

INFLUENCE OF GENETIC BACKGROUND ON ANTHOCYANIN AND
CO-PIGMENT PROFILE AND STABILITY OF COLORED CORN

A Thesis

by

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ABSTRACT

Growing consumer demand for healthy and natural snacks provides an opportunity for utilization of pigmented corn in the tortilla chip market. This study determined the effect of phenotype¹ on anthocyanin and co-pigment composition and processing stability of several experimental hybrid varieties of corn from four phenotypes (red, purple, blue, and red/blue). The goal was to determine if genetics/phenotype can be utilized to selectively breed for pigmented corn lines with greater stability during thermo-alkaline processing. The pigment and co-pigment composition were determined using LC-MS/MS. Total and monomeric anthocyanin contents were quantified using pH differential method. Effect of thermo-alkaline processing on anthocyanin profile and color stability was measured using the aforementioned methods, as well as with a colorimeter.

The blue and red/blue phenotypes had similar pigment profiles with the more stable acylated anthocyanin cyanidin-3-(6''-malonylglucoside) present in greatest proportion and as such were hypothesized to have greater stability during processing. The purple phenotype contained a higher proportion of monomeric cyanidin-3-glucoside. Generally, anthocyanin profile was similar within phenotype. Regarding total anthocyanin content, the purple phenotype had the highest level of anthocyanins (890-3312 µg/g), and the red phenotype had the lowest (8.9-127 µg/g). For non-purple

¹Note: In this thesis, the word “phenotype” refers to “color class” of the pigmented corn variety (i.e. red, purple, blue, and red/blue are the 4 phenotypes/color classes studied).

samples, a clear effect of environment on anthocyanin content was observed, with samples grown in Weslaco, TX having consistently higher levels than samples grown in College Station, TX. This suggests specific environments may favor better accumulation of the anthocyanins.

During processing of corn into tortilla chips, the purple sample underwent the most significant change in anthocyanin content, retaining 59.1% and 32.1%, at 0.5% and 1% lime treatment levels, respectively. The blue and red/blue samples retained 88.0% and 78.6% (0.5% lime level), respectively, and 63.6% and 51.2% (1% lime level), respectively. However, chroma properties of the blue sample decreased unfavorably during processing.

Overall, the red/blue sample performed best at the alkaline pH encountered in tortilla chip processing, with good pigment retention, high lightness value (38.6) and relatively high chroma (9.3) at the 1% lime level, indicating that anthocyanin profile may predict color stability during processing. Therefore, these phenotypes could be improved for use in commercial tortilla chip processing.

DEDICATION

To my parents, Debbie and Chris Collison, to my brother and sister-in-law, Scott and Aimee Collison, as well as to Brian Harding.

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CHAPTER I

INTRODUCTION

Consumer interest in snacks that are healthy, natural, and unique provides an opportunity for innovative products in the growing tortilla chip market. Tortilla chips were a \$2.5 billion market in 2013 and accounted for approximately 14% of the US snack food market in 2010 (SOTI, 2012, 2013). One way consumer interest in healthy and natural snacks is addressed is through the use of anthocyanin-rich pigmented corns to create the blue, red, and purple tortilla chip products that consumers perceive as healthier than white corn chips. Anthocyanins provide attractive, natural color and potential health benefits, such as selective inhibition of cancer cell growth and attenuation of some life-style diseases such as hypertension and hyperglycemia (Song et al., 2012; Long et al., 2013; Shindo et al., 2007; Tsuda et al., 2003). However, when these pigmented corns are subjected to the thermo-alkaline process (nixtamalization) in order to produce tortilla chips, the alkaline pH transforms the once vibrant color into a product with a dull, faded appearance. This is due to the fact that the anthocyanin pigments in the corn are unstable and susceptible to degradation at alkaline pH (Brouillard, 1982). As color is a major factor affecting consumer opinion of food quality, minimizing undesirable color change during processing of corn into tortilla chips is essential.

While monomeric anthocyanins possess limited stability against pH increases and subsequent hydration of the anthocyanin chromophore, acylation of the anthocyanin can

greatly improve pigment stability through intramolecular co-pigmentation (Dangles et al., 1993). Other colorless organic molecules can stabilize anthocyanins through intermolecular co-pigmentation as well (Eiro and Heinonen, 2002). These associations can partially block the nucleophilic attack of water, allowing for a greater proportion of the more stable, colored flavylium cation form to persist through processing at more alkaline pH values (Giusti and Wrolstad, 2003; Castaneda-Ovando et al., 2009; Boulton, 2001). The anthocyanins present in red, blue, and purple pigmented corn have been previously identified as cyanidin-3-glucoside, pelargonidin-3-glucoside, peonidin-3-glucoside, as well as their malonated derivatives (Aoki et al., 2002; Pascual-Teresa et al., 2002; Moreno et al., 2005; Abdel-Aal et al., 2006). Recent studies have shown that the anthocyanin profile is fairly similar among different phenotypes of pigmented corn (blue, red, purple, and pink), with variation mainly in proportion. Monomeric anthocyanins were determined to be most abundant across the majority of corn phenotypes investigated, with the exception of purplish-red phenotypes, which contain a greater proportion of acylated anthocyanins (Moreno et al., 2005; Abdel-Aal et al., 2006). Since it is well known that acylated anthocyanins are more stable than their non-acylated counterparts, selective breeding of corn lines with greater proportions of these more stable acylated (malonated) anthocyanins could lead to genetic improvement of pigmented corn for food use (Saito et al., 1995).

Most studies investigating anthocyanins in pigmented corn during corn tortilla chip processing have focused on extractable anthocyanin content or changes in the profile of these compounds in a limited set of samples (De la Parra et al., 2007; Del Pozo-Insfran

et al., 2006; Mora-Rochin et al., 2010). However, no studies have investigated the potential impact of anthocyanin and co-pigment composition on stability of color during processing. The main goal of this research is to investigate the effect of pigment and co-pigment composition in genetically distinct corn hybrids on color stability during nixtamalization and tortilla chip processing.

The objectives of this study were:

1. Identify and quantify major anthocyanins and co-pigments in colored corn hybrids
2. Determine the effect of pigment and co-pigment composition on stability to thermo-alkaline processing

CHAPTER II

LITERATURE REVIEW

Corn Production and Use

The United States is the world's largest producer of corn. The US harvested over 84 million acres in 2011, accounting for 32% of world production of corn. Approximately 20% of this harvest is exported each year, making the U.S. the largest exporter of corn as well. However, only approximately 12% of all harvested corn is utilized for food (corn chips, tortillas, high fructose corn syrup, etc.). Almost 80% is used as animal feed, with the remainder having industrial applications, such as production of ethanol, drywall, and paints, among others (National Corn Grower's Association, 2013; USDA, 2013).

Corn Phenolic Compounds

While the majority of phenolic acids in fruits and vegetables are present in either their free form or as soluble conjugate glycosides, grain phytochemicals mostly occur in their insoluble bound form, with few in free or soluble conjugate form (Vinson et al., 1998, 2001; Sosulski et al., 1982). Phenolic acids are abundant in the corn pericarp and primarily bound to hemicelluloses, which are amorphous heteropolysaccharides comprised of a xylose backbone to which branches of arabinose, galactose, and/or uronic acid are attached. These heteroxylans are cross-linked by diferulic bridges and are also often partially acylated with up to 12% acetyl groups (Gonzalez et al., 2004; Saulnier et

al., 1995). In the endosperm and aleurone sections of the corn kernel, phenolic acids linked to amine groups (phenolic amines) are common, such as feruloylputrescine, p-coumaroylputrescine, diferuloylspermidine, and p-coumaroylspermidine (Naczka and Shahidi, 2006). Approximately 85% of the phenolic compounds in corn exist in the insoluble bound form, with ferulic acid as the phenolic compound present in greatest proportion (Adom and Liu, 2002; Bunzel et al., 2001). The phenolic acids, along with carotenoids, are considered the most significant sources of antioxidants in cereal grains (Montilla et al., 2011).

Anthocyanins in Corn

Anthocyanins are natural water-soluble phenolic compounds that provide the red, blue, purple, and orange colors to a wide array of fruits, vegetables, grains, and plant species (Pazmino-Duran et al., 2001). These pigment compounds have received much attention due to their potential as natural food colorants able to replace the synthetic dyes often under scrutiny (Bridle and Timberlake, 1997). Due to the perception of these anthocyanin compounds as healthy pigments, interest in creating products containing these natural colors is on the rise. Pigmented corns are currently produced in the U.S. in small quantities for their use in specialty foods (Abdel-Aal et al., 2006).

The anthocyanins of pigmented corn have been identified as cyanidin-3-glucoside, pelargonidin-3-glucoside, and peonidin-3-glucoside, as well as their malonated and succinated derivatives (Aoki et al., 2002; Pascual-Teresa et al., 2002; Abdel-Aal et al., 2006; Moreno et al., 2005). These malonated and succinated forms,

along with other acylated anthocyanins, are known to possess greater stability to pH changes than their non-acylated counter-parts (Giusti and Wrolstad, 2003).

