracted from full-fat weight to determine the yield of fat.

Anthocyanin Extraction

Anthocyanins were extracted by mixing 1.000 g ± 0.0010 defatted ground chips with 10 mL of acetone and 0.1% HCl acidified water (40:60, (v/v)). Samples were vortexed to mix and stored in the refrigerator (0-5°C) for 24 hours. The mixtures were centrifuged at 4696 *g* for 20 minutes using a Heraeus Multifuge X1R centrifuge with bucket rotor (Thermo Scientific, Waltham, MA). The chips were reextracted 3 times following the same procedure to ensure complete extraction, for a total of 4 extractions. A fifth extraction was conducted to confirm the extraction efficiency of the solvent and previous extractions. This last extraction was not added to the total volume of samples. The acetone in the combined extractions was evaporated by nitrogen gas flow at 30°C. Subsequently, water was removed by lyophilization. Dry extracts were stored at -80°C for 7 weeks until analysis. The crude extracts were reconstituted in 8 mL 40% acidified acetone and sonicated for 15 minutes to aid dissolution. Samples were filtered using Whatman No. 1 paper and brought to 15 mL in graduated cylinder using 40% acidified

acetone (Whatman Inc., Florham, NJ, USA). Extracts were further purified with $CHCl_3$ partition for HPLC-MS analysis (Jing, 2006).

Total Monomeric Anthocyanin Content

Total monomeric anthocyanin content of blue corn chips was analyzed by colorimetric analysis using the method described by Lee and others (Lee et al., 2005). The appropriate dilution factor was determined by diluting test sample with pH 1.0 potassium chloride buffer (0.025 M) until absorbance at 520 nm was within 0.2 and 1.4 AU. Using this dilution factor (1:4, (v/v)) each sample was tested diluted with pH 1.0 buffer and pH 4.5 sodium acetate buffer (0.45 M) at both 520nm and 700nm, within 20-50 minutes after preparation. Test samples were read versus a blank cuvette filled with either pH 1.0 buffer or pH 4.5 buffer.

Anthocyanin pigment concentration was calculated, expressed in units of cyanidin-3-glucoside equivalents, as follows:

Anthocyanin pigment (cyanidin-3-glucoside equivalents, mg/L) =

$$(A \times MW \times DF \times 10^3)/(\varepsilon \times 1)$$

where $A = (A_{520nm} - A_{700nm})pH\ 1.0 - (A_{520nm} - A_{700nm})pH\ 4.5$; MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside; DF = dilution factor established prior to analysis; 1 = pathlength in cm; ε = 26,900 molar extinction coefficient, in L x mol^{-1} x cm^{-1}, for cyanidin-3-glucoside; and 10^3 = factor for conversion from g to mg.

HPLC Analysis of Anthocyanins

Standard Preparation and Calibration Curve

Cyanidin-3-glucoside standard was dissolved in acidified water and used as stock solutions for generating the calibration curve. The stock solution was diluted in 0.1% HCl acidified water to allow 10 μg/mL, 5 μg/mL, 1 μg/mL, and 0.5 μg/mL solutions of cyanidin-3-glucoside. These four standard solutions were injected into the HPLC system to create a four-point calibration curve.

HPLC Conditions

Purified extracts were analyzed on a HPLC system equipped with a Waters Alliance 2690 Separations Module and a Waters-Micromass ZQ2000 Mass Spectrophotometer (Milford, MA), Waters 996 Photodiode Array Detector (Milford, MA), and MassLynx and QuanLynx software (version 4.1). The column used was a reverse phase 2.1 mm x 150 mm Atlantis T3 C18 column with 3-μm particle size, preceded by a 5 mm guard column (Waters Corp, Milford, MA). The solvents were (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The solvent gradient conditions were as follows; 0-20 min, 5% B; 20-27 min, 40% B; 27-30 min, 5% B, returning to initial conditions (run time 30 min). Other HPLC conditions included a flow rate of 0.2 mL/min; column temperature of 30°C; sample temperature of 4°C; detection of 530 nm; and injection volume of 5 μL. Individual anthocyanin peaks were identified first by determining if the peak formed at the correct R_t. Once the correct R_t was verified, spectra of each anthocyanin were checked to assure that the mass for each compound was correct. After each anthocyanin was identified, the peak area was compared to the standard curve to calculate concentration of each anthocyanin in the samples.

