IMPROVEMENT OF EXTRACTABILITY AND AQUEOUS STABILITY OF SORGHUM 3-DEOXYANTHOCYANINS

A Thesis

by

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ABSTRACT

Consumer interest in natural ingredients has increased demand for natural food colorants. 3-Deoxyanthocyanin pigments from sorghum are more stable to food processing conditions than anthocyanin analogs. However, 3-deoxyanthocyanins self-associate in aqueous solution and are difficult to extract from sorghum tissue, limiting their application as natural food colorants. The goal of this research is to improve extractability and aqueous stability of sorghum 3-deoxyanthocyanins.

Effect of gum arabic, sodium alginate (0.5g/L and 1.0g/L), and metal ions Fe^{3+} or Mg^{2+} (0.2mM) on aqueous stability of 3-deoxyanthocyanins was investigated at pH 3 and pH 5. Pigments were obtained from red sorghum leaf (predominantly luteolinidin derivatives) and sorghum leaf sheath (predominantly apigeninidin derivatives) via standard extraction procedure (1% HCl in methanol/2 h/20°C). Additionally, a black sorghum grain (luteolinidin/apigeninidin derivative mixture) was used to evaluate the effect of microwave irradiation on extractability of the 3-deoxyanthocyanins from black sorghum grain; this was investigated utilizing different power (300-1200 W) and time (0.25-40 min) settings. Blue maize and black cowpea, containing acylated and non-acylated anthocyanins pigments, respectively, were used to for comparison. Pigment yield and profile were determined using UV-Vis and UPLC-MS, respectively.

Both gums effectively stabilized 3-deoxyanthocyanins. Gum arabic stabilized apigeninidin dominant (64-96% color retention) and luteolinidin/apigeninidin extracts (59-92% color retention) but not luteolinidin dominant extracts (0-11% color retention). This was likely caused by enhanced hydrophobic interaction with apigeninidin. The

stabilizing effect of alginate was more effective at pH 5 (74% mean color retention) than pH 3 (64 mean % color retention). Thus alginate effect was likely due to ionic and hydrogen bonds. Metal ions exhibited generally negligible effect. However, luteolinidin in alginate and Fe^{3+} yielded a deep black color, possibly an Fe^{3+} mediated luteolinidin-alginate complex.

Microwave-assisted extraction increased yield of 3-deoxyanthocyanins almost three-fold (2.44 mg/g) relative to control (0.88 mg/g). The 3-deoxyanthocyanin profile exhibited generally insignificant change; however, long exposure (30–40 min) at 600-1200 W produced cyanidin in extracts, possibly due to structural transformation caused by microwave irradiation. Anthocyanin profiles of maize and cowpea exhibited extensive hydrolysis of acyl esters and glycosides. Microwave-assisted extraction increased extractability of sorghum 3-deoxyanthocyanins, improving their potential application as natural food colorants.

DEDICATION

To my parents Tim and Barb. Happy mother's day, father's day, 26th anniversary of parenting your favorite scientist, 60th birthday, and Merry Christmas.

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I am humbled to have what feels like an endless list of people to thank for the support, mentoring, and guidance I have received during the odyssey my academic and research career has become over the past decade which has culminated in this thesis.

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CHAPTER I

INTRODUCTION

Consumer demand for natural colorants in their food has sky rocketed in the past two decades. “Clean label” claims are increasingly important to consumers. More than twenty percent of new products in the US in 2014 featured “Clean label” positioning (Bizzozero 2015). Colorants are a key aspect of a clean label. Over the past forty years studies have linked the consumption of synthetic dye to hyperactivity, the first of which was the Feingold 1975 study (Feingold 1975). Thirty years later McCann et al. published a widely cited Lancet article linking hyperactivity in children to synthetic dye which has had a significant impact on public opinion of synthetic dyes in food (McCann et al 2007). Studies such as these have caused consumer concern and distrust for synthetic dyes. As a result of public demand, in July 2010 the European Union began requiring warning labels on products containing certain synthetic dyes (Decker 2014; EC 1333/2008 2008; Buchweitz 2012). This has increased food industry and consumer interest in natural alternatives. Between January 2009 and December 2011 in the United States, 66% of new product launches contained natural colors while only 34% contained synthetic or artificial colors. In this same time frame, 85% of new products in Europe contained natural colorants (Kunkel 2014).

Anthocyanins are the most common water soluble natural colorants used in food and beverage formulations. Anthocyanins are responsible for red, purple, and blue characteristic of many fruits, vegetables, and flowers. Anthocyanins for commercial use are most frequently extracted from purple potato and carrots, and berries. Anthocyanins provide vibrant color but possess three major short comings as commercial natural food colorants. First is sensitivity to conditions characteristic of food processing including: pH, heat, and oxidation. Second, anthocyanins are relatively expensive sources of colorant. Sources of anthocyanins are high in moisture content and enzyme activity causing short shelf life and difficult storage. Third, the functional strength of anthocyanins as a colorant is low resulting in large amounts of anthocyanins required for desired color in a food product.

For these reasons, there is an opportunity for alternative water soluble natural colorants. 3-Deoxyanthocyanins (3DXN) are anthocyanin analogs lacking a substitution at position 3 of heterocyclic ring (C3). The 3DXNs have comparatively greater stability to pH (Mazza and Brouillard 1987), heat (Yang et al 2014), and oxidizing environments (Ojwang and Awika 2008) associated with food processing than anthocyanins. Sorghum is the primary source of 3DXNs, which occur throughout most of the plant tissue and can be extracted from the bran, leaves, and stalk of the plant. These sources are easily

dried, and generally considered waste products of grain production making them less expensive sources of pigments.

The 3DXNs, however, have two major obstacles that limit their potential as natural food colorants:

1. Reduced hydrophilicity relative to anthocyanin analogs which causes 3DXNs pigment molecules to rapidly self-associate in aqueous systems.
2. Generally difficult to extract from sorghum tissue.

Thus, methods to stabilize the pigments in aqueous systems and improve their extractability are needed.

Anthocyanins are stabilized naturally within plants by complex mixtures of polysaccharides and metal cations. Metalloanthocyanins are supramolecules composed of 6 anthocyanin molecules, 6 flavone molecules, and 2 metal ions (Yoshida 2015). The most common example of these metalloanthocyanins are blue pigments found in flower petals. The metal ions, specifically multivalent cations interact with anthocyanin hydroxyl groups. This interaction results in stacking and formation of the large metalloanthocyanins. It is likely that by mimicking these naturally occurring matrices, aqueous stabilization of 3DXN will be possible.

The purpose of this research is to overcome the barriers limiting utilization of 3DXNs from sorghum as natural food colorants including difficult extraction and rapid

self-association. Solutions to these problems will better position 3DXNs from sorghum for use as a commercial natural food colorant.

Research Objectives

1. Determine effect of microwave energy on 3DXN extraction from sorghum bran

2. Establish effect of polysaccharide gums and multivalent cations on aqueous stability of 3DXN pigments.

CHAPTER II

LITERATURE REVIEW

Importance of Pigments to the Food Industry

Color is the sensory attribute that most influences consumer perception of quality. Color significantly influences perceived flavor, refreshment, and odor (Zellner and Durlach 2003; Clydesdale et al 1992; Johnson er al 1982; Johnson and Clydesdale 1982; Zellner and kautz 1990; Zellner and Whitten 1999; Zellner and Durlach 2003; Clydesdale 1992). Because quality attributes are directly linked to the color of a food or beverage, the color of a food holds significant influence in a consumer's purchase decision. Colorants are frequently added to food products to increase perceived quality. Traditionally this has been in the form of synthetic dyes. Synthetic dyes are vibrant, inexpensive sources of food color. The majority of synthetic dyes are organic molecules derived from petroleum. In order to increase product appeal to consumers, the amount of dye added to food continues to rise. The milligrams of dye per capita have increased fivefold over the past sixty years (Kobylewski 1992). Between 2009-2013 the natural color sector grew 77% in the US. Globally, natural colorants are out selling synthetic colorants. The growth in the natural colorant market is projected to continue to grow and by 2017 the natural colorant market is estimated to reach $1.7 billion (Decker 2014).

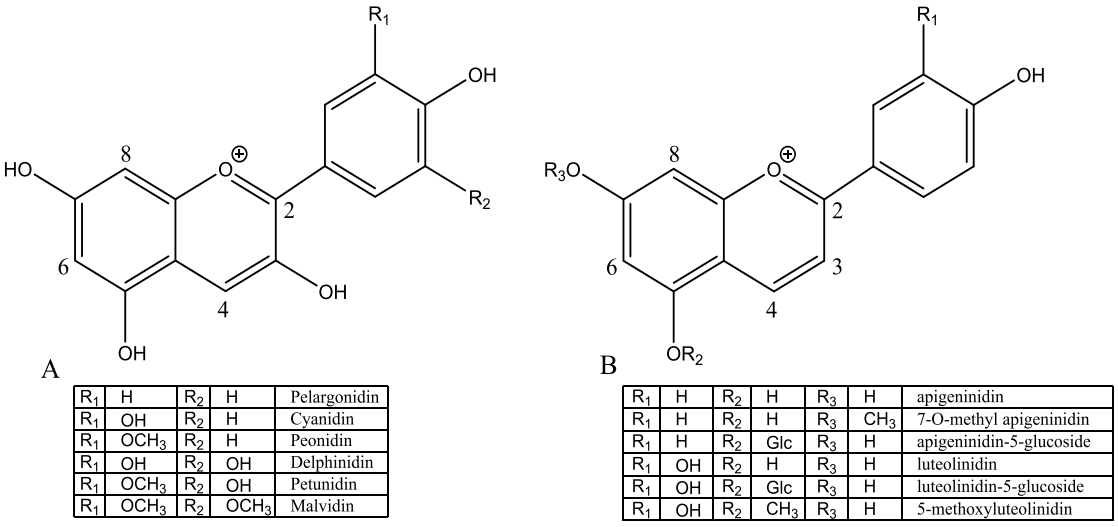
Anthocyanins as Natural Colorants

Anthocyanins are a class of polyphenolic compounds naturally occurring in plants and are responsible for a range of colors including red, orange, yellow, blue, and violet. Anthocyanins are one of a number of classes of polyphenols and are characterized as glycosylated polyhydroxy and polymethoxy derivatives of flavylium salts (Brouillard et al 1982; Motohashi 2012). Anthocyanidins are the base pigments from which anthocyanins form. Anthocyanins are glycosylated at C3 and/or C5 (Gould et al 2009) (See Fig. 1). Because of glycosylation at C3 and/ or C5, anthocyanins are significantly more stable than anthocyanidins and are thus the configuration, nearly exclusively, found in nature (Francis 1989; Lacobucci and Sweeny 1983).

Anthocyanins are one of the most common natural food colorants and are popular for their vibrant red, blue, and purple hues. These are most frequently extracted from purple carrots, purple corn, purple potatoes, and berries (Bridle and Timberlake 1997; Li et al 2013). In addition to their appeal as a natural food colorant, anthocyanins have a number of associated health benefits including functioning as an anti-inflammatory (Lietti et al 1976), anti-cancer/ chemopreventative (Yang et al 2009; Hui et al 2010; Suganyadevi et al 2013; Devi et al 2011; Shis et al 2006), and powerful antioxidants (Devi et al 2012; Benson et al2013). However, sources of anthocyanins possess high moisture content and enzyme activity making them difficult to store and

transport (Petti et al 2014). Additionally, anthocyanins have limited stability particularly to the extremes associated with food processing such as heat (Turker er al 2004; Marti et al 2002; Brouillard and Delaporte 1977), pH (Francis 1989; Brouillard and Delaporte 1977; Song et al 2013; Cemeroglu et al 1994; Turfan et al2011; Sadilova et al 2006; Kirca and Cemeroğlu 2003), and food additives such as ascorbic acid (Sondheimer and Kertesz 1953; Francis and Shrikhande 1974) and sulfites (Kozlowski 1936; Jurd 1964).

Anthocyanin Structure



R_1	H	R_2	H	Pelargonidin
R_1	OH	R_2	H	Cyanidin
R_1	OCH_3	R_2	H	Peonidin
R_1	OH	R_2	OH	Delphinidin
R_1	OCH_3	R_2	OH	Petunidin
R_1	OCH_3	R_2	OCH_3	Malvidin

R_1	H	R_2	H	R_3	H	apigeninidin
R_1	H	R_2	H	R_3	CH_3	7-O-methyl apigeninidin
R_1	H	R_2	Glc	R_3	H	apigeninidin-5-glucoside
R_1	OH	R_2	H	R_3	H	luteolinidin
R_1	OH	R_2	Glc	R_3	H	luteolinidin-5-glucoside
R_1	OH	R_2	CH_3	R_3	H	5-methoxyluteolinidin

Figure 1. A: Basic structure of an anthocyanidin. B: Basic structure of 3-deoxyanthocyanidin. Lack of substitution at C3 is the key structural difference between A and B.

Anthocyanins exist in four common species two of which are colorless. The four species are the flavylium cation AH^+, quinoidal base A, pseudobase/ carbinol B, and chalcone C. B and C are colorless species. The two colored species have different

chromophoric properties and the ratio of the two at equilibrium dictate the perceivable color. The four species exist in equilibrium (Iacobucci and Sweeny 1983; Brouillard and Delaporte 1977; Mazza and Brouillard 1987b; Brouillard and Dubois 1977; Sweeny and Iacobucci 1983).

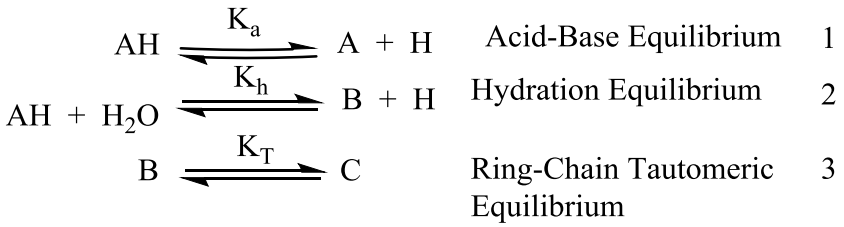


Figure 2. Anthocyanin equilibrium constants where K_a, K_h, and K_T are the equilibrium constants for acid-base, hydration, and tautomeric reactions, respectively.

The reaction equilibria of anthocyanins shift according to environment but are influenced most by pH (Brouillard and Dubois 1977). AH^+ is the predominant species at low pH, typically pH 2 and below (Brouillard and Delaporte 1977). As pH rises, rapid proton loss occurs resulting in bathochromic shift and a blue pigment (reaction 1 in Fig. 2.) (Iacobucci and Sweeny 1983). Nucleophilic attack results in hydration of the molecule and in structural change causing the molecule to lose visible color (reaction 2 in Fig. 2) (Brouillard and Delaporte 1977). After hydration, tautomerization can occur at the pyrylium ring resulting in opening of the ring (reaction 3 in Fig. 2). These reactions are further depicted in Fig. 3 below.

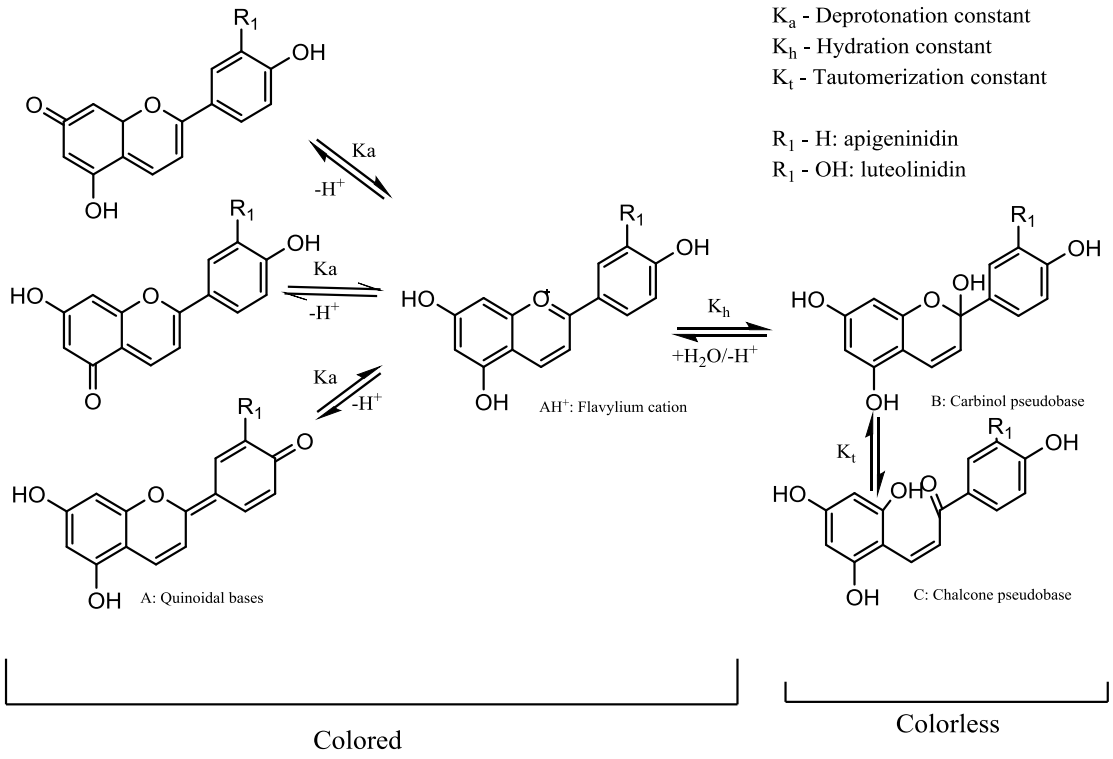


Figure 3. An illustration of the species of 3-deoxyanthocyanins that exist in varying concentrations depending primarily on the pH of solution.

Acidity constants vary by anthocyanin; anthocyanins with greater K_a or lower K_h, retain more color as pH increases.

Because the intended use of anthocyanins is as natural colorants, the colored species (flavylium cation and quinoidal base) are of greatest interest. However, because these species have limited stability and are highly influenced by environment, the application of anthocyanins as natural food colorants is limited.

3-Deoxyanthocyanins

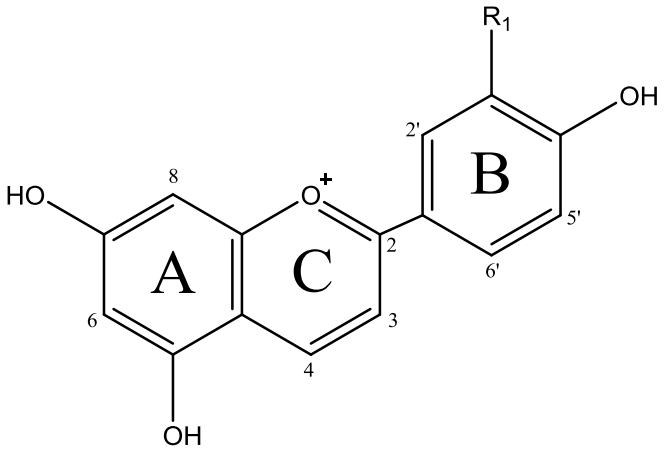


Figure 4. The basic structure of a 3-deoxyanthocyanidin

A 3DXN is an anthocyanin with no substitution at C3 (Iacobucci and Sweeny 1983; Awika et al 2004). This lack of substitution results in unique properties that enhance the stability of 3DXNs to processing conditions. The 3DXNs have greater stability to change in pHs (Mazza and Brouillard 1987; Awika et al 2004; Greea et al 2012), heat (Yang et al 2014), and the presence of oxidizing agents like ascorbic acid (Ojwang and Awika 2008) and sulfites (Geera et al 2012; Ojwang and Awika 2008) than anthocyanin analogs.