Anthocyanin Chemistry

The basic structure of the anthocyanin is the 3-ringed anthocyanidin, or aglycone (Figure 1). This structure comprises an aromatic ring (A-ring) that is bound to an oxygen-containing heterocyclic ring (C-ring), which is linked to another aromatic ring (B-ring) (Konczak and Zhang, 2004). Only 6 anthocyanidins are commonly found in foods: cyanidin, pelargonidin, peonidin, malvidin, petunidin, and delphinidin (Clifford, 2000). In nature, anthocyanidins are generally found in their glycosylated form and referred to as anthocyanins, with cyanidin-3-glucoside being the most abundant anthocyanin (Kong et al., 2003). Over 500 different anthocyanins have been discovered, with differences in structure such as type, number, and position of sugars moieties, the number of hydroxyl and methoxy groups, as well as the amount and type of aliphatic or aromatic acids attached to the sugars in the molecule (Kong et al., 2003; Wu et al., 2006). The sugars most often found in anthocyanins are glucose, galactose, xylose, rhamnose, and arabinose, as well as combinations of these monosaccharides to produce di- and trisaccharides (Brouillard, 1982; Schwartz et al., 2008). These sugar residues can be acylated by aromatic acids such as *p*-coumaric, ferulic, sinapic, and caffeic acids and/or by aliphatic acids, namely malonic, malic, succinic, acetic, and oxalic acids (Brouillard, 1982; Bueno et al., 2012; Schwartz et al., 2008).

Anthocyanin Color

Excitation of the anthocyanin molecule by visible light leads to its vibrant color. Anthocyanins contain many double bonds in their structure (Figure 1), which provide the molecule with high electron mobility, and as these double bonds are excited and shifted, color results (Schwartz et al., 2008). The conjugate double bonds in the anthocyanin molecule absorb light between 480-550 nm to produce the vibrant red, blue, and purple colors (Curtright et al., 1994). If the A- or B-rings of the molecule are substituted with electron-donating groups, such as methoxy and hydroxyl groups, a deeper hue is produced. The hue is deepened from a red/orange to a purple/blue due to a bathochromic shift in the light absorption band of the visible spectrum from a shorter wavelength to a longer one (Curtright et al., 1994). For example, in a comparison of pelargonidin (contains 4 hydroxyl groups) and delphinidin (6 hydroxyl groups), a shift from 520 nm to 545 nm and to a bluer color, for the respective compounds, is observed (Pina et al., 2012). The color of anthocyanins is also strongly affected by the number and type of sugar moieties, acylation of these sugars, pH changes, and co-pigmentation (Schwartz et al., 2008).

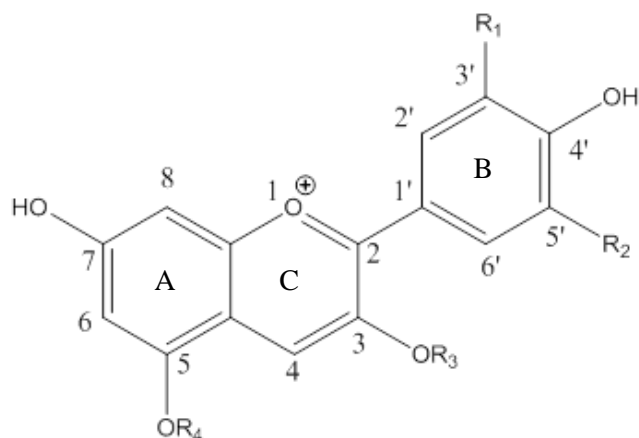


Figure 1. Basic Anthocyanin Structure. R1 and R2 = -H, -OH, or -OCH₃; R3 = -glycosyl, R4 = -H or -glycosyl.

Anthocyanin Stability

There are numerous factors which affect the stability of anthocyanins, such as pH, temperature, enzymes, oxygen concentration, as well as the presence of other phenolic compounds or metal ions (Markakis, 1982; Fossen et al., 1998). Anthocyanins are degraded during processing and storage due to many of the aforementioned factors. With an understanding of anthocyanin chemistry and stability, anthocyanin compounds can be better selected for their intended application, and processing parameters can be further optimized, in order to minimize the loss of these pigments (Scwartz et al., 2008; Zhao et al., 2008; Sidani and Makris, 2011; Jie et al., 2013).

Influence of pH

Anthocyanin molecules have different pH dependent chemical forms due to their ionic nature, and these forms correspond to varying observed colors (Brouillard, 1982; Heredia et al., 1998). The tinctorial strength of anthocyanins is highest at around pH 1, and intensity of color, as well as stability, decreases with increasing pH (Schwartz et al., 2008). At pH values below approximately 2, the flavylium cation is the main structure and it usually possesses either a red or orange color. As the pH increases, two main events occur, which are a proton transfer reaction involving the acidic aglycone hydroxyl groups, as well as the nucleophilic attack of water at position 2 on the flavylium cation C-ring. The former reaction produces the quinoidal base forms (usually blue or light purple), and the latter hydration reaction causes the generation of the colorless carbinol pseudobase, which is also in equilibrium with the pale yellow chalcone form between approximately pH 5-6 (Castañeda-Ovando et al., 2009; Fossen et al., 1998). Above pH 7, most anthocyanin molecules either become dark blue/purple/black or are degraded, unless they are the acyl-type anthocyanins or they are involved in molecular associations with other flavonoid compounds, a phenomenon known as co-pigmentation (Fossen et al., 1998; Cavalcanti et al., 2011).

Co-pigmentation

The two main types of co-pigmentation are intramolecular and intermolecular co-pigmentation (Dangles et al., 1993; Mazza and Brouillard, 1990). Intermolecular co-pigmentation is the molecular association between a colored anthocyanin and a colorless

co-pigment (flavonoid, phenolic compound, or other colorless organic molecule) in which weak hydrophobic forces or ionic interactions act as the major mechanisms (Brouillard, 1983; Mazza and Brouillard, 1990; Dangles et al., 1993). Intramolecular co-pigmentation occurs when the co-pigment is itself a part of the anthocyanin molecule through a covalent linkage to a glycosyl moiety on the anthocyanin chromophore (Dangles et al., 1993; Brouillard, 1983). As intramolecular co-pigmentation occurs through acylation of the anthocyanin, it is a more effective means of blocking the hydration of the anthocyanin chromophore, and therefore more stable to alkaline pH than intermolecular co-pigmentation (Fossen et al., 1998; Bueno et al., 2012).

Anthocyanin color can be both intensified and stabilized by co-pigmentation (Eiro and Heinonen, 2002; Boulton, 2001). Co-pigmentation can cause both a hyperchromic shift (higher absorbance values/greater intensity of color) and/or a bathochromic shift (a shift towards higher wavelength at the λ_{\max} , usually leading to a slightly bluer color) (Rein, 2005; Asen et al., 1972). Co-pigment molecules are rich in π electrons, so they are able to form weak complexes with the electron-poor flavylum cation, and this interaction can partially block the nucleophilic attack of water on the flavylum cation, allowing the complex to maintain its red or orange color (Rein, 2005; Matsufuji et al., 2003). This is an important interaction, as color is one of the main quality factors affecting consumer acceptance of food.

Anthocyanins and Human Health

In addition to the attractive colors they impart in foods, anthocyanins are a class of bioactive flavonoids possessing many potential health benefits. As a relatively high amount of these compounds are consumed in a normal, healthy diet, the interest in the biological activities of anthocyanins has intensified (Wrolstad, 2004). Anthocyanins are reported to have anti-oxidant and anti-inflammatory activities, so consumption of these compounds can reduce the risk of cancer, diabetes, cardiovascular disease, arthritis, and stroke (Wrolstad, 2004; Konczak and Zhang, 2004; Kähkönen and Heinonen, 2003; Prior and Wu, 2006).

Considering purple corn color specifically, the anthocyanin fraction has been reported to contribute to many of the same health-promoting properties, such as anti-oxidant activity, anti-carcinogenic properties, as well as to lowering the risk of developing lifestyle diseases such as hypertension, obesity, and diabetes (Cevallos-Casals and Cisneros-Zevallos, 2003; Long et al., 2013; Shindo et al., 2007; Tsuda et al., 2003).