Black Soybean Milk Extraction

Multiple attempts were made to extract anthocyanins from black soybean milk, which were ultimately unsuccessful. These methods included a chloroform partition, two-phase aqueous extraction, and base hydrolysis. The chloroform partition was conducted by adding 3 parts black soybean milk to 2 parts of acetone (Rodriguez-Saona & Wrolstad, 2001). The solution was filtered by Buchner filtration to separate the anthocyanins from insoluble material. The plant material was reextracted using acetone until a clear solution was obtained. Two volumes of chloroform were added to the clear solution in a tube and gently mixed by turning the tube upside down a few times. The two-phase aqueous extraction involved adding 1 mL black soybean milk to a tube with 3.22 g sodium phosphate monobasic monohydrate, 2.5 mL HPLC grade water, and 2.5 g EtOH (25%, w/w) (Wu et al., 2014). The total weight was brought up to 10 g by adding HPLC grade water. The solution was mixed and put in a 25°C water bath and incubated for 1 hour. Alkaline hydrolysis was also used to attempt to extract the anthocyanins where a 1 M basic solution was added to the milk and was heated for 20 minutes in a 60°C water bath. Following the water bath, the solution was filtered using Whatman No. 1 paper.

Statistical Analysis

Extractions were replicated 3 times and each replication was measured twice. Statistical significance was determined using One-Way ANOVA with Tukey's PostHoc test (SPSS ver. 22).

Results and Discussion

Lipid Extraction

The average crude fat removed from the chip samples by hexane extraction ranged between 1.5 g/10 g chips and 1.9 g/10 g chips (**Table 4**). These results represent an incomplete extraction of the lipids because only one extraction was completed. However, several extractions were not needed because lipids were not a goal of this study and the purpose for fat extraction was ease of anthocyanin extraction. Fat globules increased difficulty during extraction when the full-fat chips were used. Preliminary data showed that lipid extraction by this method improved anthocyanin extraction efficiency and eased sample handling (data not shown). Thus, defatted chips were used for anthocyanin extraction.

Table 4. Crude fat removed from chip samples.

Chip Sample	Crude Fat (g/10 g chips)
A	1.6
B	1.9
C	1.5
D	1.7
E	1.6
F	1.8
G	1.5

Total Anthocyanin Content of Tortilla Chips

The different blue corn chip brands analyzed in this study contained similar average TAC levels ranging from 141.4 mg/kg to 199.8 mg/kg full-fat chip, while the control yellow corn chip brand contained a mean TAC level of 3.8 mg/kg full-fat chip, which was expected to have 0 mg/kg (**Table 5**). As expected, the blue corn chip samples

had a significantly higher TAC than the yellow corn chip sample (**Figure 7**). Processed blue corn chips have less anthocyanins than previously reported values for raw blue corn, with processed corn chips containing an average of 162.7 mg/kg while raw blue corn contains an average of 471 mg/kg. Of the six brands, chip sample D had the highest mean amount of anthocyanins, which was 41% more than the lowest mean amount in sample E. Chip brand D was significantly higher than chip brands B, C, E, F.

Table 5. Average total anthocyanin content of blue and yellow corn tortilla chips.

Sample	TAC (mg/kg)	SD
A	177.4	10.8
B	142.1	15.3
C	162.2	19.3
D	199.8	20.4
E	141.4	10.6
F	153.8	48.6
G	3.8	3.7

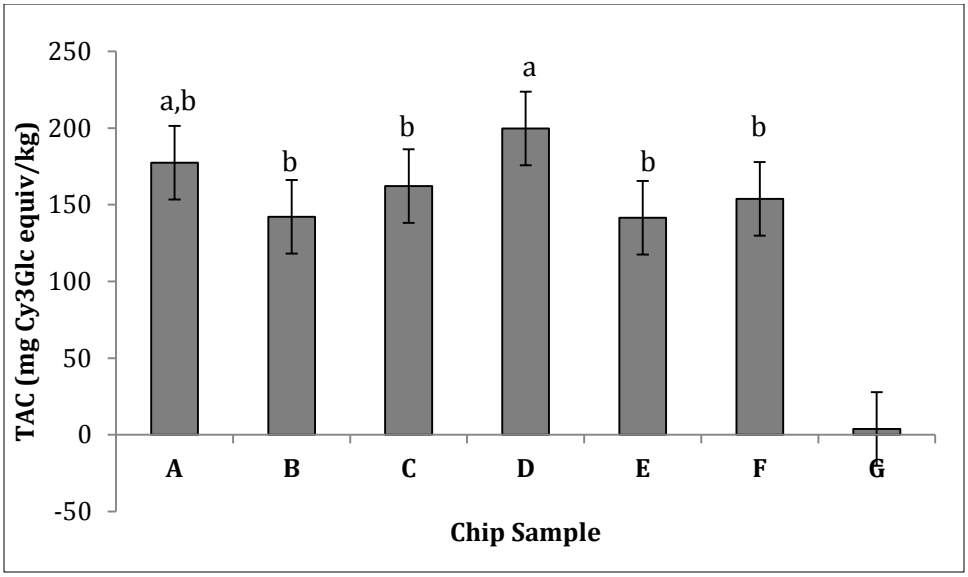

Figure 7. Total anthocyanin content of corn tortilla chips.
Samples marked with the same letter are not statistically different ($p<0.05$).

