MECHANISMS DRIVING EXTRACTABILITY AND AQUEOUS COLLOIDAL STABILITY OF 3-DEOXYANTHOCYANINS IN THE PRESENCE OF AMPHIPHILIC POLYSACCHARIDES

A Dissertation

by

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ABSTRACT

Sorghum derived 3-deoxyanthocyanin (3-DXA) are of growing interest as natural food colors due to their unique stability compared to anthocyanins. The commercial use of 3-DXA is limited due to the poor extractability and reduced hydrophilicity of 3-DXA, causing self-association and precipitation in aqueous solutions.

Previous work from our group demonstrated that microwave-assisted extraction (MAE) improved extraction efficiency of 3-DXA from sorghum. Likewise, amphiphilic polysaccharides, such as gum arabic, were reported to improve aqueous stability of 3-DXA. The goal of this work was to determine mechanisms responsible for the enhanced efficiency of MAE and aqueous stability of 3-DXA in the presence of amphiphilic polysaccharides.

The increased extractability of 3-DXA by MAE was investigated by comparing pigment and copigment profile, of both non-tannin and tannin sorghum, after MAE to conventional extraction. The mechanisms of interaction between 3-DXA and amphiphilic polysaccharides was determined by investigating the effect of gum arabic and pectins of different degree of methylation (DE 38-86), 3-DXA profile, and pH on the aqueous stability of 3-DXA. Fluorescence quenching, zeta potential, and average particle size of 3-DXA polysaccharide complexes were measured.

The 3-DXA were structurally stable to microwave energy (1200 W/30 min); MAE induced sorghum cell wall degradation releasing ferulate esters. These two mechanisms were responsible for the increased extractability under MAE (2-3X) compared to conventional extraction.

Preliminary work showed that gum arabic stabilized more hydrophobic 3-DXA (apigeninidin) than hydrophilic 3-DXA (luteolinidin). Fluorescence quenching confirmed apigeninidin had a larger binding constant ($19.2x10^3$ M^{-1}) with gum arabic compared to luteolinidin ($1.72x10^3$ M^{-1}) and a stable 3-DXA-gum arabic complex was formed. Pectin (DE 54) was also more effective at stabilizing extracts with a higher proportion of apigeninidin compared to extracts higher in luteolinidin in aqueous solutions (38-86% vs 10-67%, respectively). Zeta potential was lower (-13 mV avg) in 3-DXA-pectin or -gum arabic solutions than without 3-DXA (-11 mV avg) suggesting that 3-DXA were encapsulated into hydrophobic regions of the polysaccharides. These results show that extracts with a greater proportion of apigeninidin than luteolinidin interact more efficiently with amphiphilic polysaccharides, suggesting hydrophobic interactions were a major interaction between the two components.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Dr. Joseph Awika (advisor) of the Department of Soil and Crop Sciences, Dr. William Rooney of the Department of Soil and Crop Sciences, Dr. Carmen Gomes of the Department of Biological and Agricultural Engineering (Texas A&M University) and the Department of Mechanical Engineering (Iowa State University), and Dr. Bimanagouda Patil of the Department of Horticultural Sciences.

Shreeya Ravisankar contributed to Chapters 3 and 4 by identifying ferulic acid compounds and by helping to elucidate the structure of 7-O-methylcyanidin.

All other work for the dissertation was completed independently by the student.

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1. INTRODUCTION

The color of food is a key indicator of overall quality and a driver of consumer purchases, but preserving color throughout the food manufacturing and handling process is a difficult task because of the sensitivity of color to food processing (Diehl, 2008; Thorngate, 2001). Artificial and natural food colors are added to foods to mitigate color deterioration or to impart an uncharacteristic, but desired, color. Synthetic colorants have specific advantages compared to natural colorants including consistent hues, dye strength, color intensity, stability, cost, and ease of incorporation into foods. However, growing consumer desire for "clean label" natural ingredients have created a demand for natural food colors. Although more difficult to produce and use in food, natural colors offer a unique advantage over artificial colors in that most of the compounds (e.g., carotenoids, anthocyanins, and betanins) may also have beneficial health effects (Socaciu, 2008; Stintzing & Carle, 2004). Typically, the main sources of natural colorants are fruits, vegetables, and grains.

Anthocyanins (ACN) are one of the most widely used natural colorants and impart red, purple, and blue colors to foods. ACN are water-soluble and found in a wide range of crops (e.g. strawberries, red cabbage, and purple corn). These colorants are not without limitations, however. The color shade and intensity of color is highly dependent on the matrix to which they are added, changing as pH increases above 3. Further, they are susceptible to bleaching by other food ingredients and are also heat labile, especially those that are non-acylated. These factors limit the use of ACN as food colorants, creating a need for more stable naturally-derived food colors.

3-Deoxyanthocyanins (3-DXA), which differ from ACN due to lack of substitution on C3 of the heterocyclic ring, have shown greater stability in the conditions that limit the success of ACN as a natural food colorant. The 3-DXA are less susceptible to color fading with increasing

pH (Awika, Rooney, & Waniska, 2004; Mazza & Brouillard, 1987a), bleaching in the presence of sulfites or ascorbic acid (Geera, Ojwang, & Awika, 2012; Ojwang & Awika, 2010), and degradation from heat treatment (Yang, Dykes, & Awika, 2014). Specifically, the lack of substitution at C3 appears to be key to the enhanced stability of 3-DXA as it reduces susceptibility to nucleophilic attack of C2 that can lead to color fading and subsequent degradation. However, the lack of substitution at C3 causes the 3-DXA to be more hydrophobic, resulting in rapid self-association in aqueous solutions. The self-association of 3-DXA leads to precipitation of pigments. Previous work in our lab has shown that this self-association can be mitigated using food gums. For instance, after 10 weeks, gum arabic or sodium alginate resulted in >95% 3-DXA suspension in aqueous solutions compared to < 40% of 3-DXA in solutions without added gums (Herrman, 2016). However, the mechanisms behind this improved aqueous stability by the gums are unknown. Understanding the mechanisms will lead to strategic use of 3-DXA in food formulations for optimum performance and color impact.

Another major limitation of 3-DXA as natural food colorants is the relatively low extraction efficiency from sorghum, the only major food source of 3-DXA. Our lab has attempted to improve extraction efficiency with accelerated solvent (Barros, Dykes, Awika, & Rooney, 2013) and enzymatic extraction (Njongmeta, 2009) with limited success. Extraction of pigments from cereal grains is difficult because the cell wall matrix is extensively crosslinked via phenolic esters that confer strength to the cell wall structure (Carpita, 1996). A recent study in our lab showed that microwave-assisted extraction (MAE) effectively increased extraction efficiency of 3-DXA from whole grain sorghum by approximately 3-fold (2.44 mg/g compared to 0.88 mg/g from control extraction method) (Herrman 2016). The mechanism behind this dramatic increase in 3-DXA yield due to MAE should be established. However, significant quantities of anthocyanidins (aglycone)

previously undetected in mature sorghum grain or plant tissue were found in the MAE extract. It is unclear whether these compounds were the result of MAE-induced structural transformation of 3-DXA (or other flavonoids from sorghum). Thus, the source of such compounds should be investigated. Of further interest is the structure and extractability of copigments from sorghum during MAE. Phenolic compounds present in sorghum (e.g. ferulic acid) have been shown to act as copigments and improve stability of 3-DXA over time (Awika, 2008). Thus, it is of interest to determine how MAE could affect extractability of copigments and their impact on 3-DXA properties.

The overall goal of this research is to increase the potential usage of 3-DXA from sorghum as natural food colorants. The specific objectives for this research are:

1. To determine the effect of microwave energy on the structure and extractability of sorghum 3-DXA and copigments
2. To determine the effect of tannins on the profile and extractability of pigments from sorghum and the effect of the tannins on the stability of the extracted pigments
3. To elucidate the mechanisms of interaction between gum arabic and 3-DXA in aqueous solutions
4. To determine the effect of pectin degree of esterification and 3-DXA profile on the aqueous stability of 3-DXA

2. LITERATURE REVIEW

2.1. Current status of food colors

Humans and animals alike respond to color stimuli as a primary indicator of quality, so color is one of the most significant drivers of consumer purchases. Color also gives an indication of flavor and overall appeal of a food product (Simon et al., 2017). Because of this, food manufacturers formulate with added colorants to impart the most appealing hue and to mitigate color deterioration during food processing. Food color additives from natural sources comprised 31% of the $1.45 billion global color market in 2009, with 5% annual growth since 2007 compared to 1% growth from artificial colors (Simon et al., 2017).

Color additives fall into two broad categories: natural or synthetic. Synthetic colors have some distinct advantages over naturally occurring colorants as they are relatively robust to food processing, stable at a range of pH values, have high tinctorial strength, and are relatively inexpensive to manufacture (Sigurdson, Tang, & Giusti, 2017). Natural food colors, meaning colored compounds naturally occurring or extracted from living tissue, are more difficult to use than synthetic food colorants. Naturally occurring pigments tend to degrade quickly with food processing and during storage. Examples of natural coloring include ACN from berries, carotenoids from carrots, and betalains from beets.

Although synthetic colors have several functional advantages, the biggest limitation of their use stems from negative consumer perception. Consumers are concerned about potential negative health effects caused by artificial colors, e.g. red 40 food dyes repeated influence on hyperactivity of children (McCann et al., 2007). Numerous recent studies have concluded that normal consumption of synthetic colorants will have a detrimental effect on human health

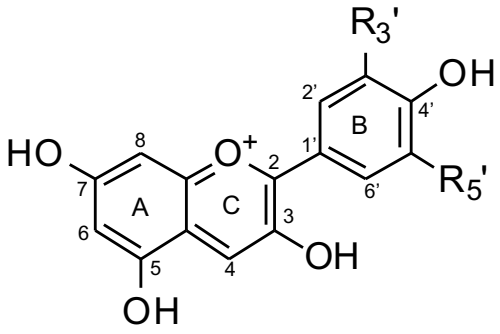
(Amchova, Kotolova, & Ruda-Kucerova, 2015); however, negative consumer perception persists. Specifically, the Nielsen Global Health & Ingredient Survey (January to March 2016) said that 50% of consumers in North and South America reported trying to avoid artificial colorants. This number was higher across the Atlantic Ocean. Approximately 60% of Europeans, 62% of Middle East/Africa, and 65% of Asia Pacific residents try to avoid synthetic colorants (Simon et al., 2017). This negative consumer perception has driven up demand for natural colorant alternatives. Companies such as General Mills and Mars, Inc. have responded to this by pledging to remove artificial colors from their products.

2.2. Anthocyanins and 3-deoxyanthocyanins

ACN are among the most widely used type of naturally-occurring colored compounds that can be used in foods to impart color. These compounds are found in fruits, vegetables, and grains with red, purple, and blue colors – such as grapes, strawberries, red cabbage, purple carrots, and purple corn. ACN are polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrylium with 2 benzoyl rings (A and B) separated by a heterocyclic (C) (Figure 1). ACN are most commonly found in nature glycosylated and/or further acylated with aliphatic or hydroxycinnamic acids on the glycosidic moieties (Figure 1). Anthocyanidins, the aglycone form of ACN, rarely occur naturally in plants. There are six common anthocyanidins that make up the backbone of most anthocyanin molecules; glycosylation and acylation generate greater diversity of ACN (Giusti & Wrolstad, 2003; He & Giusti, 2010). Anthocyanidins are poorly water-soluble whereas ACN, with hydrophilic glycosides, are more water-soluble.

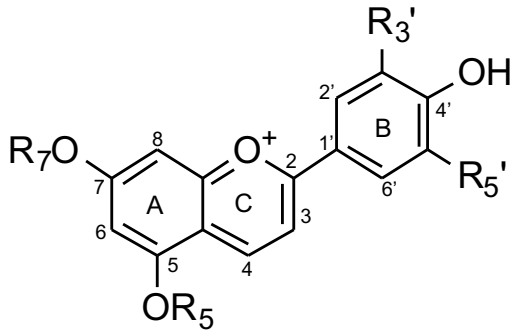
3-Deoxyanthocyanins (3-DXA) are also naturally occurring color compounds that share similar yet distinct characteristics with ACN. The distinct structural feature of 3-DXA is the lack

of substitution on the third carbon of the C-ring compared to ACN that are substituted at C-3 (Figure 1). The lack of substitution at C-3 causes three main characteristics of 3-DXA. The first is the effect on pigment color. The conjugated bonds within the chromophore absorb light at a lower (hypsochromic) wavelength than that of ACN, resulting in 3-DXA perceived as yellow-orange instead of red-purple at acidic pH. The second effect is on solubility. The lack of substitution at C3 causes the 3-DXA to be less polar than ACN, and consequently less water-soluble than ACN. The third effect is on stability. The electron density of 3-DXA in the C-ring is greater than that of ACN, resulting in resistance to nucleophilic attack and hydration, the first step of degradation and subsequent color loss (Yang et al., 2014).



Anthocyanidin

Anthocyanidin	R_3'	R_5'
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvinidin	OCH_3	OCH_3



3-Deoxyanthocyanidin

3-Deoxyanthocyanidin	R_3'	R_5'	R_5	R_7
Apigeninidin	H	H	H	H
Luteolinidin	OH	H	H	H
5-Methoxyapigeninidin	H	H	CH_3	H
7-Methoxyapigeninidin	H	H	H	CH_3
5-Methoxyluteolinidin	OH	H	CH_3	H
7-Methoxyluteolinidin	OH	H	H	CH_3

Figure 1 Substitution pattern of common anthocyanidins and 3-deoxyanthocyanidins.

2.3. Behavior of anthocyanins and 3-deoxyanthocyanins in aqueous solutions

The color of ACN is determined by multiple factors including the hydroxylation and methoxylation pattern of the anthocyanidin structure. Higher hydroxyl substitution causes a bathochromic shift of λ_{max} whereas a higher number of methoxyl substitution induces a hypsochromic shift of λ_{max} (Mazza & Brouillard, 1987b). Extrinsic pH is a major determinant of ACN color because ACN exist in a dynamic structural equilibrium; the ratio of structures varies depending on the pH of the solution (Figure 2). The same ACN may display a broad range of colors and intensities based on the specific pH of their environment. At any pH, ACN exist in equilibrium of four structures: the flavylium cation (AH^+), carbinol pseudobase (B), quinoidal base (A), and chalcone (C). At pH < 2, the (red) colored flavylium cation (AH^+) dominates. As pH increases (between 3-4), parallel reactions occur: hydration at C2 or C4 followed by proton loss to colorless carbinol pseudobase (B) and/or pale yellow chalcone species (C), or deporotonation to form the blue-purple quinoidal bases (A). The kinetic reaction is proton loss, but the more thermodynamically stable product is the chalcone. The colored quinoidal base structures dominate at slightly acidic or near neutral pH but eventually transform into the colorless carbinol pseudobase structure (the more thermodynamically stable structure). Thus, over time, ACN will become colorless at elevated pH (Castaneda-Ovando, de Lourdes Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009; Timberlake, 1980).

Similar to ACN, the color properties of 3-DXA are dependent on the substitution pattern and pH; however, 3-DXA are much more robust to increasing pH. As pH increases, 3-DXA have reduced propensity to hydration and structural transformation to colorless carbinol pseudobases (B) or chalcones (C) (Figure 2). The predominant reaction in aqueous solutions as pH increases is deprotonation to a quinoidal base (Figure 2). This was illustrated by Awika et. al. (2004) where at

pH 7, luteolinidin and apigeninidin lost 60% of color but the anthocyanidin analogs, cyanidin and peonidin, lost over 80% color at pH 7 (Awika et al., 2004). The favored transformation of 3-DXA from colored flavylium cation to colored quinoidal base as pH increases is a significant advantage for 3-DXA color stability in comparison to ACN.

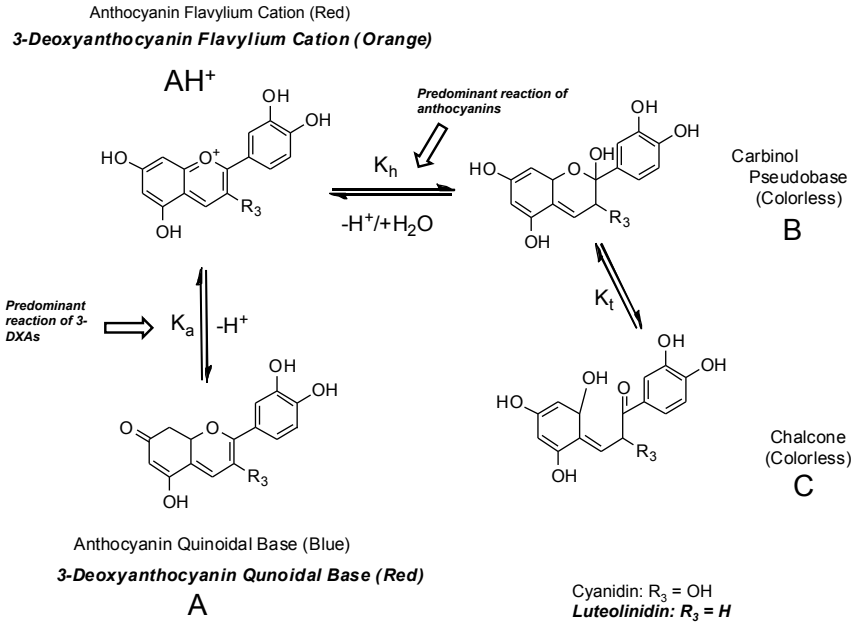


Figure 2 Transformation reactions of cyanidin (anthocyanidin) and luteolinidin (3-deoxanthocyanidin) in aqueous solutions.

2.4. Stability of anthocyanins and 3-deoxyanthocyanins

The stability of ACN is dependent on both intrinsic and extrinsic factors. The intrinsic structure of the ACN itself affects its stability, with glycosylated and acylated ACN having higher structural stability (Giusti & Wrolstad, 2003). In neutral pH conditions, additional hydroxyl or

methoxyl substitution will decrease aglycone stability whereas monoglycosides and diglycoside derivatives are more stable (Castaneda-Ovando et al., 2009).

Extrinsically, stability is affected by the presence of other compounds (e.g. flavonoids or metal ions) that may interact with ACN through copigmentation. Copigmentation can be intermolecular (ACN + non-colored copigment), intramolecular (ACN fold upon itself) , or consist of self-association (ACN + ACN) and typically results in an increased stability and color intensity (hyperchromic shift) of ACN (Trouillas et al., 2016). Copigmentation stabilizes ACN in their flavylium cation form, usually through charge-transfer or pi-pi bond overlap. With pi-pi bond overlap, the copigment sterically hinders nucleophilic attack on the ACN and thus reduces hydration and subsequent formation of colorless chalcones (Trouillas et al., 2016). In addition to increasing color stability of ACN, copigmentation may provide a protective effect during the processing of ACN. For instance, added phenolic acid copiments (such as protocatechuic acid, p-courmaric acid, and ferulic acid) increased the half-life of ACN from blackberry wine residues by 9.5, 12.7, and 16.8 days, respectively, compared to the control with thermal treatment (50°C).

Copigmentation is likely to occur within ACN or 3-DXA extracts because flavonoids and other phenolics that can act as copigments are naturally present in the plant materials used as pigment sources. Of specific interest to the stability of 3-DXA are ferulic acid and proanthocyanidins, both naturally found in sorghum (Dykes, Rooney, Waniska, & Rooney, 2005). Ferulic acid was found to increase stability of non-methoxylated and mono-methoxyaled 3-DXA over 4.5 months of exposure to fluorescent light (Awika, 2008). Proanthocyanins (condensed tannins) occur in some varieties of sorghum and have been shown to cause stabilization of ACN via copigmentation. This is especially true with wine, where tannin-anthocyanin interactions are widely studied and are partially responsible for the stability of grape ACN (Boulton, 2001;

González-Manzano, Dueñas, Rivas-Gonzalo, Escribano-Bailón, & Santos-Buelga, 2009; Salas, Fulcrand, Meudec, & Cheynier, 2003; Singleton & Trousdale, 1992). However, the efficacy of proanthocyanin (PA) monomers, dimers, and trimers may be limited because of their small size compared to the higher polymeric and relatively linear PA present in sorghum. For instance, PA monomers, dimers, and trimers did not extend half-life (thermal degradation) for acai fruit ACN compared to control solutions (Pacheco-Palencia & Talcott, 2010). Sorghum PA are primarily polymerized to units greater than 10; the interactions of PA with other organic compounds has been shown to increase as the degree of polymerization increases (Renard, Watrelot, & Le Bourvellec, 2017). The extraction of 3-DXA from tannin sorghum could cause a natural copigmentation effect and enhance the stability of these pigments.

In contrast to the stabilizing effect of copigmentation, there are ingredients that can reduce the stability of ACN, such as sulfites or ascorbic acid. Both sulfites and ascorbic acid often serve as preservatives but these ingredients can degrade anthocyanins and lead to color loss/food bleaching. The primary reason for color bleaching of ACN is the loss of structural electron resonance. This occurs after nucleophilic attack at a carbon within the heterocyclic ring structure. In the presence of sulfites, nucleophilic attack occurs at C4 of the anthocyanin, forming a colorless bisulfite adduct (Berké, Chèze, Vercauteren, & Deffieux, 1998; Ojwang & Awika, 2010). Color loss of ACN from ascorbic acid in the presence of oxygen can also be explained by two theories: the first is oxidative cleavage of the pyrylium ring, and the second is through a direct condensation reaction (Ojwang & Awika, 2008). Despite the induced degradation of ACN by sulfites and ascorbic acid, 3-DXA have been shown to be relatively resistant to the negative effects of these ingredients. Ojwang & Awika (2008) found that crude sorghum pigment extracts were stable in the presence of ascorbic acid at pH 2 (31% color loss) and showed color intensity increases at both

pH 3.2 and 5.0 over time compared to a red cabbage (anthocyanin) extract that lost 30-85% of its color in the presence of ascorbic acid (Ojwang & Awika, 2008). Similarly, 90% of plum ACN were lost upon addition of ascorbic acid in model juices (Hernández-Herrero & Frutos, 2015). With sulfites, extracted 3-DXA were found to be highly resistant (20% color loss) to bleaching from nucleophilic attack by sulfite and retained over 85% absorbance over 28 days at pH 1, 3, 5, and 7 (Geera et al., 2012). The resistance of 3-DXA to degradation by sulfites and ascorbic acid compared to ACN furthers the potential of 3-DXA as natural food colorants.

Another major limitation of ACN is their susceptibility to degradation by heat. Sadilova et al. (2006) reported that 50% of elderberry pigments degraded after 3 h of heating at 95°C (Sadilova et al., 2006). Dobson et al. (2017) found that pasteurization reduced total anthocyanin content by 15% in black currant juice model (Dobson et al., 2017). Hager et al. (2008) reported significant degradation of ACN with thermal treatment, up to 73% in black raspberry juices (Hager et al., 2008). Fernández-López et al. (2013) reported 63.8, 46.1, and 26.7% color retention for elderberry, red cabbage, and hibiscus extracts after 6 h of incubation at 95°C (Fernández-López et al., 2013). Degradation of ACN typically follows first-order kinetics, and therefore, it is preferable to extract ACN in ways that minimize exposure heat. Yang et al. (2014) investigated the thermal stability of 3-DXA and reported 79-89% color retention after 2 h at 95°C. The color retention after heating indicates greater 3-DXA stability to high temperatures as compared to ACN. It was hypothesized that the relatively greater resistance of 3-DXA to thermal degradation is because 3-DXA have a higher protonation constant (k_a) instead of hydration constant (k_h), which reduces chalcone formation. Chalcone formation is the considered to be the first step of ACN thermal degradation (Sadilova, Carle, & Stintzing, 2007).

2.5. Limitations of 3-deoxyanthocyanins as natural colorants

Although 3-DXA have superior resistance to degradation by heat, sulfites, preservatives, and low acid pH compared to ACN, there are two main limitations for their use as natural food colorants. The first is that 3-DXA are more hydrophobic than ACN, i.e. 3-DXA are sparingly water-soluble compared to ACN. Due to the lack of substitution at C3, there is a relatively large area (C4-C5') of 3-DXA structure that is hydrophobic and thus, when in aqueous media, 3-DXA will rapidly self-associate and eventually precipitate. Additionally, 3-DXA are predominately aglycones, a structure that is inherently less water-soluble than ACN that most commonly are glycosylated. Herrman (2016) showed that at pH 3, 0% of 3-DXA were retained in aqueous solution after 10 weeks. The poor aqueous stability of 3-DXA was not due to structural degradation but instead due to precipitation and sedimentation of the 3-DXA. To facilitate the use of 3-DXA in water-based foods, such as beverages, there is a need to stabilize and prevent precipitation of 3-DXA in aqueous systems.

The second major limitation of the use of 3-DXA as food colorants is their relatively poor extraction efficiency from sorghum. Previous literature has shown that conventional extraction methods with acidified methanol yield the highest concentration of pigments compared to other solvents, accelerated solvent, or enzymatic extraction methods (Awika et al., 2004; Barros et al., 2013; Njongmeta, 2009). Recent work in our lab has shown that microwave-assisted extraction can increase yield of 3-DXA from sorghum grain by 3X (Herrman 2016). There is a need to establish the mechanism for such enhanced extraction efficiency under MAE to better exploit the technology.

2.6. Effect of polysaccharide gums on the stability of anthocyanins and 3-deoxyanthocyanins

Poor aqueous stability leading to pigment precipitation is somewhat unique to 3-DXA. ACN, as they occur predominately in glycosylated forms, are much more water-soluble than 3-DXA and therefore remain in suspension in aqueous solutions to a much greater extent than 3-DXA. However, ACN are highly susceptible to structural degradation and color loss, but this can be mitigated using soluble polysaccharides (gums). ACN and other flavonoids can complex with cell wall polysaccharides (C Le Bourvellec & Renard, 2012; Mazzaracchio, Pifferi, Kindt, Munyaneza, & Barbiroli, 2004; Renard, Baron, Guyot, & Drilleau, 2001; Renard et al., 2017). This phenomenon has led to the application of polysaccharides to foods to help stabilize and preserve ACN during food processing. Gum arabic has been shown to enhance color of ACN and protect ACN from ascorbic acid condensation or from oxidation by hydrogen peroxide during storage (Chung, Rojanasasithara, Mutilangi, & McClements, 2016). The addition of gum arabic was also found to improve color stability, increasing ACN half-life (Guan & Zhong, 2015). Similarly, alginate, carrageenan, and pectin were all found to stabilize ACN in the presence of iron during thermal incubation (Tachibana, Kimura, & Ohno, 2014). Black currant anthocyanin stability was improved with the addition of pectin (Buchweitz, Speth, Kammerer, & Carle, 2013a) as were strawberry ACN (Buchweitz, Speth, Kammerer, & Carle, 2013b). The interaction mechanisms between the gums and ACN were proposed to be hydrogen bonding and hydrophobic interactions.

Food gums not only offer protective effects for ACN, but also can act as both emulsifiers and emulsion stabilizers, which is of importance for the usage of 3-DXA. Emulsifiers are surface active and reduce the interfacial tension between two immiscible liquids. Emulsion stabilizers act as viscosity modifiers and reduce the movement of particles within the continuous phase (Schmidt, Koch, et al., 2015). Polysaccharides with both hydrophobic and hydrophilic moieties function both

as emulsifiers and emulsion stabilizers. Examples include gum arabic, pectin, and octenyl succinic anhydride (OSA) starch. The surface-active groups of polysaccharides are either hydrophilic sugar moieties (like amylopectin in OSA starch or galacturonic acid of pectin) and the hydrophobic proteins (gum arabic and pectin) or methoxyl groups (pectin). The emulsifying properties of pectin are attributed to both the presence of hydroxyproline rich proteins and methoxylated carboxyl groups on the galacturonic acid residues (Mirhosseini et al., 2008).

Previous work from our lab has shown that 3-DXA self-association and precipitation can be reduced using polysaccharides, specifically gum arabic and sodium alginate. Gum arabic and sodium alginate (1.0 g/L) stabilized 3-DXA (prevented 3-DXA precipitation) in aqueous solutions at both pH 3 and 5 for 10 weeks (Herrman 2016). The 3-DXA stabilization was found to be dependent on pH, gum structure, and 3-DXA profile. Gum arabic more effectively stabilized extracts high in apigeninidin than those high in luteolinidin. Unlike with gum arabic, the stabilizing effect of sodium alginate was dependent on pH instead of 3-DXA profile. Alginate was more effective at pH 5 than pH 3. The difference in gum effectiveness suggested different stabilization mechanisms. However, the exact mechanisms contributing to improved aqueous stability were not determined (Herrman, 2016). Thus, there is a need to elucidate the mechanism contributing to aqueous stability of 3-DXA to be able to successfully stabilize 3-DXA in food products like beverages.

2.7. Extraction of anthocyanins and 3-deoxyanthocyanins

Extraction of ACN, and other flavonoids, from fruits and vegetables is often as simple as soaking the plant material in a solvent (usually water) after grinding. ACN are polar molecules and are therefore soluble in water and other common organic solvents such as methanol, ethanol,

or acetone. Acidified solvents are most commonly used because the low pH stabilizes the flavylium cation (Castaneda-Ovando et al., 2009). Although relatively easy to extract, extraction efficiency of ACN has been improved with more complex extraction methods including enzymatic extraction, supercritical fluid extraction, ultrasound-assisted extraction, pressurized liquid extraction, or microwave-assisted extraction (Silva, Costa, Calhau, Morais, & Pintado, 2017). Increasing the temperature of extraction is the most common way to improve extraction efficiency but ACN are notoriously heat-labile and thus there is an upper limit to the temperature at which ACN can be extracted without concurrent degradation. For instance, extraction at 30-35°C resulted in the highest yield of ACN from black currants compared to higher temperatures (Cacace & Mazza, 2003).

Extraction of 3-DXA from sorghum has not been as widely studied as extract ion of ACN from other fruits, vegetables, and grains. The most common cited extraction method occurs in ambient conditions with acidified methanol for approximately 2h. Other tested solvents have not been as effective as acidified methanol for the extraction of 3-DXA from sorghum grain. Njongmeta (2009) extensively investigated the extractability of 3-DXA with aqueous ethanol acidified by citric, acetic, or tartaric acids but extracted 50% less 3-DXA from sorghum bran than acidified methanol (Njongmeta, 2009). Kayodé et. al. (2012) optimized extraction of 3-DXA from sorghum leaf sheath and found that the addition of HCl to aqueous ethanol resulted in almost 5-fold increase in both apigeninidin and luteolinidin yields compared to aqueous ethanol extraction without HCl (Kayodé, Bara, Dalodé-Vieira, Linnemann, & Nout, 2012). Extraction of 3-DXA with acetone is not considered an effective method as it was reported to lead to structural modification of apigeninidin and luteolinidin (Awika et al., 2004; Awika, Rooney, & Waniska, 2005; Kayodé et al., 2012). Enzymatic extraction (with cellulase, protease, hemicellulose, beta glucanase, and

xylanase) produced a highly-colored gel matrix from which phenolic compounds could be extracted but 3-DXA were not able to be extracted nor quantified (Njongmeta, 2009).

Barros et al. (2013) tested the effect of accelerated solvent extraction (ASE) on the yields of phenolic compounds from sorghum brans. Accelerated solvent extraction utilizes high temperatures and pressures above 1000 psi causing solvent superheating. The high temperature and pressure increased solvent diffusion rates, disrupting strong solute-matrix interactions, and decreasing solvent viscosity to improve matrix penetration by the solvent and thus can improve extraction yields. Using 50% ethanol in water, ASE at 120°C and 150°C extracted similar amounts of phenols and 12% more antioxidants from sorghum compared to acidified methanol. However, for 3-DXA, all combinations of temperatures and pressures and differing solvents were not effective at increasing extraction efficiency. Conventional acidified methanol extraction yielded 3730 μg/g 3-DXA from black sorghum bran, whereas the highest ASE yield was 820 μg/g with 70% ethanol in water (Barros et al., 2013). Although ASE wan not efficient compared to the conventional acidified methanol extraction, an important trend was identified in extraction of 3-DXA from sorghum: increased temperature corresponded with higher 3-DXA yield. As temperature increased from 120°C to 150°C using 50% and 70% ethanol in water, the yield of 3-DXA increased almost 25%. This relationship between temperature and yield suggests 3-DXA can withstand high temperature extraction (unlike ACN).

2.8. Microwave-assisted extraction of anthocyanins and 3-deoxyanthocyanins

Microwave-assisted extraction (MAE) has been found to effectively increase the extraction efficiency of ACN from fruits, vegetables, and grains because it can greatly reduce extraction inputs (e.g. time or solvent requirements). During MAE, microwave energy is transformed into

heat within both the extraction solvent and biological matrix via dipole rotation and ionic conduction. Both of these movements occur instantaneously, creating friction that rapidly increases temperatures and pressure within the plant tissue, causing further matrix permeability and eventual cell rupture that facilitates diffusion of compounds into the solvent, increasing extractability (Routray & Orsat, 2012). This cell damage can facilitate higher extraction yields and diffusion of ACN into the extraction solvent. In this regard, Sun et. al. (2007) was able to extract 39.06 mg/100 g ACN from fruit in 12 minutes with MAE compared to 34.15 mg/100 g after 60 min of conventional extraction (Sun, Liao, Wang, Hu, & Chen, 2007). Zou et. al. found similar results. MAE for 132 s resulted in 54.72 mg/g ACN from mulberry compared to 44.82 mg/g after 60 min of conventional extraction (Zou et al., 2012). MAE has been shown to increase extraction efficiency by reducing extraction time, the overall impact on anthocyanin yield is mixed, primarily due to poor structural stability of ACN to MAE (Zhao, Li, et al., 2013).

MAE of ACN from grain products has also not seen much success. In grains, Abdel-Aal et. al. (2014) used MAE for ACN from blue wheat, purple corn, and black rice. They reported lower total ACN yield at all temperatures, wattages, and times compared to CE, while finding significant differences in the ACN composition between extraction methods (Abdel-Aal, Akhtar, Rabalski, & Bryan, 2014). Overall, ACN readily degrade with heat treatments, and it is likely that degradation of ACN contributed to the decreased extraction efficiency with MAE of grains.