According to Wu et al., (2006) the average daily intake of anthocyanins in the United States is estimated to be around 12.5 mg/day. Depending on the food source, whether a fruit, vegetable, tuber, legume, or cereal, the concentrations and types of anthocyanins can vary drastically (Bridle and Timberlake, 1997). Cyanidin is the anthocyanin thought to contribute most to anthocyanin intake (approximately 45%), and this is reasonable considering its glycosylated form (cy-3-glucoside) is the most abundant anthocyanin in nature (Wu et al., 2006; Kong et al., 2003). Certain berries,

such as chokeberry and elderberry, are extremely rich in anthocyanins with concentrations ranging from around 2-10 mg/g fresh weight. Other fruits also rich in anthocyanins are black currants, black raspberries, and red grapes. (Bridle and Timberlake, 1997; Clifford, 2000; Wu et al., 2006). Red radish and red cabbage are vegetable sources with high anthocyanin contents, containing around 1.15 mg/g fresh weight (Wu et al., 2006). Pigmented grains, such as black rice (3.28 mg/g), purple corn (1.28 mg/g), scarlet red corn (0.61 mg/g), and purple wheat (0.50 mg/g) are also significant sources of anthocyanins (Abdel-Aal et al., 2006; Hosseinian et al., 2008).

Current Natural Anthocyanins Used in Food Industry

Due to the many attractive colors of anthocyanins, these compounds have great potential for use as natural colorants. The majority of commercial anthocyanin-based colorants come from fruit and vegetable sources such as elderberry, black chokeberry, black currant, red cabbage, red radish, black carrot, red-fleshed sweet potato, and purple sweet potato; Purple corn is an important grain-based source of natural color (Downham and Collins, 2000).

Most of the anthocyanins derived from fruits and purple corn are mono- and diglucosides, and therefore possess only limited stability to processing factors, such as pH and temperature changes (Giusti and Wrolstad, 2003; Brouillard et al., 1989). As previously mentioned, acylation of anthocyanins provides increased stability through the mechanism of intramolecular copigmentation, so it is important to note natural colorant sources high in acylated anthocyanins, which have food industry applications: red

radishes (Giusti and Wrolstad, 1996), red cabbage (Dyrby et al., 2001), red potatoes (Bassa and Francis, 1987), purple sweet potatoes (Fossen and Andersen, 2000), and black carrots (Stintzing et al., 2002).

Alkaline Processing of Corn

Nixtamalization

Nixtamalization, a traditional process developed by the Aztecs, is the primary processing step in the production of many corn products, such as table tortillas and corn tortilla chips. The process involves the alkaline cooking and steeping of corn kernels in a calcium hydroxide solution. The alkaline cooked corn (nixtamal) is then washed to remove excess lime and to partially remove the pericarp before the nixtamal is ground into masa, which can then be used to make tortillas, tortilla chips, and related corn products (Pflugfelder et al., 1988). This alkaline treatment leads to many changes in the physical structure of the grain, the chemical composition, as well as the nutrition of the processed product (de la Parra et al., 2007).

Physical, Chemical, and Nutritional Implications of Nixtamalization

A significant change due to the alkaline cooking and steeping involves the softening and partial removal of the outer covering of the grain (pericarp) through partial solubilization of the hemicelluloses present in the pericarp and cell walls, as well as the physical washing step (Gomez et al., 1989). This allows the alkaline solution to enter the

grain, facilitating the diffusion of calcium and water into the corn kernel. As the tortilla and other nixtamalized products are the primary source of calcium in the diets of many in Mexico and Central America, the amount of calcium incorporated into the nixtamal which will be ground into masa is of high nutritional significance (Gutierrez et al., 2007). Nixtamalization also causes starch and protein modifications of the grain, affecting the functional properties of the final product, such as texture, flexibility, crispiness, and shelf-life (Gomez et al., 1989). Another outcome of the alkaline cooking and steeping stages is the loss of solids, such as polysaccharides, fiber, starch, and protein, as well as phytochemical compounds, to the wastewater (nejayote) (Pflugfelder et al., 1988; Gutierrez-Urbe et al., 2010).

Effect of Nixtamalization on Anthocyanin Content of Pigmented Corns

According to de la Parra et al. (2007) and in confirmation of previous studies, a majority of the anthocyanins present in pigmented corn are lost during steeping to the wastewater. In the same study it was determined that the nixtamalized blue corn and red corn lost 80% and 23%, respectively, of the anthocyanins present in their raw corn counterparts. A comparison of total anthocyanin content between the latter stages of processing (masa, tortillas, and tortilla chips) of both the red and blue corns showed no significant differences among the stages. In contrast, the anthocyanin losses in blue corn were found to further increase when the raw kernels were processed into nixtamal, tortillas, and chips (losses of 37%, 54%, and 75%, respectively) (Del Pozo-Insfran et al., 2006). This and other studies suggest that in addition to the diffusion of anthocyanins

into the wastewater, structural degradation of the anthocyanin compounds also occurs during nixtamalization due to the harsh combination of a high cooking temperature (90 °C) and alkaline pH (approximately 11) (Salinas-Moreno et al., 2003). With increasing lime concentration, and therefore increasing pH of the cooking solution, greater anthocyanin losses are incurred in pigmented corn (Cortes et al., 2006; de la Parra et al., 2007).

In order to counter the negative pH effect of nixtamalization on anthocyanin losses, acidification treatments have been investigated (Del Pozo-Insfran et al., 2006). This treatment did cause a slightly greater retention of anthocyanins in blue corn through the stages of processing (nixtamal, tortillas, and chips) due to their heightened stability in acidic environments. However, the acidic treatment alters the desired and expected lime-cooked flavor (Del Pozo-Insfran et al., 2006).

An additional factor dictating total anthocyanin losses is the true location of the pigments in the maize kernel. When pigments are located strictly in the outer pericarp layer, essentially all of the anthocyanins are either degraded or lost through the washing and removal of the pericarp layer. However, if the pigments are present in the aleurone and pericarp layers, the aleurone pigments are physically retained through the nixtamalization process (Salinas-Moreno et al., 2003).

Overall Goal of this Research

Many studies on the anthocyanins of pigmented corn focus on extractable anthocyanin content, as well as degradation of these pigments and changes in the profile

of these compounds through processing into masa, tortillas, and chips. However, to our knowledge, no literature exists on how the anthocyanin and co-pigment composition affects color stability during processing. Thus, the overall goal of this research is to study the effect that pigment and co-pigment composition has on color stability of genetically distinct, newly developed pigmented corn hybrids during nixtamalization and corn tortilla chip processing.

CHAPTER III

IDENTIFICATION AND QUANTIFICATION OF MAJOR ANTHOCYANINS AND CO-PIGMENTS IN COLORED CORN HYBRIDS

Introduction

Consumer interest in healthy, natural snacks provides an opportunity for growth and innovation in the tortilla chip market. Consumers perceive colored corn chips, such as blue or purple corn chips, to be healthier than traditional white or yellow corn chips (SOTI, 2012). Anthocyanins, a class of water-soluble flavonoids, are the main pigments in colored corn (Abdel-Aal et al., 2006). In addition to providing appealing colors in foods, anthocyanins also possess potential health benefits, such as cancer cell growth inhibition and prevention of other diseases, including hypertension and cardiovascular disease (Song et al., 2012, Long et al., 2013, Shindo et al., 2007, Wrolstad, 2004).

Among pigmented corn phenotypes, monomeric anthocyanins such as cyanidin-3-glucoside, peonidin-3-glucoside and pelargonidin-3-glucoside have been characterized, as well as their malonated counterparts (Moreno et al., 2005; Aoki et al., 2002; Pascual-Teresa et al., 2002; Abdel-Aal et al., 2006). A majority of pigmented corn lines contain mostly monomeric anthocyanins, with the exception of purplish-red phenotypes, which have been shown to possess a larger proportion of acylated anthocyanins (Moreno et al., 2005; Abdel-Aal et al., 2006). As it is well known that acylated anthocyanins are more stable than their non-acylated forms at higher pH, identification of phenotypes or specific varieties with greater proportions of these stable

pigments could lead to improved selection of colored corn for food use (Saito et al., 1995). However, the influence of genetics/phenotype and environment on the pigment and co-pigment composition of pigmented corn is not very well understood.

The aim of this portion of the study was to characterize the major anthocyanin and co-pigment profiles of a set of genetically distinct pigmented corn hybrids to understand how genetics/phenotype affects the composition and content of anthocyanins and co-pigments relevant to food processing. The goal was to determine if genetics/phenotype can be used to select corn that inherently contains a pigment profile that would provide greater stability during thermo-alkaline processing. From this information, corn lines that contain a higher proportion of acylated anthocyanins can be selected for food applications, which could lead to a greater retention of natural pigments during processing.