HPLC – Individual Anthocyanin Content Blue Corn Chips

The calibration graph was obtained by plotting peak area (y) against concentration of standard dilutions (x). The regression equation for the standard was y= 35,067,802x + 3,344 with a correlation coefficient r = 0.998.

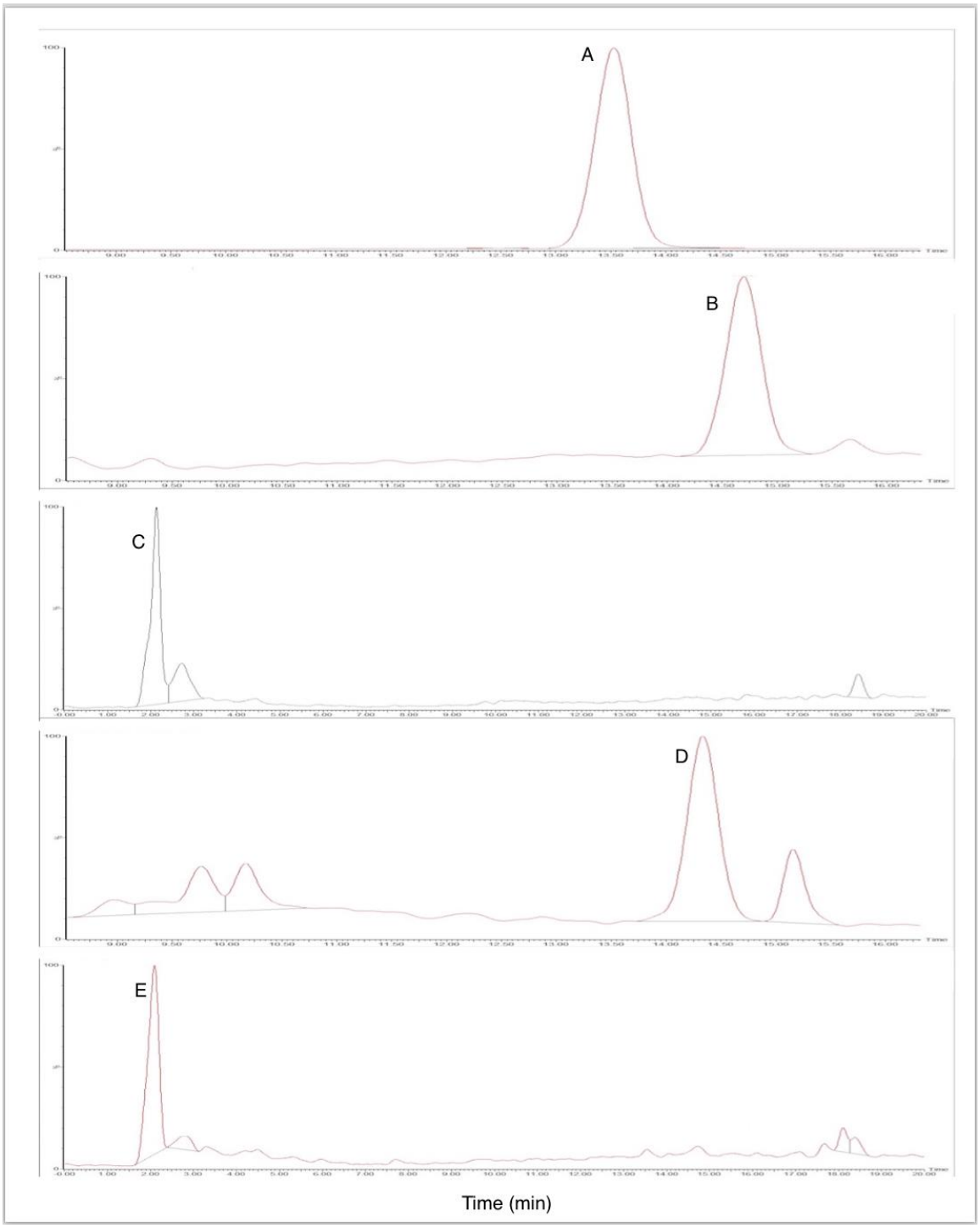

Figure 8. Individual chromatograms of anthocyanins in blue corn tortilla chips.
(A: cyanidin-3-glucoside; B: peonidin-3-glucoside; C: petunidin-3-glucoside; D: pelargonidin-3-glucoside; E: delphinidin-3-glucoside)