Herrman (2016) previously investigated effect of microwave energy on the extractability of pigments from sorghum (3-DXA), black cowpea (ACN), and blue corn (ACN). From sorghum grain, the application of microwave energy resulted in dramatically increased yields of 3-DXA, 2.46 mg/g compared to the CE that yielded 0.88 mg/g 3-DXA. The 3-DXA profile after MAE was similar to that of the CE, indicating that 3-DXA were stable to microwave energy. However, the

profile also showed the unexpected presence of anthocyanidins. Pigment profile after MAE of black cowpea and blue corn showed extensive ACN hydrolysis of acyl esters and glycosides. As a result, the yield of ACN from corn was not increased whereas the yield of ACN from cowpea increased 3-fold for extraction under 10 min, after which yield decreased as time increased. MAE is a promising technology for improved extraction efficiency (higher 3-DXA yield in shorter time compared to conventional extraction) but the impact of microwave energy on 3-DXA structure and copigments needs to be established.

2.9. Practical application of 3-deoxyanthocyanins

Food colors are used to attenuate color loss during food processing, enhance and correct the original color of foods, standardize raw materials, and give color to an otherwise colorless food (Sigurdson, Robbins, Collins, & Giusti, 2016). The usage of 3-DXA as natural food colorant is currently limited compared to ACN. To further the commercial usage of 3-DXA as a natural food color, the stability of 3-DXA in a complex food matrix should be assessed. It is difficult to predict the stability of 3-DXA in complex food matrix based on the evidence in isolated systems because stability can be compromised (or enhanced) in the presence of other ingredients or based on the processing method. There is potential for synergistic effects from ingredients and processing, such as enhanced encapsulation of 3-DXA by hydrocolloids during heating that would potentially increase the stability of 3-DXA (and prevent color loss thus extending shelf life). Sweeteners can induce degradation of ACN. When sucrose (table sugar) or other sweeteners are heated, degradation products, like furfurals, can induce degradation of ACN (Türkyılmaz, Hamzaoğlu, & Özkan, 2019). The complexity of food processing and the variability in pigment stability is illustrated by the use of jambolan ACN in a beverage model (Sari, Wijaya, Sajuthi, & Supratman,

2012). ACN from jambolan were more stable in a beverage model with added phenolic acid copigments, at both room and refrigeration temperatures, and with exposure to white fluorescent light, but copigmentation did not confer a protective effect during exposure to high temperatures (80°C and 90°C) (Sari et al., 2012). Results may be further muddled with the addition of polysaccharides needed to stabilize 3-DXA in solution, as polysaccharides such as gum arabic may behave differently after pasteurization due to denaturation and unfolding of the protein moiety (Buffo, Reineccius, & Oehlert, 2001). These unknowns can only be addressed by investigating the use of 3-DXA as a color additive in a food product.

3. STABILITY OF 3-DEOXYANTHOCYANIN PIGMENT STRUCTURE RELATIVE TO ANTHOCYANINS FROM GRAINS UNDER MICROWAVE ASSISTED EXTRACTION*

3.1. Introduction

Consumer desire for "natural" and "clean label" ingredients has spurred increased interest in plant-derived food colorants. Among these, anthocyanins are the most popular water-soluble colorants from plants currently used in foods. Anthocyanins are structurally diverse and thus provide a wide array of color properties for different food applications. Furthermore, dietary anthocyanins can benefit chronic disease prevention. For example, a recent meta-analysis of 32 randomized controlled trials (1491 participants) that evaluated the effects of anthocyanins on glycemic control and lipid metabolism demonstrated that anthocyanin intake improved glycemic response (reduced both fasting and postprandial glucose, as well as glycated hemoglobin), and lipid profile (reduced both total cholesterol and LDL) (Yang et al., 2017). However, a key challenge to anthocyanin use is their relatively low stability to food processing conditions (Chung, Rojanasasithara, Mutilangi, & McClements, 2017).

3-Deoxyanthocyanins (3-DXA) are anthocyanin analogs that are unsubstituted at C-3 of the heterocyclic ring (Figure 3). The 3-DXA are rare in nature and are most abundantly found in sorghum (*Sorghum bicolor*), which accumulates the 3-DXA are the primary secondary pigments in different tissues, including grain (Awika, Rooney, et al., 2005), sheath (Geera et al., 2012; Kayodé et al., 2011); and leaves (Petti et al., 2014). The lack of substitution at C-3 confers the 3-DXA greater resistance to nucleophilic attack of the flavylium cation and subsequent structural

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degradation and color loss compared to anthocyanins (Awika, 2008; Mazza & Brouillard, 1987a). Because of this, 3- DXA maintain color stability over a wider pH range than anthocyanins (Awika et al., 2004), have superior resistance to heat (Yang et al., 2014), and are stable in the presence of common food additives like ascorbic acid and sulfites (Ojwang & Awika, 2008, 2010). For this reason, the 3-DXA are increasingly of interest as natural colorants and are finding applications in foods (Girard & Awika, 2018).

The 3-DXA structure also confers them unique photochromatic properties; luteolinidin and apigeninidin were recently shown to reversibly transform into colorless trans-chalcones under light-shielded conditions, and re-generate under UV-irradiation back to the colored species (flavylium cation and quinoidal bases) via cis-chalcone tautomerization in aqueous organic system (Yagishita, Mihara, Kohno, & Shibata, 2016). This property is not common in other natural pigments and has potential diverse applications beyond food, including cosmetics, and industrial uses such as molecular switching devices (e.g., to control enzyme activity), data storage, and solar energy storage (Xiong, Zhang, Warner, & Fang, 2019). Furthermore, the 3-DXA show enhanced biological activity relative to anthocyanin analogs (Shih et al., 2007; Yang, Browning, & Awika, 2009); the compounds are currently under development for medical use against ocular diseases (Fontaine, Lafont, Sahel, & Veillet, 2018).

At present the most economically viable source of the 3-DXA pigments is black sorghum grain, which accumulates high levels of the compounds localized in the bran (Awika, McDonough, & Rooney, 2005). Because the bran is a byproducts of sorghum milling, the compounds could be readily valorized from a food processing waste. However, a major challenge is poor extractability of the compounds from sorghum bran tissue. For example, use of subcritical fluid or elevated temperature (Barros et al., 2013) proved ineffective at improving extractability of 3-DXA from

sorghum relative to conventional acidified methanol method. As grasses (monocots), cereal grain cell walls are highly cross-linked via ferulate ester bonds (de O. Buanafina, 2009), making it relatively difficult to disrupt the cell wall and release the 3-DXA from cell matrix. Methods that improve extraction efficiency of 3-DXA could tremendously enhance their potential commercial applications.

Microwave-assisted extraction (MAE) of anthocyanins and related phenolic compounds has been the subject of numerous recent studies. Microwave energy induces instantaneous dipole rotation and ionic conduction leading to rapid heating and pressure buildup in the plant tissue, increasing matrix permeability and eventual cell rupture (Routray & Orsat, 2012), However, MAE is not very effective at improving extractability of anthocyanin pigments from plant tissue (Abdel-Aal et al., 2014), primarily due to the structural instability of anthocyanins to microwave energy. Nucleophilic attack leading to hydration at C-2 is believed to be a major mechanism of MAE-induced anthocyanin degradation (Zhao, Luo, et al., 2013) (Zhao et al., 2013). It is not known whether the relative resistance of the 3-DXA heterocyclic ring to nucleophilic attack (Awika, 2008; Mazza & Brouillard, 1987a) can protect their structure during MAE. Because of the structural stability of the 3-DXA relative to anthocyanin analogs, we hypothesize that MAE will uniquely improve extraction efficiency and yield of the 3- DXA from sorghum without structural degradation of the compounds. This study aimed to investigated the effect of microwave energy on structural stability and extractability of 3-DXA from sorghum grain in relation to anthocyanin analogs from representative monocot and dicot grains.

3.2. Materials and methods

3.2.1. Plant materials

A non-tannin black sorghum (Tx430) grown in College Station, TX in 2017 was used as the source of 3-DXA; this sorghum variety is a known rich source of these compounds (Awika, McDonough, et al., 2005). To compare the relative effect of microwave energy on the 3- DXA vs anthocyanin analogs, a black cowpea (TK1092-S), and blue maize (RY-288) were also used. The maize is a source of acylated anthocyanins (Collison, Yang, Dykes, Murray, & Awika, 2015) and has a cell wall structure more similar to that of sorghum (both are monocots). Cowpea anthocyanins are primarily non-acylated (Ojwang, Dykes, & Awika, 2012), and the cell wall structure of cowpea (a dicot) differs from that of monocots (Vogel, 2008). The whole grains were ground to pass through a 1 mm mesh using a UDY mill (model 3010-030, UDY Corporation, Fort Collins, CO) and stored at –20 °C before extraction.

3.2.2. Chemicals and reagents

3-Deoxyanthocyanidin standards (apigeninidin and luteolinidin chloride, > 97% purity) used to model MAE stability of the 3-DXA were purchased from AlsaChim (Strasbourg, France). Cyanidin and peonidin chloride was purchased from Extrasynthese (Genay Cedex, France). All chemicals and reagents were of analytical grade.

3.2.3. Extraction of pigments

3.2.3.1. Conventional extraction

For conventional extraction (CE), ground samples were extracted in 1% HCl in methanol (v/v%) at room temperature for 2 h on a shaker (VWR Shaker model 3500). Samples were

extracted in a 10:1 solvent to sample ratio. After extraction, samples were centrifuged for 10 min and the supernatant was collected and stored at −20 °C until analyzed.

3.2.3.2. Microwave-assisted extraction (MAE)

The ground samples were subjected to microwave energy for 1–30 min at 300, 600, or 1200 W in a Microwave Accelerated Reaction System (MARS) 5 Xpress (CEM Corporation, Matthews, NC). Samples were extracted in a 10:1 solvent to sample ratio. The maximum temperature for extraction was set to 100 °C. After extraction, samples were centrifuged, and the supernatant was collected and stored at −20 °C until analyzed. To determine the effect of microwave energy on the structure of 3-DXA, apigeninidin and luteolinidin standards (1.75 uM), along with the luteolinidin analog, cyanidin, were similarly subjected to microwave energy (1200 W) for up to 30 min with maximum temperatures of 100 °C.

3.2.4. Effect of MAE on pigment yield and extractable phenol content

Total anthocyanin content (TAC) was quantified using a UV–Vis spectrophotometer (Shimadzu 2450, Shimadzu Scientific Instruments North America, Colombia, MD). Samples were diluted to an absorbance of 1 ± 0.05 at λmax and scanned from 400 to 700 nm. Subtraction of the absorbance at 700 nm eliminated the effect of any natural haze in the sample. Pigments were quantified using Equation 1 (Fuleki & Francis, 1968) and converted to mg/g sample:

Equation 1

$$\textit{Total anthocyanin content} = (abs * MW * DF)/(\varepsilon * g)$$

Abs is the absorbance at λ_{max} minus absorbance at 700 nm, and *MW* is molecular weight of luteolinidin or cyanidin, for 3-DXA or anthocyanin quantification, respectively. *DF* is the dilution factor, ε is the molar extinction coefficient of luteolinidin or cyanidin, and *g* is the extracted sample weight. Browning index was determined from diluted extracts as $(ABS_{420nm} - ABS_{700nm})/(ABS_{\lambda max} - ABS_{700nm})$ (Reyes & Cisneros-Zevallos, 2007). Extractable phenols were determined by the modified Folin-Ciocalteu as previously described (Barros et al., 2013).

3.2.5. Effect of MAE on pigment and copigments structure and profile

To determine effect of MAE on the 3-DXA profile or anthocyanin profile, along with co-extracted phenolics, a Waters ACQUITY UPLCTQD-MS system (Waters Corp., Milford, MA) equipped with a photodiode array eλ (PDA) detector, and ESI source was used following a previously described method (Ojwang et al., 2012; Yang et al., 2014) with minor changes. A Kinetex C18 column (100 × 2.10 mm, 2.6 μm) (Phenomenex, Torrance, CA) thermostated at 40°C was used for separation; flow rate was 0.4 mL/min. For pigment analysis, the mobile phases consisted of 1% formic acid in water (A), 1% formic acid in acetonitrile (B). Solvent B gradient was 0–2 min 5%, 2–8 min 20%, 8–15 min 70%, 15–20 min 70%, 20–23 min 5%, and 23–27 min 5%. For copigment profile, solvent A was 0.05% formic acid in water while solvent B was 0.05% formic acid in acetonitrile. The monitoring wavelength for 3-DXA was at 480 nm and 520 nm for anthocyanins; copigments were monitored at 280 nm, 325 nm, and 360 nm. MS data was acquired in positive mode for pigments, and in negative mode for copigments. Mass parameters were optimized, for both positive and negative ionization, as follows: capillary voltage 3.5 kV, cone voltage 40 V. Compounds were identified as described by Yang et al. (2014).

3.2.6. Statistical analysis

Extractions and analyses were replicated at least twice. Analysis of means was completed using JMP Pro (Version 12.0.1, SAS Institute, Inc., Cary, NC) with one-way analysis of variance and Tukey's HSD used to compare treatment means. Differences in treatments were determined at 5% significance level.

3.3. Results and discussion

3.3.1. Effect of microwave energy on 3-deoxyanthocyanin pigment extraction from sorghum

Microwave conditions resulted in significantly improved extraction efficiency (yield and speed) of 3-DXA from sorghum compared to conventional extraction (Figure 3A). For example, within 1 min of MAE, 3- DXA yield (1570–2230 μg/g) was similar or higher than the 2 h conventional extraction (1520 μg/g). In general, pigment yield increased with increasing extraction time at all MAE power levels used, suggesting stability of the 3-DXA pigments to degradation under prolonged microwave energy exposure. However, in contrast to the 300 and 600 W power treatments, 3-DXA yield plateaued within 5 min at 1200 W power treatment (Figure 3A). This suggests that the maximum impact on grain tissue to release pigments at 1200 W was relatively rapid, but may also suggest that prolonged exposure to MAE at this power setting may have led to partial degradation of some 3-DXA to negate any additional increase in pigment yield with extraction time. The maximum 3-DXA yield of 3100 μg/g was obtained at 600 W/ 30 min, although this was not significantly different from yield at 600 W/10 min or 1200 W/5 min (2870 μg/g) (Figure 3A). Thus, MAE doubled the 3-DXA pigment yield from sorghum, while increasing extraction speed by approx. 12–24X. Compared to reports for anthocyanins from various grain matrices (Abdel-Aal et al., 2014), the MAE data for sorghum pigments is very promising and

suggests high stability of the 3- DXA to microwave induced pigment degradation. For example, under optimized MAE conditions, Liazid, Guerrero, Cantos, Palma, & Barroso, (2011) obtained a maximum yield advantage of 17.6% for anthocyanins form grape skin compared to conventional extraction, whereas Abdel-Aal et al. (2014) reported mostly a decline in anthocyanin yield from different pigmented grains under MAE. Because our previous work with accelerated solvent system (high temperature and pressure) did not significantly increase 3-DXA yield (Barros et al., 2013), the MAE data indicates that the large increase in 3-DXA yield is likely induced by microwave irradiation and not merely increase in temperature of extraction. The fact that pigment yield from sorghum was doubled from what has been previously possible using MAE suggests this technology could provide a viable path to broader commercialization of the unique sorghum 3-DXA for food and other uses. We anticipate that the process could be optimized to improve efficiency even further by modifying solvent: sample ratio, pH, and particle size of plant tissue, among others.

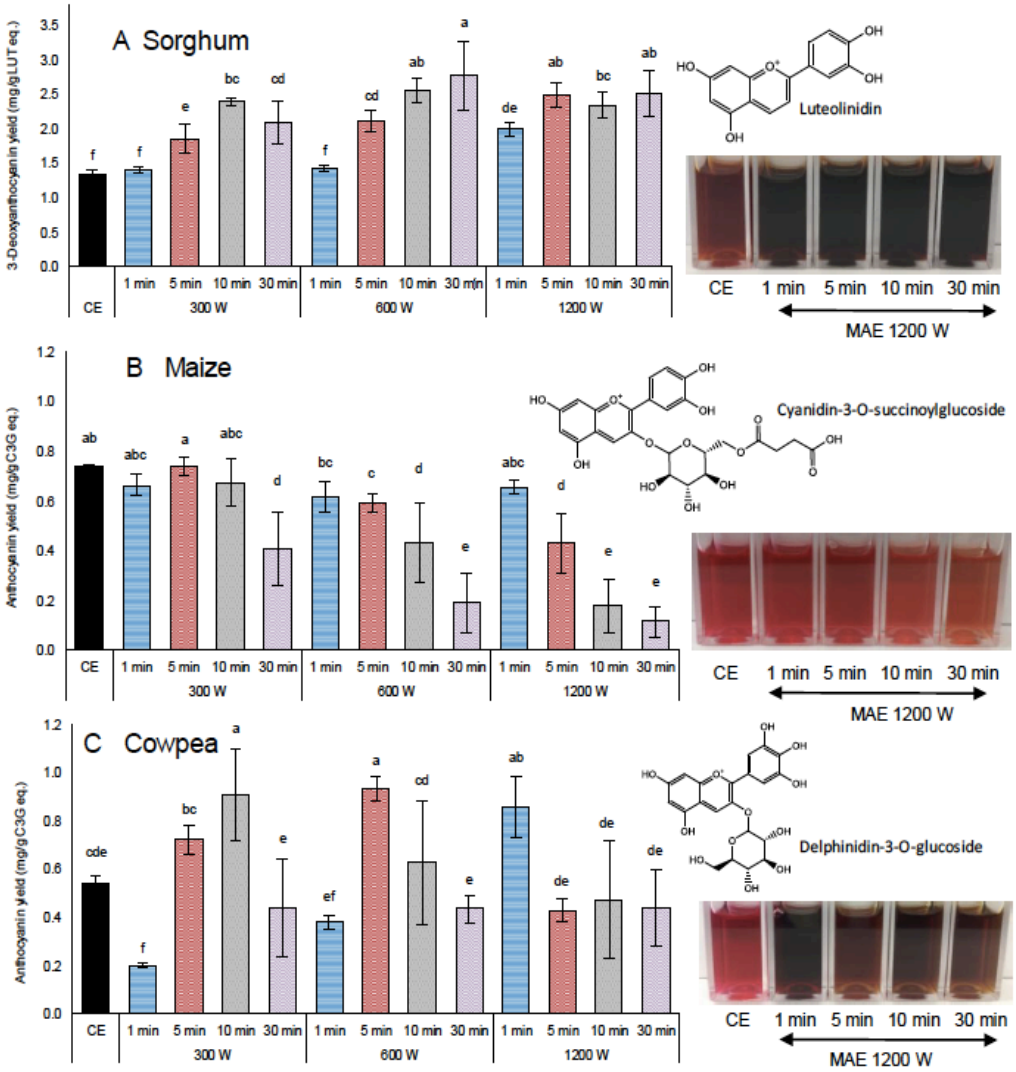


Figure 3 Effect of microwave assisted extraction (MAE) conditions on pigment yield from sorghum vs maize and cowpea, and representative structures of the major pigment compounds identified in each grain. Representative images of the pigments obtained at 1200 W power setting also shown. Maximum temperature during microwave-assisted extraction was 100°C. Extracting solvent was 1% HCl in methanol. CE = conventional extraction (2 h/24°C). Error bars illustrate ± SD. Different letters within each grain bar graph indicate significant differences (Tukey's HSD, $p < 0.05$).

3.3.2. Effect of microwave energy on anthocyanin extraction from maize and cowpea

To establish relative advantage of MAE in extracting 3-DXA from sorghum grain compared to other anthocyanins from grains, we compared the behavior and yield of anthocyanin pigments from maize and cowpea under similar conditions used for 3-DXA. Markedly different trends were observed for maize vs cowpea pigment yields (Figure 3B, C). For maize, MAE did not increase pigment yield compared to conventional extraction under any MAE condition used (Figure 3B). Under the milder MAE conditions (1–10 min at 300 W and 1 min at 600 & 1200 W) maize pigment yields were similar to conventional extraction method, whereas the rest of MAE conditions significantly decreased pigment yield (Figure 3B). Generally, the more severe the MAE conditions, the higher the decrease in pigment yield observed for maize. For example, at 1200 W, pigment yield from maize decreased from 710 μg/g at 1 min to 125 μg/g at 30 min. When comparing equivalent extraction time of 10 min, pigment yield from maize was 730, 470, and 190 μg/g at 300, 600, and 1200 W, respectively (Figure 3B). This data confirms that anthocyanins from maize are unstable to MAE, as was previously reported for a purple maize and other cereal grains (Abdel-Aal et al., 2014).

In contrast to maize, for cowpea, relatively mild MAE conditions (300 W/5–10 min, 600 W/5 min, and 1200 W/1 min) significantly increased pigment yield compared to conventional extraction (Figure 3C). For example, 300 W/10 min increased cowpea pigment yield by approx. 68% relative to conventional extraction (from 590 to 990 μg/g) (Figure 3C). This was a somewhat surprising finding, since such high yield advantage due to MAE has not been reported for anthocyanins. A plausible explanation is that because the cowpea variety used in this study is known to contain proanthocyanidins (Ojwang, Yang, Dykes, & Awika, 2013), the microwave induced heating likely resulted in partial depolymerization of the proanthocyanidins and their

oxidation to anthocyanidins, catalyzed by the acidified alcohol used for extraction (Porter, Hrstich, & Chan, 1985; Ravisankar, Abegaz, & Awika, 2018). If this is the case, the formed anthocyanidin aglycones would increase overall pigment yield, but would have limited food applications due to their known poor stability relative to naturally occurring glycosylated anthocyanins.

In fact, the cowpea pigments yield significantly decreased at all power settings when subjected to prolonged extraction times (30 min at 300 W, ≥10 min at 600 W, and ≥5 min at 1200 W) (Figure 3C). However, unlike maize pigments that consistently declined in yield with increased extraction time under MAE, the cowpea pigments appeared to stabilize after initial decline as can be seen for 1200 W treatment (Figure 3C). Visual observation, however, showed that the cowpea pigments became noticeably more brown with prolonged MAE treatment (Figure 3C); this likely confounded the absorbance readings used to estimate pigment yield (Lao & Giusti, 2016).

The browning index (BI) data confirmed the visual appearance for cowpea pigments under MAE (Table 1). The cowpea extract had the most severe browning; for example, at 1200 W, its BI increased 3X within 1 min treatment vs conventional extraction (0.86 vs 0.29), and were 8 – 12X higher at 5–30 min treatments (2.3–3.4). By comparison, at 1200 W, the BI for maize remained relatively low at MAE treatments up to 10 min (0.3–0.7 vs 0.3 for conventional extraction), but was comparably high at 30 min treatments (1.0–3.5) (Table 1). On the other hand, the BI for sorghum extracts remained relatively low regardless of MAE conditions, ranging between 0.4 and 0.7, vs 0.4 for CE. This indicates that MAE of sorghum did not lead to reactions that induce significant browning in the 3-DXA extracts. The observed pigment yields of maize and cowpea extracts thus likely overestimate anthocyanin content to a greater extent than the sorghum pigments because of the higher presence of brown colored compounds formed during MAE. Pigment browning can be attributed to the degradation or polymerization of anthocyanins or tannins into

melanoidin-type compounds (Jiang et al., 2019). The unstable nature of anthocyanins to MAE likely results in easier formation of reactive intermediaries that participate in the browning reactions.

Table 1 Effect of microwave-assisted extraction on extractable phenols and browning index of sorghum, maize, and cowpea.

Microwave power		300W		600W		1200W	
Sample	**Extraction Time (min)**	**Extractable phenols (mg/g GAE)**	**Browning index**	**Extractable phenols (mg/g GAE)**	**Browning index**	**Extractable phenols (mg/g GAE)**	**Browning index**
Sorghum	**CE**	**6.67 ± 0.87**	**0.39**				
	1	$5.10 \pm 1.05^{c*}$	0.40	$5.46 \pm 0.34^{b*}$	0.40	$9.75 \pm 1.43^{c*}$	0.47
	5	6.94 ± 0.48^{b}	0.42	$8.30 \pm 0.92^{a*}$	0.51	$12.98 \pm 1.99^{b*}$	0.60
	10	$8.63 \pm 0.34^{a*}$	0.52	$8.32 \pm 0.71^{a*}$	0.59	$12.63 \pm 2.03^{b*}$	0.61
	30	$8.73 \pm 0.29^{a*}$	0.59	$8.19 \pm 1.04^{a*}$	0.65	$15.03 \pm 3.04^{a*}$	0.64
Maize	**CE**	**3.86 ± 0.37**	**0.25**				
	1	3.67 ± 1.09^{c}	0.28	$3.26 \pm 0.25^{c*}$	0.30	$6.89 \pm 0.74^{ab*}$	0.49
	5	$4.58 \pm 0.49^{b*}$	0.34	$5.89 \pm 0.31^{a*}$	0.49	$7.30 \pm 1.03^{a*}$	0.49
	10	$6.71 \pm 0.43^{a*}$	0.47	$5.70 \pm 0.4d^{ab*}$	0.71	$6.21 \pm 1.60^{b*}$	0.65
	30	$6.60 \pm 0.48^{a*}$	0.98	$5.41 \pm 0.71^{b*}$	1.52	$7.64 \pm 1.25^{a*}$	3.48
Cowpea	**CE**	**5.12 ± 0.69**	**0.29**				
	1	$3.11 \pm 0.98^{b*}$	0.29	$4.00 \pm 0.37^{b*}$	0.29	$8.18 \pm 1.44^{c*}$	0.86
	5	$5.99 \pm 0.52^{a*}$	0.34	$7.39 \pm 0.50^{a*}$	0.52	$11.07 \pm 2.36^{b*}$	2.28
	10	$6.53 \pm 0.30^{a*}$	0.47	$7.45 \pm 0.74^{a*}$	0.72	$12.02 \pm 3.67^{ab*}$	2.48
	30	$6.24 \pm 0.95^{a*}$	1.06	$7.77 \pm 0.61^{a*}$	1.49	$13.37 \pm 3.37^{a*}$	3.41

Microwave extraction maximum temperature was set to 100 °C; CE, conventional extraction (2 h/24 °C); acidified methanol (1% HCl) was used for all extractions. GAE, gallic acid equivalents. Browning index was calculated as Abs_{420}/Abs_{480} (sorghum extracts) or Abs_{420}/Abs_{530} (maize and cowpea extracts). Data are expressed as mean ± standard deviation. Values with different letters within a row are significantly different ($p < 0.05$).

3.3.3. Effect of microwave energy on sorghum 3-deoxyanthocyanin profile and structure

As expected, all major pigments identified in sorghum were luteolinidin and apigeninidin derivatives, with the 3-DXA aglycones dominating (Figure 4A, Table 2). Compared to conventional extraction, the profile of the sorghum 3-DXA was largely preserved after MAE (Figure 4A, Table 3). The relative proportions of the four major sorghum pigment compounds, luteolinidin, apigeninidin, and their 7-O-methylderivatives remained virtually the same during MAE, even after 30 min at 1200 W at 100 °C (not shown) indicating overall stability of these compounds to microwave irradiation. The most noticeable effect of MAE on 3-DXA compounds was the significant decrease in the 3-DXA glycosides beyond 1 min MAE treatment (Figure 4A, Table 2). This suggests that the MAE readily hydrolyzed the glycosidic bond of the 3-DXA to release the aglycones.

However, unequivocally establishing relative stability of the 3-DXA to MAE during the extraction process based on above evidence is not possible since any microwave induced degradation could be partially masked by enhanced extractability. For this reason, we exposed pure forms of the dominant 3-DXA aglycone compounds, apigeninidin and luteolinidin, to microwave energy using similar conditions used for sorghum extraction. Both apigeninidin and luteolinidin were remarkably stable to microwave energy under the most severe conditions tested (1200 W for 30 min, 100 °C maximum temperature) (Figure 4B). On the other hand, when the luteolinidin analog, cyanidin, was tested, it was completely degraded within 5 min at 1200 W (not shown). Thus, the lack of substitution at C-3 of the 3-DXA molecules appears to considerably enhance their stability to microwave irradiation.

Quantitative data revealed that within 1 min of MAE at 1200 W, the yield of virtually all the detected 3-DXA compounds were similar to or higher than the 2 h conventional extraction

(Table 3). This indicates that MAE can provide an efficient means to rapidly extract the compounds from sorghum bran tissue. After 5 min MAE at 1200 W, the content of all 3-DXA glycosides significantly decreased relative to both 1 min MAE and conventional extraction. The decrease in the 3-DXA glycosides at 5 min relative to 1 min MAE was 40–85% (Table 2). On the other hand, all the 3-DXA aglycones significantly increased after 5 min vs 1 min MAE at 1200 W (by 14–54%), and were also on average 33% higher than conventional extraction. Because the glycosides are a minor component of sorghum 3-DXA (Awika et al., 2004), making up only about 8% of the pigments in the present study (Table 3, Figure 4A), the loss of 3-DXA glycosides likely did not significantly affect overall sorghum pigment yield under MAE. Thus, the increase in sorghum pigment yield under MAE observed relative to maize and cowpea (Figure 3) can be largely attributed to the stability of the 3-DXA aglycones to microwave energy.

The stability of 3-DXA aglycones to microwave energy is likely contributed by their thermal stability (Yang et al., 2014) and relative resistance to nucleophilic attack compared to anthocyanins (Awika, 2008; Mazza & Brouillard, 1987a). The lack of substitution at C-3 of the 3-DXA heterocyclic ring reduces their hydration constant (R Brouillard, Iacobucci, & Sweeny, 1982), which disfavors hydrolytic attack at C-2 (Awika, 2008). The consequence is that the 3-DXA are much more resistant to chalcone formation, and heterocyclic ring fission and subsequent structural degradation at elevated temperatures compared to anthocyanins (Yang et al., 2014). Another mechanism believed to contribute to microwave-induced anthocyanin degradation is the decomposition of water molecules, leading to formation of hydrogen peroxide and subsequent nucleophilic attack on anthocyanins (Zhao, Luo, et al., 2013). The increased resistance of 3-DXA C-ring to nucleophilic attack would therefore enhance their microwave stability. Furthermore, because microwave heating depends on ionic conduction and dipole rotation that acts on polar

molecules to a greater extent than lower-polarity molecules, the reduced polarity of the 3-DXA relative to anthocyanins may further contribute to their better stability to MAE vs anthocyanins. The fact that the sorghum 3-DXA molecules are structurally unchanged by microwave energy indicates that MAE would be a viable technology to improve isolation of these compounds from plant tissue for targeted medical and industrial uses.

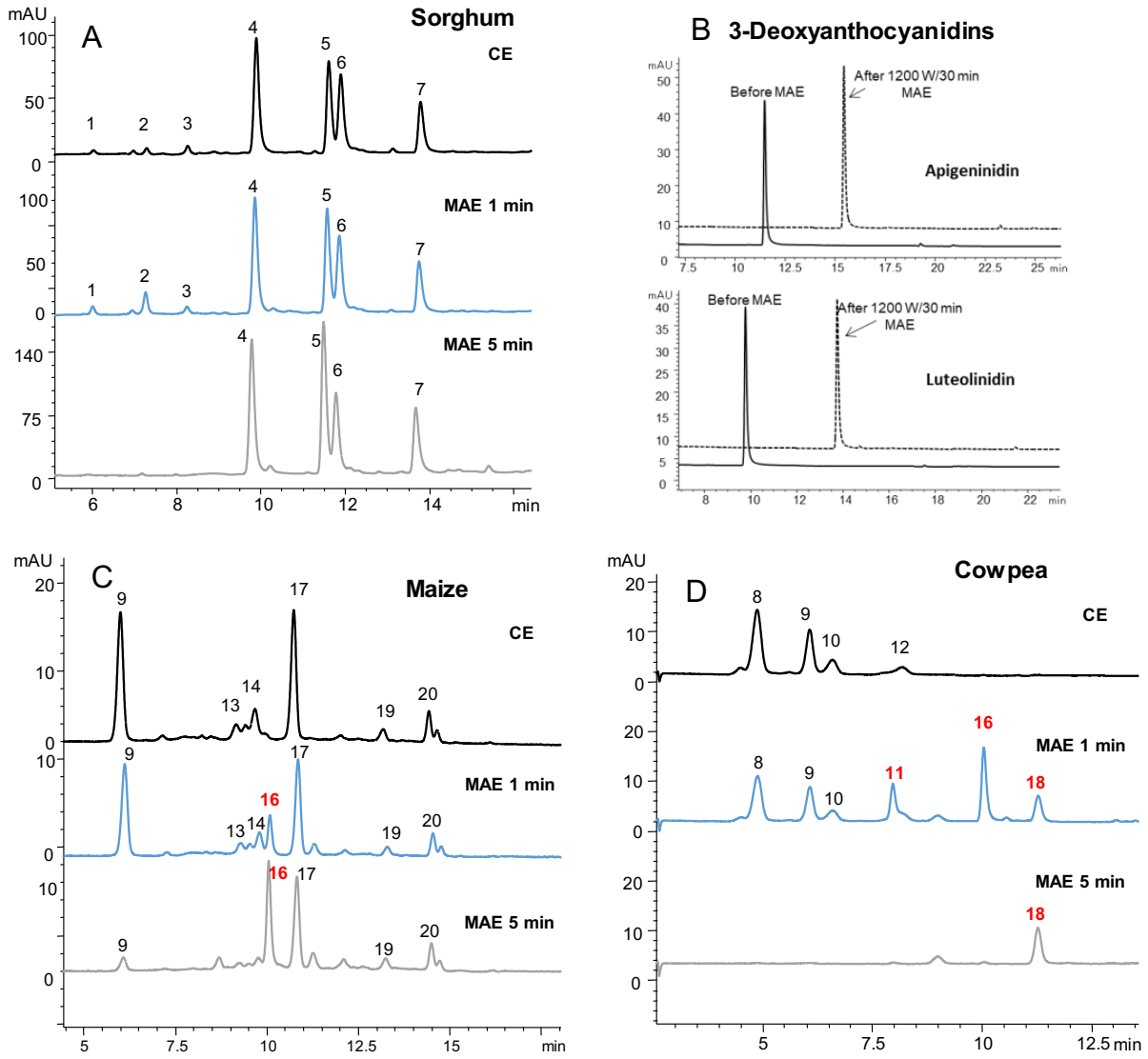


Figure 4 Effect of microwave-assisted extraction (MAE) at 1200 W on HPLC profile of sorghum 3-deoxyanthocyanins (480 nm), and anthocyanins from maize and cowpea (520 nm). Maximum temperature during MAE was 100°C. CE = conventional extraction (2 h/24°C). Acidified methanol (1% HCl) was the extracting solvent. Pure apigeninidin and luteolinidin peaks offset by 10%. Peak numbers identified in Table 2. Red highlights are peaks not present in the CE.