Materials and Methods

Materials

Pigmented Corn Kernels. Pigmented corn kernels with varying genetic backgrounds from four different phenotypes (red, blue, purple [also identified as maiz morado], and red/blue) were provided by the Texas A&M University Quantitative Genetics and Maize Breeding Program. Two colored corn inbred lines were crossed to make experimental hybrids, and the corn samples used in this study were the grain of these hybrid plants

that had been self-pollinated in two different environments (College Station, TX, 2013 and Weslaco, TX, 2011). The corn kernel genetic variety, phenotype, and growing location/year are listed in Table 1 and additional information can be found in Mahan et al. (2013). Prior to analysis whole grain samples were pre-ground using a coffee grinder (Cuisinart, Model DCG-20N series), ground with a UDY cyclone sample mill equipped with a 0.1 mm screen (Model 3010-030, UDY Corporation, Fort Collins, CO), and stored in a freezer at -20°C until extraction. A moisture analyzer (Model HB43-S, Mettler-Toledo, LLC, Columbus, OH) was used to determine the moisture contents of the ground samples, which were confirmed using the AACC Air-Oven Method of moisture content determination (method 44-15.02, AACC International, 1999).

Chemicals and Reagents. All reagents used were of analytical grade. Cyanidin chloride and pelargonidin chloride standards were purchased from Extrasynthese Natural Products, Genay Cedex, France. Ferulic acid and caffeic acid were purchased from Spectrum Chemical Mfg. Corp., Gardena, CA; *p*-coumaric acid was purchased from Sigma-Aldrich Chemicals, St. Louis, MO.

Table 1. Characteristics of pigmented corn varieties selected for this study.

Genetic hybrid variety	Phenotype	Growing location/year
LAMA Red R10 x Red Hybrid Ear R3	Red	Weslaco, TX/2011, College Station, TX/2013
Red Ear R4 x LAMA Red R10	Red	Weslaco, TX/2011, College Station, TX/2013

Table 1- Continued

Genetic hybrid variety	Phenotype	Growing location/year
Wenwei1 R5 x Red Hybrid Ear R3	Red	Weslaco, TX/2011, College Station, TX/2013
LH287 R8 x Wenwei1 R5	Red	College Station, TX/2011, College Station, TX/2013
Wenwei2 R6 x Red Hybrid Ear R3	Red	College Station, TX/2011, College Station, TX/2013
Red Ear R4 x Wenwei1 R5	Red	Weslaco, TX/2011, College Station, TX/2013
Wenwei2 R6 x LH287 R8	Red	Weslaco, TX/2011, College Station, TX/2013
LH195 R7 x Red Hybrid Ear R3	Red	College Station, TX/2011, College Station, TX/2013
Wenwei2 R6 x Maize Morado R11	Purple	Weslaco, TX/2011, College Station, TX/2013
Wenwei1 R5 x Maize Morado R11	Purple	Weslaco, TX/2011, College Station, TX/2013
LH195 R7 x Maize Morado R11	Purple	Weslaco, TX/2011, College Station, TX/2013
Red Ear R4 x Maize Morado R11	Purple	Weslaco, TX/2011, College Station, TX/2013
Red Hybrid Ear R3 x Maize Morado R11	Purple	Weslaco, TX/2011, College Station, TX/2013
Lfy Blue R1 x Maize Morado R11	Purple	Weslaco, TX/2011, College Station, TX/2013
Lfy Blue R1 x Ethiopia Blue R2	Blue	Weslaco, TX/2011, College Station, TX/2013
Lfy Blue R1 x Ethiopia Blue2 R9	Blue	Weslaco, TX/2011, College Station, TX/2013
Ethiopia Blue2 R9 x Ethiopia Blue R2	Blue	Weslaco, TX/2011
Lfy Blue R1 x Red Ear R4	Red/Blue	Weslaco, TX/2011, College Station, TX/2013
Red Hybrid Ear R3 x Ethiopia Blue2 R9	Red/Blue	College Station, TX/2011, College Station, TX/2013
Wenwei1 R5 x Ethiopia Blue R2	Red/Blue	Weslaco, TX/2011, College Station, TX/2013
Wenwei2 R6 x Lfy Blue R1	Red/Blue	Weslaco, TX/2011, College Station, TX/2013
Red Ear R4 x Ethiopia Blue2 R9	Red/Blue	Weslaco, TX/2011, College Station, TX/2013
Ethiopia Blue2 R9 x Red Ear R4	Red/Blue	Weslaco, TX/2011, College Station, TX/2013
Ethiopia Blue R2 x Red Ear R4	Red/Blue	Weslaco, TX/2011, College Station, TX/2013

Methods

Extraction. Ground corn flour (5 g) was extracted in 1% HCl in methanol (10 mL) with continuous shaking for 2 h at room temperature. The samples were then centrifuged (9,900 g-force for 8 min) using a Heraeus Megafuge 11R Centrifuge (Thermo Fisher Scientific, Asheville, NC) and the supernatants were collected. A portion of each supernatant was then passed through a syringe filter with a 0.45 μm nylon membrane before being injected (3 μL) onto the HPLC column.

HPLC Analysis. Individual anthocyanins and co-pigments were separated and quantified using an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) equipped with a ZORBAX Stable Bond Eclipse XDB-C18 column (4.6 \times 150 mm, 5 μm ; Agilent Technologies, MD, USA), with a guard column (4.6 \times 12.5 mm, 5 μm). The mobile phase comprised 2% formic acid in water (A) and 2% formic acid in acetonitrile: methanol (50:50) (B). The elution gradient (B) was: 0-2 min, 10% isocratic; 2-4 min, 10-20%; 4-24 min, 20-50%; 24-28 min 50% isocratic; 28-30 min, 50-10%; 30-37 min, 10% isocratic. Separation was achieved at a flow rate of 1 mL/min with the column thermostat held at 40°C. Compounds were monitored at wavelengths of 280, 320, 360 nm (for co-pigments), and 520 nm (for anthocyanins) using a diode array detector.

UPLC-ESI/MS Analysis. Analysis was carried out using a Waters-ACQUITY UPLC/MS system (Waters Corp., Milford, MA), which was equipped with a binary

solvent manager, sample manager (autosampler), column heater; and photodiode array $e\lambda$ detector (PDA). This was interfaced with a mass spectrometer equipped with a tandem quadrupole (TQD) electrospray ionization (ESI) detector. A Kinetex C18 column (150 mm x 2.10 mm, 2.6 μm , Phenomenex, Torrance, CA) was utilized for separation with a column temperature of 50 °C for anthocyanins, which were monitored at 520 nm, and a temperature of 40° C for other phenolics, which were monitored at 280 nm, 320 nm, and 360 nm. Mass spectrometric data for anthocyanins were acquired in positive mode and in negative mode for other phenolics. Empower 2 software (Waters Corp., Milford, MA) was employed to obtain and analyze the data. The MS scan was recorded in the range of 100-1000 Da for anthocyanins and other phenolics. Nitrogen was the drying and nebulizing gas used, and argon was used as the collision gas (AOC, Bryan, TX). The nitrogen gas flow conditions were 800 L/h and 50 L/h for desolvation and at the cone, respectively. Source block temperature and desolvation temperature were set at 150°C and 400°C, respectively. The intensity of the mass signals of protonated/deprotonated molecules and aglycone fragments was used for optimization of ionization conditions, and was performed for each individual compound/peak detected. Mass parameters were optimized as follows: capillary voltage, 3.5/3.0 kV; and cone voltage, 40/30 V for positive/negative ionization, respectively. The MS/MS scan was optimized as follows: cone voltage, 40/30-55 V; and collision energy, 20/15-35 V for anthocyanins/co-pigments, respectively.

Samples were prepared in identical fashion to those for HPLC analysis, but the final filtration prior to injection was through 0.2 μm PTFE filters, with 1 μL injected

onto the LC column for analysis. The solvents used were 2% formic acid in water (solvent A) and acetonitrile (solvent B) for anthocyanin analysis, and 0.05% formic acid in water (solvent A) and acetonitrile (solvent B) for the co-pigments. The gradient, at a flow rate of 0.4 mL/min, was as follows: 5% B from 0 – 2 min, 5 – 75% B from 2 – 27 min, 75 % B isocratic from 27 – 30 min, 75 – 5 % B from 30 – 31 min, followed by 5% B isocratic for 5 min to allow for column equilibration before the next injection.

Quantification of Major Anthocyanins and Co-pigments. Major anthocyanins and co-pigments were quantified based on standard curves calibrated using pure standards.

However, when standards were not available, content was determined using a molecular weight correction factor and based on the assumption that derivatives possess molar absorptivities similar to their aglycones. Concentrations of identified compounds were expressed as $\mu\text{g/g}$, dry basis.

Determination of Total Anthocyanin Content and Proportion of Monomeric

Anthocyanins. Total anthocyanin content (TAC), total monomeric anthocyanin content (TMA), as well as proportion of monomeric anthocyanins, was determined using the pH differential method as described by Lee et al. (2005). Samples were extracted in 1% HCl in methanol (5 g sample/10 mL MeOH) and diluted appropriately to reach A_{520} of around 1.0 using pH 1.0 buffer. Using this determined dilution factor, 2 dilutions of the sample were prepared (one with pH 1.0 buffer and one with pH 4.5 buffer). Samples were scanned from 800-200 nm using a UV-Vis spectrophotometer (Shimadzu Scientific

Instruments, Columbia, MD) and absorbance data was collected at 520 and 700 nm.