Cyanidin-3-glucoside was found to be the most prominent anthocyanin in the blue corn chip samples (**Table 6**). Values for cyanidin-3-glucoside ranged from 13.3 mg/kg full-fat chip to 29.5 mg/kg full-fat chip, with a mean of 20.8 ± 4.6 mg/kg. Pelargonidin-3-glucoside was the next highest amount of anthocyanins with a range of 0 mg/kg full-fat chip to 2.4 mg/kg full-fat chip, with a mean of 0.5 ± 0.9 mg/kg. Delphinidin-3-glucoside was not detected in all blue corn chip samples, but was the third highest anthocyanin. The amounts ranged from of 0 mg/kg full-fat chip to 0.8 mg/kg full-fat chip, with a mean of 0.0 ± 0.4 mg/kg. Peonidin-3-glucoside had a minimum of 0 mg/kg full-fat chip and a maximum value of 0.6 mg/kg full-fat chip, with a mean of 0.0 ± 0.4 mg/kg. Petunidin-3-glucoside was the anthocyanin that was found in the least abundance in the blue corn chip samples ranging from 0.0 mg/kg full-fat chip to 0.2 mg/kg full-fat chip, with a mean of 0.0 ± 0.3 mg/kg. Malvidin-3-glucoside was not found in any of the blue or yellow corn chip samples. Petunidin-3-glucoside was the only anthocyanin found in the yellow corn chip sample, however the amount is extremely small.

Studies looking at the health benefits of anthocyanins list a range of values that could potentially lead to increased health benefits (Prior, 2004; Thomasset et al., 2009). This range includes 139 to 1200 mg/day of anthocyanins to provide health benefits in humans, such as vasoprotective, antioxidant, and chemoprotective effects. However, most of the studies use doses of anthocyanins that well-exceed that of a normal diet without supplementation. It is shown that doses of anthocyanins that do not exceed ~140 mg tend to not provide any significant outcomes (Prior, 2004; Thomasset et al., 2009). Based on both the TAC and HPLC results, all of the studied blue corn tortilla chip samples do not

meet this threshold. While most studies suggest individuals consume "high" intakes of anthocyanins, there is not yet a nationally accepted, or recommended, dose amount of anthocyanins that is most likely to have an effect.

Several human studies investigate to determine what dose level of anthocyanins would produce increased health outcomes. A crossover study with 12 hypercholesterolemic participants found that 320 mg of anthocyanins improves endothelium-dependent vasodilation (Zhu et al., 2011). Another study found that the anthocyanins in blueberries increased serum antioxidant status and have the ability to prevent low-density lipoprotein oxidation (Mazza, Kay, Cottrell, & Holub, 2002). Yet, the limitation to this study was the use of freeze-dried wild blueberry powder that contained a 1200 mg dose of anthocyanins. While it does not provide a specific number, a large study looking at myocardial infarction in women found that a high intake of anthocyanins might reduce myocardial infarction risk in predominately young women (Cassidy et al., 2013). This study suggested the combined intake of two anthocyanin-rich foods, such as blueberries and strawberries, was associated with the decreased risk. However, results between studies are inconsistent. The only suggestion that can be made from the current research is the consumption of high intakes of anthocyanin-rich foods. Even with the low amounts of individual anthocyanins, there is still the evidence that shows blue corn tortilla chips can contribute to a high dietary intake of anthocyanins.

One limitation of these results is the large discrepancy in anthocyanin content values between TAC and HPLC analysis. One potential explanation may be the possibility of some degradation during storage, but this would not fully explain the

dramatic difference, because precautions were taken during storage to prevent degradation. Studies consistently show that degradation occurs over time, especially at higher temperatures (23-100°C) (Patras, Brunton, O'Donnell, & Tiwari, 2010; Wang & Xu, 2007), with samples experiencing a >50% anthocyanin loss when stored for at least 6 months at room temperature in several studies (Patras et al., 2010). However, one study reported that anthocyanins stored at 4°C will stabilize the anthocyanins for over 12 months (Gössinger et al., 2009). The blue corn anthocyanins were stored for 7 weeks at -80°C, and accordingly should have experienced minimal loss during that time. According to the previous study, storage at this temperature and time, the anthocyanins in the blue corn tortilla chips should not have degraded as much is seen. Another explanation for the difference in values could be that the TAC assay also absorbs color from any anthocyanidins present in the blue corn tortilla chips, whereas the HPLC-MS was only programmed to look for the 6 glycoside forms listed above. Additionally, if other anthocyanidins are present that were not measured by HPLC, such as capensinidin, europhinidin, and rosinidin, which could account for a higher TAC value.

Another factor to consider is the amount of chips an individual would have to eat to see benefits. A serving of blue corn tortilla chips is 28 g, which is approximately 6 chips. However, this is a low amount of chips and it can be assumed that most Americans are eating more than one serving of blue corn tortilla chips in one sitting. In a 100 g portion, blue corn tortilla chips provide 18.5 mg of anthocyanins, using the results from the total anthocyanin content. If an individual wanted to consume this amount of anthocyanins, by eating blue corn tortilla chips, they would have to eat approximately 22

chips, based on the number of chips per serving. This is not an inconsiderable amount of blue corn tortilla chips.