Table 2 Identification of major 3-deoxyanthocyanins and anthocyanins in sorghum, maize, and cowpea extracts based on UPLC-MS/MS.

Peak no.	t_R (min)	λ_{max}	$[M+H]^+$	$[M-H]^-$	MS/MS fragments	Identification
3-Deoxyanthocyanins (sorghum only)						
1	6.95	487	433		271	Luteolinidin glucoside
2	7.27	469	417		255	Apigeninidin glucoside
3	8.26	487	431		269	7-O-Me-apigeninidin glucoside
4	9.88	487	271		-	Luteolinidin
5	11.60	474	255		-	Apigeninidin
6	11.89	487	285		270, 242	7-O-Me-luteolinidin
7	13.77	469	269		254, 226	7-O-Me-apigeninidin
Anthocyanins (maize and cowpea)						
8	4.88	525	465		303	Deplphinidin-3-O-glucosoide
9	6.07	519	449		287	Cyanidin-3-O-glucoside
10	6.62	526	479		317	Petunidin-3-O-glucoside
11	7.99	520	303		-	Delphinidin
12	8.08	520	493		331	Malvidin-3-O-glucoside
13	9.19	502	433		271	Pelargonidin-3-O-glucoside
14	9.46	513	535		287	Cyanidin-3-O-(3”-malonylglucoside)
15	9.71	529	463		301	Peonidin-3-O-glucoside
16	10.06	524	287		-	Cyanidin*
17	10.80	519	549		449, 287	Cyanidin-3-O-succinylglucoside
18	11.29	525	301		-	7-O-methylcyanidin*
17	13.25	525	549		301	Peonidin-3-O-malonylglucoside
20	14.49	524	565		317	Petunidin-3-O-malonylglucoside
Copigments (sorghum and maize)						
21	13.87	325		339	324, 193, 177, 175, 133	Feruloylrhamnoside*
22	14.22	325		339	324, 193, 177, 162, 133	Feruloylrhamnoside*
23	22.05	324		723	385, 263, 233, 193, 162	Dehydrotriferulic acid rhamnoside*

Retention time (t_R); m/z in positive mode for anthocyanin, negative mode for phenolic acid esters. *Compounds present only in microwave extracted samples.

Table 3 Effect of microwave-assisted extraction (MAE) compared to conventional extraction on content of 3-deoxyanthocyanins from sorghum and anthocyanins from maize and cowpea.

Compound	CE	MAE 1 min	MAE 5 min
3-Deoxyanthocyanins from sorghum			
Luteolinidin glucoside	16.6 ± 0.9[a]	16.4 ± 0.7[a]	10.0 ± 4.1[b]
Apigeninidin glucoside	21.9 ± 1.2[b]	67.6 ± 2.8[a]	10.3 ± 1.0[c]
7-O-Me-apigeninidin glucoside	31.2 ± 1.1[a]	29.7 ± 0.7[a]	10.0 ± 4.2[b]
Luteolinidin	256.4 ± 3.2[b]	263.8 ± 6.2[b]	319.9 ± 27.7[a]
Apigeninidin	204.8 ± 2.8[c]	224.1 ± 7.0[b]	343.8 ± 16.3[a]
7-O-Me-luteolinidin	203.7 ± 8.8[ab]	189.5 ± 6.7[b]	216.4 ± 21.6[a]
7-O-Me-apigeninidin	126.5 ± 1.1[c]	124.6 ± 1.9[c]	171.8 ± 19.5[a]
Total	861 ± 18.9[c]	916 ± 24.8[b]	1090 ± 94.5[a]
Ferulate esters from sorghum			
Ferulic acid rhamnodside*	*nd*	-	519.1 ± 26
Ferulic acid rhamnodside**	*nd*	-	959.1 ± 59
Dihydrotriferulic acid rhamnoside	*nd*	-	336.6 ± 44
Anthocyanins from maize			
Cyanidin-3-O-glucoside	145.0 ± 3.6	104.7 ± 4.2	9.5 ± 3.6
Pelargonidin-3-O-glucoside	16.0 ± 1.4	11.6 ± 1.2	6.1 ± 3.1
Cyanidin-3-O-(3”-malonylglucoside)	16.7 ± 1.6	11.5 ± 0.7	8.0 ± 1.3
Peonidin-3-O-glucoside	35.9 ± 0.6	25.5 ± 1.8	6.7 ± 1.8
Cyanidin	*nd*	21.4 ± 3.8	36.5 ± 14.0
Cyanidin-3-O-succinylglucoside	162.0 ± 6.3	126.2 ± 10.4	88.0 ± 17.0
Peonidin-3-O-malonylglucoside	14.3 ± 0.6	10.5 ± 2.0	11.1 ± 1.0
Petunidin-3-O-malonylglucoside	32.7 ± 1.6	24.5 ± 1.5	23.2 ± 2.38
Total anthocyanins	423 ± 15.6[a]	336 ± 25.6[b]	189 ± 44.0[c]
Ferulic acid rhamnodside*	*nd*	-	1,243.3 ± 83
Ferulic acid rhamnodside**	*nd*	-	2,300.5 ± 102
Dihydrotriferulic acid rhamnoside	*nd*	-	768.7 ± 70
Anthocyanins from cowpea			
Delphinidin-3-O-glucoside	150.6 ± 3.8	127.2 ± 20.3	*nd*
Cyanidin-3-O-glucoside	84.2 ± 2.3	65.7 ± 3.3	*nd*
Petunidin-3-O-glucoside	33.3 ± 2.5	23.2 ± 6.7	*nd*
Delphinidin	*nd*	33.0 ± 1.9	*nd*
Malvidin-3-O-glucoside	17.9 ± 3.3	16.9 ± 4.5	*nd*
Cyanidin	*nd*	60.5 ± 6.1	*nd*
7-O-Methylated cyanidin	*nd*	31.8 ± 2.6[b]	48.2 ± 5.2[ab]
Total anthocyanins	286 ± 11.9[b]	358 ± 15.6[a]	48 ± 15.6[c]

Solvent was 1% HCl in methanol. Conventional extraction was 120 min in ambient conditions; microwave-assisted extraction (MAE) was for 1 min or 5 min with 1200 W at 100°C. Data are expressed as mean ± standard deviation. Different letters within each row indicate significant differences (Tukey’s HSD, $p < 0.05$). nd = not detectable in extract. *Peak 21; **Peak 22. ‘-’ not measured.

3.3.4. Effect of microwave energy on maize and cowpea anthocyanin profile

In contrast to the sorghum 3-DXA profile, the anthocyanin profile of both maize and cowpea were dramatically affected by MAE in comparison to conventional extraction (Figure 4C, D). For example, in the maize pigment extract, an unusual presence of cyanidin aglycone was observed in the 1 min/1200 W MAE extract which was absent in the conventional extract (Figure 4C). Anthocyanin aglycones are extremely rare in nature, which suggest the cyanidin was the product of MAE (Abdel-Aal et al., 2014). The cyanidin peak size actually increased in the 5 min MAE extract, in contrast to all other anthocyanin peaks that declined in 5 min vs 1 min MAE extracts. Quantitative data confirmed the significant increase of cyanidin in the 5 min extract (36.5 μg/g) vs the 1 min extract (21.4 μg/g) (Table 3). At the same time, one of the 2 dominant pigments identified in the conventional and 1 min MAE maize extract, cyanidin-3-O-glucoside, almost completely disappeared in the 5 min MAE extract (Figure 4C, Table 3). This strongly suggests that, as observed for sorghum, the MAE readily hydrolyzed the anthocyanin glucosides to their aglycones. However, it appears that the acylated maize anthocyanins are more stable than the glycosides to MAE. For example, the other dominant peak in the maize anthocyanin extract, cyanidin-3-O-succinylglucoside decreased by 30% (from 126 to 88 μg/g) in 5 min vs 1 min MAE extract; by contrast cyanidin-3-O-glucoside declined 91% (from 145 to 9.5 μg/g) in 5 min vs 1 min MAE extract (Table 3). Thus, the overall known better stability of the acylated anthocyanins to different food processing conditions remains true for microwave assisted extraction. The poor stability of anthocyanin glycosides to MAE was prominently visible in the cowpea extract where all the major anthocyanins in the conventional extract were glycosides (no acyl-glycosides detected) (Figure 4D, Table 1). The dominant anthocyanins in the conventional extract were delphinidin-3-O-glucoside and cyanidin-3-O-glucoside. However, within 1 min/1200 W MAE

extraction, significant quantities of delphinidin and cyanidin aglycones were detected (Figure 4D, Table 3); this agrees with the observation for maize (Figure 4). One unusual observation was that the amount of cyanidin aglycone observed in the 1 min MAE cowpea extract was especially high relative to the amount of cyanidin-O-glucoside present in the conventional extract. Assuming a similar degradation pattern observed in the maize extract, it is very likely that the 'excess' cyanidin aglycone was the result of acid-catalyzed depolymerization of cowpea procyanidins (Ojwang et al., 2013) to release cyanidin (Porter et al., 1985). Furthermore, an additional anthocyanin-type compound peak with UV signal identical to cyanidin, but a mass matching peonidin (m/z 301) was identified in the cowpea MAE extracts (peak 18, Figure 4D, Table 2). Comparison to authentic peonidin compound (m/z 301) eliminated this as a possibility. Additional fragmentation of the compound led us to conclude it was methylated on the A-ring, and was thus tentatively identified as a 7-O-methylcyanidin. Because it was present only in the cowpea MAE extracts, the compound is also likely a depolymerization product of the cowpea proanthocyanidins. Overall, the proanthocyanidin degradation products partly explain the higher than expected yield of cowpea pigments in the MAE extract of cowpea under mild conditions (Figure 3).

Another interesting observation was that despite almost complete disappearance of all anthocyanin peaks in cowpea extract at ≥5 min MAE at 1200 W (Figure 4D), the pigment content of the cowpea extract remained relatively stable at about 55% relative to the 1 min/1200 W MAE, or 80% relative to conventional extraction (Figure 3). This suggests that extensive polymerization of anthocyanin and proanthocyanidin degradation products (Jiang et al., 2019) was responsible for most of the color in the MAE cowpea extracts, which agrees with the high browning index of the cowpea extract (Figure 3C, Table 1). The carbocation intermediates that result from acid-catalyzed proanthocyanidin degradation are highly unstable and likely to readily participate in

polymerization reactions with C-2 hydrated anthocyanidin molecules and their chalcones during microwave irradiation. This likely explains the different behaviors of the maize vs cowpea pigment extracts under MAE treatment (Figure 3). It is also possible that some alcohol-soluble proteins (and to a lesser extent, reducing sugars) co-extracted by MAE may react with the phenolics in the different extracts to influence browning patterns. However, the fact that sorghum and maize, which generally have similar protein and carbohydrate profiles behaved very differently suggest the browning was primarily due to reaction of anthocyanin degradation products with other phenolic copigments. Nevertheless, the composition of non-phenolic components in the MAE extracts should be established.

3.3.5. Microwave-assisted extraction releases cell wall bound phenolics from sorghum and maize

Based on the dramatically enhanced extractability of 3-DXA from sorghum grain tissue by MAE, we hypothesized that the MAE induced rapid cell wall rupture to release the pigments from cell matrix. Even though this theory has been proposed before (Chan, Yeoh, Yusoff, & Ngoh, 2016; Desai, Parikh, & Parikh, 2010), direct evidence for it is yet lacking, with the closest evidence we could find being visual observation of altered cell wall matrix appearance (Dandekar & Gaikar, 2002). In this work, we aimed to identify cell wall-specific phenolic acid derivatives that could provide direct evidence for MAE-induced cell wall degradation. Three major cinnamic acid derivatives were identified in MAE extracts of maize and sorghum, but not cowpea (Figure 5, Table 2); these compounds were not present in the conventional extracts, leading us to conclude that they were likely derived from the cereal grain cell wall.

All three compounds were derivatives of ferulic acid based on MS/ MS fragmentation patterns, and all were esters of a methylpentose (parent compound m/z –146 u); tentatively identified as rhamnose) (Table 2). Two of the three compounds had the same m/z at 339 and were identified as ferulic acid esters of methylpentose, whereas one compound had m/z 723, and was identified as dehydrotriferulic acid ester of methylpentose. Ferulic acid and its dimers/trimers are major components of cereal grain cell wall (Dobberstein & Bunzel, 2010; Saulnier & Thibault, 1999) but are generally rare in dicots like cowpea (Awika, Rose, & Simsek, 2018). The three ferulic acid derivatives were considerably more abundant in the maize MAE extract than sorghum extract (Table 3). It is not clear whether this indicates a more extensive cross-linking of maize cell wall structure vs sorghum cell wall.

The presence of these ferulate esters after MAE is indicative of likely cell wall degradation, which contributed to increased phenols in the extracts (Table 1). The cell wall degradation allowed for greater release of 3-DXA during extraction. Because of rapid generation of heat within cells caused by microwaves, pressure also increases, likely leading to cell rupture liberating pigments from cells that were otherwise difficult to access and extract. Further, the release of ferulate copigments may benefit the 3-DXA color applications. Copigments, including ferulic acid, were shown to improve color stability of 3-DXA over time in aqueous solutions (Awika, 2008).

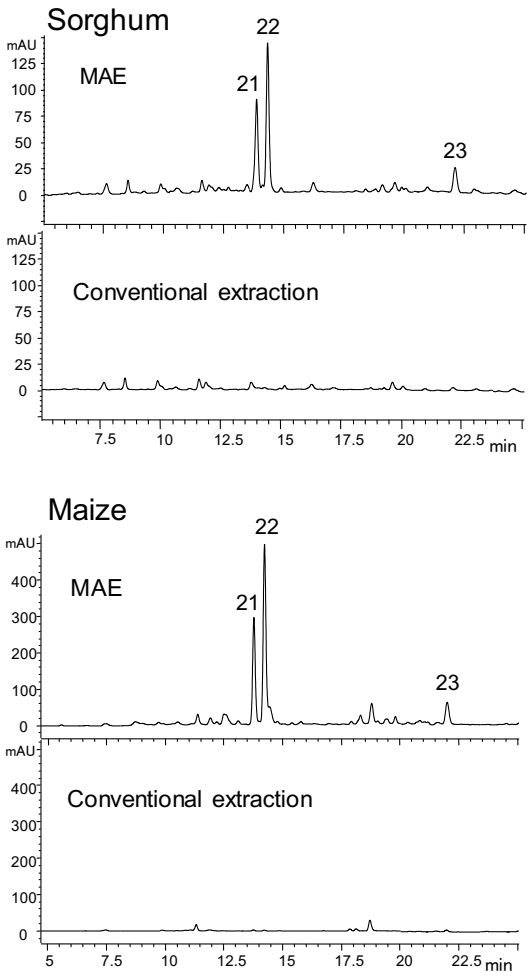


Figure 5 HPLC profile (325 nm) of cell wall ferulic acid esters observed in sorghum and maize after microwave-assisted extraction (MAE) at 1200W/100°C/5 min. Conventional extraction was for 2 h at 24°C. Acidified methanol (1% HCl) was the extracting solvent. Peak numbers identified in Table 2.

3.4. Conclusion

Microwave assisted extraction appears to be uniquely suitable for significantly improving the extraction efficiency and yield of sorghum 3-DXA pigments. This is a new and highly relevant finding because attempts to improve extraction efficiency of anthocyanins (3-DXA analogs in other plants) with MAE have not been very successfully in the past, largely due to their poor

structural stability under MAE. The poor stability of anthocyanins is confirmed in our side-by-side comparative study. MAE effectively improved extractability of 3-DXA from sorghum for two reasons. Firstly, the 3-DXA structure remains stable under relatively severe microwave conditions, which is partly due to their thermal stability as well as resistance to heterocyclic-ring cleavage. Secondly, MAE induced sorghum cell wall degradation, enhancing the release of the 3-DXA from within the cell matrix. The improved extraction efficiency of 3-DXA from sorghum by microwave energy is a key finding that should facilitate further development of their use as natural food colorants, and other high value applications. Technological and economic feasibility of microwave technology to extract the 3-DXA from sorghum bran and other high pigment sorghum tissues on a pilot scale should be explored.

4. EFFECT OF SORGHUM TANNINS ON MICROWAVE-ASSISTED EXTRACTABILITY AND COLOR PROPERTIES OF 3-DEOXYANTHOCYANINS

4.1. Introduction

The use of naturally-derived colors in foods and beverages has been on the rise as a result of consumer desire for "cleaner" food labels that are free from intimidating, synthetic, or unknown ingredients (Nachay, 2019; Wrolstad & Culver, 2012). In response, food manufacturers are formulating products with natural food colors (Martins, Roriz, Morales, Barros, & Ferreira, 2016) in place of synthetic food colors. Although the acceptability among consumers is greater compared to synthetic food colors, natural food colors have multiple disadvantages: low tinctorial strength, batch inconsistencies, requirement of extraction and purification from biological materials, limited color range, and most importantly, easy degradation during food processing.

Anthocyanins are one example of a widely used, water soluble natural food colorant, but they are unstable in low-acid foods and degrade with heat, preservatives, and exposure to light (Rodriguez-Amaya, 2016). Thus, there is a need for natural, water-soluble food colors that have greater performance in foods.

The naturally occurring anthocyanin analogs, sorghum 3-deoxyanthocyanins (3-DXA), have promising potential as natural food colorants due to unique stability in conditions that degrade anthocyanins. Recent work has shown that 3-DXA are relatively stable in low acid and alkaline pH (Akogou, Kayodé, den Besten, & Linnemann, 2018; Akogou, Kayodé, den Besten, Linnemann, & Fogliano, 2018; Awika et al., 2004), in the presence of ascorbic acid, pyruvic acid, and sulfites (Geera et al., 2012; Ojwang & Awika, 2008, 2010), and high temperature (Yang et al., 2014).

Unlike anthocyanins, 3-DXA are unsubstituted at C-3 (of the C-ring) which reduces their susceptibility to nucleophilic attack of the flavylium cation and subsequent structural changes that would lead to color change or color loss (Yang et al., 2014). For example, in the presence of ascorbic acid at pH 2, a commercial (red cabbage) anthocyanin extract lost 85% of its color compared to a (sorghum) 3-DXA extract that lost only 31% after 21 days (Ojwang & Awika, 2008). Due to their stability, there is rising interest in using 3-DXA as a novel natural food color. However, the commercial use of 3-DXA as a natural food color is limited by the poor extractability of 3-DXA from sorghum grain tissue (Herrman, Brantsen, Ravisankar, Lee, & Awika, 2020). Cereal grain cell wall matrix is highly cross-linked (de O. Buanafina, 2009), reducing solvent accessibility and efficient diffusion of 3-DXA out of the cell vacuole during extraction.

Microwave-assisted extraction (MAE) was recently demonstrated as viable technology to increase extractability of sorghum 3-DXA (Herrman et al., 2020). During MAE, microwave energy induces dipole rotation and ionic conduction, instantaneously increasing temperature and pressure within an extraction vessel. Because of the quick heating, MAE is an especially effective method for heat-labile compounds since overall extraction time is reduced considerably. In Chapter 3, we repored a 2X increase in 3-DXA yield with 5 min MAE compared to the 120 min conventional extraction method (CE) (Herrman et al., 2020). The 3-DXA were stable to microwave energy, and the grain cell wall was degraded during MAE; both factors contributed to effectiveness of MAE at extracting 3-DXA from sorghum compared to anthocyanins from other grains (Herrman et al., 2020).

During MAE pigment extraction, non-pigment phenolics (copigments) are co-extracted. The structure and stability of these compounds to microwave energy could have a significant impact on pigment yield and properties. In sorghum, the presence of tannins could potentially

affect 3-DXA extraction under MAE in major ways. First, tannins could protect 3-DXA via copigmentation, thus increasing 3-DXA yield and stability. On the other hand, tannins could be detrimental to the color stability of MAE extracted 3-DXA due to the potential for oxidative depolymerization of condensed tannins into anthocyanidins catalyzed by the heat generated during MAE (Porter et al., 1985; Ravisankar et al., 2018). Anthocyanidins are undesirable in a color extract because of their extremely poor stability in aqueous solutions (Awika et al., 2004). Because high 3-DXA sorghums recently developed for commercial uses tend to also contain tannins (Rooney, Rooney, Awika, & Dykes, 2013), it is important to establish how tannins affect the extractability, color properties, and overall stability of 3-DXA. We hypothesize that oxidative depolymerization of condensed tannins will extensively occur during MAE of tannin sorghum, resulting in increased apparent pigment yield relative to non-tannin sorghum, but negatively impacting subsequent color stability of extracted pigments. The objective of this work was to determine the effect of tannins on the profile and extractability of pigments from sorghum and, subsequently, the effect of the tannins on the stability of the extracted pigments. This work provides valuable information to enhance the potential use of sorghum 3-DXA as natural coloring agents with added functional value for the food industry.

4.2. Materials and methods

4.2.1. Sorghum brans and purified sorghum tannins

Bran was obtained from three different sorghum varieties with differing concentration of 3-DXA and tannins were used in this work. The first bran was from a non-tannin black sorghum (Tx430; provided by Texas A&M AgriLife Research) that has been reported to have high concentrations of 3-DXA. The non-tannin bran was used as the control. The second bran was from

a tannin black sorghum (Atx3363/Tx3362; provided by Texas A&M AgriLife Research) that contains both condensed tannins and high 3-DXA. The third bran was from commercial high tannin sorghum that contains condensed tannins but has low levels of 3-DXA (provided by Texas A&M AgriLife Research). The brans were obtained by decorticating 1 kg batches of sorghum in a PRL mini-dehuller (Nutama Machine Company, Saskatoon, Canada) and were separated with a KICE grain cleaner (model 6DT4-1, KICE Industries, Wichita, KS). The brans (10% yield from whole grain) were ground to pass through a 1 mm mesh using a UDY mill (model 3010-030, UDY Corporation, Fort Collins, CO) and stored at -20°C before extraction.

To establish how the MAE conditions affected sorghum tannin structure and properties, a purified tannin extract was used. The tannins were extracted with 70% acetone (v/v%) from high tannin bran using a previously described method (Barros, Awika, & Rooney, 2012). Solid-phase extraction with Sephadex LH-20 (Global Life Sciences Solutions USA, Pittsburg, PA) was used to remove low molecular weight flavonoids and concentrate the condensed tannins. Tannin content of brans and purified tannin extract were determined using normal phase HPLC as described by Girard, Castell-Perez, Bean, Adrianos, & Awika (2016). The tannin black bran and high tannin bran contained 141 mg/g and 91 mg/g of condensed tannins, respectively. The purified tannin extract contained 512 mg/g of condensed tannins.

4.2.2. Chemicals and reagents

Standards of 3-DXA (apigeninidin and luteolinidin chloride, >97% purity) were purchased form AlsaChim (Strasbourg, France). Cyanidin and peonidin chloride were purchased from Extrasynthese (Genay Cedex, France). All chemicals and reagents were of analytical grade.

4.2.3. Extraction of pigments from bran

4.2.3.1. Conventional extraction

For conventional extraction (CE), ground bran was extracted in 1% HCl in methanol (v/v%) at room temperature for 2 h on a shaker (VWR Shaker model 3500). Samples were extracted in a 40:1 solvent to sample ratio. After extraction, samples were centrifuged (10,000 x g for 10 min) using a Heraeus Megafuge 11R Centrifuge (Thermo Fisher Scientific, Asheville, NC) at 20 ºC. The supernatant was collected and stored at -20°C until use.

4.2.3.2. Microwave-assisted extraction

Each bran and the purified tannins were subjected to microwave-assisted extraction (MAE) for 1-30 min at 300, 600, or 1200 W in a Microwave Accelerated Reaction System 5 Xpress (CEM Corporation, Matthews, NC) in a 40:1 solvent to sample ratio. The solvent was 1% HCl in methanol (v/v%). The highest temperature of extraction was set at 100°C. The purified tannin extract was solubilized in 1% HCl in methanol (v/v%) before microwaving. The MAE treatment conditions were selected based on previous work (Chapter 3). After microwaving, samples were centrifuged and stored at -20°C until use.

4.2.4. Total pigment yield

Total 3-DXA content was quantified using a UV-Vis spectrophotometer (Shimadzu 2450, Shimadzu Scientific Instruments North America, Colombia, MD). Extracts were diluted to an absorbance of 1 ± 0.05 at λ_{max} and scanned from 400-700 nm. Subtraction of the absorbance at 700 nm eliminated the effect of any natural haze in the sample. Pigments were quantified using Equation 2 (Fuleki & Francis, 1968) and converted to mg/g bran:

Equation 2

*Total pigment content = (abs*MW*DF)/(ε*g)*

Total pigment content from non-tannin black bran and tannin black bran were calculated as luteolinidin equivalents. For the calculation of luteolinidin equivalents using Equation 2, *abs* is the absorbance at visible λ_{max} minus the absorbance at 700 nm, *MW* is the molecular weight (g/mol) of luteolinidin (MW = 271), *DF* is the dilution factor, ε is the molar extinction coefficient of luteolinidin (31,700 L/(mol*cm)) (Awika et al., 2004), and *g* is the extracted bran weight. Total pigment content from high tannin bran was calculated as cyanidin equivalents because the majority of pigments detected from tannin bran after MAE were anthocyanidins. For the calculation of cyanidin equivalents using Equation 2, *abs* is the absorbance at visible λ_{max} minus the absorbance at 700 nm, *MW* is the molecular weight (g/mol) of cyanidin (MW = 287), *DF* is the dilution factor, ε is the molar extinction coefficient of luteolinidin (24,800 L/(mol*cm)) (Awika et al., 2004), and *g* is the extracted bran weight.

4.2.5. Changes in visual color

The effect of tannins on visible spectra and color was determined for each extract using a UV-Vis spectrophotometer (400-700 nm) and a Minolta CT-410 colorimeter (Konica Minolta Inc. Mahwah, NJ), respectively. The *L*a*b** coordinates were measured with illuminant D65 to calculate hue angle (H^o), using Equation 3 (Reyes & Cisneros-Zevallos, 2007):

Equation 3

$$H^\circ = \tan(a^2 / b^2)^{-1}$$

4.2.6. Effect of MAE on pigment profile

To determine the effect of MAE on the pigment profile, an Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA) with a quaternary pump with degasser, an autosampler, a column compartment, and a diode array detector was used. A Zorbax Eclipse Plus C18 column (4.6 mm x 150 mm, 5.0 μm, Agilent, Santa Clara, CA) was used to carry out separation with solvent A as 1% formic acid in water (v/v %) and solvent B as 1% formic acid in acetonitrile (v/v %). The elution profile by percentage of solvent B was 0 min, 10%; 25 min, 30%; 30 min, 60%; 35 min, 10%. Pigment identification was based on matching HPLC retention profile, UV-vis spectra of authentic standards (when available), or based on reports from literature. The monitoring wavelengths were 480 nm for 3-DXA and 520 nm for anthocyanidins. Pigments were quantified using a standard curve of luteolinidin chloride (for 3-DXA) or cyanidin chloride (for anthocyanidins). The structure of pigments was determined using a Waters ACQUITY UPLC-TQD-MS system equipped with a photodiode array eλ (PDA) detector and ESI source (Waters Corp., Milford, MA), following the method described by Yang et al., 2014. A Kinetex C18 column (100 x 2.10 mm, 2.6 μm) (Phenomenex, Torrance, CA) was used to separate pigments via gradient with 1% formic acid in water (A) and 1% formic acid in acetonitrile (B). The monitoring wavelength for 3-DXA was at 480 nm and 520 nm for anthocyanidins. MS data was acquired in positive mode for the pigments; capillary voltage was 3.5kV, and cone voltage was 40V.

4.2.7. Determination of extractable phenol content

Extractable phenol content was determined by Folin-Ciocalteu assay as described by Kaluza et al. (1980) and modified by Barros et al. (2013). Crude extracts were diluted 50-fold before analysis. Briefly, 0.1 mL of sample in 1.1 mL distilled water was reacted with 0.4 mL Folin reagent and 0.9 mL 0.5 M ethanolamine for 20 min at room temperature. The absorbance of the samples was then measured using a UV-visible spectrophotometer (Shimadzu UV-2450) at 600 nm against a reagent blank. The phenol content was expressed as mg of gallic acid equivalents/g of sample (mg GAE/g) on dry weight basis based on calibration curve of gallic acid.

4.2.8. Color stability of extracted pigments

The stability of extracted pigments in in different pH solutions was determined by measuring changes in absorbance over time at λ_{max} using UV-Vis spectrophotometer (Shimadzu 2450). The HPLC profile was also monitored to determine relative stability of individual compounds. Extracts produced using CE and MAE (600 W, 100°C, 5 min) were diluted in buffers with ethanol (40 v/v%) to improve solubility of 3-DXA and anthocyanidins. The buffers used were hydrochloric acid-potassium chloride at pH 1 and citric acid-sodium citrate at pH 3 and 5. The solutions were sonicated for 20 s and left to equilibrate for 30 min. The extracts were scanned from 400-700 nm and left under laboratory light until subsequent scans were taken after 14 days.

4.2.9. Statistical analysis

Extractions and analyses were done in duplicates and replicated at least twice. Analysis of means was completed using JMP Pro (Version 12.0.1, SAS Institute, Inc., Cary, NC) with one-

way analysis of variance and Tukey's HSD used to compare treatment means. Differences in treatments were determined at a 5% significance level.

4.3. Results and discussion

4.3.1. Effect of tannins on pigment yield and color properties of extracts from sorghum bran under microwave-assisted extraction

Microwave-assisted extraction (MAE) significantly increased extraction efficiency of pigments from sorghum compared to conventional extraction (Table 4). After 5-10 min MAE, pigment yield from non-tannin black bran (12.2 mg/g) increased 2X compared the 2 h CE (7.2 mg/g), similar to observations in Chapter 3. On the other hand, the relative increase in pigment yield due to MAE was more pronounced for tannin brans (3-10X) compared to CE (Table 4). The black tannin bran pigment yield increased from 3.3 mg/g (CE) to 10.1 mg/g with MAE. The high tannin bran pigment yield increased from 0.3 mg/g (CE) to 2.9 mg/g with MAE. The higher increases in pigment yield of tannin brans relative to non-tannin bran suggests that the presence of tannins significantly impacted pigment extraction from sorghum bran.

Table 4 Effect of the presence of tannins on pigment yield and extractable phenols from sorghum brans subjected to microwave-assisted extraction (MAE).

	Extraction		Total pigment yield (mg/g)	Extracted phenols (mg/g)
Non-tannin black bran	Conventional		7.2 ± 0.17^{b}	35.9 ± 1.7^{c}
	MAE	1 min	5.8 ± 0.33^{c}	39.8 ± 4.2^{b}
		5 min	11.7 ± 0.48^{a}	56.4 ± 2.9^{a}
		10 min	12.2 ± 0.45^{a}	58.6 ± 3.0^{a}
Tannin black bran	Conventional		3.2 ± 0.04^{c}	56.2 ± 5.9^{d}
	MAE	1 min	3.2 ± 0.28^{c}	71.7 ± 2.6^{c}
		5 min	9.6 ± 0.53^{b}	111.2 ± 4.2^{b}
		10 min	10.1 ± 0.38^{a}	116.8 ± 4.2^{a}
High tannin bran	Conventional		0.3 ± 0.01^{c}	35.8 ± 3.6^{c}
	MAE	1 min	0.7 ± 0.02^{b}	46.8 ± 3.9^{b}
		5 min	2.9 ± 0.24^{a}	70.5 ± 3.4^{a}
		10 min	2.6 ± 0.40^{a}	72.3 ± 2.5^{a}

Total pigment yield was calculated as luteolinidin (non-tannin black bran and tannin black bran) or cyanidin (high tannin bran) equivalents. Extracted phenols were calculated as gallic acid equivalents. Conventional extraction (CE) was 2 h/24°C; microwave-assisted extraction (MAE) was 600 W at 100°C.). Data are expressed as mean ± standard deviation. Different letters indicate significant difference between treatments (Tukey's HSD, $p<0.05$).

The improved extraction efficiency of 3-DXA by MAE from sorghum is due to unique stability of 3-DXA structure to microwave energy, and MAE-induced cell wall degradation that enhances diffusion of 3-DXA out of the cell (Herrman et al., 2020). However, the more dramatic increase in pigment yield from tannin sorghum brans suggests additional mechanisms are involved in MAE pigment extraction in the presence of tannins. One such mechanism is the aforementioned oxidative depolymerization of condensed tannins to anthocyanidins that is catalyzed at high temperature in acidified alcohol. We confirmed that this occurred during MAE using purified tannins, which developed significant color after MAE treatment (Figure 6D). The presence of anthocyanidins after MAE could artificially inflate the pigment yield from tannin sorghums compared to non-tannin sorghum. Anthocyanidins absorb light at the wavelength used to calculate

3-DXA yield (λ_{max} between 485-495 nm); the overlap in light absorption by 3-DXA and anthocyanidins thus can confound pigment yield calculation.