Proportion of monomeric anthocyanins was only determined for a subset of purple and red/blue samples. The remaining samples had total pigment contents that were too low, so proportion monomeric could not be determined. Total anthocyanin content was determined for all samples. For total anthocyanin content, absorbance was calculated as:

$$A_{TAC} = (A_{520 \text{ nm}} - A_{700 \text{ nm}}) \text{ pH } 1.0$$

Whereas for total monomeric anthocyanin content absorbance was calculated as:

$$A_{TMA} = (A_{520 \text{ nm}} - A_{700 \text{ nm}}) \text{ pH } 1.0 - (A_{520 \text{ nm}} - A_{700 \text{ nm}}) \text{ pH } 4.5$$

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

$$TAC = (A_{TAC} \times MW \times DF \times 10^3) / (\epsilon \times 1)$$

$$TMA = (A_{TMA} \times MW \times DF \times 10^3) / (\epsilon \times 1)$$

$$\% \text{ Monomeric anthocyanins} = (TMA/TAC) \times 100$$

Where A_{TAC} is absorbance for total anthocyanin content at 520 nm, A_{TMA} is absorbance for total monomeric anthocyanin content at 520 nm, MW is molecular weight of Cy-3-glu (449.2 g/mol), DF is dilution factor, 1 is the pathlength in cm, and ϵ is molar extinction coefficient of Cy-3-glu (26900).

All data were reported in $\mu\text{g/g}$ (dry basis).

Statistical Analysis. Data analysis was carried out using one-way ANOVA to detect differences among the means, and Fisher's Least Significant Difference was used to

separate means with $P < 0.05$. Statistical Analysis was done using JMP Pro (Version 10.0.0, SAS Institute, Cary, NC).

Results and Discussion

Identification of Major Anthocyanins

Reverse phase HPLC and UPLC-ESI/MS analysis were used to identify the major anthocyanins present in the pigmented corn hybrids by comparing the molecular ions and MS/MS fragment ions with those of published studies (Abdel-Aal et al., 2006 and Aoki et al., 2002). When possible, the identities of compounds were further confirmed through the comparison of HPLC retention times and UV-*vis* characteristics of the sample compounds with those of pure standards. The representative LC-MS characteristics of a purple corn extract are summarized in Table 2.

Table 2. Identification of anthocyanins in a purple corn extract (Wenwei1 R5 x Maize Morado R11) based on UPLC retention time (t_R), UV-*vis* spectroscopic characteristics (λ_{max}), MS and MS/MS spectroscopic pattern. ESI was performed in the positive mode. Peak numbers are referenced to Figure 3. The structures of the peaks are in Figure 2.

Peak no.	t_R (min)	λ_{max} (nm)	$[M+H]^+$ (m/z)	MS/MS fragments (m/z)	Proposed identification
1	5.224	515	449	287	Cyanidin-3-glucoside
2	5.767	502	433	271	Pelargonidin-3-glucoside
3	5.997	513	535	287	Cyanidin-3-(3''-malonylglucoside)
4	6.130	520	463	301	Peonidin-3-glucoside
5	6.503	519	535	287	Cyanidin-3-(6''-malonylglucoside)
6	7.023	503	519	271	Pelargonidin-3-malonylglucoside
7	7.308	521	549	301	Peonidin-3-malonylglucoside

When anthocyanins are analyzed by ESI/MS in the positive mode, the molecular ion $[M+H]^+$ conveys the true molecular weight of the ion because anthocyanins naturally carry a positive charge, and fragment ions occur in the form of $[M+H-X]^+$. The fragment ion is the aglycone form of the anthocyanin. Three major aglycones were observed with the associated MS/MS fragment ions: cyanidin, m/z 287, pelargonidin, m/z 271, and peonidin, m/z 301. The representative UPLC chromatograms of a purple corn sample (Wenwei1 R5 x Maize Morado R11), a red/blue corn sample (Wenwei2 R6 x Lfy Blue R1), and a blue corn sample (Lfy Blue R1 x Ethiopia Blue R2) are shown in Figure 3. In general, 7 anthocyanin peaks were identified, with generally similar patterns among phenotypes (Abdel-Aal et al., 2006; Aoki et al., 2002). **Peak 1** ($t_R = 5.224$, $\lambda_{max} = 515$) had a $[M+H]^+$ at m/z 449. The MS/MS resolved only one fragment ion (100% intensity) at m/z 287. The loss of 162 amu signifies the loss of a hexose, and the aglycone of m/z

287 indicated cyanidin. As glucose is the most common hexose reported in corn anthocyanins, it was the hexose assumed in this study. Therefore, **peak 1** was identified as cyanidin-3-glucoside. **Peak 2** ($t_R = 5.767$, $\lambda_{max} = 502$) had a $[M+H]^+$ at m/z 433. The MS/MS fragmentation showed a dominant ion at m/z 271, signifying that pelargonidin was the aglycone and that a loss of a hexose moiety had occurred (M-162 amu), so **peak 2** was identified as pelargonidin-3-glucoside.

Peak 3 ($t_R = 5.997$, $\lambda_{max} = 513$) had a $[M+H]^+$ at m/z 535 with the dominant fragment ion at m/z 287. The aglycone was thus identified as a cyanidin; the loss of 248 amu signified that this was due to the presence of a malonylhexose fragment, so **peak 3** was identified as cyanidin-3-(3''-malonylglucoside) (Abdel-Aal et al., 2006). **Peak 4** ($t_R = 6.130$, $\lambda_{max} = 520$) showed a molecular ion $[M+H]^+$ at m/z 463 with a fragment ion (100% intensity) at m/z 301. The loss of 162 amu signifies the loss of a hexose unit, so **peak 4** was identified as peonidin-3-glucoside. **Peak 5** ($t_R = 6.503$, $\lambda_{max} = 519$) had a $[M+H]^+$ at m/z 535 with the MS/MS fragmentation showing a dominant ion at m/z 287, which matched the pattern of **peak 3**; however, the later elution suggests a different attachment position for the malonyl moiety. Based on literature, we identified **peak 5** as cyanidin-3-(6''-malonylglucoside) (Pascual-Teresa et al., 2002). **Peak 6** ($t_R = 7.023$, $\lambda_{max} = 503$) had a $[M+H]^+$ at m/z 519. The MS/MS fragmentation showed a dominant ion at m/z 271; the loss of 248 amu indicated malonylglucoside is attached to a pelargonidin aglycone. **Peak 6** was thus identified as pelargonidin-3-malonylglucoside (Pascual-Teresa et al., 2002). **Peak 7** ($t_R = 7.308$, $\lambda_{max} = 521$) had a $[M+H]^+$ at m/z 549. The MS/MS showed the dominant fragment ion at m/z 301 (M-248 amu, loss of

malonylglucoside), indicating a peonidin aglycone, so **peak 7** was identified as peonidin-3-malonylglucoside (Pascual-Teresa et al., 2002).

All 7 of the anthocyanins were identified in the purple phenotype. The two dominant anthocyanins in all phenotypes were cyanidin-3-glucoside and cyanidin-3-(6''-malonylglucoside). In the red phenotype, only the dominant cyanidin-3-glucoside and cyanidin-3-(6''-malonylglucoside) could be identified, with several red samples containing undetectable levels of anthocyanins. The red/blue and blue phenotypes exhibited similar anthocyanin profiles to each other. Under conditions used in this study, anthocyanin profiles were generally similar within phenotypes (purple, red, red/blue, and blue), suggesting that the genetic background has a major influence on anthocyanin profile in pigmented corn.

Peak no.	Anthocyanin structure	Substituents	
		R1	R3
1	Cyanidin-3-glucoside	OH	Glu
2	Pelargonidin-3-glucoside	H	Glu
3	Cyanidin-3-(3''-malonylglucoside)	OH	MalGlu
4	Peonidin-3-glucoside	OCH3	Glu
5	Cyanidin-3-(6''-malonylglucoside)	OH	MalGlu
6	Pelargonidin-3-malonylglucoside	H	MalGlu
7	Peonidin-3-malonylglucoside	OCH3	MalGlu

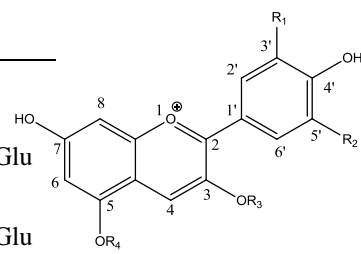


Figure 2. Chemical structures of the seven anthocyanins found in pigmented corn. See Table 2 for molecular masses, maximum absorption wavelengths and peak retention times. R2 = H; R4 = H.