Table 6. HPLC analysis of individual anthocyanins in blue corn chips.

	Cyanidin	Peonidin	Pelargonidin	Delphinidin	Petunidin	Malvidin
Sample	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**
A	23.7 (5.4)	0.0 (0.4)^{a,b,c}	0.0 (0.3)^{a,b}	ND^{b,c}	ND^{b}	ND
B	15.3 (1.8)	ND^{a,b,c}	ND^{a}	0.4 (0.4)^{c}	0.1 (0.1)^{b,c}	ND
C	19.4 (4.2)	0.3 (0.3)^{c}	ND^{a}	ND^{a}	ND^{a}	ND
D	22.5 (5.0)	0.1 (0.3)^{b,c}	1.8 (0.7)^{c}	ND^{b,c}	0.2 (0.1)^{c}	ND
E	21.2 (3.8)	ND^{a}	0.8 (0.3)^{b,c}	ND^{b,c}	ND^{a}	ND
F	22.7 (4.3)	ND^{a,b}	1.1 (0.4)^{c}	ND^{b}	0.1 (0.1)^{b,c}	ND
G	ND^{a}	ND^{a,b,c}	ND^{a,b}	ND^{b,c}	0.0 (0.1)^{b,c}	ND
TOTAL	20.8 (4.6)	0.0 (0.4)	0.5 (0.9)	0.0 (0.4)	0.0 (0.3)	ND

Values in units of mg cyanidin-3-glucoside equivalents/kg full-fat chip).
ND = Not Detectable; "TOTAL" row is the mean and standard deviation of all blue corn samples for that specific anthocyanin (excludes yellow corn G).
Values with no letters in the same column are not statistically different ($p<0.05$).
Values with the same letter in the same column are not statistically different ($p<0.05$).

Conclusion

Blue corn tortilla chips provide anthocyanins in the form of a shelf-stable, processed food. Based on values previously reported for anthocyanins content of raw, whole grain blue corn, these numbers estimate that approximately half of anthocyanins from raw blue corn survive processing and can be consumed. Compared to a yellow corn tortilla chip, there is a significant amount of anthocyanins contained in the blue corn chips. When compared to fresh fruit, blue corn tortilla chips provide a similar amount of anthocyanins as a plum, based on the data from the TAC analysis. In a 100 g portion, blue corn tortilla chips provide 18.5 mg while a plum provides 13.4 mg. However, these numbers seem low compared to fruits and vegetables known to have higher amounts of anthocyanins such as strawberries (24.4 mg/100g), raspberries (35 mg/100g), and radishes (35.5 mg/100g). The advantage of anthocyanins present in blue corn tortilla chips, or similar products, is the convenience and stability of these products. While, fresh fruits and vegetables may yield more anthocyanins, they will not last nearly as long as processed products, allowing consumers to buy without the worry of waste. Blue corn tortilla chips, and similar products, provide an alternate way to consume anthocyanins, especially for populations that may not have access to fresh fruits and vegetables, or low-income populations.

These results suggest that, with all else being nutritionally equivalent between yellow and blue corn chips, the blue corn chips have the added advantage of providing potential health benefits. However, this is not to be seen as a nutritional recommendation

to initiate consumption of corn chips, but rather a preference for the pigmented product when consumption is occurring.

Future research directions include analyzing other processed pigmented grain products, such as black soybean milks and pastas, understanding the bioavailability of anthocyanins in food, and also understanding how shelf-stability affects anthocyanin content in processed foods. As anthocyanins are studied more, especially in processed foods, more alternate options to consume anthocyanins can be used by the public.

References

A More Colorful Diet Is A Healthier Diet. (2001, March 19). *The Food Institute Report*, p. 2.

Bò, C. D., Ciappellano, S., Klimis-Zacas, D., Martini, D., Gardana, C., Riso, P., & Porrini, M. (2010). Anthocyanin Absorption, Metabolism, and Distribution from a Wild Blueberry-Enriched Diet (*Vaccinium angustifolium*) Is Affected by Diet Duration in the Sprague−Dawley Rat. *Journal of Agricultural and Food Chemistry*, *58*(4), 2491–2497. http://doi.org/10.1021/jf903472x

Bueno, J. M., Sáez-Plaza, P., Ramos-Escudero, F., Jiménez, A. M., Fett, R., & Asuero, A. G. (2012). Analysis and Antioxidant Capacity of Anthocyanin Pigments. Part II: Chemical Structure, Color, and Intake of Anthocyanins. *Critical Reviews in Analytical Chemistry*, *42*(2), 126–151. http://doi.org/10.1080/10408347.2011.632314