The lack of substitution at C3 results in a region between C5 to C4' that has greater hydrophobicity than their anthocyanin analogs and is less reactive with hydrophilic molecules (Fig. 4.). This region is unique to 3DXNs and is one of the main

contributing factors for the unique chemical and biochemical properties of 3DXNs (Mazza and Brouillard 1987; Yang et al 2014; Awika et al 2004; Greea et al2012; Awika 2008). This region causes 3DXNs to be less susceptible to nucleophilic attack which results in bonds breaking, hydration, and conformational change at C2 (Mazza and Brouillard 1987; Yang et al 2014; Brouillard 1982; Brouillard and Dubois 1977; Brouillard 1982). The result is an equilibrium shift favoring colored species. 3DXNs are less susceptible to nucleophilic attack and subsequent color loss than anthocyanin analogs, making them well suited for use as natural food colorants.

The advantages of 3DXNs as natural food colorants have been well documented (Yang et al 2014; Ojwang and Awika 2008; Awika et al 2004; Greea et al 2012; Ojwang and Awika 2010). Obstacles however, remain in the way of practical commercial use of 3DXNs as natural food colorants. For example, while 3DXNs are a water-soluble pigment, the same region of the molecule that provides enhanced stability to processing conditions, also results in rapid self-association. This self-association prohibits 3DXNs from being used as a natural food colorant in an aqueous application as-is and requires some method of stabilization to prevent self-association to keep the pigment evenly dispersed throughout the solution.

Source of 3-Deoxyanthocyanins

3DXNs are predominantly found in pigmented sorghums. They are located in the

bran, leaves, leaf sheaths and stalk (Petti et al 2014; Geera et al 2012; Awika et al 2004; Kayodé et al 2011; Dykes and Rooney 2010). There are two predominant forms of 3DXNs that occur in sorghum: apigeninidin and luteolinidin. They are accompanied by their most common methoxylated derivatives 7-methoxy-apigeninidin, and 5-methoxy-luteolinidin, as well as other less common derivative forms (Geera et al 2012; Khalil 2010). The two forms differ by one hydroxyl group at C3' of the B-ring; luteolinidin has a catechol group on the B ring. The two forms exist in ratios that vary by sorghum type.

3-Deoxyanthocyanins are relatively inexpensive sources of natural pigments. They are extracted from leaves, leaf sheaths, stalk, and bran all of which are generally considered a waste product of sorghum (Petti et al 2014; Awika et al 2004; Geera et al 2012; Dykes and Rooney 2010). These sources are dried easily allowing for inexpensive transport and long-term storage.

Extraction of 3-Deoxyanthocyanins

The location of 3DXN pigment molecules particularly within the bran of the sorghum grain can make extraction difficult. The pigments are held tightly within cell vacuoles. To reach the pigments in the vacuole the cell wall must be broken. Breaking through the cell wall is difficult and requires a significant amount of agitation. For benchtop extraction of anthocyanins and other phenolic compounds, three primary methods exist for extraction: conventional solvent, accelerated solvent extraction, and

microwave assisted extraction. Conventional solvent extraction for 3DXNs typically use acidified organic solvent (typically methanol) and prolonged agitation (Geera et al 2012; Awika et al 2004; Awkia et al 2009; Dykes and Rooney 2010). This process requires a substantial amount of time, typically at least two hours (Awika et al 2004; Dykes and Rooney 2010). Accelerated solvent extraction (ASE) utilizes high pressure and heat to increase the rate at which pigment is extracted (Barros et al 2013). ASE is more time efficient than the conventional solvent extraction method because extractions require significantly less time with similar or greater extraction yields (Barros et al 2013; Abdel-Aal et al 2014). Finally, a less common method of pigment extraction is microwave-assisted extraction (MAE). MAE uses microwave irradiation to rapidly and precisely heat the extraction system resulting in faster extraction with less solvent (Dang et al 2014; Lamble and Hill 1998). MAE itself is not uncommon, however, its use for extraction of pigments is a fairly new practice and has never been utilized for extraction of 3DXN pigments from sorghum.

MAE is an extraction process using microwave energy to excite polar molecules to increase yield of extraction either by generation of heat or by rapid movement of polar molecules resulting in liberation from otherwise strong chemical bonds (Guo et al 2001). MAE is time efficient and reduce solvent consumption making it both environmentally and economically advantageous (Desai et al 2010).

Microwaves penetrate into cellular structure causing vaporization of water molecules and volatile materials causing cells to rupture (Desai et al 2010). Once cell walls have been disrupted, compounds which were previously bound and not extractable are liberated and extracted resulting in a process with substantially higher efficiency. Dang et al. used MAE to enhance extraction of proanthocyanidins from grape seed. Less time and solvent were required for phenolic extraction and maintained greater than 99.3% recovery rate (Dang et al 2014). Proestos and Komaitis compared reflux extraction with MAE for extraction of plant phenolic compounds. Results showed MAE reduced time and solvent necessary for extraction and increased the phenolic compounds extracted (Proestos and Komaitis 2008).

It is likely that the same benefits experienced with MAE in these extractions of phenolic compounds could also be translated into 3DXN pigment extraction. Dabiri et al (2005) used MAE to optimize pigment extraction from Rubia tinctorum resulting in an extraction process with more than 80% greater recovery than previously utilized method requiring substantially less time. Similarly, when Abdel-Aal et al (2014) compared MAE, ASE, and conventional solvent extraction methods for extraction of anthocyanins from cereal grains, MAE yielded the greatest extraction of anthocyanins. MAE however, resulted in structural change of anthocyanins. This could be a result of poor heat stability of anthocyanins, or the glycosidic structure of anthocyanins causing them to be more

influenced by microwaves, or perhaps the microwaves caused cleavage between the sugar at C3 and anthocyanin resulting in dramatically reduced stability of the anthocyanin. It is possible that with greater heat stability (Yang et al 2014), 3DXNs would be more effectively extracted using MAE.

Aqueous Stabilization of Anthocyanins

In nature, anthocyanins are stabilized in the plant cell vacuoles via multivalent metal ions which create ionic bridges with the anthocyanin molecules (Bayer et al 1991; Goto and Kondo 1991; Hayashi et al 1958) and polysaccharides (Yonekura-Sakakibara et al 2009). It is likely that by mimicking these matrices it would be possible to stabilize 3DXNs in aqueous solution.

Multivalent Cations as Anthocyanin Stabilizers

Part of natural stabilization of anthocyanins is complexation with multivalent metal ions forming ionic bridges between anthocyanins and multivalent ion (Vázquez and Ramírez de León 2012). This supramolecule is referred to as a metalloanthocyanin and is composed of six molecules of anthocyanin, six molecules of flavone, and two metal ions (Mori et al 2008). These metealloanthocyanins are most common in blue flowers.

A number of multivalent cations have been used in an attempt to stabilize anthocyanins in aqueous solution by forming metallic anthocyanin complexes. Bayer et

al (1966) examined color enhancement of Co, Ni, Ca, Ba, Fe^{3+}, Al, Mg, Sn(IV), Ti(III), Cr(III), and UO_2^{2+}. Fe(III), Al, Sn(IV), Ti(III), Cr(III), and UO_2^{2+} enhanced pigment quality at weakly acidic to neutral pH. Takeda et al (1994) applied K, Ca, Mn, Co, Cd, Zn, Ni, Al, and Fe^{2+} to anthocyanin, and found that Mg, Mn, Co, Cd, Zn, and Ni formed metallic anthocyanin complexes with protodelphinidin. Dangles et al (1992) complexed aluminum-anthocyanin in aqueous solution. When increasing the pH of the solution, Al^{3+} chelated with the flavylium ion (AH^+) and shifted the molecule to the quinoidal base resulting in a purple solution. This happened at pH 3-4.4, which would otherwise result in a hydrated colorless pseudo base or chalcone. It also became evident the Al chelation was in competition with hydration, resulting in the dehydration of pseudo base and return to colored quinoidal base (Brouillard 1992).

Magnesium is a divalent cation common in anthocyanin stabilization. Mg^{2+} is known for its role in stabilizing blue pigment in a number of flowers (Takeda et al 1984; Takeda et al 1994; Mori et al 2008; Takeda 2006; Shiono et al 2005). Takeda et al (1984) explored the molar ratios of anthocyanin to Mg *in vitro*. Greater concentration of Mg yielded greater concentration of blue pigment. The magnesium: anthocyanin ratio reached maximum pigment formation at 0.5. This ratio was observed in *Salvia patens* flowers (Takeda et al 1994). Within the pigment extracted from the *Salvia patens* flower, a stable complex was not attained without the presence of Mg (Takeda et al

1994). Mori et al (2008) analyzed the blue pigment found in the *Salvia uliginosa* flower and found Mg, Al, and Fe; however, the Al and Fe were only in trace amounts and Mg was the predominant ion present. Furthermore, when Mg was removed from the pigment components, color quality reduced and the hue became unstable.

Given the ability of multivalent cationic metals to form metallic anthocyanin complexes and improve color quality and stability, it is likely that the metal ions will enhance the stability of 3DXNs in aqueous solution. However, because the purpose of stabilizing 3DXNs in aqueous solution is to be used as a natural food colorant, only metal ions which are safe for consumption are practical to use. For this reason Mg and Fe are prime candidates for use in 3DXN stabilization in aqueous solution.

Structure of Metal-Anthocyanin Complexes

Many different anthocyanins have been studied for metal-anthocyanin formation, primarily pelargonidin-3-glucoside (Buchweitz et al 2012; Tachibana et al 2014), cyanidin-3-glucoside (Takeda et al 1984; Tachibana et al 2014), delphinidin-3-glucoside (Mori et al 2008; Buchweitz et al 2012; Tachibana et al 2014), cyanosalvianin (Mori et al 2008), and malonylawonanin (Takeda et al 1994 Takeda et al 1984). For formation of a metal-anthocyanin complex, the multivalent metal cation requires at least two hydroxyl groups in ortho position on the B ring (refer to Fig. 5). This was observed by Buchweitz et al. via raman spectroscopy (Buchweitz et al 2012). They observed a similar shift in

wavelength in cyanidin-3-glucoside, and delphinidin-3-glucoside upon addition of metal ion that was not observed with pelargonidin-3-glucoside. Tachibana et al. (2014) had similar results when comparing pelargonidin-3-glucoside, cyanidin-3-glucoside, and delphinidin-3-glucoside and their chelation with Fe^{3+}. Pelargonidin-3-glucoside had significantly lower color intensity and showed no change in color intensity upon addition of Fe^{3+}, contrary to cyanidin-3-glucoside, and delphinidin-3-glucoside, which had markedly increased color intensity as a result of metal ion addition.

Multivalent cations are able to form metal-anthocyanin complexes through interaction with hydroxyl groups on the B ring as mentioned. This means that for stabilization of 3DXNs, luteolinidin may form a complex with metal ions while apigeninidin will not. This will likely result in different degrees of stabilization depending on the source of 3DXNs as the ratio of the two 3DXNs varies from source to source.

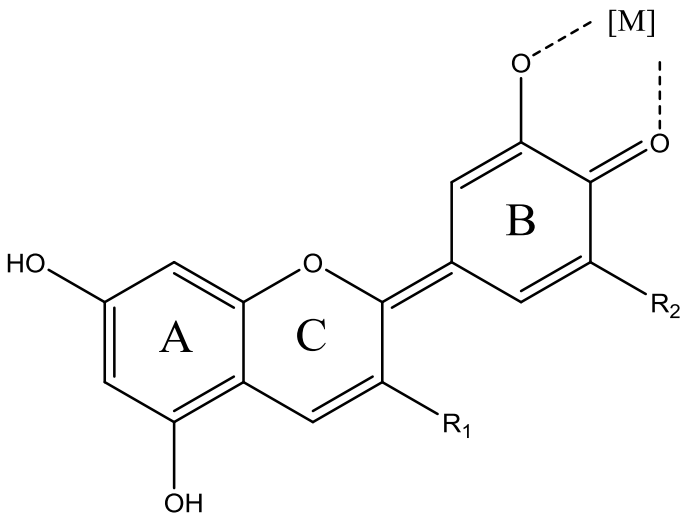


Figure 5. Structure of metal-anthocyanin complex

Polysaccharides as Anthocyanin Stabilizers

Anthocyanins need stabilization primarily to prevent nucleophilic attack and hydration. 3-Deoxyanthocyanis, on the other hand, need stabilization matrices to mitigate self-association. Some polysaccharides such as pectin, glucose, fructose, and maltose are known to improve stability and color retention of anthocyanins (Tachibana et al 2014; Lewis et al 1995; Chung et al 2016), while others such as amylose, amylopectin, α-cyclodextrin, β-cyclodextrin, and polydextrose are ineffective (Lewis et al 1995). The efficacy of many of these polysaccharides is likely a result of modification of water activity rather than an interaction with the anthocyanin. These polysaccharides are added in very high concentration, up to 50% (Lewis et al 1995). When limited water

is available for reactions in a system, there is limited potential for hydration reactions with anthocyanins and subsequent color loss (Lewis et al 1995).

For stabilizing anthocyanins for beverage colorant use, it is necessary for the polysaccharide to possess key characteristics including but not limited to: low impact on viscosity and flavor, chemical interaction with the anthocyanin, and efficacy at low concentration. These characteristics rule out many polysaccharides utilized in previous work to stabilize anthocyanins (Tachibana et al 2014; Lewis et al 1995; Chung et al 2016). For 3DXNs, it may be important that the polysaccharide has both hydrophilic and hydrophobic moieties. This is because of the unique structural variation at C3 in 3DXNs as opposed to anthocyanin analogs (See Fig. 1.). The lack of substitution at C3 results in a large region of the molecule that is relatively hydrophobic and prone to rapid self-association in aqueous solution. A polysaccharide with emulsifying properties has the potential to stabilize the pigment by interacting with the hydrophobic region of the molecule and water molecules to reduce self. Additionally, a negatively charged polysaccharide may also be effective in interacting with 3DXNs in acid environments as they carry a positive charge. Two polysaccharides with potential for 3DXN stabilization are sodium alginate and gum arabic.

Sodium Alginates

Alginates are high molecular weight polymers that are extracted from brown seaweed and carry a negative charge. Alginates are composed of the building blocks β-D-mannuronic acid and its C5 epimer, α-L-guluronic acid. The two link together forming a linear polysaccharide with (1,4)-glycosidic bonds and the proportion of the two dictates physical characteristics (Helgeurd et al 2009; McHugh 1987). Alginates increase viscosity in aqueous solution and are used as both a thickening and gelling agent in the presence of calcium (Nishinari 1993; Braccini and Perez 2001). In food alginate is used in products such as syrup, gravy, and pie filling (Helgerud et al 2009).

Viscosity modification aside, alginate can participate in stabilization matrixes for compounds such as anthocyanins by way of its anionic charge. Tachibana et al. (2014) used Sodium alginate and Fe^{3+} to stabilize cyanidin-3-glucoside with great success. Effect of sodium alginate alone was not investigated however the combination of sodium alginate and metal ion yielded the most successful stabilization matrix of those examined. It was proposed that the anionic charge of sodium alginate allowed for the formation of a stable complex between alginate, metal ion, and anthocyanin. Given these chemical and physical properties of sodium alginate, it may be possible to form a stable 3DXN in aqueous solution.

Gum Arabic

Gum Arabic is a proteinaceous polysaccharide known for its stabilizing characteristics of oil in water emulsions in acidic environments (William and Phillips 2008; Ma et al 2015). It is one of the most common food hydrocolloids. Gum arabic is composed primarily of 1, 3-linked β-D-galactopyranosyl (Patel and Goyal 2015) and to a lesser extent, arabinopyranose, arabinofuranose, rhamnopyranose, glucuropyranosyl uronic acid, and 4-O methyl glucuropyranosyl uronic acid (Islam et al 1997). In addition to polysaccharide chains, gum arabic has a protein content ranging from 1.5-3% (Ma et al 2015) comprised primarily of hydroxyproline and serine (Islam et al 1997). The combination of large hydrophilic polysaccharide blocks and hydrophobic protein backbone results in emulsification properties (refer to Fig. 6) (Dror et al 2006).

Gum arabic is a unique gum in that it contributes insignificantly to solution viscosity, exhibiting Newtonian behavior in a 50% solution (Islam et al 1997). Because of these properties it is utilized by the food industry to stabilize hydrophobic flavors in beverage systems and used at a concentration of approximately 1% (Jafari et al 2012). Gum arabic does not significantly influence viscosity or flavor and thus is an excellent potential 3DXN stabilizer.

Gum arabic is known to enhance stability of anthocyanins in aqueous solution. Chung et al. utilized gum arabic to enhance the color stability of anthocyanins in a

beverage model system (Chung et al 2016). Gum arabic enhanced stability of anthocyanins in the concentration range of 0.05%-1.50%. Gum arabic also enhances thermal stability of anthocyanins in mildly acidic solution (Guan and Zhong 2015). Furthermore, fluorescent spectra and atomic force spectra suggested there was a chemical interaction between gum arabic and anthocyanins (Tachibana et al 2014; Guan and Zhong 2015). These characteristics provide compelling evidence for suitability of gum arabic as a stabilizer of 3DXNs.

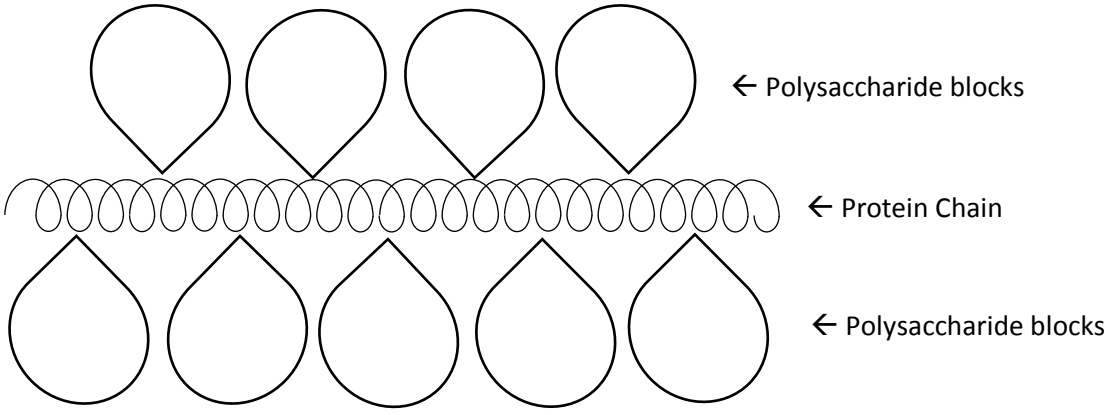


Figure 6. Illustration of gum arabic structure. Polysaccharide blocks are hydrophilic and interact with water molecules in aqueous solution. Protein chain is hydrophobic. The presence of hydrophilic and hydrophobic constituents result in emulsification properties.

Overall Goal of the Research

Several studies have demonstrated a variety of functional advantages of 3DXNs as potential natural food colorants. However, a need exists to improve extraction efficiency and stability of 3DXNs in aqueous solution to advance commercialization of these food colorants. This work investigates the potential of MAE to enhance extractability of the 3DXA pigments, and the ability of polysaccharide gums and metal cations to stabilize the pigments in aqueous solution.