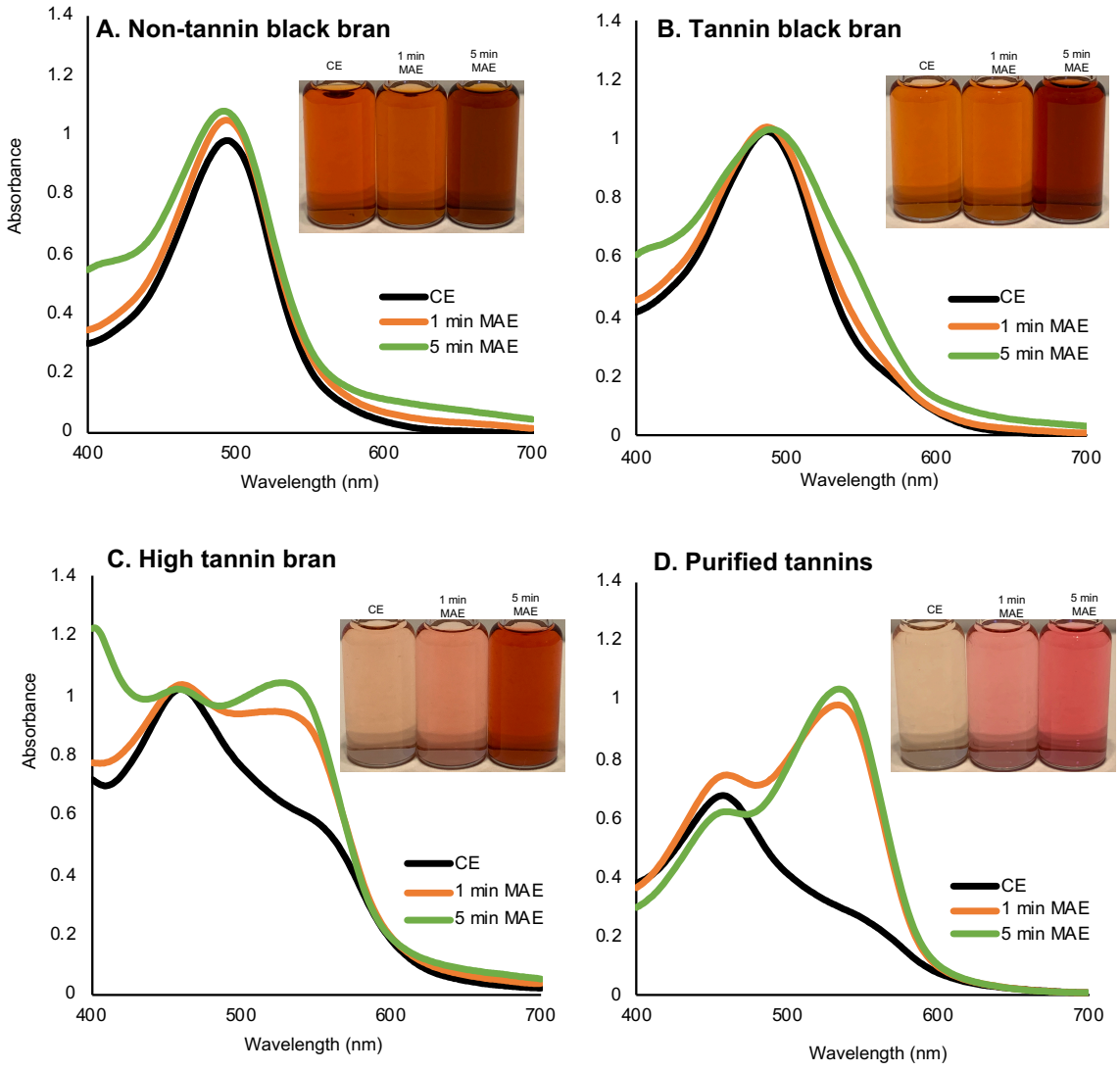


Figure 6 Effect of microwave energy on UV-Vis spectra and appearance of extracts from non-tannin black sorghum (a), tannin black sorghum (b), high tannin sorghum (c), and purified tannins (d) after microwave-assisted extraction (MAE) compared to conventional extraction (CE). Conventional extraction (CE) was 2 h/24°C; microwave-assisted extraction (MAE) was at 600 W, 100°C.

The hue angle ($H°$) of tannin MAE extracts decreased to a greater extent after MAE relative to non-tannin extracts (Table 5). After 5 min MAE, $H°$ of tannin black extracts decreased 11°, whereas a 25° decrease was observed for high tannin sorghum extracts (relative to CE). In contrast, the $H°$ of the MAE extract from non-tannin black bran increased by 5° compared to CE. Hue ($H°$) is measured in a color circle, with $H° = 0$ for a red color and $H° = 60°$ for a yellow color. As $H°$ increases from 0° to 60°, the respective color changes from red to orange and then to yellow. The decrease in $H°$ for MAE extracts of tannin sorghum indicate the extracts became redder compared to CE. The reddening of the extracts suggests that tannin degradation to anthocyanidins significantly contributed to the color of the extracts. The purified tannins subjected to microwave energy treatment directly confirmed this. The $H°$ of purified tannins decreased 67° after microwave treatment (Table 5).

Table 5 Effect of microwave-assisted extraction (MAE) on major 3-deoxyanthocyanin (3-DXA) and anthocyanidins, hue (H°), and λ_{max} of extracts from non-tannin black bran, tannin black bran, high tannin bran, and purified tannins.

	Non-tannin black bran (μg/g)		Tannin black bran (μg/g)		High tannin bran (μg/g)		Purified tannins (μg/g)	
	CE	5 min MAE	CE	5 min MAE	CE	5 min MAE	CE	5 min MAE
Luteolinidin glucoside	82 ±1	106 ±4	60 ±1	104 ±7	*nd*	*nd*	*nd*	*nd*
Apigeninidin glucoside	77 ±2	200 ±11	*nd*	136 ±10	*nd*	60 ±3	*nd*	*nd*
Luteolinidin	1,194 ±27	1,134 ±58	444 ±13	652 ±39	*nd*	78 ±4	*nd*	*nd*
Cyanidin	*nd*	*nd*	*nd*	518 ±28	*nd*	392 ±30	*nd*	18,651 ± 2,075
7-O-Methyl-cyanidin	*nd*	*nd*	*nd*	370 ±19	*trace*	368 ±18	2,046 ± 722	29,134 ± 4,388
Apigeninidin	216 ±6	550 ±45	304 ±37	709 ±37	*nd*	129 ±9	*nd*	*nd*
7-O-Methyl-luteolinidin	1,328 ±27	1,426 ±64	25 ±51	377 ±17	*nd*	59 ±3	*nd*	*nd*
7-O-Methyl-apigeninidin	270 ±6	342 ±17	133 ±4	282 ±11	*nd*	*nd*	*nd*	*nd*
Total 3-DXA	3,167	3,758	966	2,260	0	326	0	0
Total anthocyanidins	0	0	0	888	0	760	2,046	47,785
Total pigments	3,167	3,758	966	3,148	0	1,086	2,046	47,785
Extract *H°*	52 ± 0.54[a]	58 ± 0.6[b]	54 ± 0.1[a]	43 ± 0.3[b]	59 ± 0.5[a]	35 ± 0.4[b]	79 ± 0.3[a]	12 ± 0.7[b]
Extract λ_{max} (nm)	494	494	486	490	459	527	458	535

Conventional extraction (CE) was 2 h/24°C; microwave-assisted extraction (MAE) was for 5 min at 600 W, 100°C. 3-DXA were calculated as luteolinidin equivalents; anthocyanidins were calculated as cyanidin equivalents. *nd* = not detected. Data are expressed as mean ± standard deviation. Different letters indicate significant difference among treatments (Tukey's HSD, $p < 0.05$).

The UV-vis spectra of the extracts also showed clear differences between the CE and MAE due to the presence of tannins (Figure 6). After 5 min MAE, the tannin black extract spectrum showed a distinct spectral hump between 500-600 nm that was not in the spectrum of the CE extract (Figure 6B). Furthermore, there was a bathochromic shift in λ_{max} from 486 nm for the CE

extract to 490 nm for the 5 min MAE extract. More interestingly, after 5 min MAE, the high tannin extract showed a distinct spectral peak at λ_{max} of 527 nm, which was not in the spectrum of the CE extract (Figure 6C). A similar spectrum was observed for purified tannins subjected to microwaves with a more prominent peak λ_{max} of 525 nm (Figure 6D). No distinct change in UV-Vis spectra was observed for non-tannin extracts (Figure 6A). The observed λ_{max} of the tannin sorghum and purified tannin microwaved extracts (near 520-530 nm) is typical of anthocyanins. The evidence clearly indicates that significant amounts of anthocyanidins were formed from tannin-containing samples subjected to MAE, partly explaining the higher relative increase in pigment yield for tannin sorghum extracted under MAE.

In addition to the effect on yield, the presence of both tannins and anthocyanidins will have an effect on the color quality and stability of the extract if used in food applications like beverages. Tannins could polymerize with the pigments, form yellow compounds if degradation occurs, or act as copigments (González-Paramás et al., 2006; Malien-Aubert, Dangles, & Amiot, 2002). Anthocyanidins are inherently unstable, especially at pH 3-5 (Raymond Brouillard, 1982) and therefore, it is expected that significant anthocyanidin loss would occur if used in beverages. The loss of anthocyanidins in a 3-DXA extract would also cause a reduction in $H°$, which could be an issue if a red hue is desired in a final product.

4.3.2. Effect of tannins on extracted phenol content of sorghum brans subjected to microwave-assisted extraction

Similar to pigment yield, there was an effect of tannins on the extracted phenol content (EPC) of tannin extracts compared to non-tannin extracts. There was a greater relative increase

(100%) of EPC after MAE of tannin sorghums compared to non-tannin black sorghum (60%) (Table 4). Extracts from tannin black bran had the highest EPC after both CE (56.2 mg/g) and MAE (116.8 mg/g) compared to non-tannin black sorghum and high tannin sorghum, likely due to higher levels of both tannins and 3-DXA in the sorghum. An additional consequence of MAE of sorghum is the release of cell-wall-bound phenolic acids (Chiremba, Rooney, & Beta, 2012; Herrman et al., 2020), which can further enhance total EPC (Oufnac et al., 2007). Many of the monomeric phenolics (i.e. cinnamic acids, flavonols) found in sorghum are stable to microwave energy and the temperatures used in this work (Liazid, Palma, Brigui, & Barroso, 2007) and also likely contribute to EPC. Lastly, the depolymerization of the condensed tannins to monomeric flavan-3-ols will result in higher EPC compared to CE extracts. This is because as the molar concentration of monomers increases there is a greater proportion of reactive hydroxyl groups available compared to when they are within polymerized tannins. Comparison of the condensed tannin profile after CE and MAE showed that MAE resulted in almost complete depolymerization to monomers (data not shown). The relative increase of EPC from tannin sorghum compared to non-tannin is of interest because of the potential copigmentation by other polyphenolic compounds that can affect 3-DXA stability.

4.3.3. Effect of tannins on pigment profile from sorghum bran under microwave assisted extraction

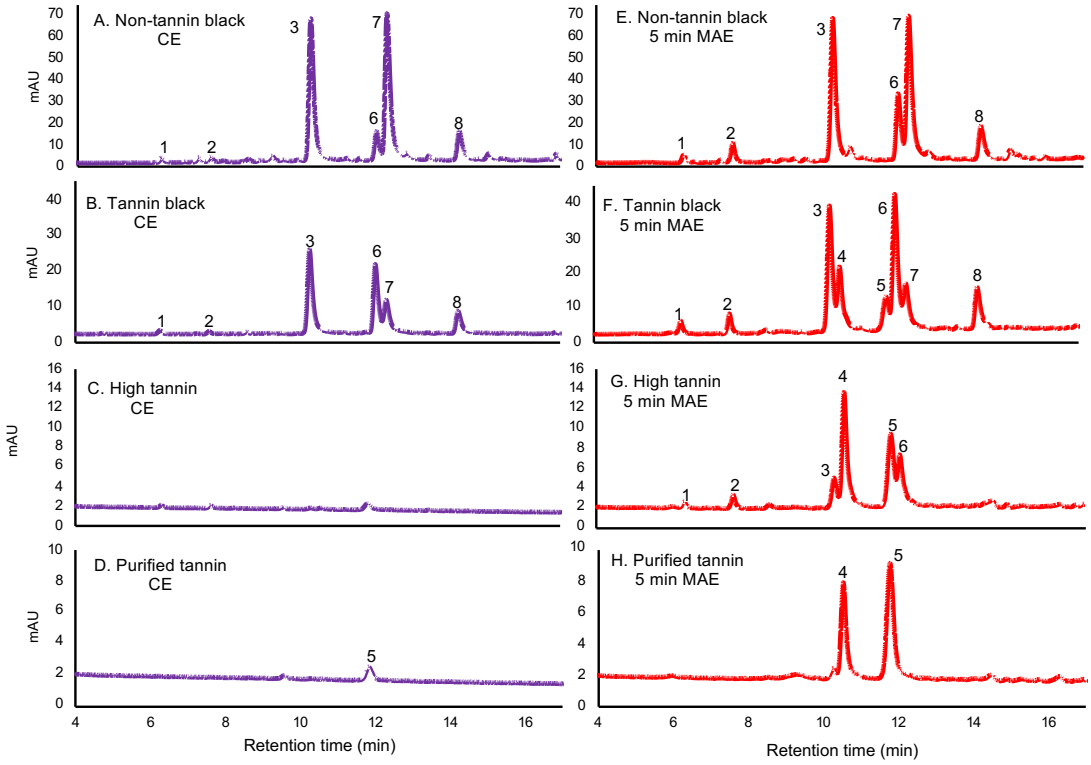
As expected, the pigment profile of CE extracts from tannin and non-tannin bran only contained 3-DXA (Table 6; Figure 7A-C). On the other hand, the pigment profile of MAE extracts from tannin brans contained both 3-DXA and two non-3-DXA pigment peaks (4 and 5) (Table 6; Figure 8F-H). Peak 4 (t_R 10.54 min, λ_{max} = 525) had $[M+H]^+$ at m/z 287. The UV-visible profile

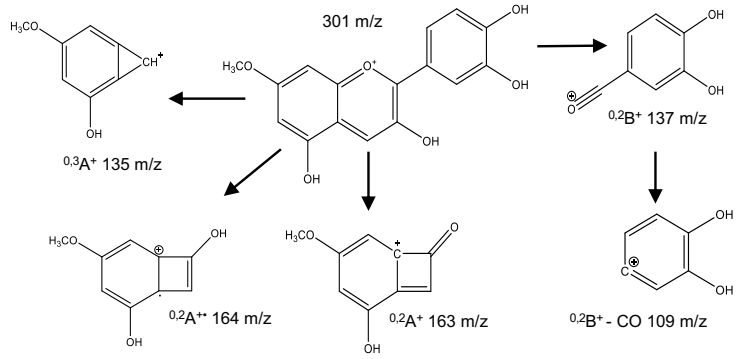
matched that of the anthocyanidin, cyanidin (authentic standard). Fragmentation produced ions at 231 (287−CO–CO), 213 (287-H_2O-CO-CO), 175 (287-C_2H_2O- C_2H_2O-CO), 165 (287-CO-CO-C_5H_6), 143 (M-H_2O-CO-CO-C_2H_2O-CO), 139 (M-H_2O-CO-CO-H_2O-CO-CO), and 137 ($^{0,2}B^+$), which are characteristic fragments for cyanidin (Barnes & Schug, 2011; Oliveira, Esperanca, & Ferreira, 2001). Therefore peak 4 was identified as cyanidin. Cyanidin was detected in brown teff after MAE (Ravisankar et al., 2018); the authors used this evidence to confirm the presence of tannins in brown teff.

Table 6 Identification of 3-deoxyanthocyanins and anthocyanidins from tannin black, non-tannin black, and high tannin sorghum extracts.

Peak no.	Retention time (t_R)	λ_{max}	$[M+H]^+$	MS/MS Fragments	Identification
1	6.29	480	433	271	Luteolinidin glucoside
2	7.63	469	417	255	Apigeninidin glucoside
3	10.32	488	271	*nd*	Luteolinidin
4	10.54	525	287	137, 139, 165, 175,143, 213, 231	Cyanidin*
5	11.78	525	301	109, 135, 137, 164, 163, 258, 269, 285	7-O-Me-Cyanidin*
6	12.03	474	255	*nd*	Apigeninidin
7	12.36	488	285	270, 242	7-O-Me-Luteolinidin
8	14.28	472	269	254, 226	7-O-Me-Apigeninidin

*Only detected in extracts of tannin sorghum produced by microwave-assisted extraction. *nd* indicates not determined. The extraction solvent was 1% HCl in methanol (v/v%).





I. Peak 5 fragmentation pattern (tentatively identified as 7-O-methyl-cyanidin)

Figure 7 Effect of microwave-assisted extraction (MAE) on HPLC profile (480 nm) of pigments from non-tannin black bran, tannin black bran, high tannin bran, and purified tannins compared to conventional extraction (CE). CE was 2 h/ 24°C; microwave-assisted extraction (MAE) was at 600 W 100°C. The fragmentation pattern of peak 5, 7-*O*-methyl-cyanidin, is also shown.

Peak 5 (t_R 11.78 min, λ_{max} = 525) had $[M+H]^+$ at 301 (Table 6; Figure 7F-I). Fragmentation of peak 5 produced ions at 285 (301-CH_3), 269 (301-CH_3-OH), 258 (301-OH-CO), 164 ($^{0,2}A^+$), 163 ($^{0,2}A^+$), 137 ($^{0,2}B^+$), 135 ($^{0,3}A^+$), and 109 ($^{0,2}B$ – CO). The increase of m/z by 14 compared to cyanidin (m/z 287) suggested the substitution of a methyl group in place of a hydroxyl group. Peonidin, an anthocyanidin, has a mass of 301 and a methyl group on the B-ring, but the retention time and fragmentation pattern of peak 5 did not match those of an authentic peonidin standard. The fragment m/z 137 is a typical fragment of a B-ring with a catechol group similar to cyanidin (Barnes & Schug, 2011; Oliveira et al., 2001); thus the methyl group was not on the B-ring. The other fragments (Figure 7I) suggested that the methyl substitution is on the A-ring. We confirmed that the methyl substitution was at C7 using a sodium acetate test (Hipskind, Hanau, Leite, & Nicholson, 1990). With the addition of 0.5 M sodium acetate, if there is a hydroxyl group on C7, there will be a bathochromic shift of λ_{max}. We did not observe this bathochromic shift of λ_{max} for isolated peak 5, indicating that the methyl substitution was on C7. This agrees with common substitution patterns of 3-DXA (Yang et al., 2009) as well as the recent identification of 7-O-methyl-catechin in sorghum (Kang, Price, Ashton, Tapsell, & Johnson, 2016).

In the tannin bran MAE extracts, cyanidin was present in slightly higher concentrations than 7-O-methy-cyanidin (Table 5). The level of cyanidin from black tannin sorghum was 0.52 mg/g compared to 0.37 mg/g of 7-O-methy-cyanidin; cyanidin from high tannin sorghum was 0.39 mg/g compared to 0.37 mg/g of 7-O-methy-cyanidin. However, after microwaving the purified tannin extract, there was a higher proportion of 7-O-methy-cyanidin (29 mg/g) compared to cyanidin (18 mg/g). This suggests a greater proportion of extension units of the polymeric condensed tannins from sorghum may be present as 7-O-methylated flavan-3-ols. Presence of a

methyl group on the A-ring alters the hydrophobicity of the tannin, which could make them more effective copigments with 3-DXA than if not methylated.

The significant presence of anthocyanidins in the tannin extracts rationalize the (previously described) differences of *H*° and UV-vis spectra when comparing to MAE extracts to CE extracts (Figure 6, Table 5). Anthocyanidins were not detected in non-tannin sorghum, and that is why there was little evidence of reddening with those extracts or changes to the UV spectra due to MAE. The significant presence of anthocyanidins in the tannin bran MAE extracts (0.8-29 mg/g) (Table 5) suggests that the color stability of tannin containing extracts pigments may be negatively impacted, which would in turn limit their food applications.

As expected, 3-DXA compounds in the non-tannin and tannin sorghum extracts increased after MAE relative to CE (Table 6) but the increase was higher in the tannin brans. The concentration of 3-DXA (based on HPLC) in non-tannin black sorghum increased (18%) from 3,167 μg/g (CE) to 3,758 μg/g (MAE). From tannin black sorghum, 3-DXA compounds increased 135% with MAE (from 966 μg/g to 2,269 μg/g). From high tannin sorghum the concentration of 3-DXA was 326 μg/g after MAE despite no 3-DXA compounds detected from high tannin sorghum after CE. The relative greater increase of 3-DXA from tannin black sorghum compared to non-tannin black sorghum may be due partly to the protective effect of tannins during MAE. Another possible explanation is that there may be physiological differences between tannin grain and non-tannin grain pericarp cell wall structure. Differences of pericarp thickness and/or cell wall cross-linking could have an effect on the extent of cell wall degradation during MAE. The exact mechanism should be further investigated.

4.3.4. Effect of tannins on color stability of sorghum pigments extracted by microwave-assisted extraction

Tannins generally improved the stability of 3-DXA (Figure 8A-F, Table 7). The effect of tannins on color stability of 3-DXA was most evident in solutions at pH 1 compared to non-tannin extract solutions (Figure 8A, D). There was a greater hyperchromic shift of 137% (490 nm) after 14 days for tannin black CE extract solutions compared to 110% (490 nm) for non-tannin black CE extract solutions (Table 7, Figure 8A, D). On the other hand, absorbance of solutions made with black tannin MAE extracts slightly faded (89% abs 490 nm), similar to non-tannin black MAE extract (86% abs 490 nm) after 14 days.

The hyperchromic shift of the tannin sorghum CE extract at pH 1 suggests that copigmentation or polymerization occurred between 3-DXA and tannins, enhancing the color of the solution similar to the mechanism reported in red wine (Boulton, 2001; González-Manzano et al., 2009). The high molecular weight tannic acid was also reported to stabilize 3-DXA compounds better than monomeric phenolics (Awika, 2008). The hyperchromic shift of non-tannin sorghum suggests that 3-DXA intermolecular copigmentation also occurred with other, non-tannin compounds present in the extracts (Awika, 2008).

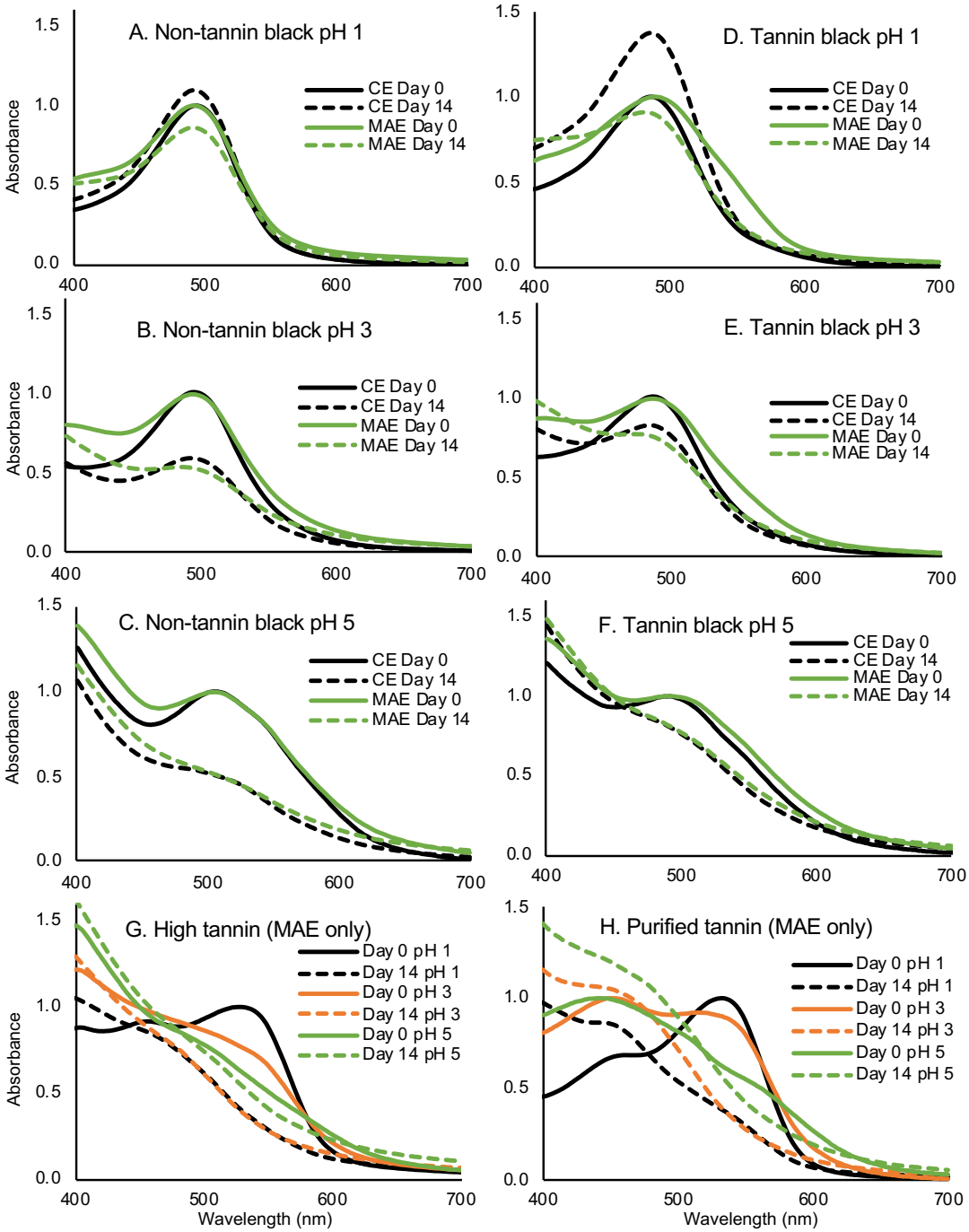


Figure 8 Effect of tannins on visible spectra of sorghum extracts after microwave-assisted extraction (MAE) compared to conventional extraction (CE). CE was 2 h/ 24°C. MAE was 1 or 5 min at 600 W 100°C.

Table 7 Percent retention of major 3-deoxyanthocyanin (3-DXA) and anthocyanidin compounds in non-tannin vs tannin sorghum extracts after 14 days at different pH levels.

		% Retention at day 14 relative to day 0						% Abs at day 14 relative to day 0		
	pH	Luteolinidin	Cyanidin	7-O-Me-Cyanidin	Apigeninidin	7-O-Me-Luteolinidin	7-O-Me-Apigeninidin	430 nm	490 nm	530 nm
Non-tannin black sorghum										
CE	1	97 ± 0.4	*nd*	*nd*	69 ± 2.6	90 ± 0.3	91 ± 0.4	116 ± 3.7^{a}	110 ± 4.3^{b}	*np*
	3	67 ± 1.8	*nd*	*nd*	94 ± 5.8	65 ± 2.0	78 ± 1.5	84 ± 5.0^{c}	60 ± 5.0^{f}	*np*
	5	20 ± 4.7	*nd*	*nd*	64 ± 1.3	33 ± 0.3	0	78 ± 0.2^{d}	57 ± 0.9^{f}	*np*
MAE	1	96 ± 1.8	*nd*	*nd*	94 ± 0.1	89 ± 8.1	87 ± 0.6	91 ± 0.8^{b}	86 ± 0.5^{cd}	*np*
	3	49 ± 0.4	*nd*	*nd*	77 ± 0.2	99 ± 1.3	0	76 ± 1.5^{d}	54 ± 2.4^{f}	*np*
	5	12 ± 4.2	*nd*	*nd*	45 ± 7.1	49 ± 8.9	0	77 ± 1.0^{d}	57 ± 1.0^{f}	*np*
Tannin black sorghum										
CE	1	99 ± 0.4	*nd*	*nd*	97 ± 0.6	89 ± 1.1	94 ± 0.4	146 ± 3.6^{a}	137 ± 2.0^{a}	*np*
	3	83 ± 3.9	*nd*	*nd*	85 ± 0.9	76 ± 1.7	79 ± 2.0	108 ± 1.1^{b}	82 ± 5.9^{cd}	*np*
	5	39 ± 5.1	*nd*	*nd*	62 ± 0.7	48 ± 3.7	0	111 ± 0.4^{b}	81 ± 2.9^{de}	*np*
MAE	1	98 ± 0.8	16 ± 0.7	53 ± 1.9	86 ± 14.7	74 ± 6.7	94 ± 0.3	107 ± 0.3^{b}	89 ± 2.5^{c}	65 ± 0.8^{b}
	3	83 ± 4.8	43 ± 0.1	24 ± 3.7	88 ± 0.2	64 ± 1.3	72 ± 3.1	97 ± 0.9^{d}	75 ± 1.0^{e}	66 ± 0.6^{b}
	5	43 ± 4.7	0	55 ± 9.0	64 ± 1.9	88 ± 0.4	0	102 ± 0.6^{c}	81 ± 1.1^{de}	71 ± 0.9^{a}
High tannin sorghum										
MAE	1	*nd*	0	33 ± 5.1	*nd*	*nd*	*nd*	108 ± 0.6^{a}	*np*	40 ± 1.3^{c}
	3	*nd*	*nd*	11 ± 5.7	*nd*	*nd*	*nd*	98 ± 1.2^{b}	*np*	51 ± 0.9^{b}
	5	*nd*	*nd*	37 ± 5.5	*nd*	*nd*	*nd*	107 ± 1.0^{a}	*np*	86 ± 0.3^{a}
Purified tannins										
MAE	1	*nd*	0	56 ± 0.3	*nd*	*nd*	*nd*	150 ± 0.9^{a}	*np*	40 ± 2.2^{c}
	3	*nd*	0	16 ± 0.2	*nd*	*nd*	*nd*	113 ± 3.4^{c}	*np*	46 ± 0.9^{b}
	5	*nd*	*nd*	40 ± 1.4	*nd*	*nd*	*nd*	127 ± 0.6^{b}	*np*	93 ± 1.3^{a}

Values for individual pigments as % was based on HPLC data after 14 days compared to the initial measurements (day 0). Content was quantified by HPLC at 480 nm for 3-DXA and 520 nm for anthocyanins. %Abs was calculated as percent absorbance at the specific wavelength on day 14 compared to day 0. *nd* Indicates pigment was not detected in the solutions on day 0. *np* Indicates no peak. Conventional extraction (CE) was 2 h/24°C; microwave-assisted extraction (MAE) was for 5 min at 600 W 100°C. Data are expressed as mean ± standard deviation.

Because of the significant presence of anthocyanidins in the MAE extracts, the absorbance at 530 nm (near the λ_{max} of anthocyanidins) (Figure 8D) was monitored to understand the degradation of anthocyanidins relative to 3-DXA (Table 7). The change in absorbance at 530 nm for the tannin black MAE pH 1 solutions was greater (-35%) than that at 490 nm (-11%) (Table 7). This indicates that the anthocyanidins degraded more readily than 3-DXA in the mixed pigment solution. In fact, the absorbance at 490 nm of pH 1 solution made with non-tannin black MAE extracts (85%) was similar to the tannin black MAE extract after 14 days, showing that the presence of anthocyanidins did not negatively affect stability of the 3-DXA. The solutions made with tannin black MAE extracts were redder than the tannin black CE extracts on day 0 (Figure 9), yet they both had relatively similar orange hue by day 14 due to the loss of anthocyanidins and dominance of 3-DXA over time.

The stabilizing effect of tannins on sorghum pigments was also clearly obvious at pH 3 and 5. At both pH 3 and 5, the absorbance loss (490 nm) after 14 days was greater for non-tannin extract solutions (40-56% loss) compared to tannin extract solutions (18-25%), independent of extraction method (Figure 8, Table 7). This again suggests that copigmentation of 3-DXA with tannins preserved color (tannin black CE solutions) and degradation of anthocyanidins did not negatively affect the color or stability of 3-DXA compounds (tannin black MAE solutions). Tannins thus appear to be effective copigments of 3-DXA even in low acid solutions. This stabilizing effect of tannins could be utilized to further enhance functionality of 3-DXA as a natural colorant.

Both MAE tannin sorghum extract and microwave treated purified tannin solutions faded significantly after 14 days at any pH (Figure 8G, H, Figure 9, Table 7). As absorbance decreased at 530 nm, there was a concurrent increase of absorbance between 400-500 nm (Figure 8G, H, Table 7). This was most noticeable in pH 1 solutions where the peak at 530 nm was most apparent in the initial spectrum. The ratio of absorbance at 530 nm to 430 nm after 14 days decreased as pH increased. For instance, absorbance of microwaved purified tannin extract solutions, after 14 days at pH 1, was 40% (of absorbance on day 0) at 530 nm compared to 150% (of absorbance on day 0) at 430 nm. At pH 5, absorbance was 93% (of absorbance on day 0) and 127% (of absorbance on day 0) at 530 nm and 430 nm, respectively (Table 7). This was because of the rapid fading of anthocyanidins at pH 5 compared to pH 1. The initial concentration of anthocyanidin flavylium cations was lower at pH 5, so therefore, the extent of fading over time was lower at pH 5 than pH 1.

The loss of absorbance at 530 nm was due to degradation of anthocyanidins or the result of copigmentation via anthocyanin-flavanol covalent coupling, resulting in a loss of red color with subsequent increase of xanthylium pigments (λ_{max} between 400-450 nm) (González-Paramás et al., 2006; Malien-Aubert et al., 2002). Anthocyanin-flavanol coupling, with oligomeric condensed tannins may limit, or delay, the loss of the red chromophore due to steric hindrance by the tannin that prevents hydration of the anthocyanidin chromophore (Malien-Aubert et al., 2002). The appearance of the solutions made with high tannin and purified tannin extracts clearly changed from red to a yellowish color as shown by the increase in hue after 14 days (Figure 9). The increase in absorbance

at 430 nm at day 14 relative to day 0 was mostly over 100% for tannin-containing samples (Table 7), again suggesting that anthocyanidin-flavanol coupling occurred in the solutions made with tannin sorghum extracts.

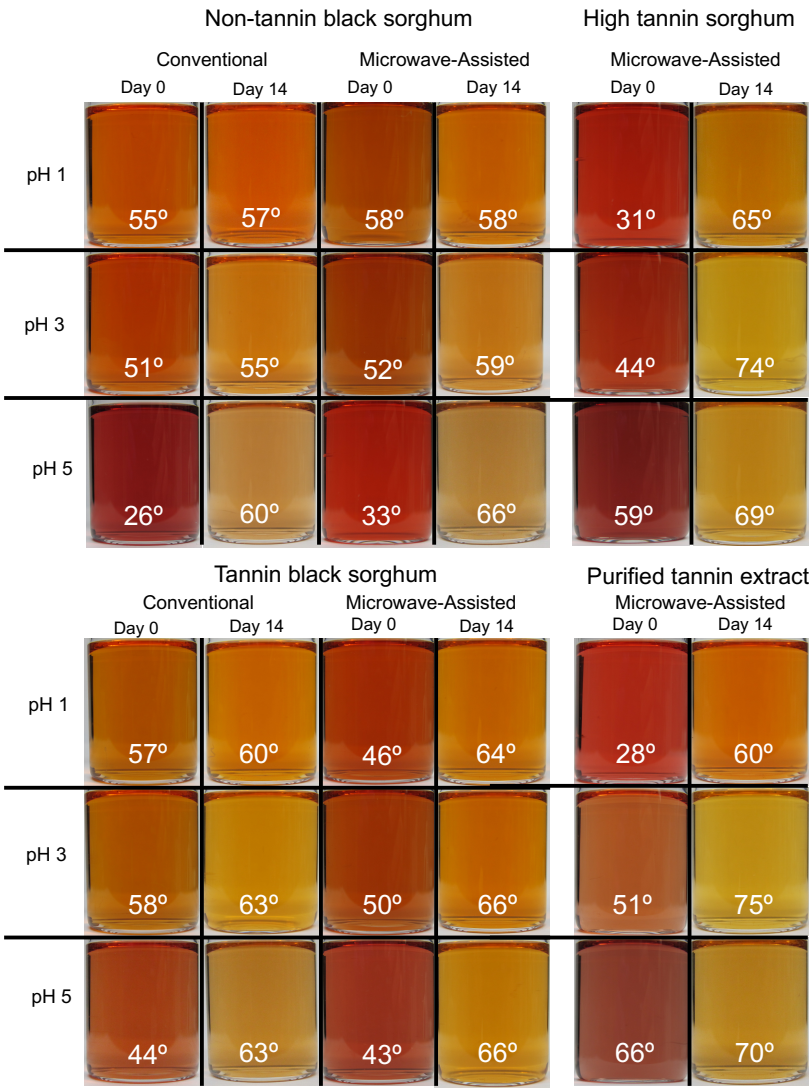


Figure 9 Effect of tannin on visual color of solutions made with sorghum extracts subjected to conventional or microwave-assisted extraction. Solutions were stored at room temperature (25°C) under laboratory fluorescent lighting. Hue angle (*H*°) of the solution is indicated on each picture.