Cassidy, A., Mukamal, K. J., Liu, L., Franz, M., Eliassen, A. H., & Rimm, E. B. (2013). High Anthocyanin Intake Is Associated With a Reduced Risk of Myocardial Infarction in Young and Middle-Aged Women. *Circulation*, *127*(2), 188–196. http://doi.org/10.1161/CIRCULATIONAHA.112.122408

Castañeda-Ovando, A., Pacheco-Hernández, M. de L., Páez-Hernández, M. E., Rodríguez, J. A., & Galán-Vidal, C. A. (2009). Chemical studies of anthocyanins: A review. *Food Chemistry*, *113*(4), 859–871. http://doi.org/10.1016/j.foodchem.2008.09.001

Clifford, M. N. (2000). Anthocyanins–nature, occurrence and dietary burden. *Journal of the Science of Food and Agriculture*, *80*(7), 1063–1072.

Cortés, G. A., Salinas, M. Y., Martín-Martinez, E. S., & Martínez-Bustos, F. (2006). Stability of anthocyanins of blue maize (Zea mays L.) after nixtamalization of seperated pericarp-germ tip cap and endosperm fractions. *Journal of Cereal Science*, *43*(1), 57–62. http://doi.org/10.1016/j.jcs.2005.05.003

Crozier, A., Del Rio, D., & Clifford, M. N. (2010). Bioavailability of dietary flavonoids and phenolic compounds. *Molecular Aspects of Medicine*, *31*(6), 446–467. http://doi.org/10.1016/j.mam.2010.09.007

Damodaran, S., Parkin, K. L., & Fennema, O. R. (Eds.). (2008). *Fennema's Food Chemistry* (4th ed.). Taylor & Francis Group, LLC.

Del Bo', C., Riso, P., Brambilla, A., Gardana, C., Rizzolo, A., Simonetti, P., … Porrini, M. (2012). Blanching Improves Anthocyanin Absorption from Highbush Blueberry (Vaccinium corymbosum L.) Purée in Healthy Human Volunteers: A Pilot Study. *Journal of Agricultural and Food Chemistry*, *60*(36), 9298–9304. http://doi.org/10.1021/jf3021333

Escalante-Aburto, A., Ramírez-Wong, B., Torres-Chávez, P. I., Figueroa-Cárdenas, J. D., López-Cervantes, J., Barrón-Hoyos, J. M., & Morales-Rosas, I. (2013). Effect of extrusion processing parameters on anthocyanin content and physicochemical properties of nixtamalized blue corn expanded extrudates. *CyTA - Journal of Food*, *11*(sup1), 29–37. http://doi.org/10.1080/19476337.2013.764929

Escalante-Aburto, A., Ramírez-Wong, B., Torres-Chávez, P., López-Cervantes, J., de Dios Figueroa-Cárdenas, J., Barrón-Hoyos, J., … Gutiérrez-Dorado, R. (2014). Obtaining Ready-to-Eat Blue Corn Expanded Snacks with Anthocyanins Using an Extrusion Process and Response Surface Methodology. *Molecules*, *19*(12), 21066–21084. http://doi.org/10.3390/molecules191221066

Felgines, C., Talavéra, S., Gonthier, M.-P., Texier, O., Scalbert, A., Lamaison, J.-L., & Rémésy, C. (2003). Strawberry anthoycanins are recovered in urine as glucuro- and sulfoconjugates in humans. *The Journal of Nutrition*, *133*(5), 1296–1301.

Felgines, C., Talavéra, S., Texier, O., Besson, C., Fogliano, V., Lamaison, J.-L., … Galvano, F. (2006). Absorption and metabolism of red orange juice anthocyanins in rats. *British Journal of Nutrition*, *95*(5), 898–904. http://doi.org/10.1079/BJN20061728

Fernandes, I., Faria, A., Calhau, C., de Freitas, V., & Mateus, N. (2014). Bioavailability of anthocyanins and derivatives. *Journal of Functional Foods*, *7*, 54–66. http://doi.org/10.1016/j.jff.2013.05.010

Gomez, A. C., Pérez, J. L. J., Orea, A. C., & Martinez, E. S. M. (2006). Photoacoustic Analysis of Blue Corn Pigments in Nixtamalized Flours. *International Journal of Thermophysics*, *27*(4), 1274–1280. http://doi.org/10.1007/s10765-006-0085-7

Gössinger, M., Moritz, S., Hermes, M., Wendelin, S., Scherbichler, H., Halbwirth, H., … Berghofer, E. (2009). Effects of processing parameters on colour stability of strawberry nectar from puree. *Journal of Food Engineering*, *90*(2), 171–178. http://doi.org/10.1016/j.jfoodeng.2008.06.018

Grotewold, E. (2006). The genetics and biochemistry of floral pigments. *Annu. Rev. Plant Biol.*, *57*, 761–780.