CHAPTER III

MATERIALS AND METHODS

Materials

Sources of 3-Deoxyanthocyanin Pigments

The three sources of pigment were chosen because they have different profiles of 3DXNs. Apigeninidin and luteolinidin and their derivatives are the 3DXNs present in sorghum. Examination of extracts with different 3DXN composition may lend greater insight into functionality of various stabilization components. For example, will stabilizations of luteolinidin be more effective in the presence of ion than apigeninidin because of the ortho configuration on the B ring? In addition to the different 3DXN ratios, different copigments and natural stabilization matrixes exist in each of the three sources.

Sorghum Leaf Sheath

Dried red sorghum leaf sheath was obtained from Health Forever Products, Lagos, Nigeria. This material was previously described by Yang et al (2014) and Geera et al (2012). Samples were extracted using the conventional extraction method. Leaf sheath pigment is predominantly apigeninidin and apigeninidin derivatives.

Red Sorghum Leaf

This is a mutant sorghum that accumulates 3-deoxyanthocyanins its red leaves. The material was previously described by Petti et al (2012). The leaf pigment is predominantly luteolinidin.

Black Sorghum Grain

A black sorghum grain (Tx430) with a mixture of luteolinidin and apigeninidin was obtained from Texas A&M AgriLife Research. Whole grain sorghum was ground to a fine powder using a coffee grinder. Whole grain was stored in freezer (-20 °C). Prior to grinding, samples of whole grain were transferred to plastic bag, sealed and allowed to equilibrate to room temperature prior to grinding. After grinding samples were transferred to a plastic bag a sealed until time of extraction.

Chemicals and Reagents

All reagents were analytical grade. Gums used for stabilizations were gum arabic (gum arabic, powder) which was purchased from Acros Organics and sodium alginate (alginic acid sodium salt M/G ratio 1.56) which was purchased from Sigma Aldrich. $FeCl_3$ and $MgCl_2$ were obtained from Sigma Aldrich. Sodium citrate dihydrate crystal and citric acid monohydrate granule, used for buffers, were obtained from Mallinckrodt Chemicals (Phillipsburg, New Jersey).

Methods

Extraction Methods

Conventional

3DXNs were extracted in a 45 mL centrifuge tubes on a shaker (VWR Shaker model 3500, speed #4). Samples were extracted for two hours at 1 atm and 25°C (room temperature). Extraction solvent was acidified methanol (hydrochloric acid: methanol= 1:99). Samples were extracted in a 10:1 solution of solvent and raw pigment source. After extraction, samples were centrifuged for 8 min at 8000 rpm. The supernatant was recovered and stored in 45 mL centrifuge tubes at −20 °C until further use. Prior to stabilization work, extracts were concentrated by 50% under vacuum at 40°C using a Multivapor system (Büchi, Flawil, Switzerland) in order to minimize solvent added to each sample thus minimizing the extracts impact on the stabilization matrix.

Microwave-assisted extraction

Microwave-assisted extraction was carried out on a Microwave Accelerated Reaction System (MARS) (CEM MARSXpress, Matthews, NC). Extraction time varied from 0.25-120 min. Power for extractions was 300, 600, and 1200 W. Microwave chamber was thermostatic at 100 °C for all extractions. Extraction solvent was acidified methanol (hydrochloric acid: methanol= 1:99). Samples were extracted in a 10:1 solution of solvent and raw pigment source respectively. Each sample were extracted in

CEM MARSXpress tubes. Sample tubes were placed in microwave vessel which was balanced with tubes filled with equal volume water blanks for all vessel openings remaining after samples were loaded. This was to ensure even microwave exposure for all extractions regardless of number of samples in a given extraction. After extraction samples were centrifuged for 8 min at 8000 rpm. The supernatant was recovered and stored in 45 mL centrifuge tubes at −20 °C. Prior to stabilization work, extracts were concentrated by 50% under vacuum at 40°C using a Multivapor system (Büchi, Flawil, Switzerland) in order to minimize solvent added to each sample thus limiting the extracts impact on the stabilization matrix.

Buffer Formulation

Sodium citrate and citric acid were used to make pH 3 and pH 5 buffer. This allows for examination of stabilization at a pH range representative of many aqueous food products. One liter of 0.1M citric acid was made by adding 21.01 g citric acid monohydrate to 800 mL distilled deionized water in a 1 L volumetric flask. Citric acid was dissolved and distilled deionized water was added to volumetric flask to bring volume to 1 L. One liter of 0.1M sodium citrate was made by adding 29.41 g sodium citrate dihydrate to 800 mL distilled deionized water in a 1 L volumetric flask. Sodium citrate was dissolved and distilled deionized water was added to volumetric flask to bring volume to 1 L.

For pH 3, 46.5 mL of citric acid solution was added to a 100 mL flask. Next, 6.3 mL sodium citrate was added to the flask. Finally distilled deionized water was added to the flask to bring the volume to 100 mL. For pH 5, 20.5 mL of citric acid solution was added to a flask. Next, 29.5 mL sodium citrate was added to the flask. Finally distilled deionized water was added to the flask to bring the volume to 100 mL.

Upon addition of all buffer components, solution was gently swirled for 30 seconds. pH of buffer was verified using a pH meter. Either citric acid or sodium citrate was added to buffer solution to ensure buffer is within 0.05 units of desired pH.

Polysaccharide Formulation

All stabilization matrices had either 0.0 g/L, 0.5 g/L, or 1.0 g/L gum added to solution. Gum was added to sample solutions from a 100 g/L stock solution. The 100 g/L stock solution was made by adding 50 g of gum to 400 mL distilled and deionized water in a 1 L beaker. The beaker was placed on stir plate with stir bar and stir set to level 5 on a 10-level dial. Gum was incrementally added to water 2-5 g at a time every 10 min ensuring gum was fully solubilized and no clumping occurred. After all gum was added, solution was covered and left in beaker to stir overnight. After solution stirred overnight, it was transferred into a 500 mL volumetric flask. Water was added to solution to bring volume to 500 mL. Ten mL aliquots of distilled deionized water were used to rinse 1 L beaker and were added to volumetric flask to reach 500 mL.

Metal Ion Formulation

Metal ions were added to stabilization matrix at a concentration of 0.2mM. Stock solution was made by serial dilution as described in Table 1.

Table 1. List of serial dilutions for metal ions

	$MgCl_2$			$FeCl_3$		
	Molecular weight: 203.3 g/mol			Molecular weight: 162.20 g/mol		
	Solution Volume (mL)	Conc. (M)	Added from stock or previous dilution	Solution Volume (mL)	Conc. (M)	Added from stock or previous dilution
Stock	25	0.1	0.5082 g $MgCl_2$	25	0.1	0.4055 g $FeCl_3$
1/100	25	0.01	2.5 mL	25	0.01	2.5 mL
1/1000	25	0.001	2.5 mL	25	0.001	2.5 mL
2/10000	10	0.0002	2.0 mL	10	0.0002	2.0 mL

Where 0.5082 g $MgCl_2$ or 0.4055 g $FeCl_3$ were added to a 25 mL volumetric flask filled with 15 mL distilled deionized water. After ion was added, distilled deionized water was added to bring volume to 25 mL. Solution was mixed until ion was completely dissolved. After total dissolution of ion, aqueous solution was poured from volumetric flask into 100 mL beaker for a micropipette to reach stock solution with a concentration of 0.1M. Using a 5 mL micropipette, 2.5 mL from stock solution was transferred to a new 25 mL volumetric flask and distilled deionized water was added to bring volume to 25 mL and create a 0.01M solution. This procedure was repeated to achieve a solution with a concentration of 0.001M. From the 0.001M solution, using a 5

mL micropipette, 2 mL of solution was added to 10 mL stabilization sample resulting in a final ion concentration of 0.2 mM.

Stabilization and Sample Preparation of Pigment Stabilization Matrix

Samples were prepared to a final volume of 10 mL and kept in 15 mL glass vials in buffers of pH 3 or 5. Buffer was made using 0.1 M stock solutions of sodium citrate and citric acid. Stock solutions (10%) of acacia gum and alginate were used to make samples of 0.05% or 0.10% gum. Multivalent metal ions, $FeCl_3$ and $MgCl_2$ were added in a final concentration of 0.2 mM. Extract (0.02 mL) was added to sample to obtain an abs. of ~1.0 at 480 nm measured by spectrophotometer. Three samples of each treatment was made. Samples were sonicated at 40% output energy for 30 s using a tip probe (6 mm diameter) sonicator (VibraCell 40, Sonics & Materials Inc., Danbury, CT) to improve initial homogeneity of stabilization matrix, then were immediately scanned on a spectrophotometer and photographed.

UV-Vis Analysis

Stabilization was evaluated by percent color retention determined by change in absorbance at λ_{max} compared to absorbance recorded at day 0. Immediately following sonication on day 0, a 3 mL aliquot of each sample was analyzed via spectrophotometer (Shimadzu UV 2450, Shimadzu Scientific Instruments North America, Columbia, MD). Samples were scanned from 200-799 nm in triplicate and the average was recorded. As

pigment precipitated from solution it settled at the bottom of the vial. To measure efficacy of stabilization the 3mL aliquots used for spectrophotometric analysis were extracted from the upper most portion of the 10 mL sample. Caution was used to minimize sample disruption which would redistribute portions of the precipitated pigment resulting in greater absorbance than what is accurate. Absorbance was recorded every two weeks for ten weeks (2.5 months).

Determination of Total 3-Deoxyanthocyanin Content

To calculate total 3DXNs, supernatant was diluted to have an absorbance of ~1.0 at λ_{max}. Absorbance was recorded at 480 nm and 700 nm using UV-Vis spectrophotometer (Shimadzu UV 2450, Shimadzu Scientific Instruments North America, Columbia, MD). Absorbance was calculated using the following equation:

$$Abs=(A_{480nm}- A_{700nm})$$

where A_{480nm} is absorbance at 480 nm and A_{700nm} is absorbance at 700nm. A_{700nm} is measured and subtracted from A_{480nm} in order to eliminate effect of natural haze occurring in sample.

3DXN pigment concentration was calculated as luteolinidin equivalents and converted to mg/g via the following equation:

$$TDC= (abs *MW* DF)/ (\varepsilon*g)$$

where Abs is absorbance, MW is molecular weight of luteolinidin, DF is dilution factor, ε is the molar extinction coefficient of luteolinidin (31700), and g is the extraction sample weight on a dry basis.

HPLC Analysis

HPLC was used for pigment profiling. Using the method described by Yang et al (2014). Samples were prepared by passing through a syringe and 0.45 μm filter with a 0.45 nylon membrane prior to HPLC analysis. HPLC analysis was performed on an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) which includes a quaternary pump (with degasser), an autosampler, a column compartment, and a diode array detector (DAD). A Luna C-18 column (150 mm × 4.6 mm, 5.0 μm, Phenomenex, Torrance, CA) was used to carry out the separation of crude extract with a two solvent gradient: Solvent A 1% formic acid in water and solvent B 1% formic acid in acetonitrile. Column thermostat was held at 40 °C for the duration of analysis. Crude extracts were monitored at 480 nm to examine 3DXNs and at 360 nm for copigments. The injection volume was 10 μL for crude 3DXN extract.

Experimental Design

The experiment conforms to a completely random design with three replications for the stabilization and microwave assisted extraction studies. For pigment stabilization, measurements occurred at time zero and at two week intervals for 10 weeks. Pigment

stabilization was calculated as a ratio comparing spectral absorbance at time zero to absorbance at the end of the experiment. The data from both studies was analyzed using a mixed model analysis. For pigment stabilization, pigment was treated as a random effect and ion, gum, gum concentration, and pH as fixed effects. Data was analyzed using SAS software (ver. 9.2, SAS Institute, Cary, NC). ANOVA was used to detect treatment effect; $\alpha=0.05$. Tukey's Test was used to separate means.

CHAPTER IV

EFFECT OF MICROWAVE-ASSISTED EXTRACTION ON 3-DEOXYANTHOCYANIN EXTRACTABILITY FROM BLACK SORGHUM

Effect of Microwave-Assisted Extraction on Total Pigment Yield

The purpose of this research was to determine what effect microwave-assisted extraction has on 3DXN pigment extractability from sorghum. Others have shown that microwave-assisted extraction causes significant degradation of anthocyanin pigments from other pigmented grains (Abdel-Aal et al 2014). Because 3DXNs have greater thermal stability than anthocyanin analogs (Yang et al 2014), we hypothesize that microwave-assisted extraction may be significantly more effective for sorghum 3DXN extraction than anthocyanins. In this work, microwave-assisted extractability of 3DXNs from sorghum was evaluated relative to anthocyanins from blue maize (RY-288) and black cowpea (ITK 1092-1), containing mainly acylated and non-acylated anthocyanins pigments, respectively.

Compared to control, extractability of 3DXNs from sorghum significantly increased for all power settings and extraction times (Figure 7; Appendix A, Table 1). Both length of extraction and microwave power affected the pigment yield of the 3DXNs. The longer the extraction, the greater the pigment yield. Higher microwave power also tended to result in greater pigment yield. The maximum pigment yield was

obtained at 600 W for 30 min where extraction yield increased almost three-fold (2.44 mg/g) relative to control (0.88 mg/g).

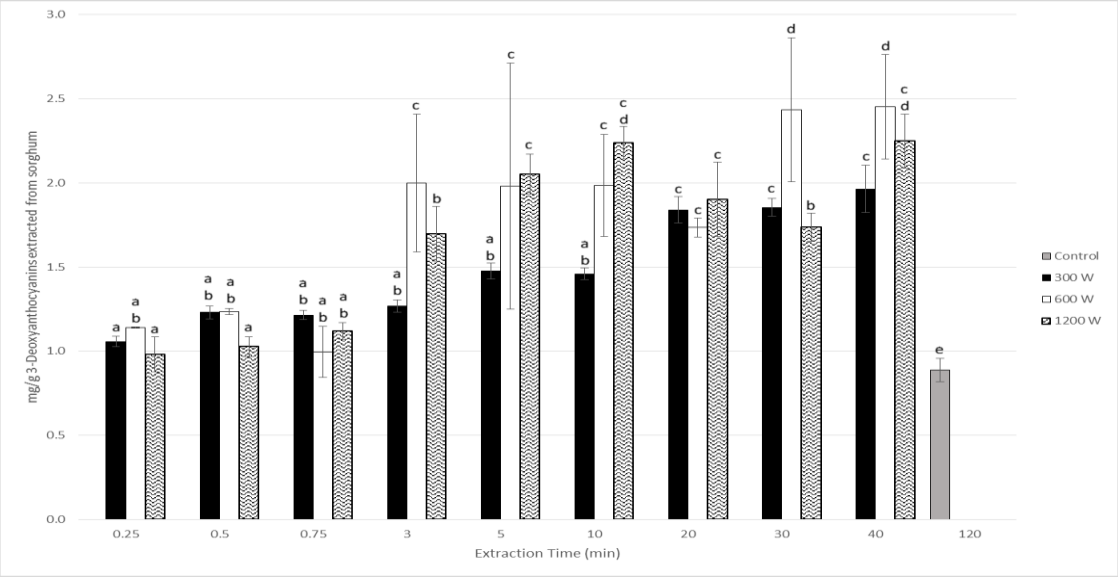


Figure 7. Effect of microwave power setting and extraction time on 3-deoxyanthocyanin pigment yield from black sorghum, as determined by UV-Vis spectroscopy at 480 nm. Pigment yield was calculated as luteolinidin equivalents. Error bars indicate ± standard deviation. Different letters indicate significance ($p<0.05$, Tukey's HSD).

Microwave-assisted extraction treatments yielded significantly greater extraction of 3DXNs from sorghum relative to anthocyanins from maize and cowpea. Microwave-assisted extraction did not significantly impact total pigment extraction from maize (Figure 7; Appendix A Table 2). Increase in extraction time resulted in only slight increase in yield. Abdel-Aal et al (2014) utilized microwave-assisted extraction for

anthocyanins from blue wheat, purple maize, and black rice and observed similar results for maize in this study. The authors reported that the yield of anthocyanins from both blue wheat and black rice significantly decreased compared to control under microwave-assisted extraction. Purple maize anthocyanins, however, did not experience the same decrease in total extraction. Across the eight different iterations of temperature, power, and extraction time, there was a range of only 10% in total anthocyanin yield from the maize.

Pigment yield from cowpea increased three-fold under microwave extraction relative to control (Figure 9). All three power settings achieved greatest pigment yield in the 3-10 min range after which the yield decreased (Figure 9; Appendix A Table 3). Extractions exceeding 10 min exhibited a sharp declined in pigment yield at all power settings. This decrease is likely the result of pigment degradation caused by severe heat and microwave irradiation. Compared to the maize anthocyanins which are mainly

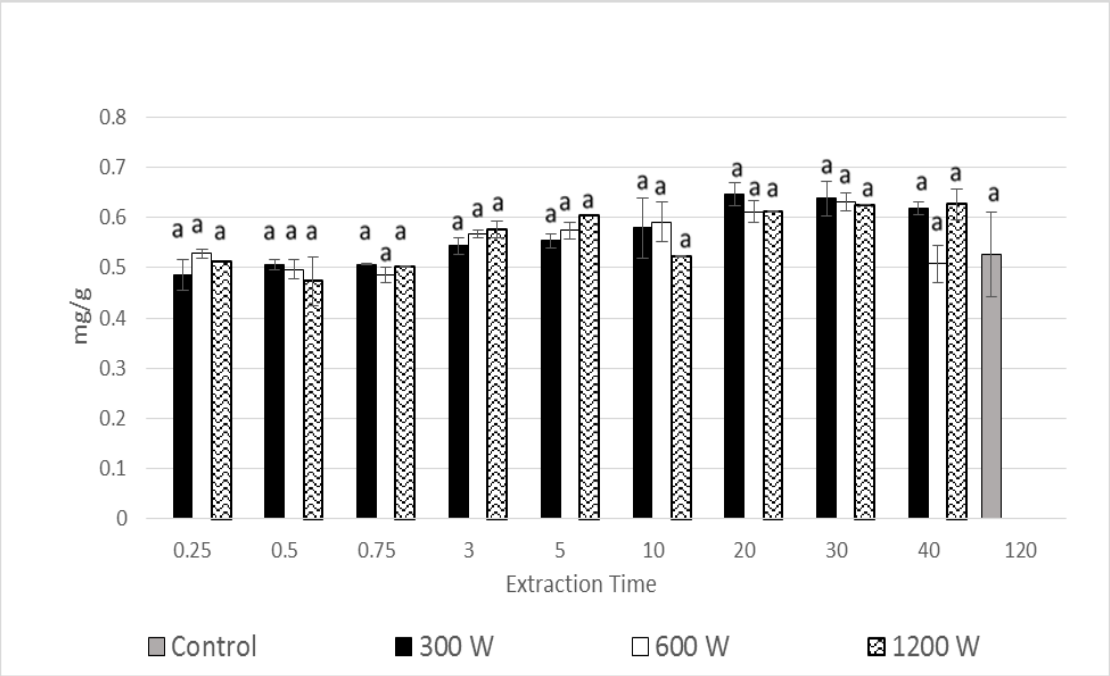


Figure 8. Effect of microwave power setting and extraction time on anthocyanin pigment yield from blue maize as determined by UV-vis spectroscopy at 520 nm. Pigment yield calculated as cyanidin equivalents. Error bars indicate ±standard deviation. Different letters indicate significance ($p<0.05$ Tukey's HSD)

acylated (Collison et al 2015), the non-acylated cowpea pigments (Ojwang et al 2012) are less stable.