4.3.5. Effect of tannins on structural stability of sorghum pigments extracted by microwave-assisted extraction

As expected, there was greater retention of individual 3-DXA at pH 1 (84% average) compared to pH 3 (72% avg.) and 5 (35% avg.) (Table 7). The presence of tannins enhanced the retention of 3-DXA at pH 3 and 5. The tannin black sorghum extracts retained 3-DXA to a greater extent (61% avg.) than those made with non-tannin black sorghum extracts (47% avg.) independent of extraction method (Table 7). For example, the retention of luteolinidin at pH 3 was 83% avg. for tannin black solutions but was 58% for non-tannin black solutions. Although the condensed tannins were almost completely depolymerized during MAE (data not shown), the enhanced stability of 3-DXA suggests that the tannin depolymerization products also had a stabilizing effect on 3-DXA. The overall greater stability of 3-DXA in solutions made with tannin extracts again highlights the potential benefit of tannins as 3-DXA copigments.

With regards to anthocyanidin stability, both anthocyanidin compounds (cyanidin and 7-O-methyl-cyanidin) degraded rapidly in solutions made with the tannin-containing MAE extract (Table 7). The 7-O-methyl-cyanidin was generally more stable than cyanidin. For example, cyanidin was undetectable in most treatments at day 14 whereas significant levels of 7-O-methyl-cyanidin (11-56%) were retained in all tannin MAE samples at day 14 (Table 7). More stable anthocyanidins would enhance hue properties of the 3-DXA extract, and thus broaden the potential for food applications.

Methylation at C-7 appears to enhance the stability of both anthocyanidins and 3-DXA; e.g. there was similar enhanced stability of 7-O-methyl-luteolinidin, the 3-DXA

analog of 7-O-methyl-cyanidin, compared to luteolinidin, the analog of cyanidin. For instance, retention of 7-O-methyl-luteolinidin in pH 5 black sorghum extracts averaged 55% vs 29% for luteolinidin. Methylation did not, however, indicate greater stability when comparing apigeninidin and 7-O-methyl-apigeninidin. At pH 5, 7-O-methyl-apigeninidin was not detected after 14 days whereas retention of apigeninidin was ≥45%. The key to enhanced stability may be both methylation and a B-ring catechol group.

The general evidence demonstrates two important findings: firstly, the presence of tannins generally improved 3-DXA stability suggesting that tannins are effective 3-DXA copigments. Secondly, structural degradation of anthocyanidins (depolymerization products of tannins) resulted in the loss of a red hue (increase in hue angle) (Figure 9) yet the color of 3-DXA remained due to their stability in the solution. Overall, MAE could be an effective method for the extraction of 3-DXA from tannin black sorghum.

4.4. Conclusion

This work showed that MAE can effectively increase 3-DXA yield from tannin black and tannin sorghum due to the protective effect of tannins on 3-DXA. However, the oxidative depolymerization of tannins to anthocyanidins will occur during MAE. The presence of anthocyanidins is of concern because they are very unstable and can lead to unreliable coloring properties if used in food or beverages. The anthocyanidins detected were cyanidin and 7-O-methyl-cyanidin, previously undetected in sorghum. These two anthocyanidins faded relatively rapidly in aqueous solutions although 7-O-methyl cyanidin was more stable than cyanidin. The overall color and structural stability of 3-

DXA was generally unaffected by the presence of anthocyanidins. CE of tannin black sorghum produced a much more stable 3-DXA extract with a greater proportion of higher molecular weight tannins than MAE. The tannins thus appeared to have a copigmentation effect on the 3-DXA. This work shows that MAE could potentially be used for the commercial extraction of 3-DXA from tannin sorghum and that tannins may be effective 3-DXA copigments in beverages or other foods.

5. INTERACTION BETWEEN AMPHIPHILIC POLYSACCARIDES AND 3-DEOXYANTHOCYANINS CONTRIBUTING TO IMPROVE AQUEOUS STABILITY OF 3-DEOXYANTHOCYANINS

5.1. Introduction

Sorghum 3-deoxyanthocyanins (3-DXA) have unique properties that make them a potential novel food colorant. The 3-DXA have shown resistance to thermal degradation (Yang et al., 2014), stability in the presence of sulfites or ascorbic acid (Geera et al., 2012; Ojwang & Awika, 2008, 2010), and are less resistant to bleaching at low acid pH (Awika et al., 2004), all of which are factors needed for use as food colors. The structure of 3-DXA is analogous to the more widespread anthocyanins, but 3-DXA are unique in that they lack substitution on the third carbon of the C-ring. This renders 3-DXA less hydrophilic than anthocyanins but also makes the compounds more resistant to hydration and nucleophilic attack (Mazza & Brouillard, 1987a). However, because of the reduced hydrophilicity of 3-DXA, when added to an aqueous solution, 3-DXA rapidly self-associate and extensive pigment precipitation is visible within 48 h. To be effective colorants in aqueous products, the poor aqueous stability of 3-DXA must be reduced.

Hydrocolloids, such as gum arabic, xanthan gum, or carrageenan, are used in the food industry to maintain colloidal stability of liquid foods (e.g. beverages, salad dressings, sauces). Depending on their structure, the gum can function as emulsifiers, stabilizers, or both. Emulsifier gums, e.g. gum arabic, function as surface active agents which reduce the interfacial tension between hydrophobic and hydrophilic phases to create

a homogenous system. Emulsion stabilizers (most gums) act as viscosity modifiers and reduce the movement of particles within the continuous phase (Schmidt et al., 2015). Hydrocolloids can also encapsulate and protect labile components from degradation in food. In particular, amphiphilic hydrocolloids have been shown to interact with and stabilize anthocyanins in aqueous solutions and other foods. For example, Chung, Rojanasasithara, Mutilangi, & McClements (2016) reported that gum arabic limited anthocyanin degradation in aqueous solutions, citing hydrogen bonding as the primary interaction between gum arabic and anthocyanins. β-Cylcodextrin improved the stability of chokeberry juice anthocyanins (Howard, Brownmiller, Prior, & Mauromoustakos, 2013) and black bean anthocyanins (Aguilera et al., 2016), via encapsulation, stabilized by hydrogen bonding and hydrophobic interactions. Based on the evidence of interactions between anthocyanins and hydrocolloids, we hypothesize that amphiphilic polysaccharides can interact with 3-DXA to improve their aqueous stability of 3-DXA, primarily via hydrophobic interactions.

Preliminary work in our lab (Herrman, 2016) established the stabilizing effect of gum arabic, an amphiphilic polysaccharide, and sodium alginate, an ionic polysaccharide, on 3-DXA. Both gum arabic and sodium alginate improved the aqueous stability of 3-DXA, but the extent was dependent on the polysaccharide, pH, and 3-DXA structure. This suggests that different interaction mechanisms were occurring between the pigments and polysaccharides. However, the exact mechanisms of these interactions are yet to be deciphered. Elucidation of these mechanisms of interaction is required to effectively and efficiently use 3-DXA as a natural food colorant. The objective of this work was to

determine the mechanisms of interaction between 3-DXA and gum arabic and sodium alginate that contribute to improved colloidal stability.

5.2. Materials and methods

5.2.1. Chemicals and reagents

All chemicals and reagents were analytical grade. Gum arabic was purchased from Acros Organics (Morris Plains, NJ). Sodium alginate (alginic acid sodium salt M/G ratio 1.56), sodium citrate dihydrate crystal, and citric acid monohydrate were purchased from Sigma-Aldrich (St. Louis, MO). Apigeninidin chloride (>97% purity) and luteolinidin chloride (>97% purity) were purchased from Alsachim (Strasbourg, France).

The nitrogen content of the gums was determined using LECO combustion method as described by Sweeney and Rexroad (1987). A factor of 6.25 was used to estimate protein content using Equation 4 (Nakauma et al., 2008). The protein content of gum arabic and sodium alginate was 1.93% and 0.40%, respectively.

Equation 4

Protein content (%) = Nitrogen content (%) x 6.25

5.2.2. Extraction of 3-deoxyanthocyanins

Red sorghum leaf sheaths (Geera et al., 2012; Kayodé et al., 2011) and red sorghum leaves (Petti et al., 2014), known to contain high levels of 3-DXA with different profiles, were used as sources of 3-DXA. The leaves and sheaths were ground to pass through a 1 mm mesh using a UDY mill (model 3010-030, UDY Corporation, Fort Collins, CO) before extraction. 3-DXA extracted in 1% HCl in methanol (v/v%) at room temperature for 2 h on a shaker plate (VWR Shaker model 3500). Samples were extracted in a 10:1 solution of solvent to ground tissue. After extraction, samples were centrifuged and the supernatant was collected and stored at -20°C. The crude extracts were filtered through 0.2 μm filters before use.

5.2.3. Profiling of 3-doxyanthocyanin extracts

To determine the profile and structures of 3-DXA, mass spectrometry was performed on a Waters ACQUITY UPLC-TQD-MS system (Waters Corp., Milford, MA) equipped with a column heater, sample manager, binary solvent manager, photodiode array (PDA) detector, and ESI source following a method similar to Yang et al. (2014) with minor changes. The separation was performed on a Kinetex C18 column (100 × 2.10 mm, 2.6 μm) (Phenomenex, Torrance, CA) at 40°C with flow rate of 0.4 mL/min. For pigment analysis, the mobile phases consisted of solvent A 1% formic acid in water, solvent B 1% formic acid in acetonitrile. The gradient of solvent B was 0-2 min 5%, 2-8 mins 20%, 8-15 mins 70%, 15-20 mins 70%, 20-23 mins 5%, and 23-27 mins 5%. The monitoring wavelength for 3-DXA was at 480 nm. Mass spectrometric data were acquired

in positive mode for pigments. Empower 2 software (Waters Corp.) was used to acquire and analyze data. The MS scan was recorded in the range of 100–1000 Da. Nitrogen was used both as a drying gas and as nebulizing gas, while argon was used as the collision gas (AOC, Bryan, TX). The nitrogen gas flow conditions were 800 and 50 L/h for desolvation and at the cone, respectively. The source block temperature was 150°C. Desolvation temperature was 400°C. Mass parameters were optimized for positive ionization, as follows: capillary voltage 3.5kV, cone voltage 40V. Compound identification was based on matching UPLC retention profile, UV–vis spectra, and MS data with authentic standards. Where standards were not available, compounds were identified based on the fragment patterns compared with reports in the literature.

5.2.4. Preparation of 3-deoxyanthocyanin-gum arabic and sodium alginate solutions

Solutions of gum arabic or sodium alginate and 3-DXA were made in pH 3 or 5 citric acid/sodium citrate buffers. The polysaccharides were solubilized overnight at a concentration of 1 g/L in either pH 3 or 5 buffer and the crude 3-DXA extracts were then added to each solution. Solutions were sonicated at 40% output energy for 30 s using a tip probe sonicator to solubilize 3-DXA in the polysaccharide solutions (VibraCell 40, Sonics & Materials Inc., Danbury, CT). The final solutions were prepared to have an initial absorbance of ~1.0 at visible λ_{max} as measured by a spectrophotometer (Shimadzu UV 2450, Shimadzu Scientific Instruments North America, Columbia, MD).

5.2.5. Characterization of 3-deoxyanthocyanin-polysaccharide solutions

5.2.5.1. Viscosity

To determine if viscosity was a contributing factor to the aqueous stability of 3-DXA, the viscosity of crude extract 3-DXA-polysaccharide solutions were measured with a rheometer (Haake Rheostress 6000) equipped with a temperature controller (Peltier TC-81) using cone and plate geometry (60 mm, 1° angle, 52 nm gap). Flow properties were evaluated at shear rates of 1-500 s^{-1} for 30 s. Temperature was maintained at 23°C.

5.2.5.2. Particle size distribution and zeta potential

The average particle size of control polysaccharide solutions was compared to that of 3-DXA-polysaccharide solutions. A larger average particle size in 3-DXA-polysaccharide solutions compared to that of solutions without 3-DXA can suggest there is a complex formed between the two compounds. The polysaccharide concentration was 0.5 g/L of solutions made with either the sheath or leaf extract and pH 3 and 5. Particle size was determined by dynamic light scattering (DLS) with a Malvern Nano ZA Zeta-sizer (Malvern Instrument Ltd., UK) with a He/Ne laser (lambda = 633 nm) at a fixed scattering angle of 173° at 25°C (± 0.1°). Aliquots of the solutions were diluted 4-fold with appropriate buffer to reduce multiple scattering phenomena.

Zeta potential of the control solutions was compared to that of 3-DXA-polysaccharide solutions. The magnitude of zeta potential gives an indication of the extent that electrostatic repulsion contributes to aqueous stability. A change in zeta potential

upon addition of 3-DXA could also indicate a conformational change in polysaccharide structure. The zeta potential values were automatically calculated from the electrophoretic mobility based on the Smoluchowski model (Tan, Celli, & Abbaspourrad, 2018) by the same Malvern Nano ZA Zeta-sizer used for particle size measurements.

5.2.6. Fluorescence quenching of gum arabic by 3-deoxyanthocyanins

Additional 3-DXA-polysaccharide solutions were prepared using pure 3-DXA for fluorescence quenching experiments to minimize the potential interference of other compounds in crude extracts on fluorescence intensity measurements. Only gum arabic was used in fluorescence quenching experiments because it has glycoproteins with the amino acid tyrosine that will fluoresce under UV light. Stock solutions of apigeninidin chloride or luteolinidin chloride (0.005 M) were made in 0.1% HCl in methanol (v/v%). Apigeninidin or luteolinidin were diluted into gum arabic solutions (0.5 g/L) to a final concentration of 0-20 μM. The concentration of 3-DXA was used to prevent inner filter effects. Gum arabic at 0.5 g/L was used because preliminary work by Herrman (2016), indicated this concentration of gum arabic improved the aqueous stability of 3-DXA, and the fluorescence intensity of 0.5 g/L gum arabic was within the range needed to ensure accurate measurements by the fluorimeter.

The fluorescence behavior of gum arabic in the presence of increasing concentration of 3-DXA standards (apigeninidin or luteolinidin) at both pH 3 and 5 was determined using a Horbia FluoroMax-4 (Horiba Scientific, Kyoto, Japan). The excitation wavelength was 278 nm, and an emission spectrum from 300-500 nm was recorded.

Although low concentrations of 3-DXA were used to minimize inner filter effects, the 3-DXA absorbed light at both the excitation and emission wavelengths. To account for this absorbance, the fluorescence intensity of each solution was corrected using the following Equation 5 (Cahyana & Gordon, 2013):

Equation 5

$$F_c = F_0^{(A_{exc} + A_{em})/2}$$

Where F_C is the corrected fluorescence intensity, and F_0 is the observed fluorescence intensity. A_{exc} and A_{em} are the absorbances at the excitation and emission wavelengths, respectively, of the 3-DXA standard solution at the corresponding concentration without gum arabic.

To understand the contribution of electrostatic interactions between gum arabic and 3-DXA, sodium chloride was added up to 0.5 M to 3-DXA-gum arabic solutions and the fluorescence intensity was recorded.

5.2.7. Statistical analysis

Each measurement was done in triplicates and replicated twice. Analysis of means was completed using JMP pro 12 (Version 12.0.1, SAS Institute, Cary, NC) with one-way analysis of variance and Tukey's HSD used to compare treatment means. Difference in treatment means were determined at 5% significance level.

5.3. Results and discussion

5.3.1. 3-Deoxyanthocyanin profile from sorghum leaves and leaf sheaths

There were distinct differences of 3-DXA profiles between the leaf sheath and leaves (Figure 10, Table 8). The sheath extract was predominantly apigeninidin, whereas the leaf extract was predominantly luteolinidin derivatives (Figure 10, Table 8). The profiles of the sheath and leaf agree with previous findings (Kayodé et al., 2011; Petti et al., 2014). Instead of describing the extracts by source, the extracts will be named for the pigment profile for the remainder of this work. The sheath extract will be API-EX (apigeninidin-dominant extract) and the leaf extract will be LUT-EX (luteolinidin-dominant extract).

The pigment profile is of interest because the structure of the pigments can affect the extent to which they interact with the hydrocolloids. Apigeninidin has 1 B-ring -OH and luteolinidin has 2 B-ring -OH, making apigeninidin more hydrophobic than luteolinidin. Thus, solutions that are made with API-EX will probably be more strongly stabilized via hydrophobic mechanisms than LUT-EX solutions. Crude extracts were used (instead of pure 3-DXA standards) to more accurately mimic the effect of naturally derived 3-DXA extracts in products.

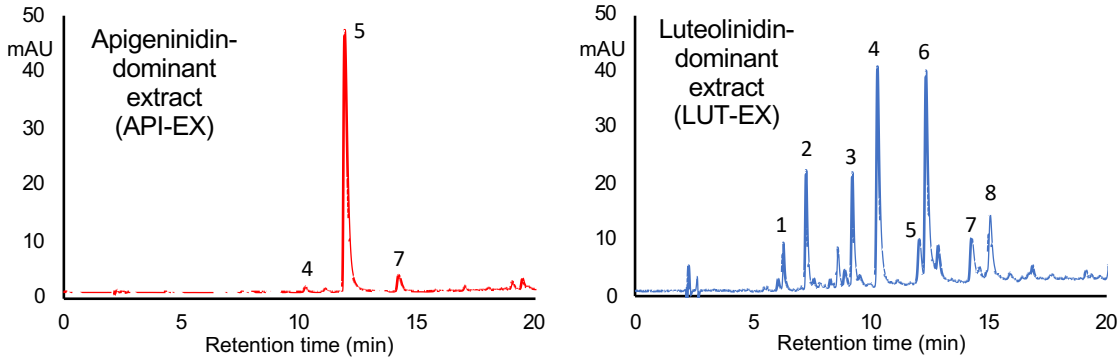


Figure 10 3-Deoxyanthocyanin profiles (480 nm) of sorghum sheath and leaf extract. Peak identity is listed in Table 8.

Table 8 3-Deoxyanthocyanins in sorghum leaf sheaths and leaves.

Peak no.	Retention time (t_R)	λ_{max} nm	$[M+H]^+$	Compound ID
1	6.29	485	433	Luteolinidin glucoside
2	7.63	470	417	Apigeninidin glucoside
3	9.18	468	431	7-O-Methyl-luteolinidin
4	10.32	485	271	Luteolinidin
5	12.03	474	255	Apigeninidin
6	12.36	485	285	7-O-Methyl-luteolinidin
7	14.28	470	269	7-O-Methyl-apigeninidin
8	15.03	489	299	5,7-O-Methyl-luteolinidin

5.3.2. Improvement of aqueous stability of 3-deoxyanthocyanins by amphiphilic polysaccharides

The work of Herrman (2016) established the effect of amphiphilic polysaccharides on the aqueous stability of 3-DXA. This section will provide a summary of the methodology and results used to determine the effect of gum arabic and sodium alginate on the aqueous stability of 3-DXA. This preliminary work is the background information for the mechanistic data collected in this Chapter.

To investigate the effect of gum arabic and sodium alginate on the aqueous stability of 3-DXA, solutions with crude 3-DXA extracts (similar to the extracts described in the previous section) and 0.5 g/L or 1.0 g/L gum were store for 10 weeks in pH 3 or 5 buffer. The aqueous stability was determined by the % absorbance retention at λ_{max} compared to the initial solutions. A portion of the results are summarized in Figure 11.

The 3-DXA in solutions without added polysaccharides almost completely precipitated after 10 weeks (Figure 11). The addition of gum arabic or sodium alginate generally improved aqueous stability of 3-DXA compared to solutions without a polysaccharide (Figure 11). Gum arabic was more effective at stabilizing the API-EX (75% avg) compared to LUT-EX extract (5% avg). Gum arabic also stabilized 3-DXA to a greater extent at pH 5 (50% avg) than pH 3 (30% avg). The increase of gum concentration from 0.5 g/L to 1.0 g/L did not improve the extent to which 3-DXA were stabilized in the solution by gum arabic. Sodium alginate, on the other hand, stabilized 3-DXA from the LUT-EX (68% avg) to a greater extent than API-EX (40% avg) (Figure 11). Sodium alginate was consistently more effective at pH 5 (74% avg) compared to pH

3 (34%). The increase of sodium alginate concentration from 0.5 g/L to 1.0 g/L also did not improve the extent to which 3-DXA were stabilized in solution.

The differences in stabilizing effect on 3-DXA by the polysaccharides suggested that different mechanisms were contributing to the aqueous stability. Both intrinsic and extrinsic factors affected the effective of either polysaccharide. To start, the pH of the solution will affect both the structure of 3-DXA and the structure of the gum. At a lower pH, a greater proportion of 3-DXA will be flavylium cations whereas the gums will have a relatively greater proportion of protonated carboxylic acid moieties (more neutral charge). At pH 5, a greater proportion of 3-DXA exist as quinoidal bases or carbinol pseudobases (Raymond Brouillard, 1982), and a greater proportion of deprotonated carboxylic acid groups exist within the polysaccharide structure (greater proportion of negative charge). Perhaps the more neutral forms of 3-DXA at pH 5 more readily associated with the gums, especially with the hydrophobic glycoproteins of gum arabic (Howard et al., 2013). The greater stability of API-EX compared to LUT-EX by gum arabic suggests the larger hydrophobic moiety of apigeninidin (Figure 1) more readily interacts with gum arabic hydrophobic glycoproteins that luteolinidin.

It is likely that hydrophobic interactions are a major driver of the interaction between gum arabic and 3-DXA, where less polar 3-DXA interact to a greater extent with gum arabic. Extracts higher in apigeninidin thus appear to be more effectively stabilized with gum arabic; this combination would thus be more soluble in beverage systems where the increase in viscosity is undesirable (e.g. sports drinks).

With sodium alginate, the greater effectiveness at pH 5 was likely due to the electrostatic repulsion of the gum which prevented self-association, allowing for greater interaction with 3-DXA. The stabilizing effect of sodium alginate on the LUT-EX suggests that in addition to hydrophobic interactions, hydrogen bonding (or other non-specific van der Waals interactions) likely contributed to interaction between the gum and 3-DXA. Thus, both gum arabic and alginate appear to effectively improve aqueous stability of 3-DXA, but by different mechanisms.

The mechanism of stability as affected by 3-DXA and polysaccharide structure is important to understand to better predict their behavior and design formulations that maximize their functionality as colorants.

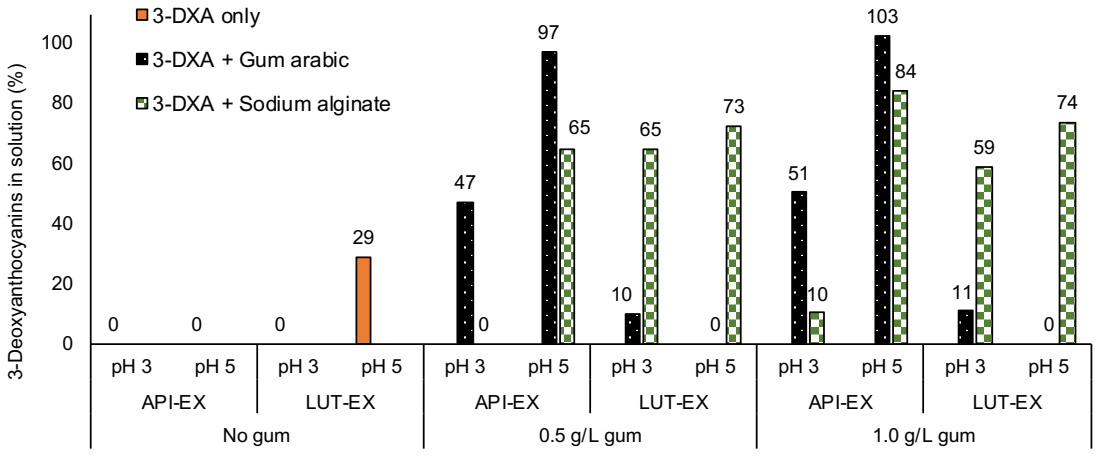


Figure 11 Effect of gum arabic and sodium alginate on aqueous stability of 3-deoxyanthocyanins in apigeninidin-dominant extract (API-EX) or luteolinidin-dominant extract (LUT-EX). Bars and numbers indicate % pigments in solutions after 10 weeks in pH 3 and 5. Adapted from Herrman (2016).

5.3.3. Effect of gum arabic and sodium alginate on viscosity of 3-deoxyanthocyanin solutions

The viscosity of the 3-DXA-polysaccharide solutions was measured to determine if viscosity was a contributing factor to 3-DXA aqueous stability. The viscosity of the 3-DXA-gum arabic solutions were not significantly different than the control (Table 9). In contrast, the 3-DXA-sodium alginate solutions were more viscous than the control solutions with slightly higher viscosity values recorded at pH 5 than 3 at both concentrations (Table 9). Negative charge of alginate will be greater at pH above pKa (~3.5) and therefore charge repulsion (preventing precipitation) will enhance water holding capacity at pH 5 vs pH 3.

The increase in viscosity by sodium alginate suggests that viscosity contributed to its stabilizing effect on 3-DXA although to a minor extent, given 1.0 g/L was not significantly more effective than 0.5 g/L alginate (Figure 11). Emulsion stabilizers increase the viscosity of the continuous phase and prevent separation of immiscible phases by slowing down the movement due to gravity or Brownian motion (McClements, 2007). Although viscosity could explain some of the stabilizing effect of alginate on 3-DXA, it does not account for the differences in stability between the API-EX and LUT-EX samples. It is likely that hydrogen bonding in addition to the stabilizing effect of viscosity contributed to the improved aqueous stability of 3-DXA by sodium alginate, especially considering its distinctly superior stabilizing effect on LUT-EX at pH 3 (Figure 11).

With gum arabic, because viscosity was similar for each solution (Table 9), the stability data (Figure 11) suggest it partitioned between the 3-DXA and aqueous phase.

The functionality of gum arabic with 3-DXA is derived from its amphiphilic structure including hydrophobic glycoproteins and hydrophilic polysaccharide chains, meaning it can interact with both hydrophilic and hydrophobic components, creating a more homogenous solution. The effectiveness of gum arabic with 3-DXA, especially with API-EX, suggest hydrophobic interactions contributed to the aqueous stability of 3-DXA.

Table 9 Effect of gum arabic or sodium alginate on the viscosity of aqueous 3-deoxyanthocyanin solutions.

	g/L polysaccharide	pH	Viscosity (mPa/s)
Control	0	3	1.10 ± 0.02
		5	1.13 ± 0.03
Gum arabic	0.5	3	1.13 ± 0.07
		5	1.18 ± 0.07
	1.0	3	1.14 ± 0.04
		5	1.13 ± 0.04
Sodium alginate	0.5	3	1.23 ± 0.04
		5	1.30 ± 0.06*
	1.0	3	1.39 ± 0.03*
		5	1.49 ± 0.06*

Data are expressed as mean ± standard deviation. *Indicates significant difference from control at the same pH (Dunnett's HSD, $p<0.05$).

5.3.4. Effect of 3-deoxyanthocyanins on particle size in gum arabic solutions

Particle size distribution of the gum arabic and 3-DXA solutions is shown in Figure 12 and Table 10. With gum arabic at 0.5 g/L, average particle size increased with the addition of the 3-DXA extracts and was higher for solutions made with LUT-EX than API-EX. The average particle size of gum arabic in solution was 178 nm at pH 3 or 145 nm at pH 5. The addition of the API-EX increased particle size to 543 and 512 nm at pH 3 and 5, respectively. Particle size with the LUT-EX addition to gum arabic solution was 1,192 nm at pH 3 and 673 nm at pH 5.

The average particle size within a solution is an indicator of colloidal stability. A larger particle size is generally indicative of a less stable solution. Larger particles can have a higher mass that causes settling and a larger size allowing for greater attractive forces that contribute to agglomeration and subsequent precipitation (Piorkowski & McClements, 2014). As shown in Figure 11, gum arabic-API-EX formed more stable complexes than gum arabic-LUT-EX complexes, evidenced by smaller particle size (Figure 12, Table 10).

Notably, average particle size was higher in LUT-EX-gum arabic solutions compared to API-EX-gum arabic solutions. This may be due to more extensive hydrogen bonding of luteolinidin (dihydroxylated B-ring) to gum arabic, perhaps inducing non-covalent hydrocolloid cross-linking compared to apigeninidin (mono-hydroxylated B-ring) resulting in a bigger particle. The lower average particle size of LUT-EX-gum arabic at pH 5 compared to pH 3 may be due to the greater charge repulsion of gum arabic at pH 5 over pH 3, reducing the effect of hydrocolloid non-covalent cross-linking.

A change in particle size indicates if two components interacted to form a new complex. Guan and Zhong reported a 2-fold increase in particle diameter when gum arabic and anthocyanins were combined compared to gum arabic alone (Guan & Zhong, 2015). The increase in particle size based on our work therefore confirms that the 3-DXA and gum arabic combined to form new complexes within the aqueous solution. The fact that gum arabic did not stabilize LUT-EX at either pH despite forming a complex suggests the interactions were weak and transitional, implying hydrophobic interactions with gum arabic protein core were negligible. On the other hand, the strong stabilizing effect of gum arabic on API-EX suggests hydrophobic interaction was a major driver of gum arabic-API-EX complex formation.

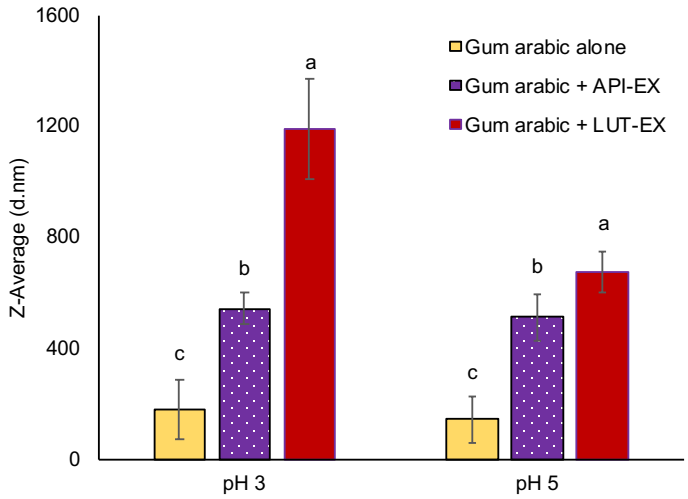


Figure 12 Effect of 3-deoxyanthocyanins on average particle size of gum arabic (0.5 g/L) solutions. Error bars illustrate ± standard deviation. Different letters indicate significant difference between treatments (Tukey's HSD, $p<0.05$).

Table 10 Effect of 3-deoxyanthocyanins on average particle size in gum arabic solutions.

pH	Extract	Average particle size (d.nm)
3	Control	178 ± 108[c]
	API-EX	543 ± 55[b]
	LUT-EX	1192 ± 182[a]
5	Control	145 ± 84[c]
	API-EX	512 ± 81[b]
	LUT-EX	673 ± 74[a]

API-EX = apigeninidin-dominant extract. LUT-EX = luteolinidin-dominant extract. Data are expressed as mean ± standard deviation. Different letters indicate significant difference (Tukey's HSD, $p<0.05$).

5.3.5. Effect of 3-deoxyanthocyanins on zeta potential in gum arabic solutions

The zeta potential (ZP) of the 3-DXA-gum arabic solutions is shown in Figure 13 and Table 11. The ZP of the gum arabic solutions was -5.99 mV at pH 3 and -7.17 mV at pH 5. ZP became more negative with the addition of 3-DXA extracts. At pH 3, ZP decreased to -12.30 with API-EX and -11.86 with LUT-EX. At pH 5, ZP decreased to -12.58 with API-EX and -12.17 with LUT-EX.

Zeta potential is a measure of the electric bilayer surrounding a particle within a solution (Bhattacharjee, 2016). A ZP that is higher in magnitude (either positive or negative) indicates a stronger electrostatic repulsion between particles. The general theory is that ZP [absolute] values of 0-10 are highly unstable, 11-20 are relatively stable, 20-30 are moderately stable, and 30+ are highly stable (Bhattacharjee, 2016). By this definition,

the absolute values of ZP of the 3-DXA-gum arabic solutions falls within the relatively stable range. The evidence thus suggests that electrostatic repulsion was partially responsible for reducing 3-DXA-gum arabic complex precipitation. Since the ZP values were not very high, the stabilizing effect of gum arabic on 3-DXA must be attributed to other factors like steric hindrance. Due to its extensive branching, steric hindrance is usually the primary driver of solution stability by gum arabic (Chanamai & McClements, 2002; Piorkowski & McClements, 2014). The ZP values, however, do not explain the difference in stability of API-EX-gum arabic solutions compared to LUT-EX-gum arabic solutions because they were not significantly different. There must be a difference in the interaction and/or strength of interaction between the 3-DXA and gum arabic, based on 3-DXA structure, that further contributes to instability.

The decrease in ZP (a more negative value) for the gum arabic samples with the addition of the 3-DXA extracts suggests that there was a conformational change of gum arabic upon addition of 3-DXA. The charge contribution of 3-DXA at pH 3 and 5 should be negligible and thus the negative charge is derived from the structure of gum arabic (Guan & Zhong, 2015). Carboxylic groups within gum arabic would carry a negative charge at pH 3 and to a greater extent at pH 5 ($pK_a \approx 3.5$). Because there was a change in ZP upon the addition of 3-DXA, gum arabic likely encapsulated the 3-DXA and a conformational change occurred to expose more negative groups within gum arabic into the continuous phase. However, as previously mentioned, it appears that LUT-EX does interact with gum arabic initially but only weak complexes that do not remain stable over time are formed (Figure 11).