He, J., Magnuson, B. A., & Giusti, M. M. (2005). Analysis of Anthocyanins in Rat Intestinal ContentsImpact of Anthocyanin Chemical Structure on Fecal Excretion. *Journal of Agricultural and Food Chemistry*, *53*(8), 2859–2866. http://doi.org/10.1021/jf0479923

Horbowicz, M., Kosson, R., Grzesiuk, A., & Dębski, H. (2008). Anthocyanins of Fruits and Vegetables - Their Occurrence, Analysis and Role in Human Nutrition. *Vegetable Crops Research Bulletin*, *68*(-1). http://doi.org/10.2478/v10032-008-0001-8

Ichiyanagi, T., Shida, Y., Rahman, M. M., Hatano, Y., & Konishi, T. (2006). Bioavailability and Tissue Distribution of Anthocyanins in Bilberry (*Vaccinium myrtillus* L.) Extract in Rats. *Journal of Agricultural and Food Chemistry*, *54*(18), 6578–6587. http://doi.org/10.1021/jf0602370

Jang, H., Ha, U.-S., Kim, S.-J., Yoon, B.-I., Han, D.-S., Yuk, S.-M., & Kim, S.-W. (2010). Anthocyanin Extracted from Black Soybean Reduces Prostate Weight and Promotes Apoptosis in the Prostatic Hyperplasia-Induced Rat Model. *Journal of Agricultural and Food Chemistry*, *58*(24), 12686–12691. http://doi.org/10.1021/jf102688g

Jing, P. (2006). *Purple corn anthocyanins: chemical structure, chemoprotective sctivity and structure/function relationships*. The Ohio State University. Retrieved from http://rave.ohiolink.edu/etdc/view?acc_num=osu1155738398

Koh, K., Youn, J. E., & Kim, H.-S. (2014). Identification of anthocyanins in black soybean (Glycine max (L.) Merr.) varieties. *Journal of Food Science and Technology*, *51*(2), 377–381. http://doi.org/10.1007/s13197-011-0493-y

Kong, J. (2003). Analysis and biological activities of anthocyanins. *Phytochemistry*, *64*(5), 923–933. http://doi.org/10.1016/S0031-9422(03)00438-2

Lee, J., Durst, R. W., & Wrolstad, R. E. (2005). Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study. *Journal of AOAC International*, *88*(5), 1269–1278.

Lee, J. H., Kang, N. S., Shin, S.-O., Shin, S.-H., Lim, S.-G., Suh, D.-Y., … Ha, T. J. (2009). Characterisation of anthocyanins in the black soybean (Glycine max L.) by HPLC-DAD-ESI/MS analysis. *Food Chemistry*, *112*(1), 226–231. http://doi.org/10.1016/j.foodchem.2008.05.056

Li, J. (2009). *Total anthocyanin content in blue corn cookies as affected by ingredients and oven types*. Kansas State University. Retrieved from http://krex.k-state.edu/dspace/handle/2097/1673

Li, J., Walker, C. E., & Faubion, J. M. (2011). Acidulant and oven type affect total anthocyanin content of blue corn cookies. *Journal of the Science of Food and Agriculture*, *91*(1), 38–43. http://doi.org/10.1002/jsfa.4173

Lila, M. A., Burton-Freeman, B., Grace, M., & Kalt, W. (2016). Unraveling Anthocyanin Bioavailability for Human Health. *Annual Review of Food Science and Technology*, *7*(1), 375–393. http://doi.org/10.1146/annurev-food-041715-033346

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*(26), 7731–7737. http://doi.org/10.1021/jf020690l

McCallum, J. L., Yang, R., Young, J. C., Strommer, J. N., & Tsao, R. (2007). Improved high performance liquid chromatographic separation of anthocyanin compounds from grapes using a novel mixed-mode ion-exchange reversed-phase column. *Journal of Chromatography A*, *1148*(1), 38–45. http://doi.org/10.1016/j.chroma.2007.02.088

Moreno, Y. S., Sánchez, G. S., Hernández, D. R., & Lobato, N. R. (2005). Characterization of anthocyanin extracts from maize kernels. *Journal of Chromatographic Science*, *43*(9), 483–487.

Navas, M. J., Jiménez-Moreno, A. M., Bueno, J. M., Sáez-Plaza, P., & Asuero, A. G. (2012). Analysis and Antioxidant Capacity of Anthocyanin Pigments. Part IV: Extraction of Anthocyanins. *Critical Reviews in Analytical Chemistry*, *42*(4), 313–342. http://doi.org/10.1080/10408347.2012.680343

Papoušková, B., Bednář, P., Hron, K., Stávek, J., Balík, J., Myjavcová, R., … Lemr, K. (2011). Advanced liquid chromatography/mass spectrometry profiling of anthocyanins in relation to set of red wine varieties certified in Czech Republic. *Journal of Chromatography A*, *1218*(42), 7581–7591. http://doi.org/10.1016/j.chroma.2011.07.027

Patras, A., Brunton, N. P., O'Donnell, C., & Tiwari, B. K. (2010). Effect of thermal processing on anthocyanin stability in foods; mechanisms and kinetics of degradation. *Trends in Food Science & Technology*, *21*(1), 3–11. http://doi.org/10.1016/j.tifs.2009.07.004

Prior, R. L. (2004). Absorption and metabolism of anthocyanins: potential health effects. *Phytochemicals: Mechanisms of Action*, 1–19.