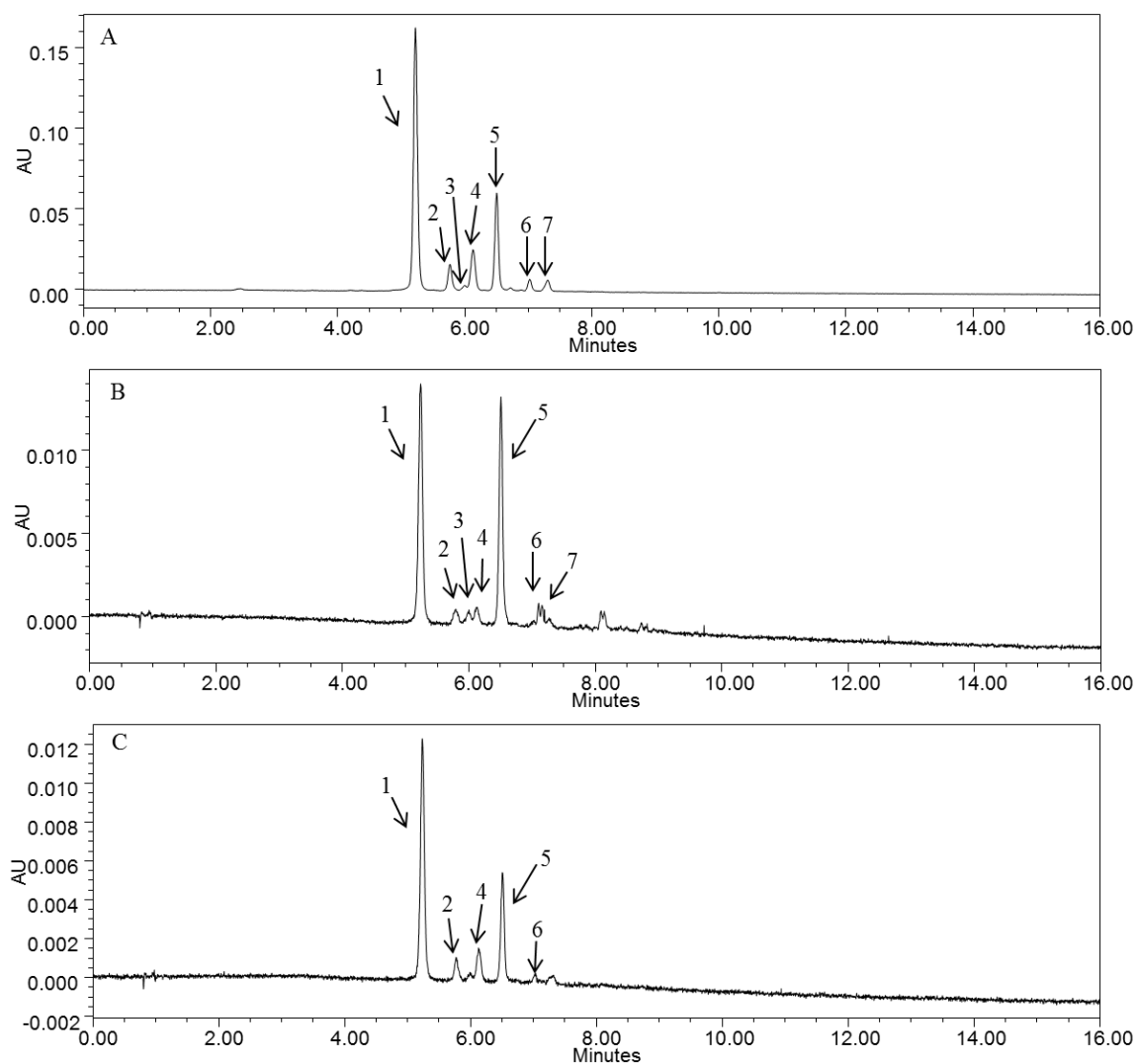


Figure 3. Representative UPLC chromatograms of anthocyanins found in the methanolic fraction from a purple corn sample (Wenwei1 R5 x Maize Morado R11) (A), a red/blue corn sample (Wenwei2 R6 x Lfy Blue R1) (B), and a blue corn sample (Lfy Blue R1 x Ethiopia Blue R2) (C) monitored at 520 nm. See Table 2 for peak identities. See Figure 2 for structures.

Compositional Differences in Anthocyanins in Pigmented Corn

All samples were analyzed by reverse phase HPLC at 520 nm to determine variability in anthocyanin profiles within and among phenotypes. Differences in pigment profile were observed between the purple and the red/blue and blue phenotypes (the blue and red/blue profiles were very similar). The main difference between these groups was the proportion of cyanidin-3-glucoside vs. proportion of cyanidin-3-(6''-malonylglucoside) (Figure 4). Overall, the purple phenotype possessed a higher proportion of monomeric cyanidin-3-glucoside, while the red/blue and blue phenotypes contained a higher proportion of acylated cyanidin-3-(6''-malonylglucoside). Based on this difference in anthocyanin profile, it was hypothesized that the blue and red/blue phenotypes would maintain more color through processing due to the higher proportion of the more stable, malonated derivatives in these phenotypes. In approximately half of the red samples, the anthocyanin levels were below the limit of detection and no peaks were observed. In the red samples with quantifiable peaks, the pigment concentrations were still much lower than in the other three phenotypes (purple, blue, and red/blue). Within each phenotype, however, the anthocyanin profiles were fairly similar among the different hybrid varieties.

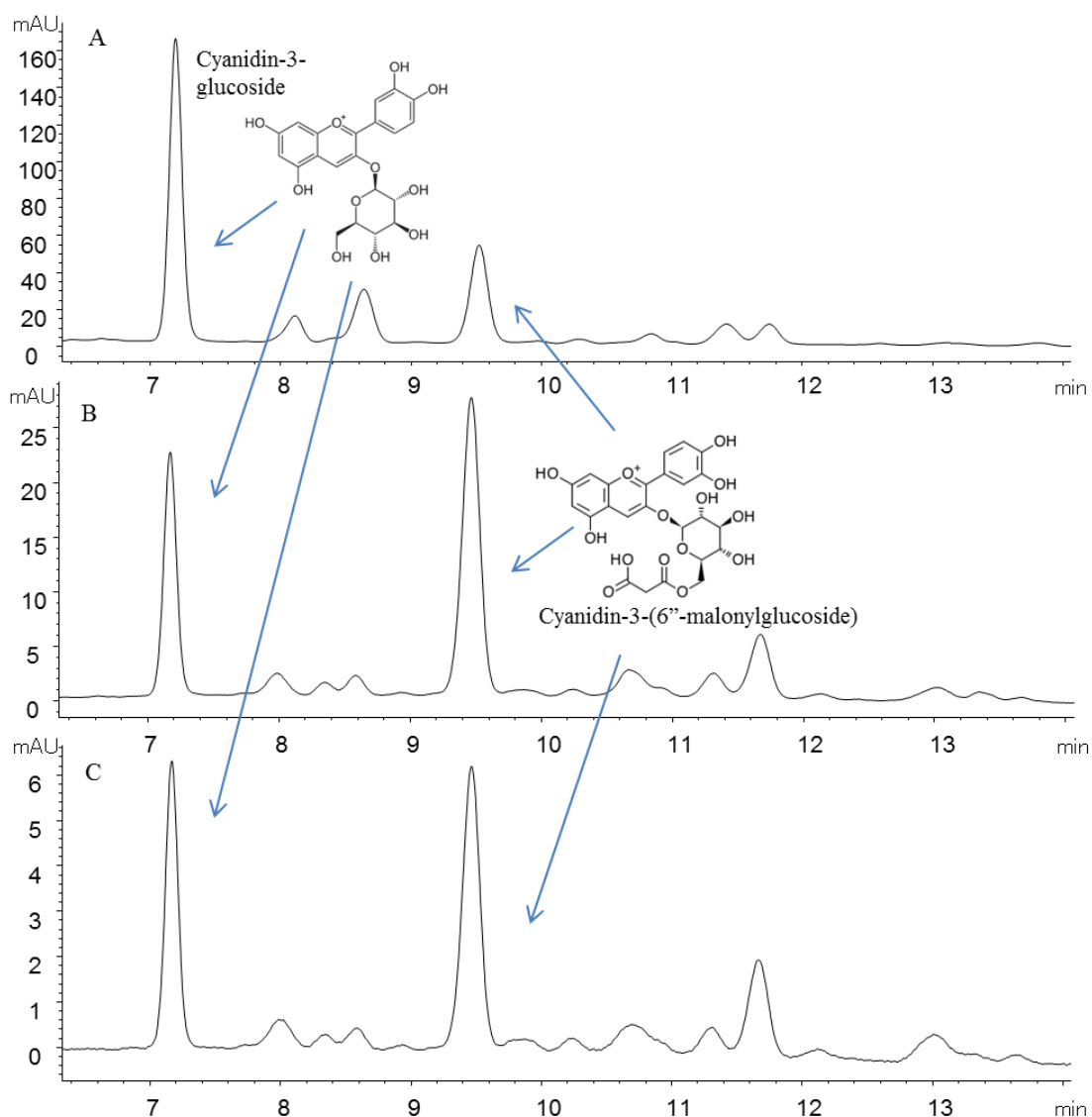


Figure 4. Representative HPLC chromatograms from the Weslaco 2011 set of samples depicting the differing anthocyanin profiles of a purple corn sample (Wenwei1 R5 x Maize Morado R11) (A) vs. a red/blue corn sample (Wenwei2 R6 x Lfy Blue R1) (B) and a blue corn sample (Lfy Blue R1 x Ethiopia Blue2 R9) (C) monitored at 520 nm. The purple phenotype contains a higher proportion of cyanidin-3-glucoside, while the red/blue and blue phenotypes have a higher proportion of cyanidin-3-(6''-malonylglucoside).