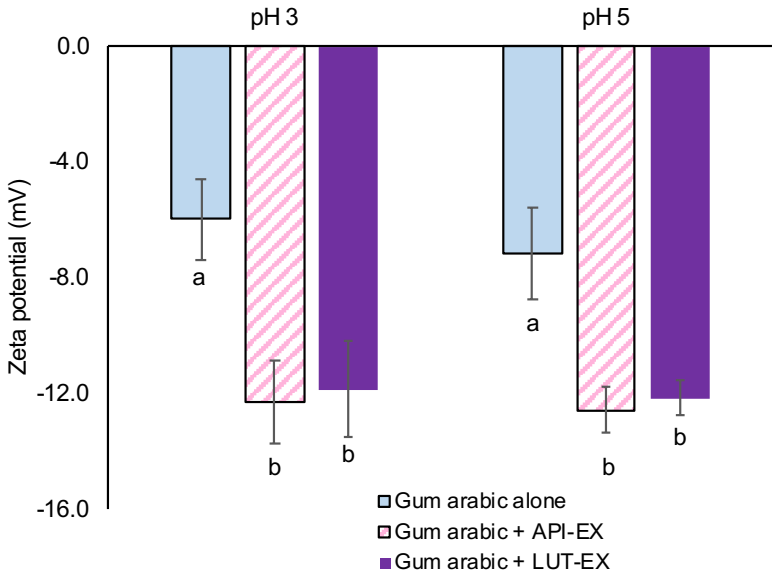


Figure 13 Effect of 3-deoxyanthocyanins on the zeta potential of gum arabic (0.5 g/L) solutions. Error bars illustrate ± standard deviation. Different letters indicate significant difference (Tukey's HSD, $p<0.05$).

Table 11 Effect of 3-deoxyanthocyanin extracts on the zeta potential in gum arabic aqueous solutions.

pH	Extract	Zeta potential (mV)
3	Control	-5.99 ± 1.41[a]
	API-EX	-12.30 ± 1.42[b]
	LUT-EX	-11.86 ± 1.68[b]
5	Control	-7.17 ± 1.58[a]
	API-EX	-12.58 ± 0.81[b]
	LUT-EX	-12.17 ± 0.62[b]

API-EX = apigeninidin-dominant extract. LUT-EX = luteolinidin-dominant extract. Data are expressed as mean ± standard deviation. Different letters indicate significant difference (Tukey's HSD, $p<0.05$).

5.3.6. Fluorescence quenching of gum arabic by 3-deoxyanthocyanins

In fluorescence quenching experiments, the fluorescence intensity of a compound is measured in the presence and absence of a quencher. The degree of fluorescence intensity loss in the presence of a quencher can give insight into the interaction mechanism between a fluorescent compound and a quencher, and the extent of the interaction relative to other compounds. In this work, the fluorescence intensity of gum arabic was measured against increasing concentration of apigeninidin and luteolinidin. Within the structure of gum arabic, there are glycoproteins that contain the fluorescent amino acid tyrosine. The glycoproteins are considered to be the primary site of interaction with other hydrophobic compounds (Reiner, Reineccius, & Peppard, 2010). This work therefore measured the change in fluorescence due to the interaction of tyrosine with 3-DXA. Changes in fluorescent behavior of gum arabic can be an indication of complex formation between gum arabic and the 3-DXA (quencher).

The fluorescence quenching of gum arabic by 3-DXA is shown in (Figure 14). There was an inverse relationship between fluorescence intensity of gum arabic and the concentration of apigeninidin and luteolinidin at both pH 3 and 5. The inverse relationship suggests an interaction between gum arabic and the 3-DXA occurred that could have been due to either collisional (dynamic) fluorescence quenching or binding-related (static) fluorescence quenching (Lakowicz, 2006). Dynamic quenching is the result of non-specific quenching in a system, when random diffusion and interactions cause a decrease in fluorescence intensity. Static quenching indicates that a stable complex was formed, and that the decrease in fluorescence intensity is attributed to the blocking of UV light by

the quencher to the fluorescent compound and subsequent reduced fluorescence emission. The distinction between dynamic or static quenching can be made with the calculation of k_q, the bimolecular quenching rate constant, from the Stern-Volmer equation (Equation 6):

Equation 6

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q \tau_0 [Q]$$

where F_0 and F are the fluorescence emission intensity of the glycoprotein (gum arabic) without and with the quencher, respectively. $[Q]$ is the concentration of the quencher (3-DXA). K_{SV} is the Stern-Volmer quenching constant, and τ_0 is the average lifetime of the fluorophore in the absence of quencher. For tyrosine, this has been reported to be around 3.37 ns (Guzow et al., 2004). Plotting F_0/F *versus* 3-DXA concentration can be used to calculate K_{SV} from the slope of the linear plot (Figure 15).

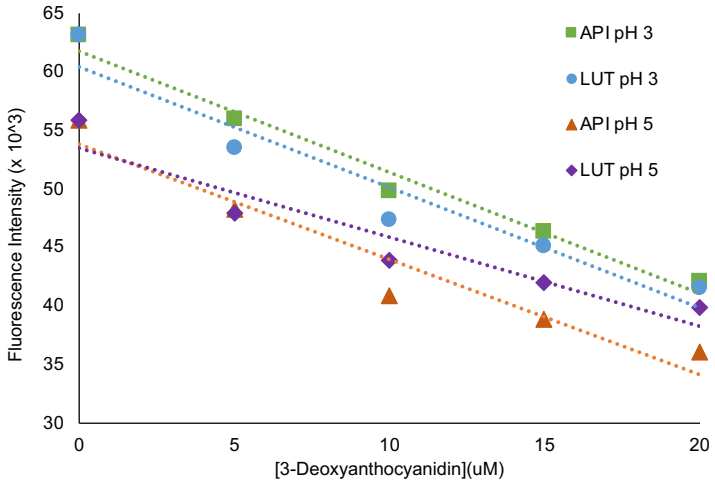


Figure 14 Effect of apigeninidin (API) and luteolinidin (LUT) on fluorescence intensity of gum arabic.

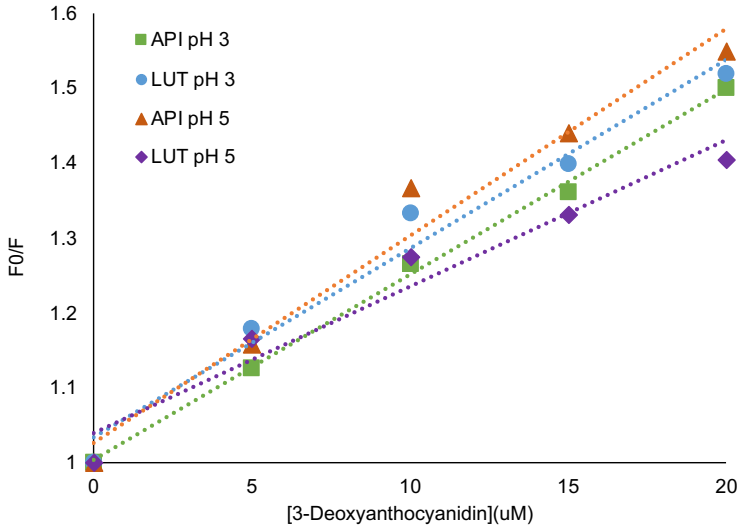


Figure 15 Stern-Volmer plot of gum arabic fluorescence intensity change with increasing apigeninidin (API) or luteolinidin (LUT) concentration.

The type of quenching can be distinguished by comparing the bimolecular quenching constant (k_q) from Equation 6 to the maximum value for diffusion limited quenching in water, i.e., 10^{10} $M^{-1}s^{-1}$ (Lakowicz, 2006). If the k_q is above this value, it suggests there is a specific interaction occurring between fluorophore and quencher, and thus, the quenching is static rather than dynamic (Chung, Rojanasasithara, Mutilangi, & McClements, 2015). The values of k_q are shown in (Table 12). Each k_q was above 10^{10} $M^{-1}s^{-1}$, indicating the quenching mechanism was static. This confirms that the 3-DXA formed a complex with gum arabic in the solution. Chung et al. (2016) reported static quenching occurred between gum arabic and purple carrot anthocyanins in model beverage solutions. Similarly, Guan and Zhong (2015) reported binding-related quenching of gum arabic with anthocyanins at pH 5.

Table 12 Fluorescence quenching Stern-Volmer constant, bimolecular quenching constant, and binding constant for gum arabic and 3-deoxyanthocyanin solutions.

	pH 3		pH 5	
	Apigeninidin	Luteolinidin	Apigeninidin	Luteolinidin
K_{SV} (M^{-1})	24.7 x 10^3	25.2 x 10^3	27.7 x 10^3	19.5 x 10^3
k_q ($M^{-1}s^{-1}$)	7.3 x 10^{12}	7.5 x 10^{12}	8.2 x 10^{12}	5.8 x 10^{12}
Log$K$ (M^{-1})	4.28	3.24	3.96	2.58
K (M^{-1})	19.2 x 10^3	1.72 x 10^3	9.08 x 10^3	0.38 x 10^3
n	0.976	0.892	0.749	0.643

K_{SV} is the Stern-Volmer quenching constant. k_q is the bimolecular quenching constant. Log$K$ (M^{-1}) is the y-intercept of Figure 16. K (M^{-1}) is the binding constant. n is the number of binding sites.

For static quenching, the apparent binding constant (*K)* and number of binding sites (n) can be calculated using Equation 7 (van de Weert & Stella, 2011):

Equation 7

$$\log\frac{F_0-F}{F} = \log K + n\log[Q]$$

F_0 and F are the fluorescence emission intensity of the protein without and with the 3-DXA quencher, respectively. From the plot of log [$(F_0 – F)/F$] vs log $[Q]$, both the binding constant (y-axis intercept) and the number of binding sites (slope) can be determined (Figure 16, Table 12).

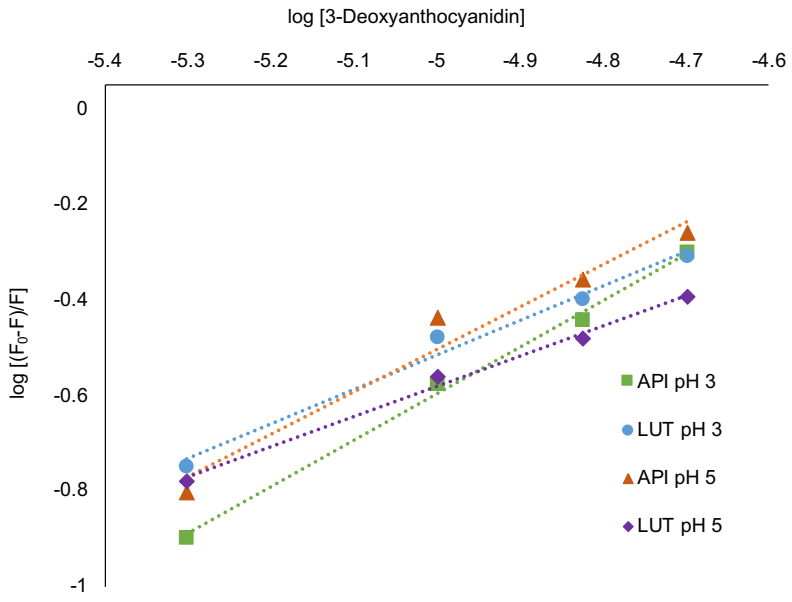


Figure 16 Plot of log [(F0-F)/F] versus log [3-deoxyanthocyanidin]. API = apigeninidin, LUT = luteolinidin.

The quenching constant and apparent binding constant for apigeninidin ($9.08x10^3$ to $19.2x10^3$ M^{-1}) was greater than that of luteolinidin ($0.38x10^3$ to $1.72x10^3$ M^{-1}) at both pH 3 and 5 (Table 12). A lower binding constant indicates that either the binding between luteolinidin and gum arabic was less favorable or the complex was less stable than that between apigeninidin and gum arabic (Acharya, Sanguansri, & Augustin, 2013). A higher binding constant therefore indicates greater affinity of apigeninidin to gum arabic than luteolinidin, and thus explains the greater aqueous stability of API-EX in gum arabic solutions than the LUT-EX as observed by Herrman (2016) (Figure 11).

The greater binding affinity of gum arabic to apigeninidin suggests that hydrophobic interactions were the dominant interaction mechanism between gum arabic and 3-DXA. Apigeninidin has a larger hydrophobic moiety within its structure because it has a single -OH substitution on the B-ring compared to luteolinidin that has two -OH substitutions on the B-ring (Figure 1).

The primary interaction mechanism between anthocyanins and tryptophan, another hydrophobic amino acid, was reported to be hydrophobic (Chung et al., 2017). Similarly, additional hydroxyl groups on the anthocyanin B-ring and an increase in pH decreased the interaction (lower association constant, K_a) of anthocyanins with human serum albumin (HSA), confirming dominant hydrophobic interactions (Cahyana & Gordon, 2013).

In a study of fluorescence quenching of human serum albumin (HSA) by phenolic acids, Zhang et al. (2018) reported that the number of hydroxy groups affected the binding constant between phenolic acids and HSA. Phenolic acids with one hydroxyl group had a higher binding affinity to HSA than two or three hydroxyl groups, suggesting that the reduced hydrophobicity of phenolic acids with multiple hydroxyl groups reduced accessibility to hydrophobic regions of HSA (Zhang et al., 2018).

The fluorescence quenching data showed that 3-DXA interacted with gum arabic to form a complex (static binding), and that apigeninidin interacted to a greater extent than luteolinidin. The greater affinity of apigeninidin to gum arabic helps explain the previously described stability data (Figure 11) that showed API-EX was stabilized to a greater extent than LUT-EX. Furthermore, the static quenching mechanism confirms that the increase in particle size of gum arabic solutions with the addition of 3-DXA was due

to the two compounds interacting to form a larger structure. The potential entrapment of 3-DXA in a hydrophobic core of gum arabic may have induced a structural change that caused negatively charge acidic groups on the gum arabic structure to be exposed to the outside solution, leading to the previously described increase of zeta potential upon addition of 3-DXA. The evidence indicates that the 3-DXA composition of sorghum pigments is a major driver of their interaction with stabilizing hydrocolloids. For example, pigment sources higher apigeninidin derivatives should be selected as colorants when gum arabic is used as a stabilizer in beverage products. Additional work to establish the interaction of diverse 3-DXA profiles with different hydrocolloids is needed.

5.3.7. Effect of sodium chloride on fluorescent quenching of gum arabic by 3-deoxyanthocyanidins

Sodium chloride (0-0.5M) was added to gum arabic-3-DXA solutions to determine the contribution of electrostatic interactions on the fluorescent quenching of gum arabic by 3-DXA and, by extension, complex formation between the two components. Overall, the addition of sodium chloride did not have an effect on the quenching of gum arabic by 3-DXA at either pH 3 (i.e. did not destabilize the complex) (Figure 17) or pH 5 (Figure 18). Therefore, electrostatic interactions were a not major contributor to the interaction between gum arabic and 3-DXA. These results align with the findings of Chung et al. (2016) reporting that the effect of sodium chloride on florescence quenching of gum arabic by anthocyanins was negligible (Chung et al., 2016). The resistance to complex

destabilization in the presence of salt suggests that gum arabic could effectively stabilize 3-DXA in beverages with relatively higher ionic strengths such as sports drinks.

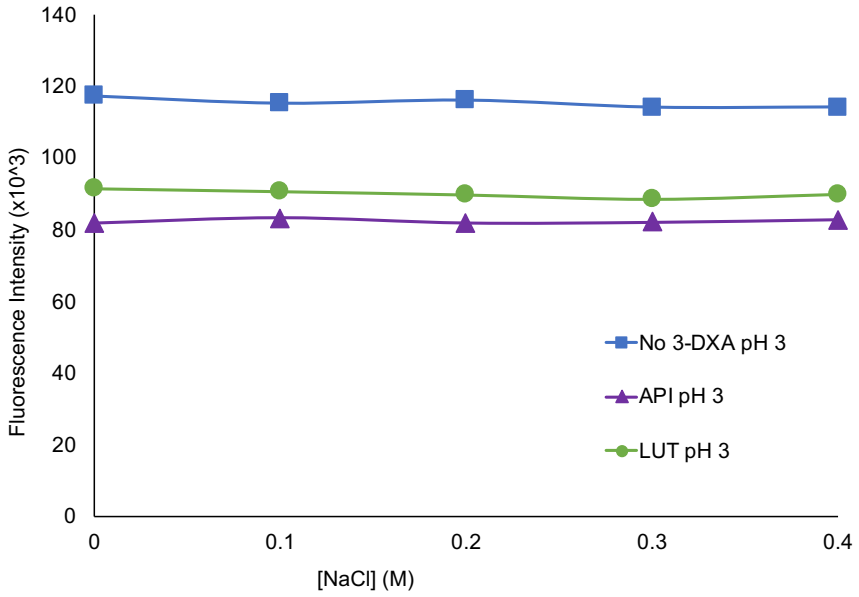


Figure 17 Effect of sodium chloride on fluorescence quenching of gum arabic by 3-deoxyanthocyanins at pH 3. API = apigeninidin, LUT = luteolinidin.

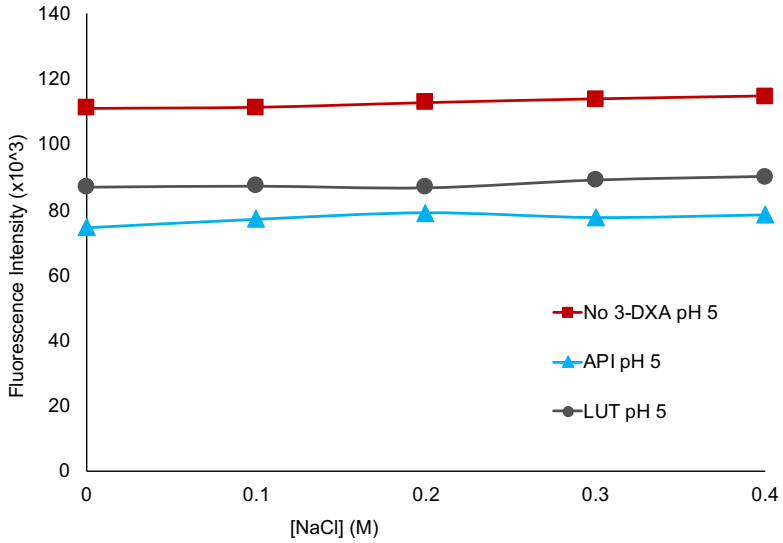


Figure 18 Effect of sodium chloride on fluorescence quenching of gum arabic by 3-deoxyanthocyanins at pH 5. API = apigeninidin, LUT = luteolinidin.

5.4. Conclusion

Gum arabic and sodium alginate act as stabilizers to 3-DXA to limit self-association to maintain pigment aqueous stability. The effectiveness of stabilization is dependent on gum structure, solution pH, and 3-DXA structure. The results of this work show that the mechanism by which gum arabic interact with 3-DXA in solution is via complex formation driven by hydrophobic interactions and to a lesser extent, hydrogen bonding. Electrostatic interactions do not appear to be a major mechanism by which gum arabic and 3-DXA interact. In beverages, both gum arabic and sodium alginate could be

used to stabilize 3-DXA, however, the choice of stabilizing gum should consider the 3-DXA profile to ensure the greatest stabilization of 3-DXA.

6. EFFECT OF PECTINS DIFFERING IN DEGREE OF ESTERIFICATION ON AQUEOUS STABILITY OF 3-DEOXYANTHOCYANINS

6.1. Introduction

The color of food is a major indicator of overall quality, but preserving color throughout the food manufacturing and handling process is a difficult task (Diehl, 2008; Thorngate, 2001). Synthetic and natural colorants are added to food products to mitigate this color deterioration or to impart an uncharacteristic, but desired, color to a food product. Synthetic colorants have specific advantages compared to natural colorants including consistent hues, high tinctorial strength, stability, cost, and ease of incorporation into foods (Kuntz, 2009; Thorngate, 2001). However, growing consumer desire for "clean label" natural ingredients has caused a shift in the industry toward the avoidance of synthetic ingredients in food products.

There are many different types of natural food colors, each with unique properties, such was water solubility, and hue (i.e. purples and reds of anthocyanins vs orange and yellow of carotenoids). One type of a natural color is sorghum 3-deoxyanthocyanins (3-DXA). These pigments are similar to the more widespread anthocyanins but lack substitution on the third carbon of the C-ring. This structural feature causes 3-DXA to have superior resistance to nucleophilic attack and structural degradation compared to anthocyanins when subjected to conditions common in food processing, such as heat (Yang et al., 2014) or ascorbic acid (Ojwang & Awika, 2008). The 3-DXA are not without limitations, however, because the same lack of substitution at the third carbon results in a

pigment that is more hydrophobic compared to the more common anthocyanins. The reduced hydrophilicity in combination with the fact that 3-DXA exist primarily in nature as aglycones, results in rapid self-association and eventual precipitation in aqueous solutions (Awika, 2011).

The use of food gums to enhance aqueous stability of hydrophobic compounds is widespread in the food industry. Food gums, or hydrocolloids, can function as emulsifiers or emulsion stabilizers to prevent the coalescence of hydrophobic compounds (Piorkowski & McClements, 2014). Emulsifiers are surface active and reduce the interfacial tension between immiscible compounds while emulsion stabilizers increase continuous phase viscosity and physically prevent the separation and aggregation of compounds (Dickinson, 2017). Pectin is a commonly used emulsifier and emulsion stabilizer (Dickinson, 2009; Verkempinck et al., 2018). It is an anionic heteropolysaccharide characterized by three major domains, homogalacturonan (HG), rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGII). HG is linear, composed of 1,4-linked alpha-D-galacturonic acids that can be methyl-esterified or acetylated, both of which influence pectin functionality and the degree to which pectin can interact with other ingredients through electrostatic interaction, hydrogen bonding, or hydrophobic interactions (Thakur, Singh, Handa, & Rao, 1997; Willats, Knox, & Mikkelsen, 2006).

With regards to the interaction mechanisms between pectin and anthocyanins, the primary mechanism is hydrogen bonding since low-methoxy pectins have been shown to stabilize anthocyanins to a greater extent than high methoxy pectins (Buchweitz et al., 2013b, 2013a). Anthocyanins with two or more hydroxyl groups on the B-ring interact to

a greater extent with pectin than anthocyanins with a monohydroxylated B-ring (Fernandes, Brás, Mateus, & de Freitas, 2014; Kopjar et al., 2009). Hydrophobic interactions play a minor role between pectin and anthocyanins. At higher pH, with a greater proportion of neutral anthocyanins (carbinol pseudobase or quinoidal base), hydrophobic interactions strengthen π-π stacking and contribute to a greater stabilizing effect on anthocyanins by pectin (Fernandes et al., 2014). Copigments, such as condensed tannins discussed in Chapter 4, could also have an effect on the aqueous stability of 3-DXA. Copigments could prevent interactions between 3-DXA and polysaccharides by blocking active sites of pectin from interacting with 3-DXA, or on the other hand, could potentially enhance the interaction and provide a synergistic-like effect on 3-DXA stabilization by pectin in aqueous solutions.

In Chapter 5, we showed that hydrophobic interactions were a primary mechanism by which 3-DXA interacted with gum arabic. However, it is likely that hydrogen bonding and other interactions contributed to the stabilizing effect of gum arabic. The objective of this work was to understand the relative contribution of hydrophobic interactions between amphiphilic polysaccharides and 3-DXA by determining the effect of pectin degree of esterification (DE) on the aqueous stability of 3-DXA. The DE of pectin influences the relative hydrophobicity (proportion of methylated carboxylic acid groups) of the polysaccharide and therefore can give insight into the type of interactions with different structures of 3-DXA. We hypothesize that high degree of esterification pectin (DE ≥ 50) will stabilize 3-DXA to a greater extent than low DE pectin. This work will explain the

relative extent of hydrophobic interactions required to stabilize 3-DXA in aqueous solutions by pectin.

6.2. Materials and methods

6.2.1. Sources of 3-deoxyanthocyanins

Two different sorghum grains and two different non-grain sorghum tissues were used as sources of 3-DXA. A sorghum leaf was obtained from Health Forever Products (Lagos, Nigeria) (Akogou, Kayodé, den Besten, Linnemann, et al., 2018; Geera et al., 2012) and sheath (Petti et al., 2014) were used because they have distinct pigment profiles (apigeninidin-dominant vs luteolinidin dominant, respectively). To determine the effect of tannin copigment on aqueous stability of 3-DXA, a non-tannin black sorghum and tannin black sorghum were also used (Texas A&M AgriLife Research Extension, College Station, TX). The grains have similar 3-DXA profiles but differ in the presence of condensed tannins (proanthocyanidins) (Chapter 3).

Sorghum grain was decorticated in 4.5 kg batches using a PRL mini-dehuller (Nutama Machine Company, Saskatoon, Canada) and separated with a KICE grain cleaner (model 6DT4-1, KICE Industries Inc., Wichita, KS) to obtain bran. The brans, sheaths, and leaves were milled to pass through a 1 mm screen using a UDY cyclone mill (model 3010−030, UDY Corporation, Fort Collins, CO) and stored at -20°C until use.

6.2.2. Chemicals and reagents

Citrus pectin with differing degrees of esterification (DE) were purchased from Sigma-Aldrich (St. Louis, MO) or provided by Danisco (Copenhagen, Denmark). Hydrochloric acid, methanol, ethanol, acetone, acetic acid, acetonitrile, and formic acid were purchased from VWR (Radnor, Pennsylvania). Folin-Ciocalteu reagent, ethanolamine, catechin, vanillin, and procyanidins B1 and C1 were purchased from Sigma-Aldrich (St. Louis, MO). Sephadex LH-20 was purchased from GE Healthcare (Uppsala, Sweden). Pure 3-DXA standards (≥95% purity) were purchased from AlsaChim (Statsbourg, France).

6.2.3. 3-Deoxyanthocyanin extraction

Ground sorghum tissue was extracted in 1% HCl in methanol (v/v%) for 2 h at room temperature on a shaker plate (VWR Shaker model 3500, speed #4). Solutions were centrifuged and the supernatant collected and stored at -20°C until use.

6.2.4. 3-Deoxyanthocyanin extract characterization

6.2.4.1. Total 3-deoxyanthocyanin, extracted phenols, and tannin quantification of sorghum tissues

Total pigment content (TPC) after extraction was quantified using a UV-Vis spectrophotometer (Shimadzu 2450, Shimadzu Scientific Instruments North America, Colombia, MD). Samples were diluted to an absorbance of 1 ± 0.05 at λ_{max} and scanned from 400-700 nm. Subtraction of the absorbance at 700 nm eliminated the effect of any

natural haze in the sample. Pigments were quantified using Equation 8 (Fuleki & Francis, 1968) and converted to mg/g from the starting material:

Equation 8

$$Total\ 3\text{-}DXA\ Content = (abs*MW*DF)/(\varepsilon*g)$$

Abs is the absorbance at λ_{max} - $Abs_{700\ nm}$, and *MW* is molecular weight of luteolinidin, *DF* is the dilution factor, ε is the molar extinction coefficient of luteolinidin (31,700), and *g* is the extracted sample weight. Extractable phenols were determined by the modified Folin-Ciocalteu as previously described and expressed as mg/g gallic acid equivalents from a standard curve of the reaction with gallic acid (Barros et al., 2013). Tannins were estimated using the modified vanillin/HCl assay as described by Price, Van Scoyoc, & Butler (1978). Results were calculated from a standard curve with catechin and expressed as mg/g catechin equivalents.

6.2.4.2. 3-Deoxyanthocyanin and condensed tannin profile

The 3-DXA profile was determined using the HPLC and UPLC-MS methods described in section 4.2.6 and 3.2.5, respectively.

The tannin black sorghum was profiled for molecular weight distribution by the normal-phase HPLC-FLD method using conditions described by Girard, Castell-Perez, Bean, Adrianos, & Awika (2016). Catechin, procyanidin B1 and C1 were used to quantify monomers, dimers, and trimers, respectively. Quantitative data for PA with a degree of

polymerization (DP) greater than or equal to four was based on procyanidin C1 (DP 3) peak response as previously described by Ojwang, Yang, Dykes, & Awika (2013).

6.2.5. Pectin degree of esterification

The DE was verified using the method of Food Chemical Codex (Committee on Codex Specifications, 2003) with modifications. Each pectin (5 g) was stirred for 10 min with 0.13 N HCl 60% ethanol. The solution was then applied to a fritted-glass filter tube and washed with the same HCl-ethanol mixture, followed by washing with 60% ethanol, and finally ethanol. The sample was dried for 2.5 h at 105°C. After drying, 0.1 g of pectin was wetted with 2 mL ethanol (to prevent clumping) and dissolved in 100 mL deionized water. Five drops of phenolphthalein indicator were added, and the sample was titrated with 0.1 M NaOH (V_1) to neutralize the free carboxyl acids. Then 20 mL of 0.5 M NaOH was added, and the solution left to sit at room temperature for 15 min to saponify methoxyl groups. Then 20 mL 0.5 M HCl was added to neutralize the solution after which another 3 drops of phenolphthalein was added. A second titration was done with 0.1 M NaOH (V_2). DE was calculated using Equation 9:

Equation 9

$$\%DE = (V_2 / (V_1+V_2)) *100$$

The three pectins were found to have a DE of 38%, 54%, and 86% and are referred to as DE 38, DE 54, and DE 86 through the remaining sections, respectively.

6.2.6. Nitrogen content of pectins

Because protein can affect the emulsifying characteristics of pectin (Akhtar, Dickinson, Mazoyer, & Langendorff, 2002), we measured the protein content of each pectin. The nitrogen content of the pectins was determined using LECO combustion method as described by Sweeney and Rexroad (1987). A factor of 6.25 was used to estimate protein content using Equation 4 (Nakauma et al., 2008).

6.2.7. Effect of pectins on aqueous stability of 3-deoxyanthocyanins

Three different pH values (1, 3, and 5) were studied to determine the effect of pH on stabilizing effect of pectins on 3-DXA. The pH will influence the relative charge distribution within pectin and determine the predominant 3-DXA structure (i.e. proportion of flavylium cations to carbinol pseudobases or other structures). Buffer solutions were prepared using potassium chloride/hydrochloric acid (pH 1), and sodium citrate/citric acid (pH 3 and pH 5). Separate 10 g/L stock solutions of the three different pectins were made and left to stir overnight for complete solubilization. Solutions were made to a final pectin concentration of 0.1 g/L, 0.5 g/L, or 1 g/L in buffer. Sodium benzoate and potassium sorbate were added for a final concentration of 0.01% to suppress microbial growth in each treatment. Each sample was sonicated for 30 s using a tip probe sonicator (VibraCell 40, Sonics & Materials Inc. Danbury, CT) to ensure homogeneity before being scanned

on a spectrophotometer at 400-700 nm. All solutions were formulated to have an absorbance of ~1 at λ_{max} on Day 0 and stored in the dark at room temperature. Aqueous stability of pigments was expressed as percent absorbance at λ_{max} after a given time relative to the initial absorbance reading (Equation 10):

Equation 10

$$\% \text{ Retained Absorbance} = [(Z - 700_z) / (W_0 - 700_{W0})] * 100$$

Where Z is absorbance at λ_{max} recorded at time point, 700_z is absorbance at 700 nm at time point, W_0 is absorbance at λ_{max} at time zero (initial reading), and 700_{W0} is absorbance at 700 nm at time zero (initial reading).

6.2.8. Viscosity

The viscosity of 3-DXA-pectin solutions was measured with a rheometer (Haake Rheostress 6000) equipped with a temperature controller (Peltier TC-81) using cone and plate geometry (60 mm, 1° angle, 52 nm gap). Flow properties were evaluated at shear rates of 1-500 s^{-1} for 30 s. Temperature was maintained at 23°C.

6.2.9. Zeta potential and particle size of pectin-3-deoxyanthocyanin solutions

Particle size and zeta potential were determined by dynamic light scattering (DLS) with a Malvern Nano ZA zeta-sizer (Malvern Instrument Ltd., UK) with a He/Ne laser

(lambda = 633 nm) at a fixed scattering angle of 173° at 25°C (± 0.1°). Aliquots of the 3-DXA-pectin solutions were diluted 4X with appropriate buffer to reduce multiple scattering phenomena. The zeta-potential values were automatically calculated from the electrophoretic mobility based on the Smoluchowski model (Tan et al., 2018).

6.2.10. Statistical analysis

Each experiment was carried out in at least triplicate. Analysis of means was done using JMP pro 12 (Version 12.0.1, SAS Institute, Inc., Cary, NC) with one-way Analysis of Variance (ANOVA). Post Hoc tests (Tukey-Kramer HSD and Dunnett's test) were used to compare treatment means ($p < 0.05$).

6.3. Results and discussion

6.3.1. Characterization of 3-deoxyanthocyanin extracts

Higher 3-DXA pigment yields were obtained from the non-grain sources compared to the brans (Table 13). The leaf sheath and leaf yielded 64.9 and 17.8 mg/g luteolinidin equivalents, respectively, whereas the non-tannin bran (NT-EX) and tannin (T-EX) bran extracts yielded 6.9 and 2.9 mg/g luteolinidin equivalents, respectively. The values agreed with previous findings (Herrman et al., 2020; Kayodé et al., 2011; Petti et al., 2014). The 3-DXA are readily extracted from non-grain tissue but extraction efficiency of 3-DXA from bran is relatively poor due to the extensive crosslinking of the grain cell wall as observed in Chapter 3.

Extractable phenols followed a similar pattern as 3-DXA pigment yield with non-grain tissue containing higher phenols than bran tissue (Table 13). Extracted phenols are of relevance because of their potential copigmentation effect on the color properties of 3-DXA (Awika, 2008). As expected, the tannin bran (a type III sorghum) was the only plant material with a significant quantity of tannins (37.6 mg/g catechin eq.). The condensed tannins primarily had a degree of polymerization >10 (Figure 19), which agrees with previous reports (Girard et al., 2016). In Chapter 4, we showed that sorghum tannins can significantly affect the stability of 3-DXA. Thus, tannins could also be copigments to 3-DXA in the presence of pectin.

Table 13 Total 3-deoxyanthocyanins, extracted phenols, tannins, distribution of 3-deoxyanthocyanins of sorghum extracts.