Rodriguez-Saona, L. E., & Wrolstad, R. E. (2001). Extraction, Isolation, and Purification of Anthocyanins. *Current Protocols in Food Analytical Chemistry*, F1.1.1–F1.1.11.

Sadras, V. O., & Moran, M. A. (2012). Elevated temperature decouples anthocyanins and sugars in berries of Shiraz and Cabernet Franc: Thermal decoupling of anthocyanins and sugars. *Australian Journal of Grape and Wine Research*, *18*(2), 115–122. http://doi.org/10.1111/j.1755-0238.2012.00180.x

Slavin, M., Kenworthy, W., & Yu, L. (Lucy). (2009). Antioxidant Properties, Phytochemical Composition, and Antiproliferative Activity of Maryland-Grown Soybeans with Colored Seed Coats. *Journal of Agricultural and Food Chemistry*, *57*(23), 11174–11185. http://doi.org/10.1021/jf902609n

Steimer, S., & Sjöberg, P. J. R. (2011). Anthocyanin Characterization Utilizing Liquid Chromatography Combined with Advanced Mass Spectrometric Detection. *Journal of Agricultural and Food Chemistry*, *59*(7), 2988–2996. http://doi.org/10.1021/jf104840v

Thomasset, S., Teller, N., Cai, H., Marko, D., Berry, D. P., Steward, W. P., & Gescher, A. J. (2009). Do anthocyanins and anthocyanidins, cancer chemopreventive pigments in the diet, merit development as potential drugs? *Cancer Chemotherapy and Pharmacology*, *64*(1), 201–211. http://doi.org/10.1007/s00280-009-0976-y

Wallace, T. C. (2011). Anthocyanins in Cardiovascular Disease. *Advances in Nutrition: An International Review Journal*, *2*(1), 1–7. http://doi.org/10.3945/an.110.000042

Wallace, T., Slavin, M., & Frankenfeld, C. (2016). Systematic Review of Anthocyanins and Markers of Cardiovascular Disease. *Nutrients*, *8*(1), 32. http://doi.org/10.3390/nu8010032

Walton, M. C., Lentle, R. G., Reynolds, G. W., Kruger, M. C., & McGhie, T. K. (2006). Anthocyanin Absorption and Antioxidant Status in Pigs. *Journal of Agricultural and Food Chemistry*, *54*(20), 7940–7946. http://doi.org/10.1021/jf061527j

Wang, W.-D., & Xu, S.-Y. (2007). Degradation kinetics of anthocyanins in blackberry juice and concentrate. *Journal of Food Engineering*, *82*(3), 271–275. http://doi.org/10.1016/j.jfoodeng.2007.01.018

Whent, M. M. (2009). *Nutraceutical Properties of Low Alpha-linolenic Soybeans Grown in Maryland*. ProQuest. Retrieved from http://books.google.com/books?hl=en&lr=&id=0tdGVzbAwwYC&oi=fnd&pg=PR2&dq=%22Table+1:+Correlation+between+18:3n-3+and+other+soybean%22+%221:+Desired+fatty+acid+profile+for+oil+used+in

+commercial+food%22+%223:+Isoflavone,+lutein,+and+tocopherol+content+of%22+&ots=ARezyc0P2N&sig=Lk5Ge5q1KuN0Q7FITb0_VL6e57w

Wu, Y., Wang, Y., Zhang, W., Han, J., Liu, Y., Hu, Y., & Ni, L. (2014). Extraction and preliminary purification of anthocyanins from grape juice in aqueous two-phase system. *Separation and Purification Technology*, *124*, 170–178. http://doi.org/10.1016/j.seppur.2014.01.025

Yue, X., & Xu, Z. (2008). Changes of Anthocyanins, Anthocyanidins, and Antioxidant Activity in Bilberry Extract during Dry Heating. *Journal of Food Science*, *73*(6), C494–C499. http://doi.org/10.1111/j.1750-3841.2008.00845.x

Zhu, Y., Xia, M., Yang, Y., Liu, F., Li, Z., Hao, Y., … Ling, W. (2011). Purified Anthocyanin Supplementation Improves Endothelial Function via NO-cGMP Activation in Hypercholesterolemic Individuals. *Clinical Chemistry*, *57*(11), 1524–1533. http://doi.org/10.1373/clinchem.2011.167361

BIOGRAPHY

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