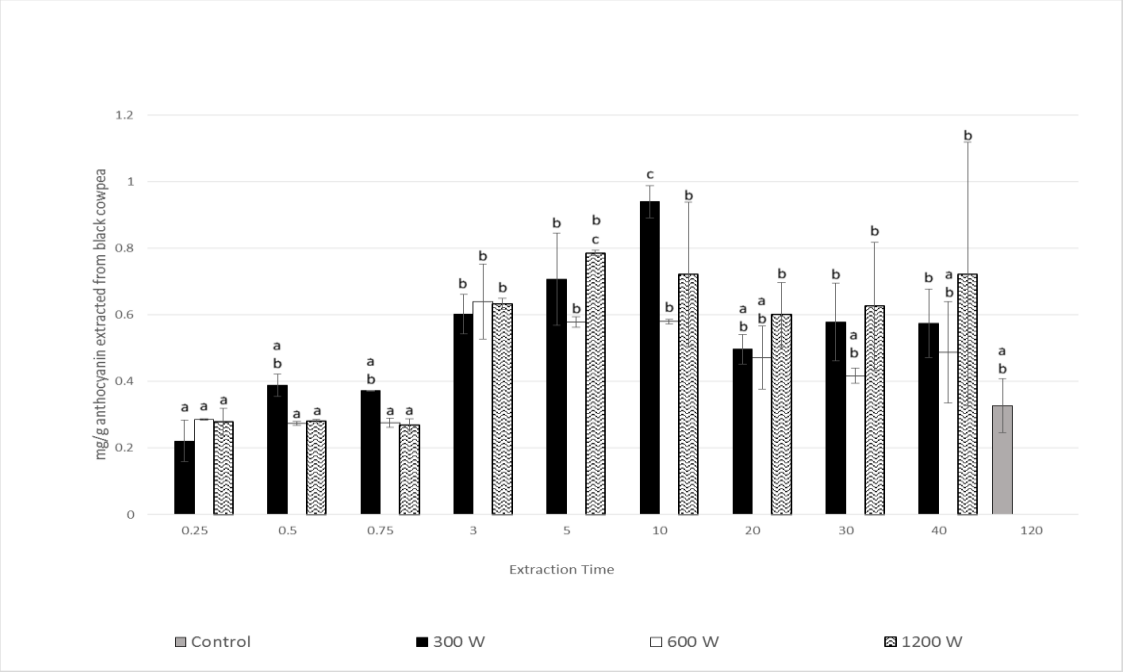


Figure 9. Effect of microwave power setting and extraction time on anthocyanin pigment yield from black cowpea as determined by UV-vis spectroscopy at 520 nm. Pigment yield calculated as cyanidin equivalents. Error bars indicate ±standard deviation. Different letters indicate significance ($p<0.05$ Tukey's HSD)

The results indicate that 3DXNs possess greater stability to microwave-assisted extraction than anthocyanin analogues and that acylated anthocyanins have greater stability to microwave irradiation than glycosylated anthocyanins. This indicates that microwave-assisted extraction is more applicable for 3DXNs and potentially acylated anthocyanins than glycosylated anthocyanins. This apparent microwave stability also has

implications for food product formulation. A food that has natural pigments added to enhance color and will require microwave heating in preparation should utilize 3-deoxanthocyanins or acylated anthocyanins to reduce potential for color degradation.

Effect of Microwave-Assisted Extraction on Pigment Profile

Few changes occurred in the sorghum pigment profile as a result of microwave-assisted extraction (Figure 10, Table 2). However, at high microwave energy exposures (600 and 1200 W; 30-40 min) some changes in pigment profile occurred, including increased prominence of 3DXN glycoside peaks (Figure 10C; Table 2). However, the most striking finding was the presence of a new pigment peak. The λ_{max} of the new peak was 523 nm (Figure 11B) which suggests an anthocyanin, and not a 3DXN which typically absorps at around 480 nm (Figure 11A). Additional analysis using UPLC-ESI/MS/MS revealed the peak had m/z 287, with fragmentation pattern that suggested cyanidin (Table 2). To the best our our knowledge, this is the first documented extraction of this anthocyanin from black sorghum. However, it is not clear whether this compound is a result of increased microwave extraction of previously non-extracted tightly bound pigments, or whether it resulted from structural transormation of the 3DXNs or other phenolics in the extract induced by the high microwave energy and long exposure. The latter explanation seems more likely, given that mineral acid was used in this work, and procyanidins and leuco-anthocyanins in sorghum can depolymerize and

form cyanidin at high temperature and low pH (Bate-Smith and Rasper 1969).

Additional work is needed to investigate this phenomenon.

Table 2. Summary of major pigments extracted from black sorghum (A) control- 120 minute conventional solvent extraction (B) lowest exposure microwave-assisted extraction; 300 W for 0.25 minute (C) and highest exposure microwave-assisted extraction; 1200 W for 40 min. Based on HPLC retention time (t_R), UV-Vis spectroscopic characteristics (λ_{max}). Peak numbers are reference to Figure 10.

Peak No.	**t_R (min)**	**λ_{max} (nm)**	**$[M-H]^+$ *(m/z)***	**MS/MS fragments *(m/z)***	**Extraction**	**Identification**
1	6.90	487.1	433	271	C	Luteolinidin glucoside
2	8.70	468.9	417	255	C	Apigeninidin glucoside
3	9.65	487.1	431	269	C	7-OMe-Apigeninidin
4	11.23	487.1	271	nf	A, B, C	Luteolinidin
5	11.63	521.2	287	185,167,157,147 ,139, 109	C	Cyanidin
6	12.78	473.7	255	nf	A, B, C	Apigeninidin
7	13.11	487.1	285	270, 242	A, B, C	7-OMe-Luteolinidin
8	16.00	468.9	269	254, 226	A, B, C	7-OMe-Apigeninidin

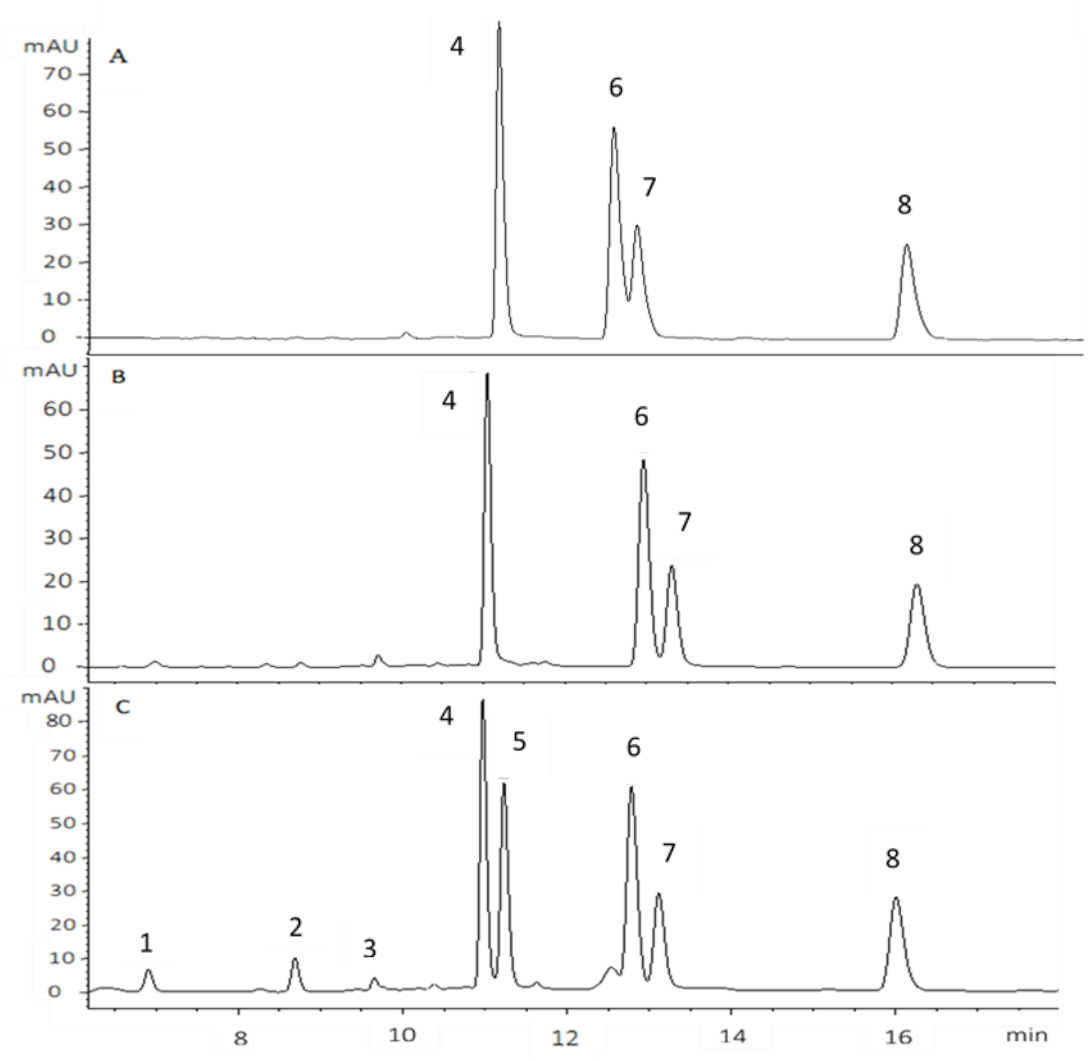


Figure 10. Representative HPLC chromatograms from whole grain black sorghum extracts monitored at 480 nm (A) control- 120 minute conventional solvent extraction (B) lowest exposure microwave-assisted extraction; 300 W for 0.25 minute (C) and highest exposure microwave-assisted extraction; 1200 W for 40 min. See Table 2 for peak identities.

Yasumatsu et al (1965) described extraction of pelargonidin from sorghum of an unspecified cultivar in 1965. Extraction of anthocyanin analogs from mature sorghum

has little other precedence in literature. At the time of extraction and identification by Yasumatsu et al (1965), HPLC was not a viable option for separation or identification. Separation and identification was conducted via thin-layer chromatography and R_f value was used for identification making comparison to the HPLC results in this research difficult.

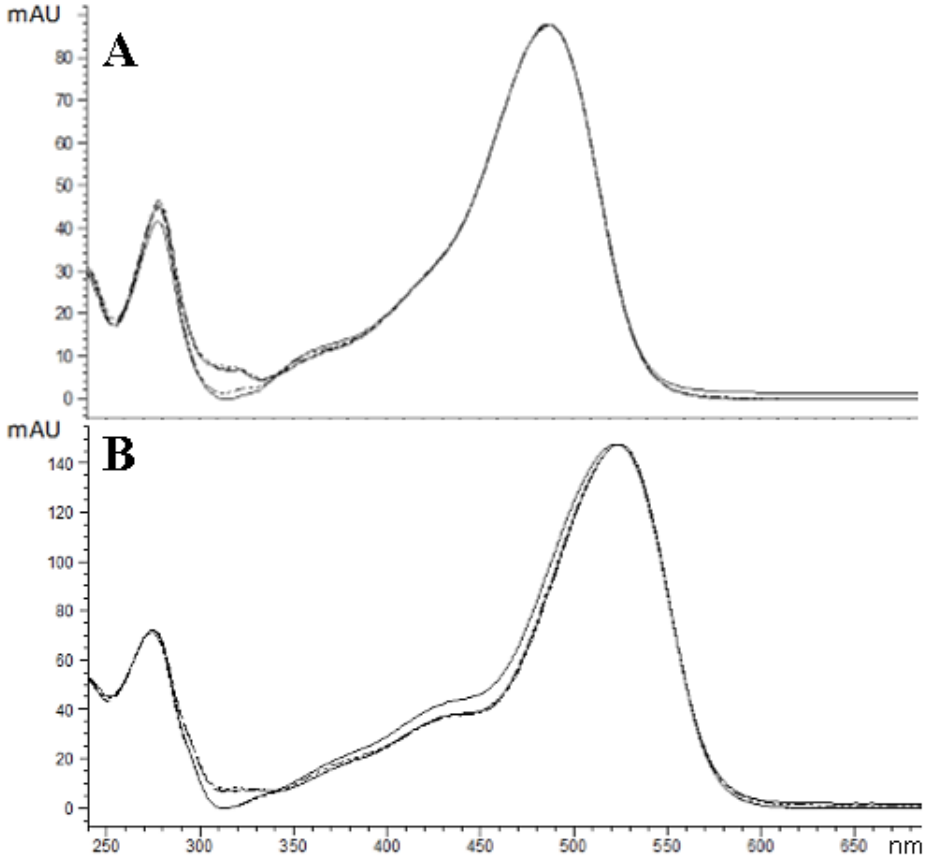


Figure 11. UV-Vis spectra from HPLC-DAD of pigment extracted from sorghum. (A) luteolinidin, corresponds to peak 1 in Figure 10. (B) the new anthocyanin compound, identified as cyanidin, corresponds to peak 2 from Figure 10.

For maize, even though no significant changes in pigment content was observed via UV-Vis spectrophotometer (Fig. 8), HPLC chromatograms indicate major change in pigment profile (Fig. 12; Table 3). The two major anthocyanins in the blue maize were cyanidin-3-glucoside and cyanidin-3-(6”-malonyl)glucoside (Figure 12), which agrees with a previous report (Collison 2014). Microwave-assisted extraction led to total loss of cyanidin-3-(6”-malonyl)glucoside peak (Figure 12 A, peak 2) after exposure to 300 W for 0.25 minutes and all extractions thereafter. Upon loss of cyanidin-3-(6”-malonyl)glucoside, a significant increase in cyanidin-3-glucoside was observed. This indicates microwave irradiation, even at minimum exposure (300 W for 0.25 minutes) resulted in complete hydrolysis of the malonyl-acyl ester linkage to cyanidin-3-glucoside. In addition to changes in cyanidin-3-glucoside and cyanidin-3(6”-malonyl)glucoside, other changes occurred including appearance of new minor peaks. Based on anthocyanin profiles of blue maize documented by Collison et al (2015) peaks 3-5 in Figure 12 C are likely pelargonidion-3-glucoside, peonidin-3-glucoside, and pelargonidin-3-malonylglucoside (Collison 2015).

Table 3. Summary of pigments extracted from blue maize (A) control- 120 minute conventional solvent extraction (B) lowest exposure microwave-assisted extraction; 300 W for 0.25 minute (C) and highest exposure microwave-assisted extraction; 1200 W for 40 min. Based on HPLC retention time (t_R), UV-Vis spectroscopic characteristics (λ_{max}). Peak numbers are reference to Figure 12.

Peak No.	**t_R (min)**	**λ_{max} (nm)**	**Extraction**	**Identification**
1	4.90	515	A, B, C	cyanidin-3-glucoside
2	6.33	513	A	cyanidin-3-(6”malonyl-glucoside)
3	6.90	502	B, C	pelargonidion-3-glucoside
4	7.47	520	B, C	peonidin-3-glucosdie
5	7.47	503	C	pelargonidin-3-malonylglucoside
6	7.83	521	A, B, C	peonidin-3-malonylglucoside

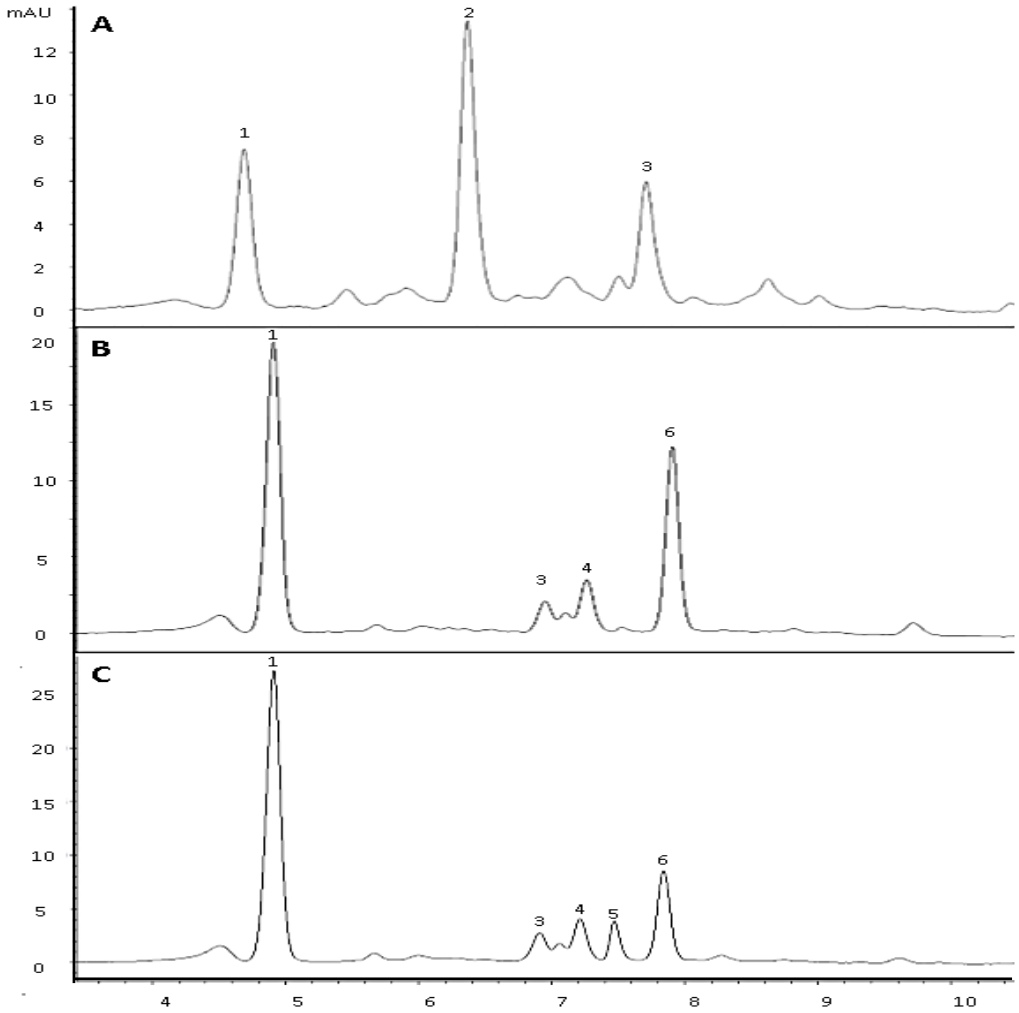


Figure 12. Representative HPLC chromatograms from blue maize extracts at 520 nm (A) control- 120 minute conventional solvent extraction (B) lowest exposure microwave-assisted extraction; 300 W for 0.25 minute (C) and highest exposure microwave-assisted extraction; 1200 W for 40 min. See Table 4 for peak identities.

In cowpea, the major anthocyanins identified were delphinidin-3-O-glucoside, cyanidin-3-O-glucoside, petunidin-3-O-glucoside, and malvindin-3-O-glucoside (Fig.