Quantification of Major Anthocyanins

Anthocyanin content varied significantly among the four phenotypes. Total anthocyanin contents of all samples from both growing years/locations are listed in Table 3.

All samples from both locations/years within the purple phenotype had significantly higher total anthocyanin contents than any sample in the remaining three phenotypes (blue, red/blue, and red) (Figures 5 and 6). Anthocyanin contents of purple samples ranged from 890-3312 $\mu\text{g/g}$, while the other three phenotypes had much lower anthocyanin contents (8.9-540 $\mu\text{g/g}$). Among the purple phenotype, which had the highest pigment content overall, the Lfy Blue R1 x Maize Morado R11 hybrid variety had the highest average anthocyanin content for both the Weslaco 2011 (WE11) set and the College Station 2013 (CS13) set ($2948 \pm 15.2 \mu\text{g/g}$ and $3312 \pm 23.8 \mu\text{g/g}$, respectively). The Red Hybrid Ear R3 x Maize Morado R11 hybrid variety had the lowest total anthocyanin content among the purple samples for both years ($890 \pm 44.7 \mu\text{g/g}$ for Weslaco 2011 and $1104 \pm 37.3 \mu\text{g/g}$ for College Station 2013).

Among the non-purple phenotypes (Figure 6), the red phenotype had the lowest total pigment content ranging from 8.9-127 $\mu\text{g/g}$ in both locations. The average anthocyanin content for red samples grown in Weslaco 2011 was 57.8 $\mu\text{g/g}$, whereas the average anthocyanin content for red samples from College Station 2013 was 14.7 $\mu\text{g/g}$. The red/blue phenotype contained samples with higher total anthocyanin contents than the red phenotype ranging from 46-368 $\mu\text{g/g}$ in both locations. The average anthocyanin content for red/blue samples grown in Weslaco 2011 was 268 $\mu\text{g/g}$, whereas the average

anthocyanin content for red/blue samples from College Station 2013 was 101 $\mu\text{g/g}$. The blue phenotype contained the highest anthocyanin content of the non-purple phenotypes ranging from 111-540 $\mu\text{g/g}$ in both locations. The average anthocyanin content for blue samples grown in Weslaco 2011 was 429 $\mu\text{g/g}$, and the average anthocyanin content for blue samples from College Station 2013 was 186 $\mu\text{g/g}$. Among the non-purple phenotypes, a clear environmental pattern existed; the samples grown in Weslaco had much higher total pigment contents on average than the samples grown in College Station (Table 3). While the way in which these specific growth environments affect the formation and accumulation of anthocyanins is not fully understood, it appears that there is an important effect of environment vs. genetics/phenotype on total pigment content in pigmented corn that must be considered.

The differences among many hybrids within a phenotype were significant, indicating a varietal effect on total pigment content. However, a clear pattern was observed, in which both phenotype and environment were useful predictors of overall anthocyanin content in the pigmented corn.

Table 3. Total anthocyanin content of raw flour from pigmented corn hybrids as determined by UV-*vis* spectrometry (Mean \pm SD, n=3).

Hybrid variety	Phenotype	Total anthocyanin content (expressed as Cy-3-glu equivalents, $\mu\text{g/g}$, db)	
		Weslaco 2011	College Station 2013
LAMA Red R10 x Red Hybrid Ear R3	Red	59.8 \pm 1.7 ^{K,L}	10.8 \pm 0.85 ^G
Red Ear R4 x LAMA Red R10	Red	37.2 \pm 6.3 ^L	24.0 \pm 0.17 ^G
Wenwei1 R5 x Red Hybrid Ear R3	Red	126.9 \pm 1.8 ^{J,K,L}	12.4 \pm 0.27 ^G
LH287 R8 x Wenwei1 R5	Red	44.0 \pm 5.3 ^{†,L}	12.7 \pm 0.74 ^G
Wenwei2 R6 x Red Hybrid Ear R3	Red	53.1 \pm 8.0 ^{†,L}	12.2 \pm 0.25 ^G
Red Ear R4 x Wenwei1 R5	Red	40.6 \pm 3.5 ^L	24.2 \pm 0.17 ^G
Wenwei2 R6 x LH287 R8	Red	57.9 \pm 0.78 ^L	12.5 \pm 0.63 ^G
LH195 R7 x Red Hybrid Ear R3	Red	42.9 \pm 2.0 ^{†,L}	9.0 \pm 0.11 ^G
Average		57.8 \pm 27.8	14.7 \pm 5.6
Wenwei2 R6 x Maize Morado R11	Purple	1015.4 \pm 36.9 ^D	3270.9 \pm 46.6 ^A
Wenwei1 R5 x Maize Morado R11	Purple	1380.4 \pm 9.9 ^C	2473.8 \pm 103.9 ^C
LH195 R7 x Maize Morado R11	Purple	2257.1 \pm 22.8 ^B	1882.1 \pm 186.0 ^D
Red Ear R4 x Maize Morado R11	Purple	2940.0 \pm 141.9 ^A	2734.3 \pm 86.8 ^B
Red Hybrid Ear R3 x Maize Morado R11	Purple	890.5 \pm 44.7 ^E	1104.4 \pm 37.3 ^E
Lfy Blue R1 x Maize Morado R11	Purple	2948.6 \pm 15.2 ^A	3312.4 \pm 23.8 ^A
Average		1905 \pm 879	2463 \pm 800
Lfy Blue R1 x Ethiopia Blue R2	Blue	506.7 \pm 10.0 ^F	261.3 \pm 16.5 ^F
Lfy Blue R1 x Ethiopia Blue2 R9	Blue	240.0 \pm 6.4 ^{H,I}	111.0 \pm 1.3 ^{F,G}
Ethiopia Blue2 R9 x Ethiopia Blue R2	Blue	540.3 \pm 3.8 ^F	n/a
Average		429 \pm 143	186 \pm 92
Lfy Blue R1 x Red Ear R4	Red/Blue	264.0 \pm 12.4 ^H	112.3 \pm 5.4 ^{F,G}
Red Hybrid Ear R3 x Ethiopia Blue2 R9	Red/Blue	160.8 \pm 0.75 ^{†,I,J,K}	45.8 \pm 0.31 ^G

Table 3-Continued

Hybrid variety	Phenotype	Total anthocyanin content (expressed as Cy-3-glu equivalents, $\mu\text{g/g}$, db)	
		Weslaco 2011	College Station 2013
Wenwei1 R5 x Ethiopia Blue R2	Red/Blue	301.6 \pm 1.2 ^{G,H}	136.5 \pm 0.67 ^{F,G}
Wenwei2 R6 x Lfy Blue R1	Red/Blue	368.0 \pm 5.9 ^G	69.4 \pm 1.9 ^G
Red Ear R4 x Ethiopia Blue2 R9	Red/Blue	205.6 \pm 8.5 ^{H,I,J}	83.5 \pm 1.0 ^G
Ethiopia Blue2 R9 x Red Ear R4	Red/Blue	211.4 \pm 11.0 ^{H,I,J}	100.1 \pm 0.76 ^G
Ethiopia Blue R2 x Red Ear R4	Red/Blue	366.1 \pm 0.97 ^G	159.4 \pm 0.69 ^{F,G}
Average		268 \pm 77	101 \pm 37

Values within the same column with no letters in common are significantly different ($p < 0.05$).