Source	3-DXA (mg/g)*	Extracted phenols (mg/g)**	Tannins (mg/g)+
Sheath (apigeninidin-dominant)	64.9 ± 0.28[a]	68.4 ± 3.67[a]	3.8 ± 1.91[b]
Leaf (luteolinidin-dominant)	17.8 ± 0.05[b]	51.9 ± 2.43[b]	2.7 ± 1.91[b]
Non-tannin bran	6.9 ± 0.01[c]	28.3 ± 1.69[d]	4.9 ± 2.10[b]
Tannin bran	2.9 ± 0.01[d]	36.2 ± 2.67[c]	37.6 ± 0.47[a]

*Total pigments quantified by UV-VIS spectroscopy as mg/g luteolinidin equivalents; **Extracted phenols measured by modified Folin-Ciocalteu as mg/g gallic acid equivalents; +Tannins measured by modified vanillin/HCl assay as mg/g catechin equivalents. Data are expressed as mean ± standard deviation. Different letters within column indicate significant difference (Tukey's HSD, $p < 0.05$).

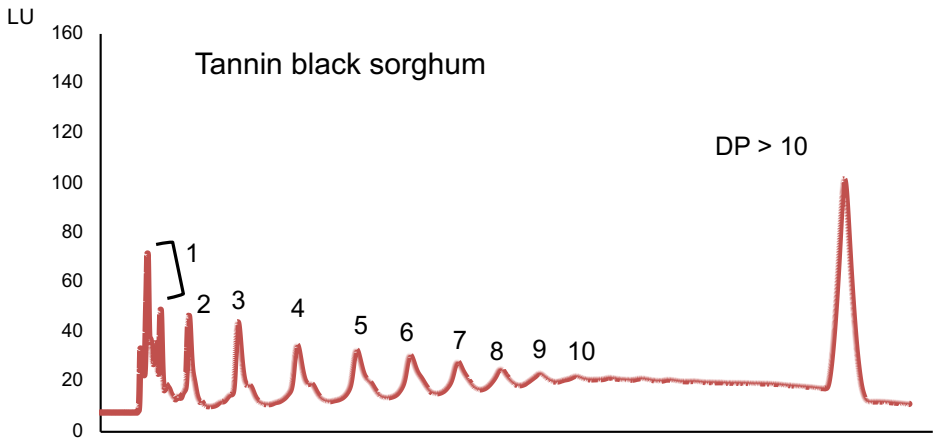


Figure 19 Normal phase HPLC profile of condensed tannins from tannin black sorghum bran. Condensed tannins were extracted from ground bran using 70% acetone. Fluorescence was measured at excitation wavelength of 230 nm and emission at 321 nm. The numbers above the peaks indicate the degree of polymerization (DP).

Profiling of 3-DXA revealed distinct differences in pigment profile from each extract (Figure 20, Table 14, Table 15). The sheath extract was composed predominately of apigeninidin and 7-O-methyl-apigeninidin (Figure 20A). In contrast, the majority of the of 3-DXA in the leaf extract were luteolinidin, 7-O-methyl-luteolinidin, or 3-DXA glucosides (Figure 20B). Because these two extracts differ significantly in 3-DXA profile, they will be referred to as apigeninidin-dominant extract (API-EX) and luteolinidin-dominant extract (LUT-EX) for the sheath and leaf extracts, respectively. Apigeninidin and luteolinidin differ by B-ring substitution pattern; apigeninidin has a single hydroxyl substitution on its B-ring whereas luteolinidin is dihydroxylated (Figure 1). A higher number of B-ring -OH groups have been found to affect the interaction of anthocyanins

with pectin (Buchweitz et al., 2013a). Delphinidin, with 3 -OH, was shown to interact more strongly with low methoxy pectin than cyanidin (2 -OH), presumably because the additional hydroxyl groups increases hydrogen bonding between pectin and anthocyanins (Fernandes et al., 2014).

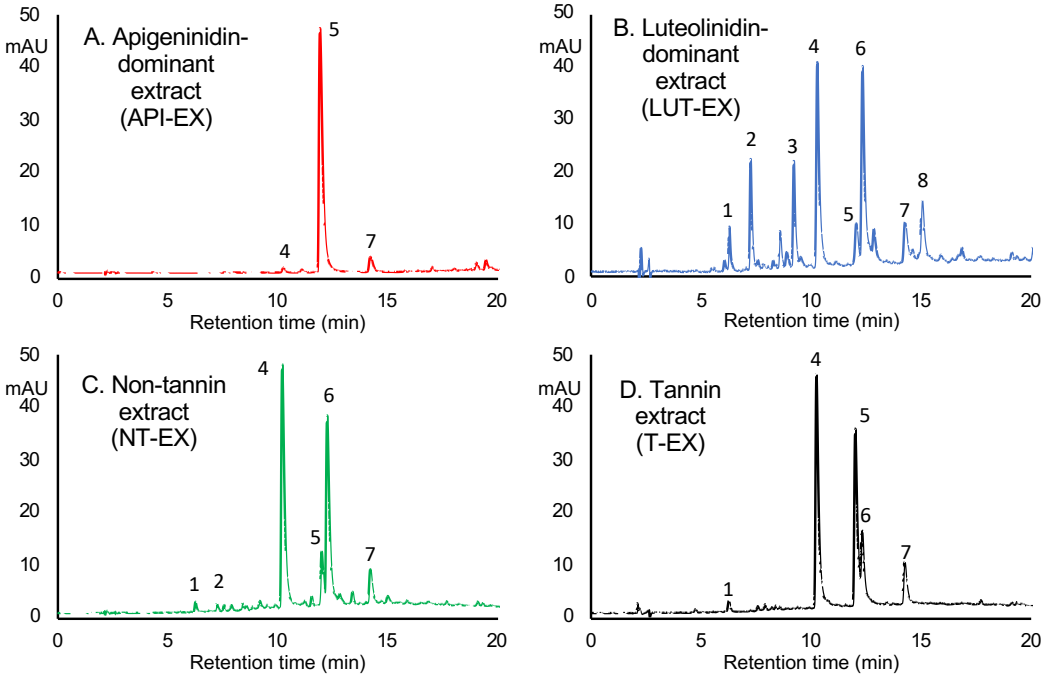


Figure 20 Pigment profile of sorghum sheath (apigeninidin-dominant), leaf (luteolinidin-dominant), non-tannin bran, and tannin bran extracts (480 nm). Peak identities are listed in Table 14.

Table 14 Identification of 3-deoxyanthocyanins of sorghum extracts.

Peak no.	Retention time (min)	λ_{max}	$[M+H]^+$	Identification
1	6.29	485	433	Luteolinidin glucoside
2	7.63	470	417	Apigeninidin glucoside
3	9.18	468	431	7-O-Methyl-luteolinidin glucoside
4	10.32	485	271	Luteolinidin
5	12.03	474	255	Apigeninidin
6	12.36	485	285	7-O-Methyl-luteolinidin
7	14.28	470	269	7-O-Methyl-apigeninidin
8	15.03	489	299	5,7-O-Methyl-apigeninidin

Table 15 Concentration of 3-deoxyanthocyanins of sorghum extracts profiled by HPLC.

	Apigeninidin-dominant (API-EX) (mg/mL)	Luteolinidin-dominant (LUT-EX) (mg/mL)	Non-tannin (NT-EX) (mg/mL)	Tannin (T-EX) (mg/mL)
Luteolinidin glucoside	*nd*	23.0 ±0.10	28.2 ±1.01	7.2 ±0.09
Apigeninidin glucoside	*nd*	51.7 ±0.12	25.7 ±2.74	*nd*
7-O-Methty-luteolinidin glucoside	*nd*	57.5 ±0.07	*nd*	*nd*
Luteolinidin	28.9 ±0.46	75.4 ±0.04	639 ±6.81	160 ±1.55
Apigeninidin	1416 ± 23	16.0 ±0.20	130 ±8.45	113 ±1.42
7-OMe-Luteolinidin	*nd*	80.1 ±0.58	604 ±10.40	67.6 ±1.03
7-OMe-Apigeninidin	123 ±2.47	20.5 ±0.28	165 ±7.99	42.7 ±0.63
Total	1568	324	1592	391

Data are expressed as mean ± standard deviation. *nd* = not detected.

The non-tannin and tannin bran extracts were relatively similar in pigment profile (Table 14, Table 15, Figure 20 C, D). The primary interest in using non-tannin vs tannin bran as a source of 3-DXA was from the hypothesis that tannins may complex with to 3-DXA as copigments, which could enhance the interaction of 3-DXA with pectin. The structure of condensed tannins, with numerous aromatic hydrophobic rings and hydroxyl groups in close proximity, lends itself to surface active properties and multiple sites for potential π-π bond overlap to function as effective copigments (Figueroa-Espinoza, Zafimahova, Alvarado, Dubreucq, & Poncet-Legrand, 2015).

6.3.2. Aqueous stability of 3-deoxyanthocyanins

The aqueous stability of 3-DXA without pectin is shown in Figure 21 with accompany photos of the solutions in Figure 22 and Figure 23. The majority of 3-DXA precipitation occurred within the first two weeks, with much slower 3-DXA precipitation rate between 2-10 weeks. It appears as if most of the 3-DXA precipitated relatively rapidly. The self-association and precipitation of 3-DXA is due to the relatively large hydrophobic area on their structure. Poor aqueous stability of 3-DXA was also observed by Herrman (2016).

There were distinct differences in aqueous stability of 3-DXA due to pH (Figure 21). The solutions at pH 1 consistently showed higher aqueous stability after 10 weeks (50% average) than those at pH 3 (33% average), which in turn was higher than aqueous stability of 3-DXA at pH 5 (13% average). At pH 1, the majority of 3-DXA exist as

flavylium cations but transform to neutral hydrated carbinol psuedobase or the quinoidal base as pH increases to 3 and 5. The incompatible positive charges of the flavylium cation limited 3-DXA self-association at pH 1; self-association occurred to a greater extent as pH increased due to greater loss of charge repulsion. Herrman (2016) reported that 0% of 3-DXA remained in solution after 10 weeks at pH 3 and 5.

An interesting observation in the solutions without pectin was the relatively high aqueous stability in LUT-EX pigments (66%) at pH 1 compared to API-EX pigments (39%) after 10 weeks. This confirms that the structure of 3-DXA influences their aqueous stability. Luteolinidin (2 -OH) is more hydrophilic than apigeninidin (1 -OH) (Figure 1). Furthermore, the greater proportion of 3-DXA-glycosides in the LUT-EX, compared to API-EX (Table 15), was probably also a major driver of the improved aqueous stability of LUT-EX. A 3-DXA extract with a higher proportion of luteolinidin and glycosides could be a more stable colorant without the need for stabilizing additives in a beverage application.

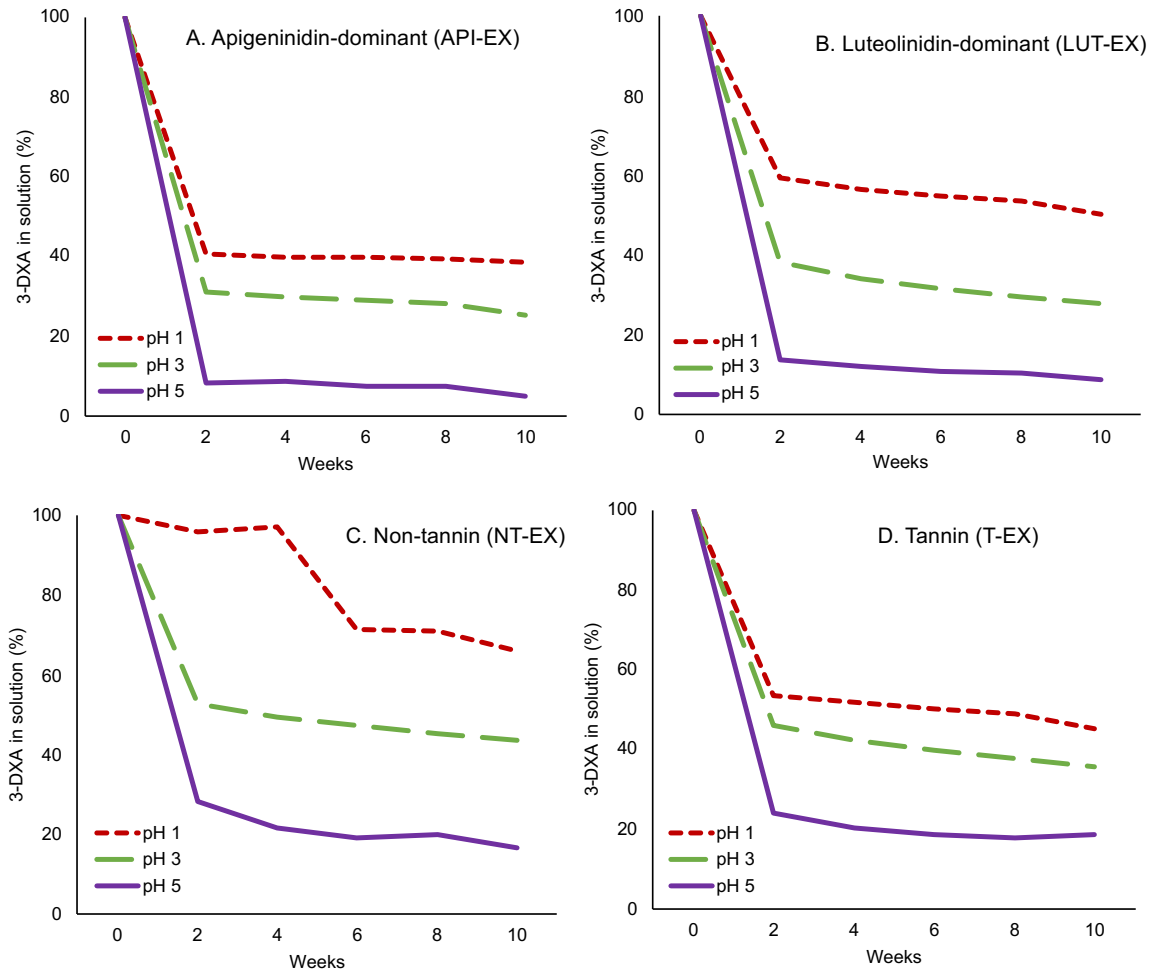


Figure 21 Aqueous stability (% remaining in solution) of 3-deoxyanthocyanins (3-DXA) in buffer solutions over 10 weeks.

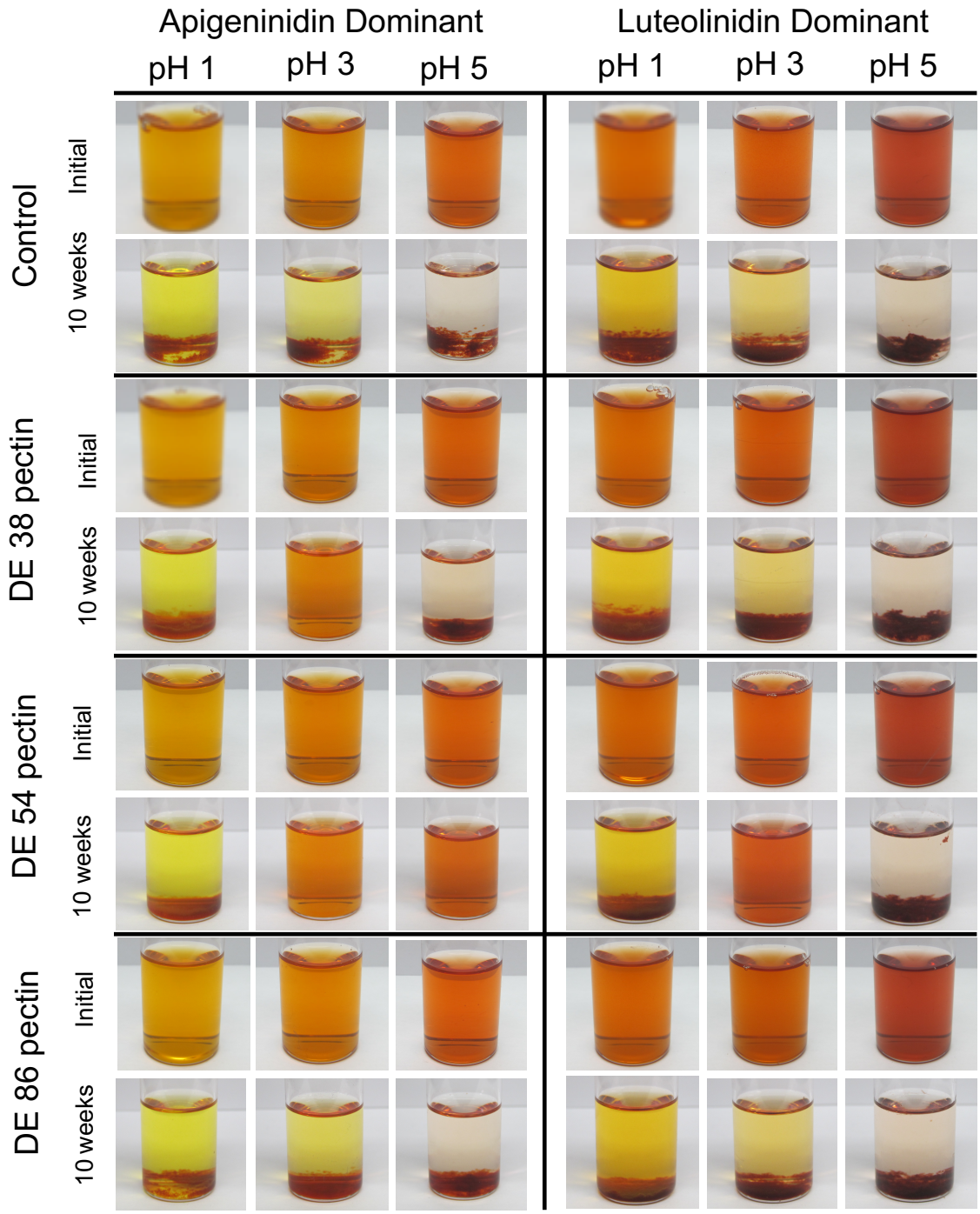


Figure 22 Effect of pectin (0.1 g/L) on the aqueous stability of 3-deoxyanthocyanins from the apigeninidin-dominant extract (API-EX) and the luteolinidin-dominant extract (LUT-EX) after 10 weeks.

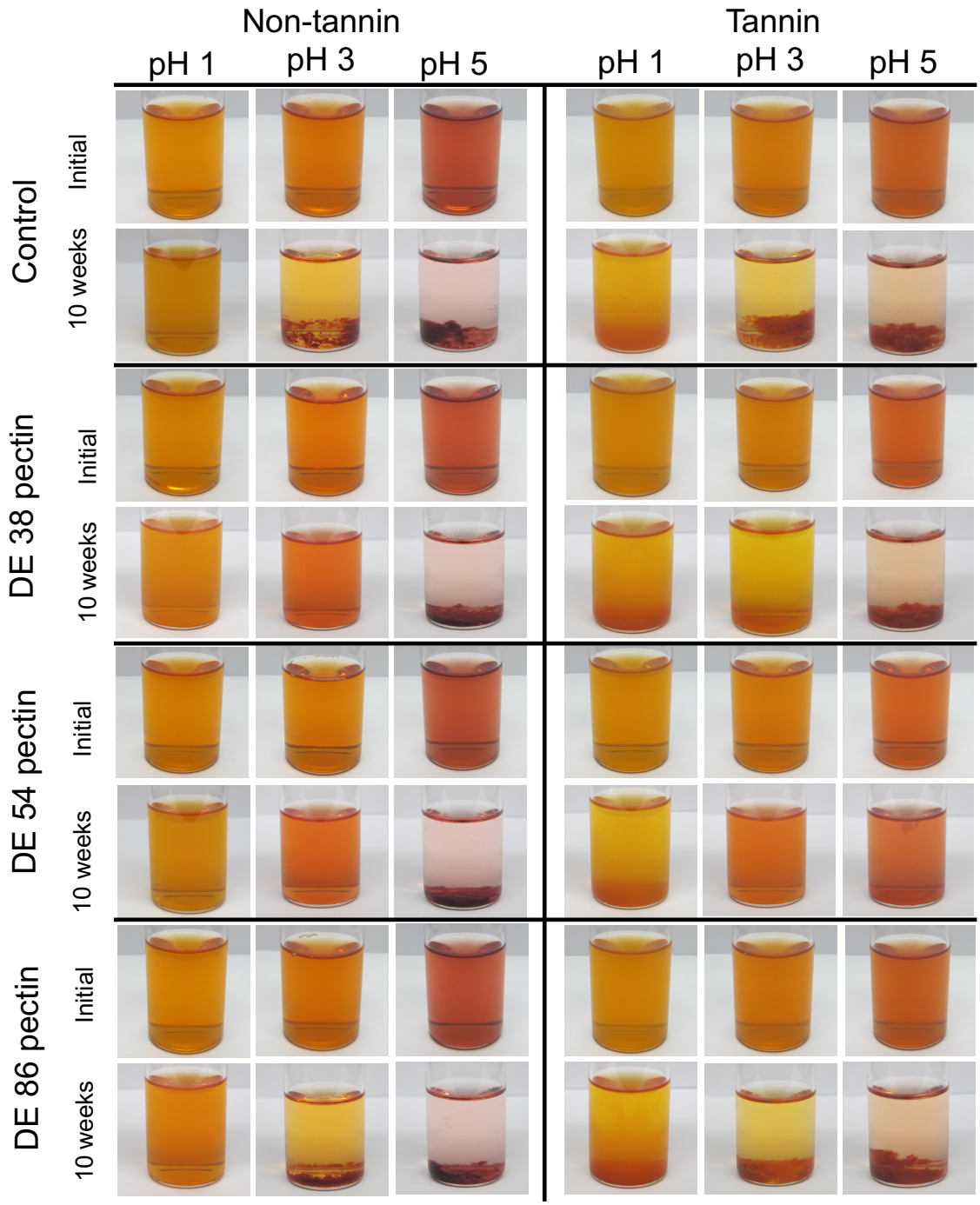


Figure 23 Effect of pectin (0.1 g/L) on the aqueous stability of 3-deoxyanthocyanins from the non-tannin bran extract (NT-EX) and the tannin bran extract (T-EX) after 10 weeks.

Table 16 Effect of pectin on the aqueous stability of 3-deoxyanthocyanins after 10 weeks.

APIGENINIDIN-DOMINANT EXTRACT (API-EX)													
NO PECTIN					DE 38 PECTIN			DE 54 PECTIN			DE 86 PECTIN		
pH	1	3	5	Pectin concentration	1	3	5	1	3	5	1	3	5
	39 ± 1.5^{c}	25 ± 2.9^{ef}	6 ± 0.9^{h}	0.1 g/L	38 ± 8.4^{c}	81 ± 11.3^{ab}	10 ± 2.5^{gh}	38 ± 0.6^{cd}	85 ± 3.7^{ab}	86 ± 1.8^{a}	38 ± 1.3^{c}	22 ± 3.6^{defg}	5 ± 1.1^{h}
				0.5 g/L	33 ± 6.5^{cde}	72 ± 13.6^{b}	78 ± 9.0^{ab}	38 ± 2.6^{c}	83 ± 1.6^{ab}	84 ± 0.9^{ab}	43 ± 7.5^{c}	37 ± 2.9^{cd}	10 ± 2.1^{gh}
				1.0 g/L	35 ± 4.5^{cd}	77 ± 10.9^{ab}	77 ± 9.8^{ab}	86 ± 0.9^{a}	80 ± 1.3^{cb}	77 ± 1.8^{ab}	86 ± 3.0^{a}	87 ± 1.4^{a}	16 ± 6.7^{fgh}
LUTEOLINIDIN-DOMINANT EXTRACT (LUT-EX)													
NO PECTIN					DE 38 PECTIN			DE 54 PECTIN			DE 86 PECTIN		
pH	1	3	5	Pectin concentration	1	3	5	1	3	5	1	3	5
	52 ± 2.8^{def}	27 ± 1.5^{h}	11 ± 2.6^{l}	0.1 g/L	50 ± 10.6^{defg}	23 ± 1.7^{hi}	15 ± 0.7^{ijkl}	49 ± 3.2^{defg}	71 ± 0.9^{a}	10 ± 1.3^{kl}	49 ± 2.9^{cdefg}	24 ± 3.5^{hij}	10 ± 2.1^{jkl}
				0.5 g/L	47 ± 6.5^{fg}	72 ± 6.6^{a}	42 ± 8.3^{g}	48 ± 3.3^{efg}	67 ± 1.0^{ab}	63 ± 0.4^{abc}	63 ± 8.8^{abc}	26 ± 5.4^{hi}	9 ± 0.7^{l}
				1.0 g/L	35 ± 4.5^{cd}	61 ± 9.2^{abcd}	62 ± 11.6^{ab}	49 ± 5.5^{defg}	67 ± 3.9^{ab}	61 ± 4.0^{abcde}	54 ± 6.8^{bcdefg}	64 ± 12.7^{ab}	23 ± 3.9^{hijk}
NON-TANNIN EXTRACT (NT-EX)													
NO PECTIN					DE 38 PECTIN			DE 54 PECTIN			DE 86 PECTIN		
pH	1	3	5	Pectin concentration	1	3	5	1	3	5	1	3	5
	69 ± 4.5^{de}	43 ± 1.8^{gh}	15 ± 2.0^{i}	0.1 g/L	72 ± 7.2^{bcde}	61 ± 4.4^{f}	17 ± 2.7^{i}	69 ± 4.1^{bcdef}	81 ± 3.5^{ab}	65 ± 4.1^{ef}	67 ± 2.7^{def}	39 ± 4.0^{gh}	15 ± 3.2^{i}
				0.5 g/L	69 ± 2.6^{def}	62 ± 3.7^{ef}	36 ± 6.2^{h}	67 ± 2.0^{def}	80 ± 2.1^{bc}	61 ± 2.1^{ef}	92 ± 7.7^{a}	46 ± 9.1^{gh}	14 ± 2.9^{i}
				1.0 g/L	66 ± 1.7^{cdef}	60 ± 3.7^{f}	36 ± 7.1^{h}	68 ± 5.7^{cdef}	78 ± 1.5^{bcd}	62 ± 3.0^{ef}	91 ± 6.5^{a}	65 ± 7.3^{ef}	49 ± 7.0^{g}
TANNIN EXTRACT (T-EX)													
NO PECTIN					DE 38 PECTIN			DE 54 PECTIN			DE 86 PECTIN		
pH	1	3	5	Pectin concentration	1	3	5	1	3	5	1	3	5
	53 ± 10.3^{def}	35 ± 10.0^{g}	18 ± 1.9^{i}	0.1 g/L	58 ± 8.2^{cdef}	65 ± 4.0^{c}	19 ± 1.6^{i}	50 ± 0.5^{def}	80 ± 12.0^{b}	91 ± 5.0^{ab}	49 ± 1.0^{def}	22 ± 3.3^{i}	19 ± 1.2^{i}
				0.5 g/L	59 ± 4.1^{cde}	63 ± 8.4^{cd}	49 ± 4.3^{ef}	51 ± 3.6^{cdef}	94 ± 1.3^{ab}	95 ± 6.2^{ab}	104 ± 6.2^{a}	91 ± 4.0^{ab}	86 ± 1.2^{b}
				1.0 g/L	57 ± 2.7^{cdef}	61 ± 10.0^{cde}	45 ± 7.6^{fg}	50 ± 4.6^{def}	93 ± 1.0^{ab}	90 ± 6.1^{ab}	104 ± 6.3^{a}	92 ± 2.3^{ab}	90 ± 4.5^{ab}

Values were calculated as percent absorbance retention after 10 weeks. Data are expressed as mean ± standard deviation. Values with different letters with one type of extract indicate significant difference between treatments (Tukey's HSD, $p < 0.05$).

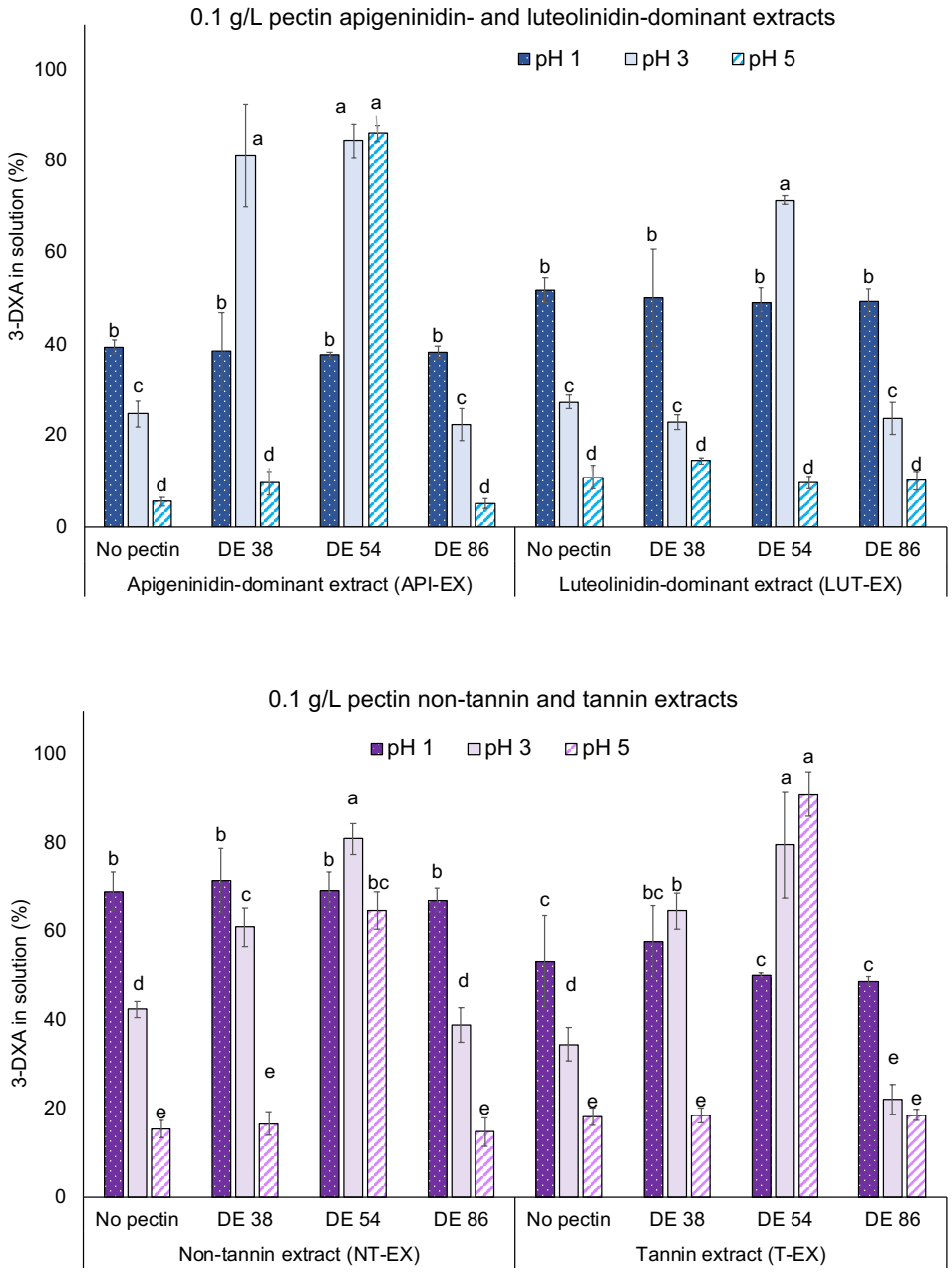


Figure 24 Effect of 0.1 g/L pectin on aqueous stability of 3-deoxyanthocyanin extracts after 10 weeks. Error bars illustrate ± standard deviation. Different letters within one type of extract indicate significant difference among treatments (Tukey's HSD, $p < 0.05$).

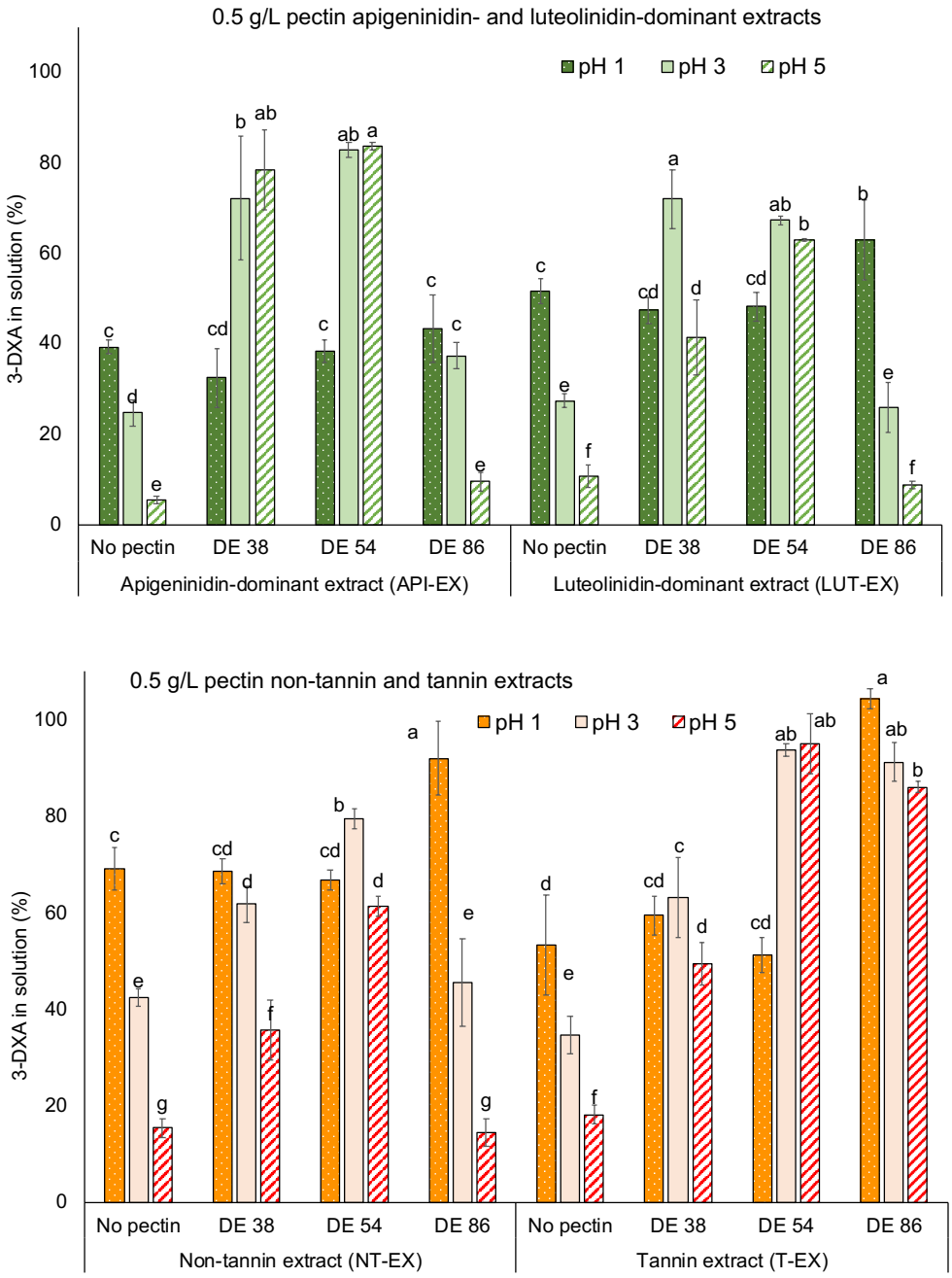


Figure 25 Effect of 0.5 g/L pectin on aqueous stability of 3-deoxyanthocyanin extracts after 10 weeks. Error bars illustrate ± standard deviation. Different letters within one type of extract indicate significant difference among treatments (Tukey's HSD, $p < 0.05$).