13, Table 4), similar to previous reports (Ojwang et al 2012). Similar to sorghum 3DXNs, low microwave-assisted extraction (300 W for 0.25 minutes) produced negligible change in anthocyanin profile of cowpea relative to control. However, at maximum exposure (1200 W for 40 minutes) four new peaks were observed (Figure 13 C). These new peaks were identified as the hydrolyzed aglycones of the four glycosides where peak 5-8 were identified as delphinidin, cyanidin, petunidin, and malvidin respectively.

Table 4. Summary of pigments extracted from black cowpea (A) control- 120 minute conventional solvent extraction (B) lowest exposure microwave-assisted extraction; 300 W for 0.25 minute (C) and highest exposure microwave-assisted extraction; 1200 W for 40 min. Based on HPLC retention time (t_R), UV-Vis spectroscopic characteristics (λ_{max}). Peak numbers are reference to Figure 13.

Peak No.	**t_R (min)**	**λ_{max} (nm)**	**Extraction**	**Identification**
1	4.13	524	A, B, C	delphinidin-3-galactoside
2	4.91	515	A, B, C	cyanidin-3-glucoside
3	5.23	526	A, B, C	petunidin-3-glucoside
4	6.15	528	A, B, C	malvidin-3-glucoside
5	6.84	520	C	delphinidin
6	7.48	527	C	cyanidin
7	7.75	520	C	petunidin
8	8.28	520	C	malvidin

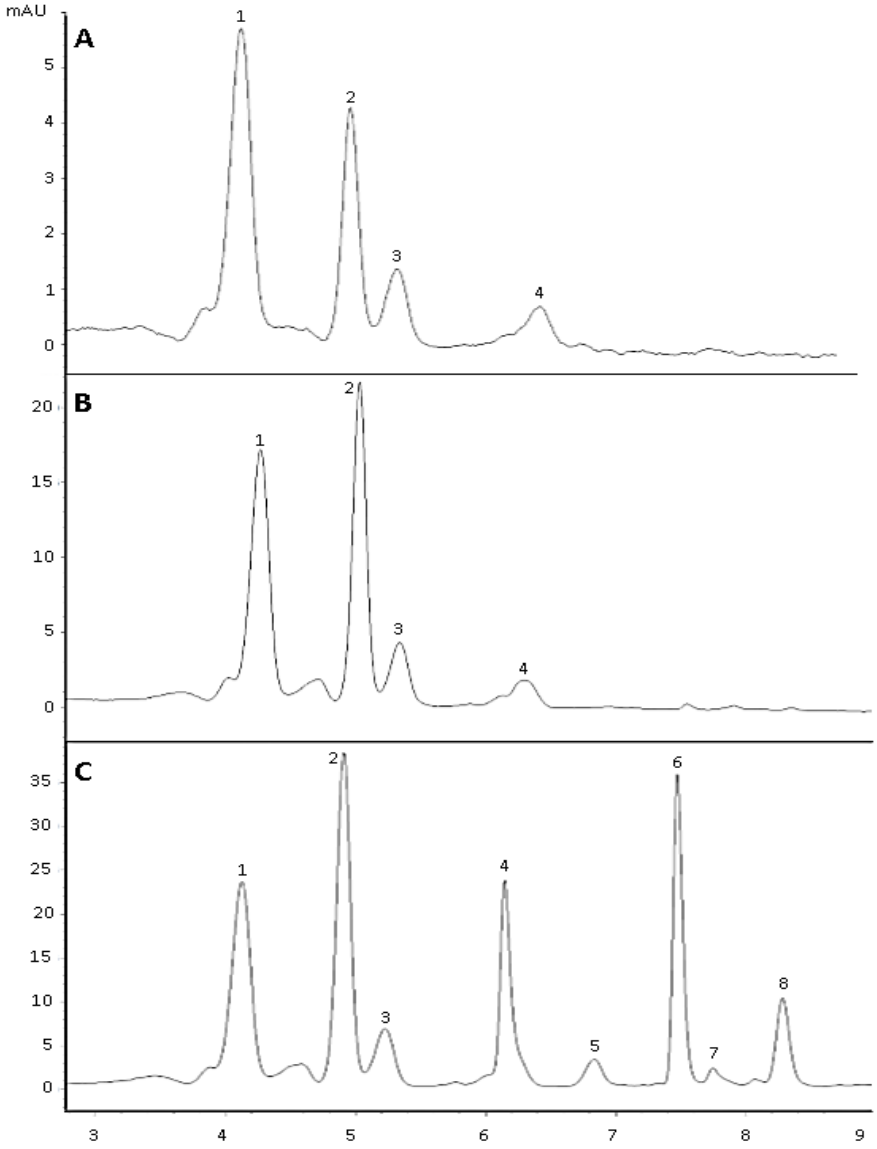


Figure 13**.** Representative HPLC chromatograms from black cowpea extracts at 520 nm (A) control- 120 minute conventional solvent extraction (B) lowest exposure microwave-assisted extraction; 300 W for 0.25 minute (C) and highest exposure microwave-assisted extraction; 1200 W for 40 min. See Table 4 for peak identities.

Deglycosylation of anthocyanins under microwave-assisted extraction has been documented by others (Abdel-Aal et al 2014; Wenkai et al 2015). Comparatively, sorghum 3-doexyanthocyanins did not undergo structural degradation. This evidence suggests greater stability of 3DXNs to microwave irradiation relative to anthocyanins.

Stability of 3-Deoxyanthocyanins from Sorghum Relative to Anthocyanins Under Microwave-Assisted Extraction

Chemical stability of 3DXA has been examined in terms of reactivity with various oxidants such as SO_2 (Geera et al 2012) or ascorbic acid (Ojwang and Awika 2008), to thermal induced nucleophilic attack (Yang et al 2014), in the presence of co-pigments, and A-ring substitution (Awika et al 2008). Chemical stability as a function of pH is also well established (Brouillard et al 1982; Sweeny and Iacobucci 1983; Mazza Brouillard 1987, Awika 2008).

No literature exists on the effect of microwave irradiation on structural stability of 3DXN pigments. Furthermore, most literature related to microwave irradiation and anthocyanins focuses on extractability rather than stability. The results of this research indicate that 3DXNs have significantly greater stability to not only heat but also the tremendous physical agitation characteristic of microwave irradiation. The trend observed in 3DXN extraction here differs from extraction trends observed for anthocyanins where significant decrease in anthocyanin yield was observed with longer

extractions time and or higher microwave power (Abdel-Aal 2014; Pap et al 2012; Zheng et al 2013; Wen et al 2015).

Anthocyanins found in maize and cowpea are characteristically less heat tolerant than 3DXNs (Barros et al 2013; Abdel-Aal et al 2014; Yang et al 2014; Reyes et al 2007) and results of this research indicate that is also the case for stability to microwave irradiation. A number of studies have also documented anthocyanin degradation caused by microwave irradiation. Abdel-Aal et al (2014) reported degradation of anthocyanins when extracted from pigmented rice, wheat, and maize via microwave-assisted extraction (Abdel-Aal et al 2014). Microwave-assisted extraction caused significant structural change and deglycosylation resulting in degradation of anthocyanins and loss of color (Abdel-Aal et al 2014). Pap et al (2012) did not explicitly document anthocyanin degradation but did observe decrease in total monomeric anthocyanins in microwave-assisted extractions as exposure time increased. Similarly, Zhend et al (2013) observed a significant decrease in anthocyanin extraction from blueberries at longer time and temperature exposure. Wen et al (2015) observed significant decrease in anthocyanin extraction with increased microwave power. Wenkai et al (2015) recorded significant hydrolysis and deglycosylation of cyanidin-3-glucoside. This type of structural transformation does not appear to have occurred in 3DXNs when subjected to similar microwave treatment conditions in the present research.

The likely cause of microwave induced pigment degradation is two-fold. The primary cause is likely greater excitation of anthocyanin molecules relative to 3DXNs because of a greater number of substituents with greater electronegativity. Greater excitation means more vibration of dipoles which may be significant enough to break weaker bonds within the pigment molecule (Sadilova et al 2006). The 3DXNs are likely less susceptible to microwave irradiation than anthocyanin analogs due to the differences in dielectric properties and relative permeability of the two molecules caused by fewer substituents, specifically at C3. As it pertains to electromagnetic radiation, the relative permeability of a molecule and dielectric constant are the degree to which the molecule can be polarized by electromagnetic energy i.e. the microwave irradiation in microwave-assisted extraction (Chémat 2013). Different molecules have different dielectric constants that are dictated by the structure and electronegativity of the given molecule. For example, water, a polar molecule, has a dielectric constant of 80.4 while hexane, a non-polar molecule is 1.9 (Chémat 2013). Because of a greater number of polar substituents, anthocyanins likely have a larger dielectric constant and greater relative permeability than 3-deoxyanthocyanins. The greater the electronegativity of a molecule, the larger the dielectric constant and the greater the excitation of dipoles will be. Greater excitation means greater vibration of dipoles and physical agitation that may cause structural change resulting in pigment degradation. Structural change could be

hydrolysis at C3 with the glycosidic substituent, or at C2 resulting in chalcone or carbinol configurations, or cleavage of phenolic rings reducing anthocyanins to other phenolic substituents (Sadilova et al 2006). Even slight change in the structure of an anthocyanin can change the color of the anthocyanin or eliminate color all together. Thus extreme agitation of anthocyanins caused by dipole excitation from microwaves cause damage to pigment molecules.

Liazid et al (2007) examined stability of phenolic compounds similar to anthocyanins subjected to microwave irradiation. They determined that phenolic compounds with fewer substituents around aromatic rings and substituents that were methoxylated rather than hydroxylated possessed greater stability to microwave irradiation. This is logical because substituents around a phenolic ring would increase polarity and relative permeability of the molecule. Additionally a hydroxyl substitution would have a greater dipole moment than a methoxylated substitution thereby increasing the relative permeability further and therefore susceptibility to dipole excitation. Thus the findings of Liazid et al (2007) conform to the theory presented; that 3DXNs have greater stability to microwave irradiation than anthocyanins because of lack of substitution at C3 which reduces their susceptibility to microwave energy.

A unique attribute of sorghum 3DXNs is the stability of the aglycone, 3-deoxyanthocyanidin, structure (Awika et al 2004). By contrast, anthocyanin aglycones

are extremely rare and unstable and all anthocyanins extracted from maize and cowpea in this experiment were glycosylated. Based on the findings of Liazid et al (2007) is it likely that glycosylation at C3 would further increase propensity for microwave induced degradation because of the size and electronegativity of C3 substituents.

The secondary contribution to pigment degradation caused by microwaves is from generated heat. The excitation of dipoles caused by microwaves results in rapid increase in temperature. The greater the susceptibility to excitation by microwaves the more heat there will be generated by said excitation. Thus increased susceptibility to microwave excitation results in greater overall pigment degradation because of both greater vibration of substitutions and greater heat generated. Therefore, because anthocyanins have characteristically poor heat stability, it is likely that if anthocyanin molecules were to remain intact notwithstanding severe vibration from dipole excitation caused by microwaves, it would likely begin to degrade because of prolonged heat exposure.

There is no way to prevent dipole excitation and rapid temperature increase within extractions other than to utilize short extraction times. This may explain the results for cowpea anthocyanin and sorghum 3DXN extraction results where extraction yield from cowpea significantly increased during short extractions but sharply decreased after 10 minutes, while sorghum extraction continued to increase with extraction time.

Comparison of Microwave-Assisted Extractability of 3-Deoxyanthocyanins from Sorghum with other Extraction Methods

The 3DXNs are generally difficult to extract from sorghum by conventional methods. Barros et al (2013) utilized accelerated solvent extraction system to increase extractability of 3DXNs from sorghum. They reported greatest increase in extraction of 3DXNs from black sorghum bran in 50% ethanol/water at 150 °C and 1500 psi for 3 minutes where a 1.7 fold increase in total 3DXN extraction was achieved (Barros et al 2013). In our study, microwave extraction at 600 W for 3 minutes resulted in a two-fold increase in pigment yield relative to control. Furthermore, a maximum of near three-fold increase in 3DXN yield under microwave-assisted extraction was achieved. Greater extraction efficiency with microwave-assisted extraction over accelerated solvent extraction indicates that the effect is due to microwave irradiation and not exclusively increase in temperature of extraction.

Microwave-assisted extraction is different than other heated extraction methods which involve conduction and/or applied pressure such as the accelerated solvent extraction method. Conductive heat increases temperature of extraction from a fixed energy source such as a hot water bath or hot plate (Cai et al 2016). Energy in the form of heat is applied over a gradient, not ubiquitously. Pressure heating undergoes similar increase in energy and subsequent increase in temperature but much more rapidly.

Increased pressure results in increased temperature of extraction by increasing molecular collision. The increase in temperature is not caused by a fixed heat source but is fundamentally the same as conductive heating. Microwaves, however, transfer energy through electromagnetic radiation. Heat is produced by excitation of dipoles and thus generated from within the cell and ubiquitously applied throughout solution almost instantaneously (Byun et al 2014; de la Hoz 2007; Mazo et al 2012; Fujii et al 2014, Jingyi et al 1997). This is an important distinction because heat from microwaves is generated by movement within a molecule rather than movement of the whole molecule as is the case with conduction or increased pressure.

Because of rapid generation of heat within cells caused by microwaves, pressure increases and cells rupture (Desai et al 2010; Elez Garofulić et al 2013). Cell rupture liberates compounds such as pigments from cells that were otherwise difficult to access and extract. This is not likely to happen by conduction or applied pressure extraction methods such as accelerated solvent extraction because neither conduction nor pressure can transfer energy and generate heat within a cell at the intermolecular level.

Conclusion

In this study, different power settings and hold times were used to evaluate the influence of microwave-assisted extraction on pigment extraction from black sorghum grain. Blue maize and black cowpea were used to evaluate possible differences in

pigment extraction and stability based on difference in anthocyanin type and profile. Microwave-assisted extraction was highly effective at extracting 3DXNs from sorghum grain resulting in a three-fold increase relative to control. Cowpea pigments degraded during long extractions while maize exhibited negligible change in total pigment yield but did experience hydrolysis of extracted anthocyanins.

Structural differences between 3DXNs and anthocyanin analogs likely resulted in different stability to microwave irradiation similar to what has been observed for heat stability of 3DXNs (Yang 2014). These differences resulted in much greater extractability of 3DXNs from sorghum relative to anthocyanins from maize and cowpea. The results of this experiment indicate that microwave-assisted extraction may be a viable method to commercially extract 3DXN pigments for food applications. Additional studies are needed to establish effect of various solvent systems on extractability of these compounds under microwave energy.

CHAPTER V

EFFECT OF POLYSACCHARIDE GUMS AND METAL IONS ON THE AQUEOUS STABILITY OF SORGHUM 3-DEOXYANTHOCYANINS

The three pigment sources used for this study have different 3DXN profiles. The leaf sheath contains primarily apigeninidin and its derivatives (Geera et al 2012). The sorghum leaf contains primarily luteolinidin and its derivatives (Petti et al 2014). The black sorghum grain has about a 60/40 mixture of luteolinidin and apigeninidin and their derivatives (Awika et al 2005). Because of the different 3DXN profiles, each pigment source reacted differently to the same stabilization matrixes based on gum and ion at different pH.

Over the course of this experiment, absorbance at 480 nm was recorded using a UV-Vis spectrophotometer for each sample and used to estimate aqueous stability. As pigment either precipitated or degraded the absorbance decreased relative to absorbance at time zero. Stabilization was quantitatively evaluated based on change in absorbance after 10 weeks relative to time zero and expressed as percent color retention. Color was also evaluated visually after 10 weeks and images recorded. The visual data is essential as slight but important changes in overall appearance of sample are not necessarily captured by UV-Vis data. Additionally, UV-Vis data does not adequately convey differences in visual appearance of different pigment sources (e.g., Figure 14).

Figure 14. Visual difference between apigeninidin (left) and luteolinidin (right) dominant extracts at pH 5.

Table 5. Effect of 1.0 g/L gum on aqueous stability of 3-deoxyanthocyanin pigments in presence or absence of metal ions at pH 3 and 5. Values reported as percentage color retention after 10 weeks compared to Day 0, determined by UV-Vis spectrophotometry at 480 nm.

	pH	Ion	Gum Arabic	Sodium Alginate	No Gum
Red Sorghum Leaf Extract	3	Fe^{3+}	14.3±6.2	61.3±10.8	0.0±0.0
		Mg^{2+}	7.4±6.3	56.8±7.8	0.0±0.0
		No Ion	10.9±0.0	58.9±0.0	0.0±0.0
	5	Fe^{3+}	0.0±0.0	80.3±7.3	28.0±0.0
		Mg^{2+}	0.0±.0.0	68.8±3.6	40.5±0.0
		No Ion	0.0±0.0	73.9±0.0	31.4±0.0
Sorghum Leaf Sheath Extract	3	Fe^{3+}	75.1±35.0	80.2±6.7	0.0±0.0
		Mg^{2+}	57.6±7.1	13.2±22.9	0.0±0.0
		No Ion	50.5±0.0	10.4±0.0	0.0±0.0
	5	Fe^{3+}	83.3±13.9	84.1±2.3	0.0±0.0
		Mg^{2+}	105.3±3.7	83.2±0.8	0.0±0.0
		No Ion	102.8±0.0	84.3±0.0	0.0±0.0
Black sorghum Grain Extract	3	Fe^{3+}	83.6±9.5	98.7±1.5	0.0±0.0
		Mg^{2+}	97.3±0.6	95.8±0.1	0.0±0.0
		No Ion	97.6±0.0	96.5±0.0	0.0±0.0
	5	Fe^{3+}	61.9±4.6	60.3±7.6	8.0±1.1
		Mg^{2+}	55.1±1.4	63.1±5.6	7.1±4.6
		No Ion	58.8±4.2	61.8±4.5	14.8±12.3

Table 6. Effect of 0.5 g/L gum on aqueous stability of 3-deoxyanthocyanin pigments in presence or absence of metal ions at pH 3 and 5. Values reported as percent color retention after 10 weeks compared to Day 0, determined by UV-Vis spectrophotometry at 480 nm.

	pH	Ion	Gum Arabic	Sodium Alginate	No Gum
Red Sorghum Leaf Extract	3	Fe^{3+}	10.2±0.9	68.5±1.6	0.0±7.0
		Mg^{2+}	10.6±0.4	70.3±11.2	0.0±0.0
		No Ion	10.0±0.0	64.9±0.0	0.0±0.0
	5	Fe^{3+}	5.5±9.4	88.3±11.8	28.8±0.0
		Mg^{2+}	9.8±8.5	66.6±4.9	29.4±0.0
		No Ion	0.0±0.0	72.5±0.0	27.0±0.0
Sorghum Leaf Sheath Extract	3	Fe^{3+}	70.5±38.3	0.0±0.0	0.0±0.0
		Mg^{2+}	81.1±39.3	0.0±0.0	0.0±0.0
		No Ion	47.1±0.0	0.0±0.0	0.0±0.0
	5	Fe^{3+}	71.8±1.3	44.9±3.8	0.0±0.0
		Mg^{2+}	97.9±3.8	12.5±21.7	0.0±15.8
		No Ion	97.4±0.0	64.7±0.0	0.0±0.0
Black sorghum Grain Extract	3	Fe^{3+}	63.9±3.5	95.8±1.4	0.0±0.0
		Mg^{2+}	94.8±4.3	94.7±0.6	0.0±0.0
		No Ion	95.3±0.0	93.8±0.0	0.0±0.0
	5	Fe^{3+}	64.2±3.8	68.0±5.2	5.2±1.0
		Mg^{2+}	58.9±4.1	64.3±3.2	7.0±0.3
		No Ion	63.3±11.0	62.8±5.1	6.7±0.5

Effect of Gum Type on Aqueous Stabilization of 3-Deoxyanthocyanins

Both gum arabic and sodium alginate were generally highly effective at stabilizing the 3DXN in aqueous solution at both concentrations used (Tables 5 & 6). The effect of the gums appeared to depend largely on pigment profile, where gum arabic

was much more effective at stabilizing the apigeninidin-dominant extract (sorghum leaf sheath pigments) and alginate more effective at stabilizing the luteolinidin-dominant extract (red sorghum leaf pigments). Both gums were equally effective at stabilizing the black sorghum grain pigments that contain luteolinidin/apigeninidin mixtures (Table 5 & 6). At the two gum concentrations used (0.5 and 1.0 g/L), no obvious differences were observed for both gums, indicating investigation of lower levels of the gums is necessary to adequately establish critical minimum gum concentration.

As expected, all 3DXA samples were unstable in aqueous environment at all pH levels in the absence of gums (Table 5 and 6; Appendix B: Figures 1, 6, and 11). In most cases, precipitation in aqueous solution was almost instantaneous. However, the luteolinidin dominant extract (sorghum leaf extract) maintained slightly greater stability in the absence of gum than either of the other pigment sources, especially at pH 5.0 (Table 5). This may be due to the more hydrophilic nature of luteolinidin relative to apigeninidin.

Stability in the Presence of Gum Arabic

In general gum arabic was very effective at stabilizing the extracts high in apigeninidin (sorghum leaf sheath and black sorghum grain extracts), but ineffective on the luteolinidin dominant extract (Tables 5 & 6, Figure 15). This stabilizing effect of

gum arabic was affected by pH; apigeninidin dominant extract was more stable at pH 5 than pH 3, whereas the opposite effect was observed for the mixed luteolinidin/ apigeninidin extract (black sorghum grain pigments). For example, in 1.0 g/L gum concentration, the apigeninidin dominant pigment had no color loss after 10 weeks at pH 5, but retained 51% absorbance at pH 3, whereas the mixed pigment retained 59% and 98% absorbance at pH 5 and 3, respectively (Table 5). Despite the measurable changes in absorbance for the two pigment extracts at the different pH values, colors remained visually appealing (Figure 8,10,13,15 of Appendix B).

Differences in stabilizing effect of gum arabic are a result of differences in structure of apigeninidin and luteolinidin. Gum arabic stabilizes by functioning as an emulsifier (Wang et al 2011). The proteinaceous backbone of gum arabic interacts with hydrophobic molecules while the polysaccharide moieties interact with water. In 3DXNs the lack of substitution at C3 makes the C4-C5' region relatively hydrophobic compared to anthocyanins. The hydrophobic proteinaceous backbone of gum arabic will likely be drawn to C4-C5' and interact with 3DXNs hydrophobically. The large hydrophilic polysaccharide moieties of gum arabic will keep the complex in aqueous solution.

The differences in gum arabic stabilization between extracts high in apigeninidin, and luteolinidin-dominant extract may be caused by the difference in substituents around the B ring. Luteolinidin possesses *ortho*-dihydroxyl substitution on the B ring (at C4'

and C3'). The *ortho*-dihydroxyl substitution increase the electronegativity of the B ring and also makes the molecule more hydrophilic, and thus reduces its affinity to the proteinaceous backbone of gum arabic. Apigeninidin is substituted at C4' only. The single B ring substitution reduces the electronegativity and repulsive force between apigeninidin and gum arabic. This increases the surface area of the molecule capable of interacting hydrophobically with the proteinaceous backbone of gum arabic thereby enhancing aqueous pigment stabilization. The stabilization relationship of apigeninidin and luteolinidin with gum arabic is depicted in Figure 16.

The lack of stabilization of luteolinidin with gum arabic would indicate that the hydrophobic region from C4-C5' of the 3DXN is insufficient surface area for effective hydrophobic interaction with gum arabic. This further implies that the ability of gum arabic to stabilize apigeninidin in aqueous solution may be unique among anthocyanins regardless of C3 substitution. This is because anthocyanin analogs have bulky hydrophilic substituents at C3. Substitution patterns of all other anthocyanins would result in enough electronegativity distributed evenly across the molecule to prevent effective interaction with gum arabic.

The results for apigeninidin and gum arabic here show unique potential to be used as a natural food colorant. Typical uses of gum arabic in industry are for flavor stabilization at concentrations of about 10 g/L. Results from this research indicate that

0.5 g/L is sufficient gum concentration to stabilize apigeninidin in aqueous solution. This indicates apigeninidin could be used as a natural colorant in products that already contain gum arabic for flavor stabilization with minimal necessary reformulations. This research may also provide valuable information for the sake of breeding. Sorghum cultivars high in 3DXN could be bred to produce predominantly apigeninidin. This would result in a natural pigment that possesses greater processing stability characteristic of 3DXN, greater color quality over a wider range of pH, and minimal reformulation necessary for application.

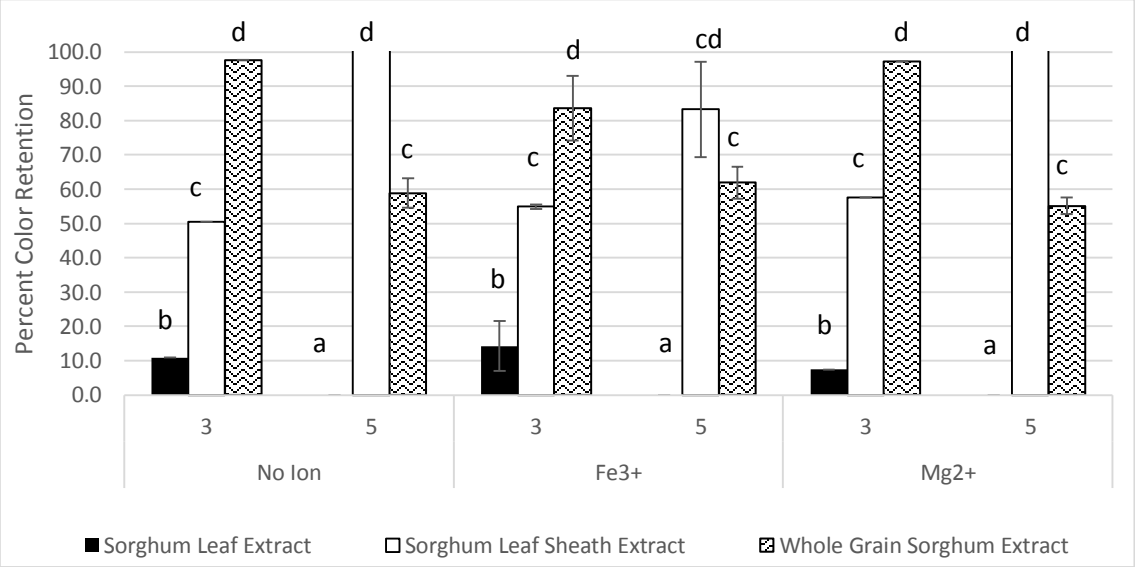


Figure 15. Effect of 1.0 g/L gum arabic on aqueous stability of 3-deoxyanthocyanin pigments in presence or absence of metal ions at pH 3 and 5. Data reported as percent color retained after 10 weeks, determined by UV-Vis spectrophotometry at 480 nm. Data are expressed as mean ± SD. Different letters indicate significance ($p<0.05$, Tukey's HSD Test)

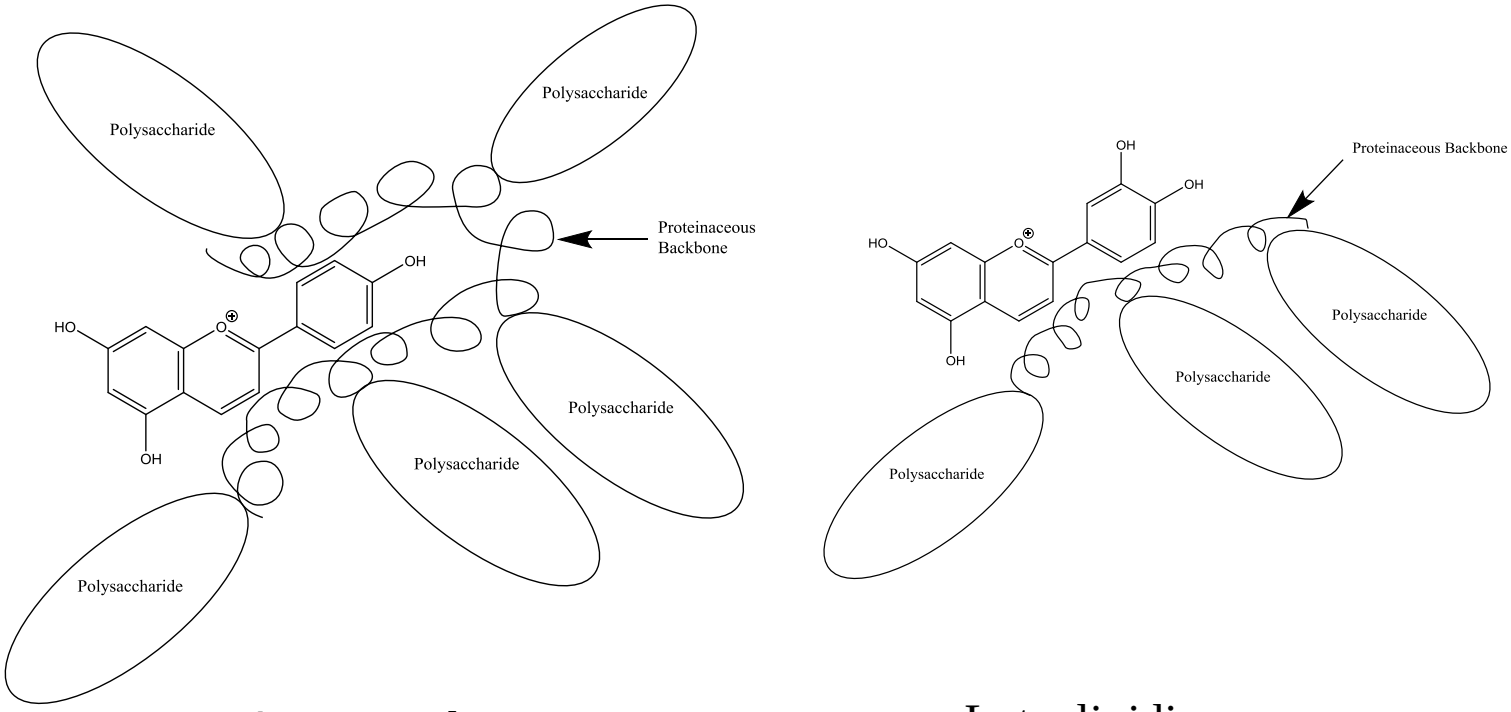


Figure 16. Proposed interaction mechanisms between gum arabic and 3-deoxyanthocyanidins. The proteinaceous backbone of gum arabic is attracted to the hydrophobic regions of the 3-deoxyanthocyanin molecules.
Left: One substitution on the B ring causes apigeninidin to have greater area which can interact hydrophobically with gum arabic.

Stability in the Presence of Sodium Alginate

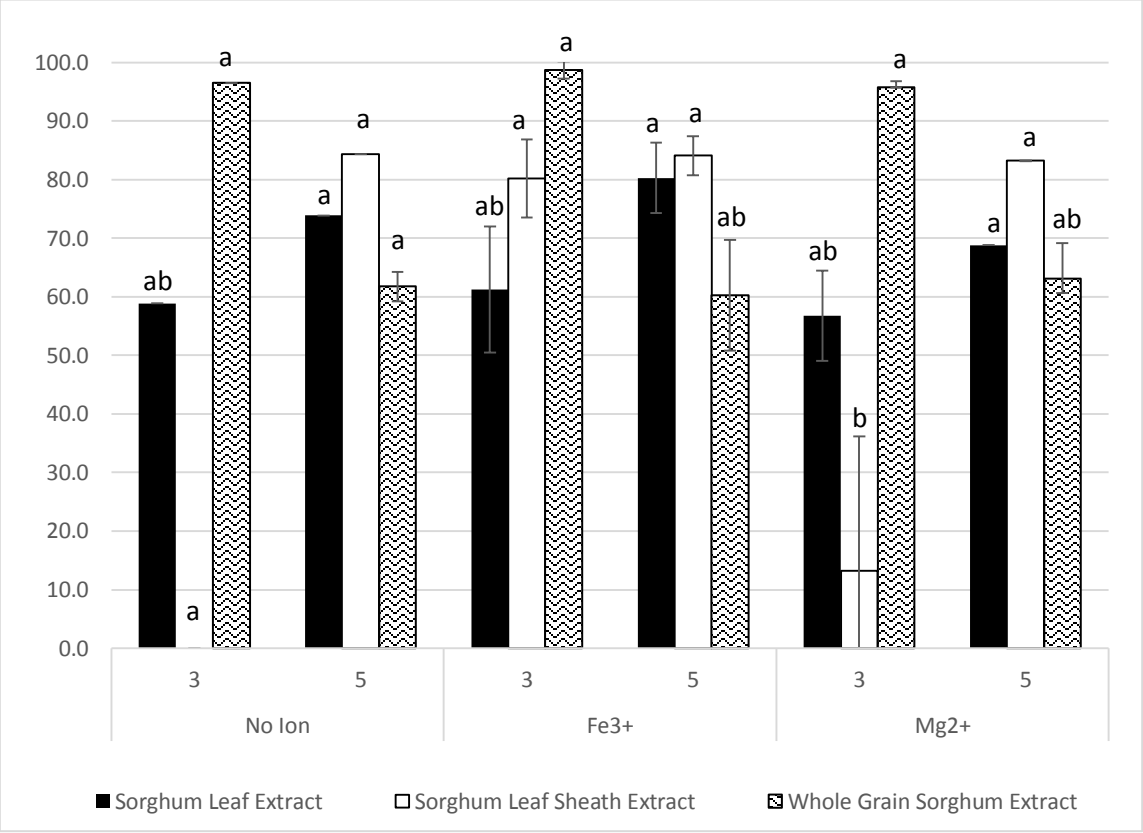


Figure 17. Effect of 1.0 g/L sodium alginate on aqueous stability of 3-deoxyanthocyanin pigments in presence or absence of metal ions at pH 3 and 5. Data reported as percent color retained after 10 weeks, determined by UV-Vis spectrophotometry at 480 nm. Data are expressed as mean ± SD. Different letters indicate significance ($p<0.05$, Tukey's HSD Test)

All pigments were effectively stabilized in presence of sodium alginate at both concentrations (Figure 17, Table 5 & 6). However, unlike gum arabic, the effect of alginate was generally less dependent on pigment composition, but mostly dependent on the pH, which suggests different stabilization mechanisms. The most important standout was that the alginate was largely ineffective at stabilizing the apigeninidin-dominant pigments (leaf sheath extract) at pH 3 (10% color retention at 1.0 g/L), but very effective

with the same pigment at pH 5 (84% color retention) (Table 5, Fig. 17). Also, the sodium alginate was a lot more effective at stabilizing the luteolinidin-dominant extract (red sorghum leaf pigments) (average color retention at 1.0 g/L, pH 3: 59.0 %; pH 5: 76.7) (Fig. 17) than gum arabic (average color retention for 1.0 g/L pH 3: 10.84%; pH 5: 0.0%) (Figure 15).

The alginate generally stabilized the sorghum pigments better at pH 5. The exception was the luteolinidin/ apigeninidin mixture extract (black sorghum grain extract) which was stabilized more effectively at pH 3 (96% color retention) than pH 5 (63%) (Figure 17). A similar trend was observed for this extract in presence of gum arabic, which suggests possible presence of other components in the extract that enhance its stability at pH 3 in presence of the gums. The difference could be caused by the method of extraction of the black sorghum grain pigments. This sample was extracted using microwave irradiation. The microwave process likely released other polyphenols and non-polyphenol grain components that may have influenced these observations. Also, the fact that an anthocyanin aglycon, cyanidin, was prominently formed in this extract (Fig. 11) may have contributed to its reduced stability at pH 5. Cyanidin has significantly less stability at higher pH than 3DXNs (Mazza and Brouillard, 1987).

Similar to gum arabic, differences between stabilizations with sodium alginate are likely the result of B ring configuration of the 3DXN. In gum arabic treatments, fewer substitutions around the B-ring resulted in greater stabilization because of fewer hydrophilic groups. However, unlike gum arabic, sodium alginate stabilizes mainly via ionic interactions and can also participate in hydrogen bonding. Thus the greater the

number of hydroxyl substitutions around the B-ring the greater the number of hydrogen bonds possible and the greater the stabilization achieved.

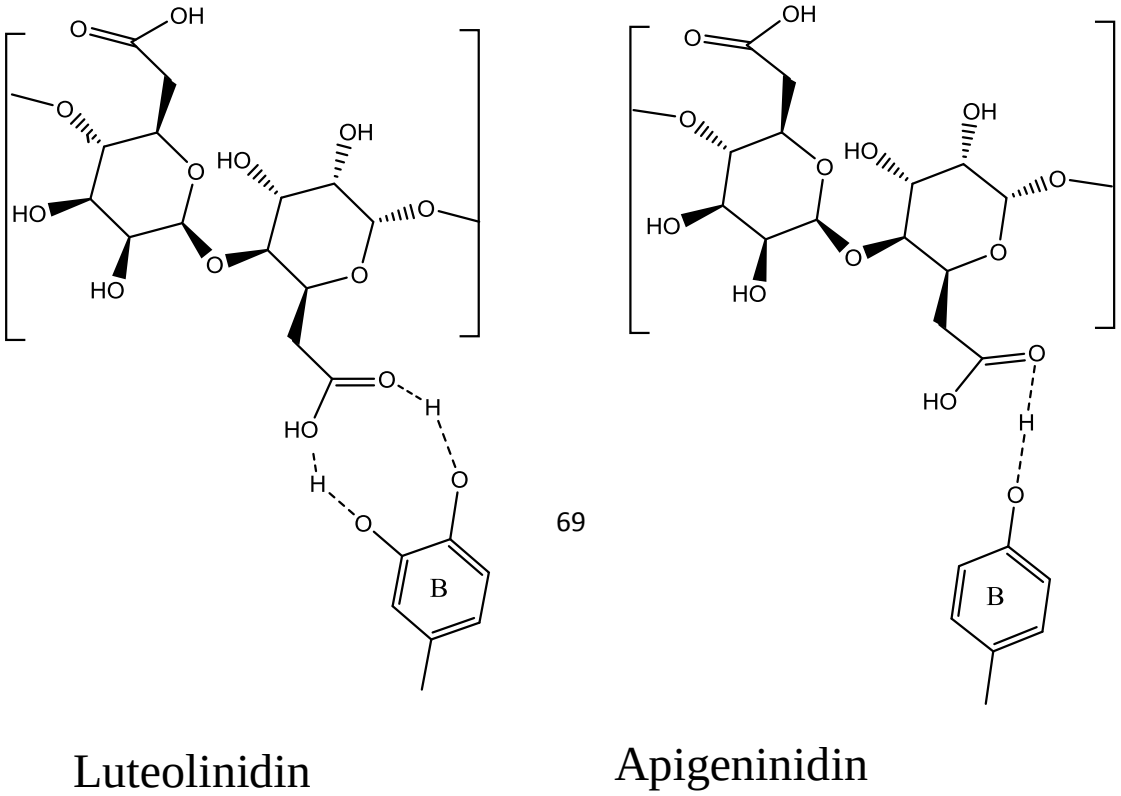
Sodium alginate may form hydrogen bonds with the hydroxyl substitutions on the B ring of luteolinidin and to a lesser extent, because of one less hydroxyl substitution, apigeninidin. This functionality has been previously observed with anthocyanin analogs. Buchweitz et al (2013) compared the stabilizing effect of different pectins using pelargoninidin-3-glucoside, cyanidin-3-glucoside, and delphinidin-3-glucoside. Pelargoninidin-3-glucoside was significantly less effectively stabilized than cyanidin-3-glucoside and delphinidin-3-glucoside. This was determined to be the result of fewer B ring substitutions.