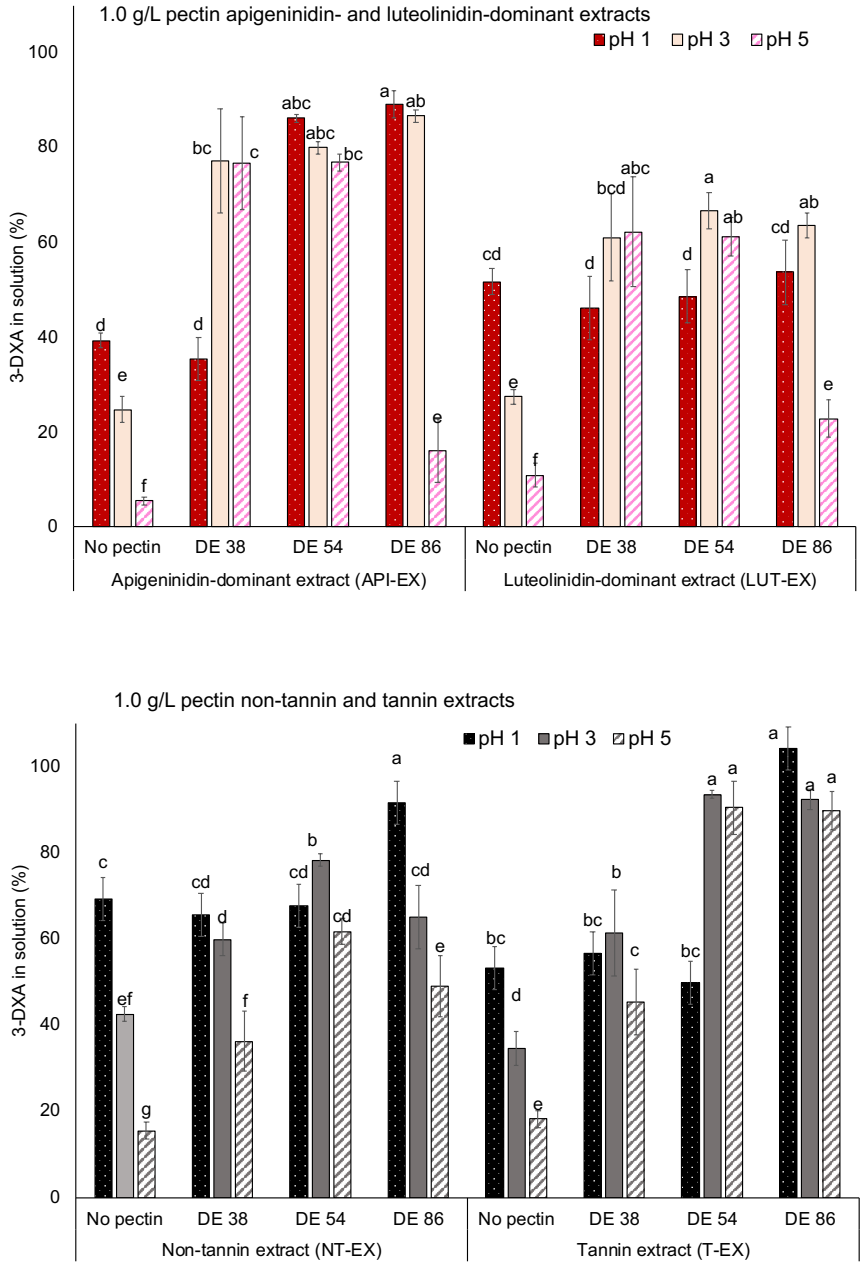


Figure 26 Effect of 1.0 g/L pectin on aqueous stability of 3-deoxyanthocyanin extracts after 10 weeks. Error bars illustrate ± standard deviation. Different letters within one type of extract indicate significant difference among treatments (Tukey's HSD, $p < 0.05$).

6.3.3. Effect of pectin on the aqueous stability of 3-deoxyanthocyanins

Overall, pectin had a stabilizing effect on 3-DXA in aqueous solutions (Table 16, Figure 22-26). Each of the variables tested (pectin concentration, pigment profile, copigment profile, pH, and pectin DE) affected the extent to which 3-DXA were stabilized. The general trend was that the 1.0 g/L pectin stabilized 3-DXA to the greatest extent compared to lower concentrations. Pectin was generally ineffective at pH 1 but highly effective at pH 3, with DE 54 pectin generally being the most effective at stabilizing 3-DXA.

The effect of pectin concentration on the viscosity of solutions is shown in Table 17. The viscosity of the solutions was not affected at 0.1 g/L or 0.5 g/L, however, 1.0 g/L pectin increased the viscosity of solutions. Due to the differences in viscosity between concentrations of pectin and because different 3-DXA stabilizing patterns were observed for the three pectin concentrations, each concentration will be discussed separately.

Table 17 Viscosity of 3-deoxyanthocyanin-pectin solutions.

pH	No Pectin	g/L	DE 38 (mPa/s)	DE 54 (mPa/s)	DE 86 (mPa/s)
1	1.15 ± 0.12	0.1	1.07 ± 0.02^{d}	1.12 ± 0.07^{de}	1.08 ± 0.02^{c}
		0.5	1.23 ± 0.02^{bc}	1.26 ± 0.11^{bcd}	1.21 ± 0.07^{abc}
		1.0	$1.56 \pm 0.04^{a*}$	$1.37 \pm 0.12^{abc*}$	1.29 ± 0.03^{ab}
3	1.14 ± 0.10	0.1	1.16 ± 0.02^{cd}	1.09 ± 0.02^{e}	1.09 ± 0.03^{c}
		0.5	1.25 ± 0.02^{bc}	1.24 ± 0.05^{cde}	1.28 ± 0.04^{ab}
		1.0	$1.55 \pm 0.17^{a*}$	$1.41 \pm 0.03^{ab*}$	$1.38 \pm 0.18^{a*}$
5	1.13 ± 0.11	0.1	1.17 ± 0.15^{cd}	1.11 ± 0.05^{de}	1.12 ± 0.18^{bc}
		0.5	1.18 ± 0.01^{cd}	1.22 ± 0.02^{cde}	1.18 ± 0.02^{abc}
		1.0	$1.35 \pm 0.04^{b*}$	$1.49 \pm 0.06^{a*}$	$1.29 \pm 0.03^{ab*}$

DE = degree of esterification. Data are expressed as mean ± standard deviation. Different letters indicate significant difference within columns (Tukey's HSD $p < 0.05$). *indicates difference from control at same pH (Dunnett's $p<0.05$).

6.3.3.1. Stabilizing effect of 0.1 g/L on 3-DXA in aqueous solutions after 10 weeks

In general, at pH 1 (0.1 g/L), pectins were ineffective at stabilizing 3-DXA (Figure 24, Table 16). The majority of carboxylic acid groups on pectin chain are uncharged at pH 1, thus it is likely that the pectins self-associated and precipitated out of solution, thus were unable to effectively interact with the 3-DXA in solution. Nevertheless, the 3-DXA were most aqueous stable at pH 1 (Figure 21), thus the need for stabilizing hydrocolloids is less important at this pH.

Pectins were generally most effective at stabilizing 3-DXA in solution at pH 3. For example, DE 54 pectin effectively preserved 71-85% of 3-DXA in solution after 10 weeks (Figure 24, Table 16). DE 38 was only effective at pH 3 with API-DE (81% 3-DXA in

solution), whereas DE 86 pectin was not effective with any extract at pH 3. A greater charge density of pectins is present at pH 3 compared to pH 1, making the polysaccharide more soluble and thus available to protect 3-DXA. However, it appears the ability of pectin to remain in solution was not enough for 3-DXA stabilization.

At pH 5, the carboxylic acid groups of pectin will carry a negative charge, yet 3-DXA will primarily be neutral quinoidal bases. Pectin, at pH 5, has a net charge repulsion higher than at pH 3 and therefore electrostatic repulsion would contribute to the stabilization of pectin to a greater extent. At pH 5, only DE 54 stabilized the 3-DXA (Figure 24), with of 65-91% pigments in solution vs controls (6-53%). The DE 38 and DE 86 pectins did not stabilize 3-DXA at this pH. This suggests a balance of hydrogen bonding, hydrophobic, and ionic interactions may be essential to stabilize the 3-DXA by pectin. For example, DE 86 may be too hydrophobic with too few sites for hydrogen bonding or ionic interactions. DE 38 may have too much charge repulsion and not enough hydrophobic regions to interact with 3-DXA.

Because the more hydrophilic LUT-EX was not stabilized at pH 5 by any of the pectins at 0.1 g/L, this suggests hydrophobic interactions are critical to a stable 3-DXA-pectin complex formation at low pectin concentration. DE 86 pectin was ineffective at 0.1 g/L. This pectin might have precipitated due to the hydrophobic structure and therefore was unable to interact with 3-DXA. The ineffectiveness of DE 86 pectin suggests that mechanisms beyond hydrophobic interactions are occurring between 3-DXA and pectin.

The broader effectiveness of DE 54 pectin suggests that a balance of both unsubstituted carboxylic acid groups and methoxyl groups are needed for proper

stabilization of 3-DXA, indicating both charge density and hydrophobic properties contribute to 3-DXA stabilization.

6.3.3.2. Effect of 0.5 g/L pectin on the aqueous stability of 3-deoxyanthocyanins after 10 weeks

The effect of pectin at 0.5 g/L on the aqueous stability of 3-DXA is show in Figure 25 and Table 16. At this concentration, pectin was overall more effective (59% average) than at 0.1 g/L pectin (46% avg) and compared to the control (33% avg).

Compared to controls and 0.1 g/L pectin, 0.5 g/L pectin did not increase viscosity (Table 17). Viscosity of 0.5 g/L solutions ranged from 1.18-1.28 mPas. Similar to the solutions with 0.1 g/L pectin, the positive effect of pectin on the aqueous stability was more so due to the surface activity of pectin partitioning between 3-DXA and water than the effect of viscosity limiting particle motion.

The most notable difference between 0.1 g/L and 0.5 g/L pectin on the aqueous stability of 3-DXA is the fact that DE 86 was highly effective at stabilizing the tannin-containing extract, T-EX (Table 16, Figure 25). In fact, it was a highly effective stabilizer of T-EX at all pH levels (86-104%) unlike at 0.1 g/L (Figure 24). This suggests that tannins had a significant stabilizing effect on both 3-DXA and pectin. Tannins are copigments to 3-DXA (Chapter 4) and have been shown to interact with polysaccharides via hydrogen bonds and hydrophobic interactions (Bautista-Ortín, Martínez-Hernández, Ruiz-García, Gil-Muñoz, & Gómez-Plaza, 2016; Le Bourvellec, Le Quere, & Renard, 2007).

Another interesting observation is the greater effectiveness of DE 38 and DE 54 pectin at 0.5 g/L (Figure 25) on LUT-EX aqueous stability compared to 0.1g/L (Figure 24) at both pH 3 and 5 (42-73%) compared to controls (27-52%). The stabilizing effect may be due to the higher concentration of pectin offering more sites for hydrogen bonding and compensating for the limited hydrophobic interactions between pectin and LUT-EX.

6.3.3.3. Effect of 1.0 g/L pectin on the aqueous stability of 3-deoxyanthocyanins after 10 weeks

The effect of 1.0 g/L pectin on the aqueous stability of 3-DXA is show in Figure 26 and Table 16. At this concentration, pectin was overall more effective (66% average) than at the other two lower concentrations (46% avg 0.1 g/L and 59% avg for 0.5 g/L), and compared to the control (33% avg).

Compared to controls, 1.0 g/L pectin solutions had a significantly higher viscosity (Table 17). For instance, viscosity was 1.56 mPa/s with 1.0 g/L compared to 1.23 mPa/s and 1.07 mPa/s for 0.5 g/L and 0.1 g/L, respectively, with DE 38 pectin. The higher viscosity could contribute to the stabilizing effect on 3-DXA by pectin, in addition to the chemical interactions between pectins and 3-DXA at 0.1 g/L and 0.5 g/L. An increase of continuous phase slows Brownian motion of particles and flocculation. Despite the higher viscosity of DE 38 and DE 54 1.0 g/L pectin solutions, the aqueous stability of 3-DXA was not majorly improved (Figure 26, Table 16). This suggests that viscosity did not have a major effect on preventing 3-DXA from precipitating out of solution similar to the observations for alginate in Chapter 5.

6.3.4. Characterization of 3-deoxyanthocyanin-pectin solutions

To understand the mechanisms by which 3-DXA interacted with pectin and the observed effect of tannins, pectin solutions with NT-EX and T-EX were characterized for zeta potential and average particle size distribution.

6.3.4.1. Zeta potential of 3-deoxyanthocyanin-pectin solutions

The zeta potentials of the pectin and 3-DXA solutions are shown in Figure 27 and Table 18. As expected, the zeta potential of pectin solutions without 3-DXA showed an inverse relationship between pectin DE and zeta potential. DE 38 pectin was the most negative (-20 to -19 mV), followed by DE 54 (-14 to -10), and then DE 86 (-7 to -4) at both pH 3 and 5. The zeta potential of each pectin was slightly less negative at pH 5 compared to pH 3. Zeta potential of pectin is such that at a pH above the pKa of pectin (~3.5-4.1), pectin with a higher DE will show a less negative zeta potential (ZP) than pectins with a lower DE, due to the greater number of (dissociated) carboxylic acid groups that will carry a negative charge (Schmidt et al., 2016). A zeta potential charge that is greater in magnitude (either positive or negative) indicates greater repulsive charges in a system, and therefore, flocculation of particles will be lower than in a system with lower magnitude of zeta potential.

With regards to the stability of pectin-3-DXA complexes in solution, DE 54 pectin was the most effective overall (Table 16), yet the values of zeta potential (-10 to -16 mV for both pH 3 and 5) were within the range where electrostatic repulsion is only considered

to be a minor contributor to colloidal stability (Guo et al., 2014). Although DE 38 had more negative zeta potential values (-14 to -24 at both pH 3 and 5), DE 38 pectin did not stabilize 3-DXA as well as DE 54 pectin (Table 16). Greater charge repulsion (via higher zeta potential) could improve stabilization of pectin in solution but appears to have a minor effect on its interaction with 3-DXA.

The zeta potential of DE 86 was highest (-7.2 to -5.0 mV) at both pH 3 and 5 despite the stability of 3-DXA by 0.5 g/L DE 86 pectin (Table 16). The high zeta potential values (of DE 86 solutions) suggest that electrostatic repulsion was not a major contributor to colloidal stability with 3-DXA and therefore steric repulsion of the 3-DXA-pectin complexes may likely have prevented complex self-association and precipitation. The zeta potential of both the non-tannin and tannin DE 86 pectin solutions were not different, so this suggests that tannin-pectin-3-DXA complexes were less predisposed to flocculate due to greater relative steric repulsion than the pectin-3-DXA complexes (without tannins) and not due to electrostatic repulsion.

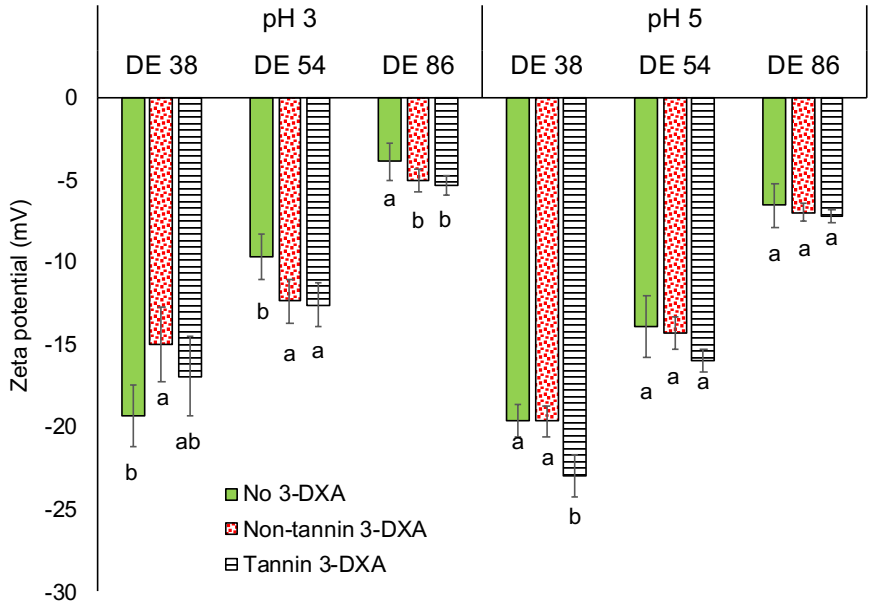


Figure 27 Zeta potential of pectin solutions (0.5 g/L) with non-tannin (NT-EX) and tannin (T-EX) 3-DXA extracts at pH 3 and 5. Error bars illustrate ± standard deviation. Different letters indicate significant difference within the same pectin and pH combination (Tukey's HSD $p<0.05$).

Table 18 Zeta potential of pectin solutions with non-tannin and tannin 3-DXA extracts at pH 3 and 5.

		Pectin without 3-DXA	Pectin with non-tannin extract (NT-EX)	Pectin with tannin extract (T-EX)
pH 3	DE 38	-19.4 ± 1.9^{b}	-15.0 ± 2.3^{a}	-17.0 ± 2.4^{ab}
	DE 54	-9.7 ± 3.4^{b}	-12.4 ± 1.3^{a}	-12.6 ± 1.3^{a}
	DE 86	-3.9 ± 1.1^{a}	-5.0 ± 0.7^{b}	-5.4 ± 0.6^{b}
pH 5	DE 38	-19.6 ± 1.0^{a}	-19.7 ± 0.9^{a}	-23.0 ± 1.3^{b}
	DE 54	-13.9 ± 1.9^{a}	-14.4 ± 1.0^{a}	-16.0 ± 0.7^{a}
	DE 86	-6.6 ± 1.3^{a}	-7.0 ± 0.6^{a}	-7.2 ± 0.4^{a}

Pectin concentration was 0.5 g/L. Data are expressed as mean ± standard deviation. Different letters indicate significant difference within the same pectin and pH combination (Tukey's HSD $p<0.05$).

6.3.4.2. Average particle size of 3-deoxyanthocyanin-pectin solutions

The average particle size of pectin solutions at pH 3 and 5, without and with 3-DXA, is shown in Figure 28 and Table 19. Particle size generally increased with the addition of 3-DXA to the pectin solutions, increasing by an average of 270-500 nm compared to solutions without 3-DXA. Comparing particle size by pH, the non-tannin-pectin solutions were generally smaller at pH 3 (1,214 nm avg.) compared to pH 5 (1,388 nm avg.) whereas for tannin-pectin solutions, the particle size was more consistent across the two pH (916-936 nm avg.). The particle sizes were similar between the pectin-NT-EX and the pectin-T-EX solutions at pH 3, however, the pectin-T-EX (930 nm avg.) solutions had consistently smaller particle size than pectin-NT-EX (1,388 nm avg.).

An increase in particle size upon addition of 3-DXA indicates that there was an interaction between the two components and some type of larger complex was formed. However, an increase in particle size is typically seen as a negative for the stability of an emulsion because larger particle sizes generally correlate with a less stable colloidal system (Akhtar et al., 2002). The smaller average particle size of pectin-T-EX solutions (at pH 5) compared to pectin-NT-EX likely contributed to the aqueous stability of 3-DXA from T-EX (Figure 25). Tannins have aromatic hydrophobic rings and hydrophilic hydroxyl groups giving them potential surface active properties (Figueroa-Espinoza et al., 2015). The amphiphilic nature of tannins in combination with their high degree of polymerization (avg. DP>10) gives multiple sites to interact with both 3-DXA (through π-π bond overlap) and pectin. Sorghum tannins may thus be effective surfactants in pectin-3-DXA solutions.

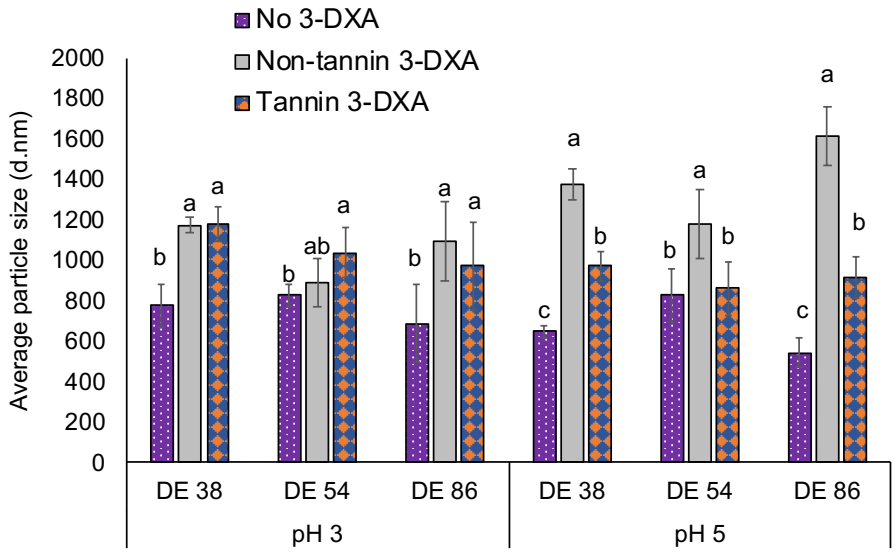


Figure 28 Average particle size of pectin solutions (0.5 g/L) with non-tannin (NT-EX) and tannin (T-EX) 3-DXA at pH 3 and 5. Error bars illustrate ± standard deviation. Different letters indicate significant difference within the same pectin and pH combination (Tukey's HSD p<0.05).

Table 19 Average particle size of pectin solutions with non-tannin and tannin 3-DXA extracts at pH 3 and 5.

		Pectin without 3-DXA	Pectin with non-tannin extract (NT-EX)	Pectin with tannin extract (T-EX)
pH 3	DE 38	775 ± 107^{b}	1174 ± 38^{a}	1177 ± 85^{a}
	DE 54	828 ± 55^{b}	886 ± 120^{ab}	1036 ± 123^{a}
	DE 86	689 ± 194^{b}	1093 ± 194^{a}	974 ± 217^{a}
pH 5	DE 38	647 ± 31^{c}	1371 ± 76^{a}	973 ± 73^{b}
	DE 54	826 ± 133^{b}	1178 ± 168^{a}	861 ± 133^{b}
	DE 86	543 ± 74^{c}	1614 ± 146^{a}	915 ± 105^{b}

Pectin concentration was 0.5 g/L. Data are expressed as mean ± standard deviation. Different letters indicate significant difference within the same pectin and pH combination (Tukey's HSD p<0.05).

6.3.5. Effect of protein on the stabilizing effect of pectin for 3-deoxyanthocyanins in aqueous solutions

The last aspect to address regarding the effect of pectin on the aqueous stability of 3-DXA is the potential effect of protein that is within the structure of pectin. The protein content of each pectin is shown in Table 20. The concentration of pectin was highest for DE 54 (1.84%), then DE 86 (1.56%), with the DE 38 having the lowest protein content (1.37%). These values generally agree with the protein content of citrus pectin that is reported in literature (Schmidt, Schmidt, Kurz, Endreß, & Schuchmann, 2015; Verkempinck et al., 2018). The stabilizing properties of pectin on 3-DXA were probably due to both protein and the amphiphilic character of the polysaccharide portion of pectin. The higher (1.84%) protein of DE 54 pectin could have been a contributing factor to its overall better efficacy, especially at low concentrations (0.1 g/L), compared to DE 38 and DE 86 pectin. For DE 86 pectin, the proteins may be less consequential due to the pectins inherent hydrophobic nature (higher DE). The effect of protein on the aqueous stability of 3-DXA should be further investigated. This could be done by comparing other sources of pectin with different levels of protein (e.g. sugar beet pectin).

The literature regarding the contribution of protein to surface activity of pectin is somewhat mixed. Some authors indicate the protein within pectin is the primary source of surface activity (i.e. it functions more like gum arabic). The protein could anchor to the interface whereas the polysaccharide chain is suspended in the continuous aqueous phase. Others conclude that the surface activity of pectin is due to the hydrophobic character given from the degree of methylation (or acetylation) along the polysaccharide chains

(Akhtar et al., 2002; Dickinson, 2017; Leroux, Langendorff, Schick, Vaishnav, & Mazoyer, 2003; Ngouémazong, Christiaens, Shpigelman, Van Loey, & Hendrickx, 2015; Schmidt, Schmidt, et al., 2015). Our data suggests that both factors may play a role to improve the aqueous stability of 3-DXA.

Table 20 Protein content of pectins.

Pectin	Protein (%)
DE 38	1.37 ± 0.12^{b}
DE 54	1.84 ± 0.06^{a}
DE 86	1.56 ± 0.26^{ab}

Data are expressed as mean ± standard deviation. Different letters indicate significant difference of protein content (Tukey's HSD ($p<0.05$).

6.4. Conclusion

The poor aqueous stability of 3-DXA can be mitigated using low concentrations pectin. The degree of effectiveness of pectin is dependent on pectin DE, solution pH, 3-DXA profile, and the presence of tannins. Overall, DE 54 pectin at pH 3 most effectively stabilized 3-DXA, likely due to the contribution of both hydrophobic interactions and hydrogen bonding of pectin with 3-DXA. Higher concentrations of pectin made DE 38 and DE 86 relatively more effective than lower concentrations. The API-EX extract was typically stabilized to a greater extent than LUT-EX suggesting more hydrophobic 3-DXA interact to a greater extent with pectin. Tannins appeared to have a synergistic effect with

pectin at stabilizing 3-DXA. Zeta potential and particle size measurements gave evidence that 3-DXA were encapsulated by pectin and that a complex was formed between the two compounds. Electrostatic interactions were likely not a major contributor to the interaction of pectin with 3-DXA. This work showed that relatively low concentrations (0.1 g/L) of pectin can effectively stabilize 3-DXA. Both pectin DE and 3-DXA profile must be considered when using pectin to stabilize 3-DXA in aqueous solutions.

7. CONCLUSIONS

7.1. Summary

Previous work in our lab showed that microwave-assisted extraction (MAE) could significantly increase the extraction efficiency (2X yield) of 3-deoxyanthocyanins (3-DXA) compared to conventional extraction (CE). Such magnitude of increased yield has not been reported for other pigments or phenolics. The improved extraction efficiency was demonstrated in the current study to be due to the fact that 3-DXA were structurally stable to microwave energy. Anthocyanins, on the other hand, were extensively degraded during MAE. Additionally, MAE degraded the grain cell wall and, because of this, 3-DXA were able to easily diffuse out into the extraction solvent, resulting in significant increases in yield compared to CE.

Because many high 3-DXA sorghums also contain tannins, tannin sorghum was also used to investigate the effect of MAE on 3-DXA in the presence of these copigments. The presence of tannins in 3-DXA extracts was of interest because of the potential copigmentation with 3-DXA and because of the potential for oxidative depolymerization of these condensed tannins to anthocyanidins.

The relative yield increase of 3-DXA from tannin sorghum (3X) after MAE was higher than that from non-tannin sorghum (2X). This suggests that tannins may have protected 3-DXA during MAE. Another theory is that the tannin sorghum pericarp is structurally different than that of non-tannin sorghum. The pericarp of tannin sorghum may be thinner than non-tannin sorghum pericarp or the cell wall structure (specifically

the covalent cross-linking by phenolic acids) is different enough that it is easier for MAE to degrade the cell wall of the tannin sorghum. The structure and composition of non-tannin and tannin sorghum pericarp and testa, specifically those with significant quantities of 3-DXA, should be further investigated to confirm this theory.

Tannins conferred a stabilizing effect on the structure of 3-DXA in aqueous solutions over time. Tannins with a higher degree of polymerization (DP) conferred a greater protective effect on 3-DXA than those with lower DP in aqueous solutions. The higher DP condensed tannins have multiple sites for π-π bond overlap with 3-DXA which essentially stabilizes 3-DXA as flavylium cations. The presence of tannins in sorghum could thus potentially enhance MAE extractability as well as functionality of 3-DXA in aqueous food systems.

During MAE, condensed tannins underwent oxidative depolymerization to anthocyanidins. Two anthocyanidins, cyanidin and 7-O-methylcyanidin, were detected after MAE of tannin sorghum. The anthocyanidins caused the 3-DXA extracts to have a redder hue compared to 3-DXA extract without anthocyanidins (from non-tannin sorghum). The two anthocyanidins degraded quickly in aqueous solutions, especially at pH 3 and 5, resulting in the change of hue from red to orange/yellow. The anthocyanidins, however, did not have a negative effect on the stability of 3-DXA. Although anthocyanidins are undesirable in 3-DXA extracts due to their unstable structure, the color imparted by 3-DXA in aqueous solutions (or potentially in beverage applications) remained stable. Thus, MAE could be used for 3-DXA extraction from tannin sorghum without concern of negative effects of anthocyanidins on 3-DXA profile.

With regards to the poor aqueous stability of 3-DXA, previous work in our lab showed that amphiphilic polysaccharides, specifically gum arabic and sodium alginate, effectively stabilized 3-DXA in solution to a greater extent than in solutions without added polysaccharides. This work aimed to elucidate the interactions between 3-DXA and amphiphilic polysaccharides that contributed to the aqueous stability of 3-DXA. To establish the mechanisms, we used gum arabic and pectins (with varying degree of esterification (DE)). We also investigated how 3-DXA pigment profile (ratio of apigeninidin to luteolinidin derivatives) and pH effected on the interactions between 3-DXA and the polysaccharides.

Three major findings on gum arabic helped to define its interaction with 3-DXA. First, the increase of particle size of 3-DXA-gum arabic compared to gum arabic alone suggested a complex formed between the two compounds. Secondly, a moderate decrease in zeta potential of 3-DXA-gum arabic compared to gum arabic alone suggested that 3-DXA were encapsulated in hydrophobic pockets of gum arabic. Fluorescence quenching of gum arabic by 3-DXA confirmed a stable complex formed between 3-DXA and gum arabic. A higher binding constant of apigeninidin with gum arabic compared to luteolinidin suggest that hydrophobic interactions were the primary interaction between 3-DXA and gum arabic. The addition of sodium chloride during fluorescence quenching did not destabilize the complex formed between 3-DXA and gum arabic, and therefore, electrostatic interactions were not a major contributor to 3-DXA-gum arabic interactions.

Similar to gum arabic, pectin also effectively stabilized 3-DXA in aqueous solutions. The stabilization of 3-DXA by pectin was influenced by pectin degree of

esterification (DE), 3-DXA profile, solution pH, and the presence of tannins. Pectin was generally more effective with extracts higher in apigeninidin derivatives, suggesting that, like gum arabic, hydrophobic interactions were important contributors to 3-DXA stabilization with pectin. Because DE 86 pectin (the most hydrophobic pectin) was generally not as effective as DE 54 pectin (more or less equal in hydrophobic and hydrophilic moieties) at stabilizing 3-DXA in solution, this suggests hydrogen bonding may also occur between 3-DXA and pectin. Another interesting observation was that 3-DXA-tannin-pectin solutions were markedly more stable, specifically with DE 86 pectin, compared to solutions without tannins. It is likely that tannins stabilized 3-DXA as flavylium cations (due to π-π cloud overlap); the 3-DXA tannin complex then were encapsulated by DE 86 pectin. Similar to gum arabic, an increase in particle size of 3-DXA and pectins suggested that a complex was formed between pectin and 3-DXA. Average particle size in 3-DXA-tannin solutions was smaller than 3-DXA-non-tannin solutions which may have contributed to greater colloidal stability of 3-DXA-tannin solutions. Zeta potential measurements again suggested 3-DXA were encapsulated by pectin. Amphiphilic polysaccharides appear to be key to 3-DXA stability; hydrophobic moieties are needed to encapsulate 3-DXA whereas hydrophilic moieties supplement complex formation via hydrogen bonding and contribute to overall solubility of the complex in aqueous solutions.

The key findings of this work are the following:

1. 3-DXA are stable to microwave energy

2. MAE breaks cereal grain cell wall, which likely increased extraction efficiency of other phenolics
3. Extractability of 3-DXA is greater from tannin sorghum than non-tannin sorghum
4. Sorghum tannins are copigments to 3-DXA and significantly contribute to their stability in aqueous solutions
5. 3-DXA interact with amphiphilic polysaccharides primarily via hydrophobic interactions, and to some extent, hydrogen bonding

The implications of these finding are:

1. MAE can valorize sorghum bran, a common waste stream, as source of 3-DXA pigments and other phenolics
2. The structure of polysaccharides key to stabilizing 3-DXA in aqueous solutions; both hydrophilic and hydrophobic moieties are needed to interact with 3-DXA and maintain colloidal stability
3. Product developers must be aware of both 3-DXA profile and gum structure during formulation to properly stabilize 3-DXA

The following work could be done to further the impact of this work:

1. Determine the effect of MAE on the copigment (other than tannins) extractability and profile from sorghum
2. Determine parameters for optimization of 3-DXA yield from MAE (power, time, and temperature)

3. Determine the effect of amphiphilic polysaccharides on 3-DXA in a complex system such as a model beverage

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