The pectin-anthocyanin stabilization described by Buchweitz et al (2013) could be the same stabilizing mechanism of sodium alginate with apigeninidin and luteolinidin. Pelargoninidin which was stabilized least effectively has the same B ring configuration as apigeninidin, while cyanindin stabilized significantly more effectively than pelargoninidin and possesses the same B-ring configuration as luteolinidin. *Ortho*-dihydroxyl substitution on the B ring of luteolinidin appears to result in greater stabilization with sodium alginate.

Sodium alginate was far more effective at stabilizing apigeninidin-dominant extract at pH 5 than pH 3 (average percent color retention in 1.0g/L pH 3: 10.4%; pH 5 83.8%). Sodium alginate is known to form gels at low pH. Gels are formed through ionic cross-links. Viscosity increases and eventually forms gels below pH 3.7, the pKa of uronic acid (Yang et al 2009, Bu et al 2005). This could explain the difference between

pH 3 and pH 5 for apigeninidin stabilizations. At pH 3 sodium alginate is dominated by intramolecular interaction, while at pH 5 sodium alginate is no longer in cross linked network and is capable of intermolecular interaction with apigeninidin. This difference is less pronounced for luteolinidin stabilizations (average percent color retention pH 3: 58.9%; pH 5: 76.7%) because luteolinidin is capable of an additional hydrogen bond and thus achieves greater stability at pH 3 despite fewer possible ionic interactions with sodium alginate.

The proposed interaction mechanism between 3DXNs and sodium alginate is depicted in Figure 18. Carboxylic groups on guluronic and mannuronic acid moieties participate in hydrogen bonds with hydroxyl substitutions on the B ring of luteolinidin and apigeninidin.



Luteolinidin Apigeninidin

69

Figure 18. Proposed alginate and 3-deoxyanthocyanin stabilized complex.
Left: *ortho*-dihydroxyl substitutions on the B ring of luteolinidin participate in more hydrogen bonds with alginate resulting in greater stability.
Right: One hydroxyl substitution on the B ring of apigeninidin participates in fewer hydrogen bonds with alginate resulting in poorer stability, especially at pH below 3.7.

Effect of Metal Ion on the Aqueous Stability of 3-Deoxyanthocyanins

In this experiment, metal ion generally did not enhance aqueous stability of the 3DXNs. Effect of Mg^{2+} on aqueous stabilization was negligible, whereas Fe^{3+} produced significant bathochromic shift in luteolinidin containing extracts. Color changed from orange-red to brown-black. (Figs 2, 4, 12-15 (A-D) of Appendix B).

The bathochromic effect of Fe^{3+} was most apparent in luteolinidin-dominant and luteolinidin/ apigeninidin mixture extracts in the presence of sodium alginate, which changed in color from the bright orange-red to dull brown or deep black (Fig. 19). Gum arabic treatments did not form the black pigment. Luteolinidin/apigeninidin mixture samples stabilized with gum arabic became orange-brown upon addition of Fe^{3+}.

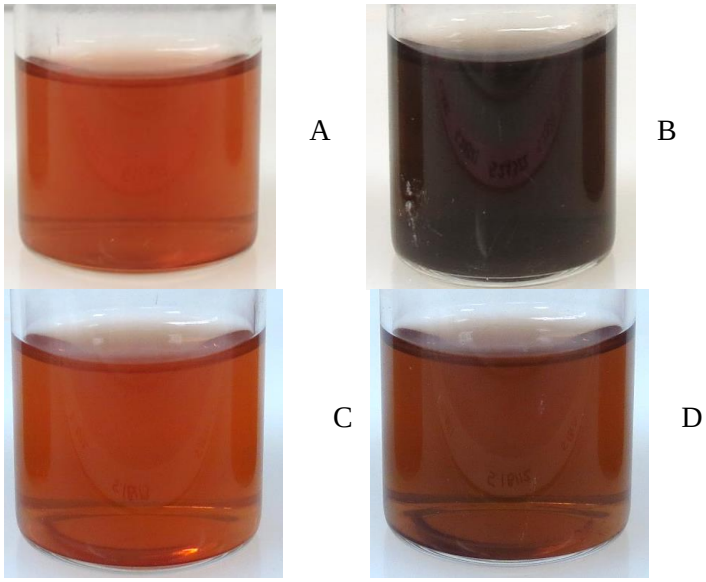


Figure 19. Effect of Fe^{3+} on 3-deoxyanthocyanin pigment color of the three sorghum samples stabilized in sodium alginate at pH 5. Samples A, C, and E are red leaf (luteolinidin dominant), black grain (luteolinidin/ apigeninidin mixture), and leaf sheath (apigeninidin-dominant), respectively. Samples B, D, and F are the corresponding pigments in presence of Fe^{3+}.

E

F

Figure 19 Continued

Fe^{3+} has been associated with enhanced stability while not significantly altering the color of the anthocyanin (Tachibana et al 2014). Instances of bathochromic shift caused by Fe^{3+} are also documented. In these instances, anthocyanin complexes typically become blue or purple (Xiong et al 2006; Bayer et al 1966; Yoshida et al 2015, Khaodee et al 2014). However, upon addition of Fe^{3+} to luteolinidin samples the metal-anthocyanin complex became black (Fig. 19 B)

Tachibana et al (2014) evaluated the thermal stability of anthocyanins in the presence of multivalent ions and gums. The greatest stability they observed was in the presence of sodium alginate and Fe^{3+} and suggested the stabilization was a result of metal-anthocyanin complex formed between C3' and C4' of cyanidin-3-glucoside with Fe^{3+} and the remaining cationic charge of Fe^{3+} interacting with a carboxylate group of alginate. This could be the same complex formed between luteolinidin, Fe^{3+}, and sodium alginate because luteolinidin possesses the same B ring configuration as cyanidin. The proposed structure of luteolinidin-Fe^{3+}-alginate is depicted in Fig. 20.

In many anthocyanin-metal complexes, bathochromic shifts result in a blue-purple pigment (Kondo et al 1998; Cheng and Crisosto 1997), not black. In many of these proposed complexes anthocyanins and metal ions are stacked and complexes formed by anthocyanin, ion, and in some cases copigment. In these complexes, commelinin for example, anthocyanins, flavones, and metal ion form in units of 6:6:2 respectively (Yoshida et al 2009). It is possible that if a luteolinidin-Fe^{3+}-alginate complex is formed (depicted in Fig. 20) that a bathochromic shift would occur similar to other anthocyanin-ion-copigment complexes; however, the chain like structure of alginate would dramatically impact that shift. When copigments are flavones, anthocyanin complexes are relatively finite and restricted to the self-assembled complexes which are limited to the available functional groups. Thus complexes like commelinin or other metalloanthocyanins exist in 6:6:2 units. The ratio of luteolinidin, alginate, and Fe^{3+} would not be 6:6:2 because of the chain like structure of alginate. It is likely that the luteolinidin: alginate: Fe^{3+} ratio would exist in a ratio of n(1:1:1) where 1 luteolinidin molecule interacts with 1 molecule of Fe^{3+} which interacts with 1 carboxylic group of alginate, and n is the number of carboxylate groups on a chain of alginate available for ionic or hydrogen bonding. Thus, because of the chain like structure of alginate this n(1:1:1) ratio could continue and be limited only by the carboxylate groups on a chain of alginate available for ionic or hydrogen bonding. Thus the greater the availability carboxylic groups on a chain of alginate the larger, and darker the pigment will become.

This complex is likely unique to luteolinidin among anthocyanins. Apigeninidin does not have the B ring structure necessary to interact with $Fe^{3+,}$ and anthocyanin analogues, such as cyanidin, which do have the appropriate B ring configuration have bulky substitutions that create steric hindrance prohibiting a continuous anthocyanin-Fe^{3+}-alginate complex. This ferric luteolinidin-alginate complex may have application in industry to serve as a natural dark pigment. While not examined in this experiment, the luteolinidin-Fe^{3+}-alginate complex may be stable enough to be used in combination with other pigments to produce darker or richer pigmentation. This may be important in food applications that use class IV caramel color (e.g., soft drinks); these products have come under increased scrutiny due to the high levels of 4-methylimidazole, a potential carcinogen, formed during class II and IV caramel manufacture. Many companies are actively seeking alternatives.

Unlike Fe^{3+}, Mg^{2+} had negligible effect on 3DXN stabilization. This is likely a combination of the different stabilization mechanisms observed in this experiment. The stabilizing effect of apigeninidin by gum Arabic is mainly hydrophobic and thus does not require ion. The stabilizing effect of alginate can be achieved with and without ion; however, when ion is involved, three charges are required to interact with pigment and gum (see Fig. 20). Therefore, as a divalent ion, Mg^{2+} would contribute negligibly in alginate-pigment complex formation.

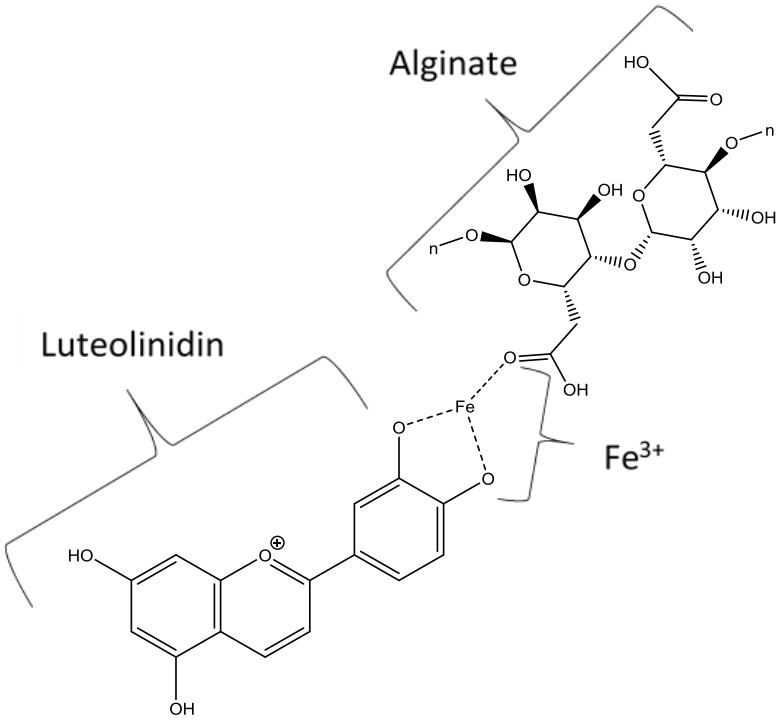


Figure 20. Proposed luteolinidin-Fe^{3+}- alginate complex. Fe^{3+} interacts with luteolinidin at C3' and C4'. The third cationic charge of Fe^{3+}interactions with the carboxylic substituent of alginate. This complex is possible for luteolinidin but not apigeninidin because of the *ortho*-hydroxy configuration. Structures similar to this have been discussed by Tachibana et al (2014) and Buchweitz et al (2013)

The purpose of this research was to evaluate the effect of polysaccharide gums and metal ions on the aqueous stability of 3DXNs. Gum Arabic and alginate both effectively stabilized 3DXNs in aqueous solution at 0.5 or 1.0 g/L. Gum arabic effectively stabilized apigeninidin-dominant pigment but was ineffective on luteolinidin-dominant pigment, suggesting hydrophobic interactions dominated the stabilizing effect of gum arabic. Metal ions did not improve stability of the pigments in presence of gum arabic. Effect of sodium alginate was less dependent on pigment composition and mostly dependent on pH; it was generally more effective at pH 5. Also, at pH 5, luteolinidin dominant pigment formed a black complex with Fe^{3+} in presence of alginate, which may

be the result of Fe^{3+} mediated polymerization of luteolinidin-ion-alginate complex. Mg^{2+} did not produce a meaningful effect.

Gum arabic stabilized apigeninidin dominant pigment extract at a concentration of 0.5g/L which is significantly lower than what is typically used for flavor stabilization in beverage applications. This provides valuable potential for apigeninidin-based sorghum pigments to be used as a natural colorant in products that routinely use gum arabic for flavor stabilization (typically used at 10 g/L). Additionally, gum arabic is not a viscosity modifier at low concentrations thus provides a wide range of application flexibility. Luteolinidin-based pigment was most effectively stabilized by sodium alginate. Thus these pigments have a potential as natural food colorant in a yogurt, ice cream, or other foods where contribution to viscosity by alginate would be an advantage. The fact that luteolinidin-based pigment formed a deep black color in presence of Fe^{3+} in alginate solution presents an interesting opportunity to expand potential application of 3-deoxyanthocyanin-based colors as alternatives to some classes of caramel.

CHAPTER VI

CONCLUSIONS AND FUTURE WORK

The goal of this work was to increase extractability and aqueous stability of 3DXNs from sorghum, results indicate both were accomplished. Microwave-assisted extraction increased the extractability of 3DXNS almost three-fold (2.44 mg/g) relative to conventional method (0.88 mg/g). Sodium alginate and gum arabic were used to enhance aqueous stability and both effectively stabilized 3DXNs in aqueous systems at low concentration (0.5- 1.0 g/L. Sodium alginate stabilized luteolinidin and apigeninidin most effectively at pH 5. Gum arabic effectively stabilized apigeninidin but not luteolinidin.

Microwave-assisted extraction was an effective extraction method for 3DXNs because of their apparent stability to microwave irradiation. In this research anthocyanins used for comparison underwent significant structural changes as a result of microwave irradiation. The 3DXNs underwent very little structural change; however, in most extreme extractions (600-1200 W; 30-40 min) cyanidin was produce in extracts. This is likely the result of structural transformation rather than a novel extraction of cyanidin from black sorghum.

Future work should focus on how microwave irradiation affects structural stability of the 3DXN pigments. This information will provide insight for how to optimize microwave-assisted extraction conditions for commercial applications. Different solvent systems should also be evaluated. Ethanol, unlike methanol, is food grade. Methanol tends to extract pigment with greater efficiency however with

microwave-assisted extraction, ethanol may increase in extraction efficacy. Other sources of plant tissue should be evaluated with microwave assisted extraction.

The stabilizing effect of both gums was largely influenced by the structure of both 3DXNs. Gum arabic stabilized apigeninidin through hydrophobic interaction. B-ring structure of luteolinidin prevented the same interaction. Stabilization occurred in concentrations of gum arabic significantly lower than what is used for flavor stabilization. The results of this research indicate that apigeninidin could be used as a natural colorant in gum arabic containing products with minimal reformulation. This is also relevant from an agronomic standpoint. Cultivars high in apigeninidin could be selectively bred to increase apigeninidin content especially in inedible, portions of the plant such as leaves and leaf sheaths.

Sodium alginate stabilized through ionic or hydrogen bonds. Luteolinidin tended to be more effectively stabilized than apigeninidin likely because of *ortho*-dihydroxy configuration around the B-ring. Pigments stabilized with alginate have a potential as natural food colorant in a yogurt, ice cream, or other foods where contribution to viscosity by alginate would be an advantage. Alginate also formed a stable deep black ferric luteolinidin pigment. Stability of the black luteolinidin-Fe^{3+}-alginate complex should be evaluated. Such dark color could serve as a novel alternative to some classes of caramel.

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APPENDIX A

PIGMENT YIELD FROM GRAINS AND PULSE EXTRACTED VIA MICROWAVE-ASSISTED EXTRACTION AND CONTROL

Table A1. 3-Deoxyanthocyanin* Yield: mg/g Sample Extracted from Whole Grain Sorghum Via Microwave-Assisted Extraction

Hold Time (min)	Control	300 W	600 W	1200 W
0.25	-	1.042 ± 0.031 [a]	1.135 ± 0.004 [ab]	0.988 ± 0.105 [a]
0.50	-	1.162 ± 0.037 [ab]	1.213 ± 0.018 [ab]	1.030 ± 0.060 [a]
0.75	-	1.194 ± 0.028 [ab]	0.992 ± 0.151 [ab]	1.101 ± 0.049 [ab]
3.00	-	1.209 ± 0.036 [ab]	1.837 ± 0.409 [c]	1.635 ± 0.162 [b]
5.00	-	1.337 ± 0.048 [ab]	1.852 ± 0.732 [c]	2.130 ± 0.118 [c]
10.00	-	1.448 ± 0.035 [c]	1.993 ± 0.304 [c]	2.189 ± 0.097 [cd]
20.00	-	1.803 ± 0.079 [c]	1.744 ± 0.057 [c]	1.874 ± 0.221 [c]
30.00	-	1.813 ± 0.054 [c]	2.418 ± 0.429 [d]	1.937 ± 0.084 [b]
40.00	-	1.922 ± 0.141 [c]	2.463 ± 0.312 [d]	2.193 ± 0.160 [cd]
120.00	0.883 ± 0.069[e]	-	-	-

*Extraction yield calculated by luteolinidin equivalents from UV-Vis abs. at 480 nm

TableA2. Anthocyanin* Yield: mg/g Sample Extracted from Whole Grain Maize Via Microwave-Assisted Extraction

Hold Time (min)	Control	300 W	600 W	1200 W
0.25	-	0.475 ± 0.031 [a]	0.533 ± 0.009 [a]	0.494 ± 0.000 [a]
0.50	-	0.497 ± 0.010 [a]	0.504 ± 0.018 [a]	0.475 ± 0.048 [a]
0.75	-	0.517 ± 0.001 [a]	0.518 ± 0.015 [a]	0.509 ± 0.002 [a]
3.00	-	0.531 ± 0.017 [a]	0.538 ± 0.008 [a]	0.561 ± 0.016 [a]
5.00	-	0.528 ± 0.013 [a]	0.553 ± 0.017 [a]	0.554 ± 0.000 [a]
10.00	-	0.549 ± 0.059 [a]	0.566 ± 0.040 [a]	0.517 ± 0.000 [a]
20.00	-	0.600 ± 0.024 [a]	0.569 ± 0.022 [a]	0.585 ± 0.002 [a]
30.00	-	0.584 ± 0.035 [a]	0.592 ± 0.018 [a]	0.585 ± 0.001 [a]
40.00	-	0.574 ± 0.013 [a]	0.511 ± 0.036 [a]	0.587 ± 0.030 [a]
120.00	0.586 ± 0.084 [a]	-	-	-

*Extraction yield calculated by cyanidin equivalents from UV-Vis abs. at 520 nm

Table A3. Anthocyanin* Yield: mg/g Sample Extracted from Whole Grain Cowpea Via Microwave-Assisted Extraction				
Hold Time (min)	**Control**	**300 W**	**600 W**	**1200 W**
0.25	-	0.247 ± 0.062^{a}	0.382 ± 0.002^{ab}	0.318 ± 0.040^{ab}
0.50	-	0.360 ± 0.033^{a}	0.314 ± 0.006^{a}	0.315 ± 0.005^{a}
0.75	-	0.409 ± 0.001^{a}	0.277 ± 0.014^{a}	0.305 ± 0.019^{a}
3.00	-	0.550 ± 0.059^{b}	0.639 ± 0.113^{b}	0.678 ± 0.017^{b}
5.00	-	0.637 ± 0.138^{b}	0.598 ± 0.016^{b}	0.762 ± 0.008^{bc}
10.00	-	0.831 ± 0.048^{c}	0.687 ± 0.007^{b}	0.658 ± 0.217^{b}
20.00	-	0.420 ± 0.044^{ab}	0.446 ± 0.095^{ab}	0.544 ± 0.097^{b}
30.00	-	0.481 ± 0.117^{b}	0.347 ± 0.023^{ab}	0.564 ± 0.191^{b}
40.00	-	0.494 ± 0.103^{b}	0.504 ± 0.152^{ab}	0.661 ± 0.395^{b}
120.00	0.409 ± 0.081^{ab}	-	-	-
*Extraction yield calculated by cyanidin equivalents from UV-Vis abs. at 520 nm				

APPENDIX B

REPRESENTATIVE IMAGES OF SAMPLES AT START AND END DATES OF EXPERIMENT

Figure B1. Luteolinidin dominant (sorghum leaf extract) stabilizations in 0.0 g/L gum			
Day 0		Week 10	
pH3	pH 5	pH 3	pH 5
A 	**B** 	**C** 	**D** 
Luteolinidin dominate (sorghum leaf extract) stabilized with 0.0g/L gum and Fe^{3+} at pH 3 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.0g/L gum and Fe^{3+} at pH 5 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.0g/L gum and Fe^{3+} at pH 3 on Week 10	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.0g/L gum and Fe^{3+} at pH 5 on Week 10
E 	**F** 	**G** 	**H** 
Luteolinidin dominate (sorghum leaf extract) stabilized with 0.0g/L gum and Mg^{2+} at pH 3 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.0g/L gum and Mg^{2+} at pH 5 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.0g/L gum and Mg^{2+} at pH 3 on Week 10	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.0g/L gum and Mg^{2+}at pH 5 on Week 10
I 	**J** 	**K** 	**L** 
Luteolinidin dominate (sorghum leaf extract) stabilized with 0.0g/L gum and no ion at pH 3 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.0g/L gum and no ion at pH 5 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.0g/L gum and no ion at pH 3 on Week 10	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.0g/L gum and no ion at pH 5 on Week 10

Figure B2. Luteolinidin dominant (sorghum leaf extract) stabilizations in 0.05 % Sodium Alginate			
Day 0		End of Experiment	
pH3	pH 5	pH 3	pH 5
A 	**B** 	**C** 	**D** 
Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L sodium alginate and Fe^{3+} at pH 3 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L sodium alginate and Fe^{3+} at pH 5 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L sodium alginate and Fe^{3+} at pH 3 on Week 10	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L sodium alginate and Fe^{3+} at pH 5 on Week 10
E 	**F** 	**G** 	**H** 
Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L sodium alginate and Mg^{2+} at pH 3 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L sodium alginate and Mg^{2+} at pH 5 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L sodium alginate and Mg^{2+} at pH 3 on Week 10	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L sodium alginate and Mg^{2+}at pH 5 on Week 10
I 	**J** 	**K** 	**L** 
Luteolinidin dominate (sorghum leaf extract) stabilized 0.5 g/L sodium	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L sodium	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L sodium

alginate and no ion at pH 3 on Day 0	sodium alginate and no ion at pH 5 on Day 0	alginate and no ion at pH 3 on Week 10	alginate and no ion at pH 5 on Week 10

Figure B3. Luteolinidin dominant (sorghum leaf extract) stabilizations in 0.05 % Gum Arabic			
Day 0		**End of Experiment**	
pH3	**pH 5**	**pH 3**	**pH 5**
A 	**B** 	**C** 	**D** 
Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L gum arabic and Fe^{3+} at pH 3 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L gum arabic and Fe^{3+} at pH 5 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L gum arabic and Fe^{3+} at pH 3 on Week 10	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L gum arabic and Fe^{3+} at pH 5 on Week 10
E 	**F** 	**G** 	**H** 
Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L gum arabic and Mg^{2+} at pH 3 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L gum arabic and Mg^{2+} at pH 5 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L gum arabic and Mg^{2+} at pH 3 on Week 10	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L gum arabic and Mg^{2+}at pH 5 on Week 10

I 	**J** 	**K** 	**L** 
Luteolinidin dominate (sorghum leaf extract) stabilized 0.5 g/L gum arabic and no ion at pH 3 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L gum arabic and no ion at pH 5 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L gum arabic and no ion at pH 3 on Week 10	Luteolinidin dominate (sorghum leaf extract) stabilized with 0.5 g/L gum arabic and no ion at pH 5 on Week 10

Figure B4. Luteolinidin dominant (sorghum leaf extract) stabilizations in 0.1 % Sodium Alginate

Day 0		**End of Experiment**	
pH3	**pH 5**	**pH 3**	**pH 5**
A 	**B** 	**C** 	**D** 
Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L sodium alginate and Fe^{3+} at pH 3 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L sodium alginate and Fe^{3+} at pH 5 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L sodium alginate and Fe^{3+} at pH 3 on Week 10	Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L sodium alginate and Fe^{3+} at pH 5 on Week 10
E 	**F** 	**G** 	**H** 

Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L sodium alginate and Mg^{2+} at pH 3 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L sodium alginate and Mg^{2+} at pH 5 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L sodium alginate and Mg^{2+} at pH 3 on Week 10	Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L sodium alginate and Mg^{2+}at pH 5 on Week 10
I 	**J** 	**K** 	**L**  **1**
Luteolinidin dominate (sorghum leaf extract) stabilized 1.0 g/L sodium alginate and no ion at pH 3 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L sodium alginate and no ion at pH 5 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L sodium alginate and no ion at pH 3 on Week 10	Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L sodium alginate and no ion at pH 5 on Week 10

Figure B5. Luteolinidin dominant (sorghum leaf extract) stabilizations in 0.1 % Gum Arabic			
Day 0		**End of Experiment**	
pH3	**pH 5**	**pH 3**	**pH 5**
A 	**B** 	**C** 	**D** 
Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L gum arabic and Fe^{3+} at pH 3 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L	Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L gum	Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L gum

	gum arabic and Fe^{3+} at pH 5 on Day 0	arabic and Fe^{3+} at pH 3 on Week 10	arabic and Fe^{3+} at pH 5 on Week 10
E 	**F** 	**G** 	**H** 
Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L gum arabic and Mg^{2+} at pH 3 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L gum arabic and Mg^{2+} at pH 5 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L gum arabic and Mg^{2+} at pH 3 on Week 10	Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L gum arabic and Mg^{2+}at pH 5 on Week 10
I 	**J** 	**K** 	**L** 
Luteolinidin dominate (sorghum leaf extract) stabilized 1.0 g/L gum arabic and no ion at pH 3 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L gum arabic and no ion at pH 5 on Day 0	Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L gum arabic and no ion at pH 3 on Week 10	Luteolinidin dominate (sorghum leaf extract) stabilized with 1.0 g/L gum arabic and no ion at pH 5 on Week 10

Figure B6. Apigeninidin dominant (sorghum leaf sheath extract) stabilizations in 0.0 % Gum

Day 0		**End of Experiment**	
pH3	**pH 5**	**pH 3**	**pH 5**
A 	**B** 	**C** 	**D** 

Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.0g/L gum and Fe^{3+} at pH 3 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.0g/L gum and Fe^{3+} at pH 5 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.0g/L gum and Fe^{3+} at pH 3 on Week 10	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.0g/L gum and Fe^{3+} at pH 5 on Week 10
E 	**F** 	**G** 	**H** 
Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.0g/L gum and Mg^{2+} at pH 3 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.0g/L gum and Mg^{2+} at pH 5 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.0g/L gum and Mg^{2+} at pH 3 on Week 10	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.0g/L gum and Mg^{2+}at pH 5 on Week 10
I 	**J** 	**K** 	**L** 
Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.0g/L gum and no ion at pH 3 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.0g/L gum and no ion at pH 5 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.0g/L gum and no ion at pH 3 on Week 10	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.0g/L gum and no ion at pH 5 on Week 10

Figure B7. Apigeninidin dominant (sorghum leaf sheath extract) stabilizations in 0.05 % Sodium Alginate

Day 0		End of Experiment	
pH3	**pH 5**	**pH 3**	**pH 5**

A 	**B** 	**C** 	**D** 
Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L sodium alginate and Fe^{3+} at pH 3 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L sodium alginate and Fe^{3+} at pH 5 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L sodium alginate and Fe^{3+} at pH 3 on Week 10	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L sodium alginate and Fe^{3+} at pH 5 on Week 10
E 	**F** 	**G** 	**H** 
Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L sodium alginate and Mg^{2+} at pH 3 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L sodium alginate and Mg^{2+} at pH 5 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L sodium alginate and Mg^{2+} at pH 3 on Week 10	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L sodium alginate and Mg^{2+}at pH 5 on Week 10
I 	**J** 	**K** 	**L** 
Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L sodium alginate and no ion at pH 3 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L sodium alginate and no ion at pH 5 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L sodium alginate and no ion at pH 3 on Week 10	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L sodium alginate and no ion at pH 5 on Week 10

Figure B8. Apigeninidin dominant (sorghum leaf sheath extract) stabilizations in 0.05 % Gum Arabic			
Day 0		End of Experiment	
pH3	pH 5	pH 3	pH 5
A 	**B** 	**C** 	**D** 
Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L gum arabic and Fe^{3+} at pH 3 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L gum arabic and Fe^{3+} at pH 5 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L gum arabic and Fe^{3+} at pH 3 on Week 10	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L gum arabic and Fe^{3+} at pH 5 on Week 10
E 	**F** 	**G** 	**H** 
Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L gum arabic and Mg^{2+} at pH 3 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L gum arabic and Mg^{2+} at pH 5 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L gum arabic and Mg^{2+} at pH 3 on Week 10	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L gum arabic and Mg^{2+}at pH 5 on Week 10
I 	**J** 	**K** 	**L** 
Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L gum	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L gum	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L gum	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 0.5g/L gum

arabic and no ion at pH 3 on Day 0	arabic and no ion at pH 5 on Day 0	arabic and no ion at pH 3 on Week 10	arabic and no ion at pH 5 on Week 10

Figure B9. Apigeninidin dominant (sorghum leaf sheath extract) stabilizations in 0.1 % Sodium Alginate			
Day 0		**End of Experiment**	
pH3	**pH 5**	**pH 3**	**pH 5**
A 	**B** 	**C** 	**D** 
Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L sodium alginate and Fe^{3+} at pH 3 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L sodium alginate and Fe^{3+} at pH 5 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L sodium alginate and Fe^{3+} at pH 3 on Week 10	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L sodium alginate and Fe^{3+} at pH 5 on Week 10
E 	**F** 	**G** 	**H** 
Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L sodium alginate and Mg^{2+} at pH 3 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L sodium alginate and Mg^{2+} at pH 5 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L sodium alginate and Mg^{2+} at pH 3 on Week 10	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L sodium alginate and Mg^{2+}at pH 5 on Week 10

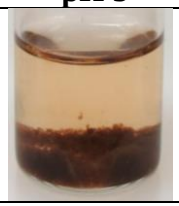
I 	**J** 	**K** 	**L** 
Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L sodium alginate and no ion at pH 3 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L sodium alginate and no ion at pH 5 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L sodium alginate and no ion at pH 3 on Week 10	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L sodium alginate and no ion at pH 5 on Week 10

Table B10. Apigeninidin dominant (sorghum leaf sheath extract) stabilizations in 0.1 % Gum Arabic

Day 0		**End of Experiment**	
pH3	**pH 5**	**pH 3**	**pH 5**
A 	**B** 	**C** 	**D** 
Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L gum arabic and Fe^{3+} at pH 3 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L gum arabic and Fe^{3+} at pH 5 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L gum arabic and Fe^{3+} at pH 3 on Week 10	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L gum arabic and Fe^{3+} at pH 5 on Week 10
E 	**F** 	**G** 	**H** 

Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L gum arabic and Mg^{2+} at pH 3 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L gum arabic and Mg^{2+} at pH 5 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L gum arabic and Mg^{2+} at pH 3 on Week 10	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L gum arabic and Mg^{2+}at pH 5 on Week 10
I 	**J** 	**K** 	**L** 
Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L gum arabic and no ion at pH 3 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L gum arabic and no ion at pH 5 on Day 0	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L gum arabic and no ion at pH 3 on Week 10	Apigeninidin dominate (sorghum leaf sheath extract) stabilized with 1.0 g/L gum arabic and no ion at pH 5 on Week 10

Table B11. Luteolinidin/ apigeninidin dominant (whole grain sorghum extract) stabilizations in 0.0 % Gum

Day 0		**End of Experiment**	
pH3	**pH 5**	**pH 3**	**pH 5**
A 	**B** 	**C** 	**D** 
Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.0g/L gum and Fe^{3+} at pH 3 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.0g/L gum and Fe^{3+} at pH 5 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.0g/L gum and Fe^{3+} at pH 3 on Week 10	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.0g/L gum and Fe^{3+} at pH 5 on Week 10

E 	**F** 	**G** 	**H** 
Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.0g/L gum and Mg^{2+} at pH 3 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.0g/L gum and Mg^{2+} at pH 5 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.0g/L gum and Mg^{2+} at pH 3 on Week 10	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.0g/L gum and Mg^{2+}at pH 5 on Week 10
I 	**J** 	**K** 	**L** 
Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.0g/L gum and no ion at pH 3 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.0g/L gum and no ion at pH 5 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.0g/L gum and no ion at pH 3 on Week 10	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.0g/L gum and no ion at pH 5 on Week 10

Table B12. Luteolinidin/ apigeninidin dominant (whole grain sorghum extract) stabilizations in 0.05 % Sodium Alginate

Day 0		**End of Experiment**	
pH3	**pH 5**	**pH 3**	**pH 5**
A 	**B** 	**C** 	**D** 

Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L sodium alginate and Fe^{3+} at pH 3 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L sodium alginate and Fe^{3+} at pH 5 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L sodium alginate and Fe^{3+} at pH 3 on Week 10	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L sodium alginate and Fe^{3+} at pH 5 on Week 10
E 	**F** 	**G** 	**H** 
Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L sodium alginate and Mg^{2+} at pH 3 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L sodium alginate and Mg^{2+} at pH 5 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L sodium alginate and Mg^{2+} at pH 3 on Week 10	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L sodium alginate and Mg^{2+}at pH 5 on Week 10
I 	**J** 	**K** 	**L** 
Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized 0.5 g/L sodium alginate and no ion at pH 3 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L sodium alginate and no ion at pH 5 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L sodium alginate and no ion at pH 3 on Week 10	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L sodium alginate and no ion at pH 5 on Week 10

Table B13. Luteolinidin/ apigeninidin dominant (whole grain sorghum extract) stabilizations in 0.05 % Gum Arabic

Day 0		End of Experiment	
pH3	**pH 5**	**pH 3**	**pH 5**

A 	**B** 	**C** 	**D** 
Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L gum arabic and Fe^{3+} at pH 3 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L gum arabic and Fe^{3+} at pH 5 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L gum arabic and Fe^{3+} at pH 3 on Week 10	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L gum arabic and Fe^{3+} at pH 5 on Week 10
E 	**F** 	**G** 	**H** 
Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L gum arabic and Mg^{2+} at pH 3 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L gum arabic and Mg^{2+} at pH 5 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L gum arabic and Mg^{2+} at pH 3 on Week 10	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L gum arabic and Mg^{2+}at pH 5 on Week 10
I 	**J** 	**K** 	**L** 
Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized 0.5 g/L gum arabic and no ion at pH 3 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L gum arabic and no ion at pH 5 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L gum arabic and no ion at pH 3 on Week 10	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 0.5 g/L gum arabic and no ion at pH 5 on Week 10

Table B14. Luteolinidin/ apigeninidin dominant (whole grain sorghum extract) stabilizations in 0.1 % Sodium Alginate

Day 0		End of Experiment	
pH3	**pH 5**	**pH 3**	**pH 5**
A 	**B** 	**C** 	**D** 
Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 1.0 g/L sodium alginate and Fe^{3+} at pH 3 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 1.0 g/L sodium alginate and Fe^{3+} at pH 5 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 1.0 g/L sodium alginate and Fe^{3+} at pH 3 on Week 10	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 1.0 g/L sodium alginate and Fe^{3+} at pH 5 on Week 10
E 	**F** 	**G** 	**H** 
Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 1.0 g/L sodium alginate and Mg^{2+} at pH 3 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 1.0 g/L sodium alginate and Mg^{2+} at pH 5 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 1.0 g/L sodium alginate and Mg^{2+} at pH 3 on Week 10	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 1.0 g/L sodium alginate and Mg^{2+}at pH 5 on Week 10
I 	**J** 	**K** 	**L** 
Luteolinidin/ apigeninidin mixture (whole grain sorghum extract)	Luteolinidin/ apigeninidin mixture (whole grain	Luteolinidin/ apigeninidin mixture (whole grain sorghum	Luteolinidin/ apigeninidin mixture (whole grain sorghum

stabilized 1.0 g/L sodium alginate and no ion at pH 3 on Day 0	sorghum extract) stabilized with 1.0 g/L sodium alginate and no ion at pH 5 on Day 0	extract) stabilized with 1.0 g/L sodium alginate and no ion at pH 3 on Week 10	extract) stabilized with 1.0 g/L sodium alginate and no ion at pH 5 on Week 10

Table B15. Luteolinidin/ apigeninidin dominant (whole grain sorghum extract) stabilizations in 0.1 % Gum Arabic

Day 0		End of Experiment	
pH3	**pH 5**	**pH 3**	**pH 5**
A 	**B** 	**C** 	**D** 
Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 1.0 g/L gum arabic and Fe^{3+} at pH 3 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 1.0 g/L gum arabic and Fe^{3+} at pH 5 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 1.0 g/L gum arabic and Fe^{3+} at pH 3 on Week 10	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 1.0 g/L gum arabic and Fe^{3+} at pH 5 on Week 10
E 	**F** 	**G** 	**H** 
Luteolinidin/ apigeninidin mixture (whole grain sorghum	Luteolinidin/ apigeninidin mixture (whole grain	Luteolinidin/ apigeninidin mixture (whole grain sorghum	Luteolinidin/ apigeninidin mixture (whole grain

extract) stabilized with 1.0 g/L gum arabic and Mg^{2+} at pH 3 on Day 0	sorghum extract) stabilized with 1.0 g/L gum arabic and Mg^{2+} at pH 5 on Day 0	extract) stabilized with 1.0 g/L gum arabic and Mg^{2+} at pH 3 on Week 10	sorghum extract) stabilized with 1.0 g/L gum arabic and Mg^{2+}at pH 5 on Week 10
I 	**J** 	**K** 	**L** 
Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized 1.0 g/L gum arabic and no ion at pH 3 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 1.0 g/L gum arabic and no ion at pH 5 on Day 0	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 1.0 g/L gum arabic and no ion at pH 3 on Week 10	Luteolinidin/ apigeninidin mixture (whole grain sorghum extract) stabilized with 1.0 g/L gum arabic and no ion at pH 5 on Week 10