n/a-sample not available

†Sample from College Station 2011

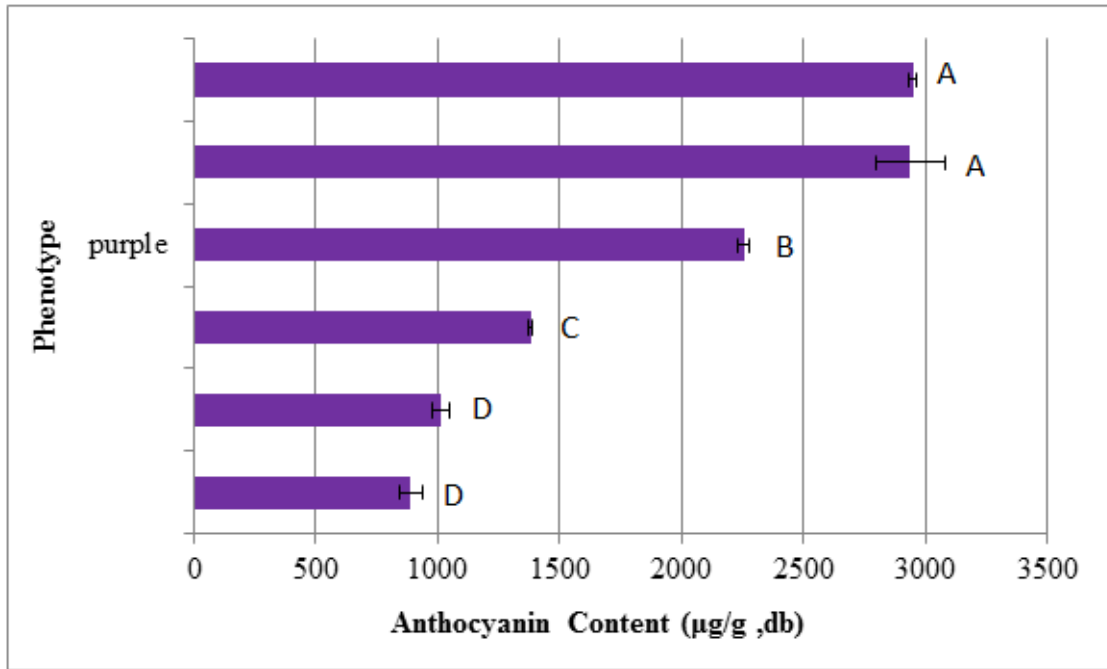


Figure 5. Total pigment content of Weslaco, TX 2011 purple samples, as determined by UV-vis spectrometry. Data are expressed as cyanidin-3-glucoside equivalents \pm SD (n=3). Columns with different letters are significantly different ($p < 0.05$).

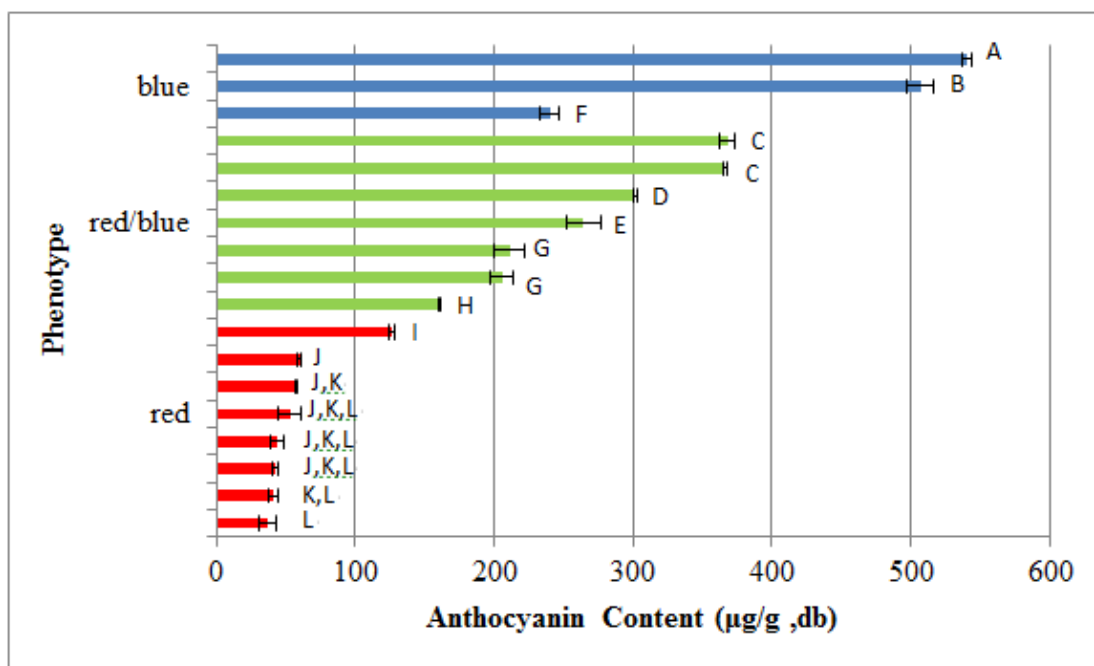


Figure 6. Total pigment content of Weslaco, TX 2011 samples, excluding purple as determined by UV-vis spectrometry. Data are expressed as cyanidin-3-glucoside equivalents \pm SD (n=3). Columns with different letters are significantly different ($p < 0.05$)

In the vast majority of the six purple genetic hybrid varieties, cyanidin-3-glucoside was the pigment present in greatest proportion over both years, with the exception of LH195 R7 x Maize Morado R11 (CS13), which contained a higher proportion of cyanidin-malonylglucoside (Table 4). As noted previously, the blue and red/blue varieties generally contained cyanidin-3-(6''-malonylglucoside) in greater proportion. However, one red/blue sample (Wenwei1 R5 x Ethiopia Blue R2, WE11) had cyanidin-3-glucoside as the most dominant pigment (Table 4). With the purple phenotype containing monomeric cyanidin-3-glucoside in greatest proportion, it is expected to be least stable to thermo-alkaline processing. The higher proportion of

cyanidin-3-(6''-malonylglucoside) in the blue and red/blue samples suggests greater color stability of this phenotype to color degradation during food processing.

The total anthocyanin content, monomeric anthocyanin content, and proportion of monomeric anthocyanins of all purple samples and two red/blue samples are given in Table 5. All of the purple samples had significantly higher proportions of monomeric anthocyanins than the red/blue samples ($p < 0.05$). Data from the pH differential method supports the HPLC pigment profile observed in the blue and red/blue lines. A significantly lower proportion of monomeric anthocyanins were present in the red/blue samples in comparison with the purple samples (Figure 7). While the red/blue class contained much lower total pigment than the purple class (Figures 5 and 6), the lower proportion of the less stable monomeric anthocyanins again suggests greater pigment stability of the red/blue and blue classes at the high pH values that would be encountered in tortilla chip processing.

Table 4. Proportion of individual anthocyanins (% of total) among the purple, blue, and red/blue phenotypes as determined by HPLC-UV-*vis*^a

Hybrid variety		Cy-3-glu ^b	Cy-3-(3''-malglu) ^b	Cy-3-(6''-malglu) ^b	Pg-3-glu ^c	Pg-3-malglu ^c	Pn-3-glu ^b	Pn-3-malglu ^b
Purple	Wenwei2 R6 x WE11	47.6	ND	25.1	6.4	3.9	11.9	5.0
	Maize Morado R11 CS13	36.8	0.36	31.6	7.3	8.1	8.7	7.2
	Wenwei2 R5 x WE11	48.0	ND	25.1	5.0	3.3	13.4	5.3
	Maize Morado R11 CS13	36.9	0.43	32.7	6.3	6.8	9.1	7.7
	LH195 R7 x WE11	42.3	ND	36.8	2.9	2.5	9.6	5.8
	Maize Morado R11 CS13	31.0	0.48	46.3	2.6	1.3	8.0	10.4
	Red Ear R4 x WE11	50.3	0.36	32.0	4.2	3.3	5.9	3.9
	Maize Morado R11 CS13	41.3	0.67	37.7	3.8	3.9	6.7	5.8
	Red Hybrid Ear R3 x WE11	55.5	ND	24.0	5.9	3.3	8.4	2.9
	Maize Morado R11 CS13	42.7	0.60	34.0	6.0	5.4	6.7	4.7
	Lfy Blue R1 x WE11	51.9	ND	17.2	5.5	2.3	18.4	4.7
	Maize Morado R11 CS13	38.3	ND	22.8	4.8	3.9	19.5	10.8
Blue	Lfy Blue R1 x WE11	33.7	ND	50.5	4.9	6.6	4.4	ND
	Ethiopia Blue R2 CS13	31.8	ND	50.2	5.0	9.1	3.9	ND
	Lfy Blue R1 x WE11	39.8	ND	60.2	ND	ND	ND	ND
	Ethiopia Blue2 R9 CS13	30.0	ND	52.2	5.2	ND	5.3	7.2
	Ethiopia Blue2 R9 x WE11	37.9	ND	46.4	4.1	7.7	4.0	ND

Table 4-Continued

Hybrid Variety		Cy-3- glu ^b	Cy-3-(3"- malglu) ^b	Cy-3-(6"- malglu) ^b	Pg-3- glu ^c	Pg-3- malglu ^c	Pn-3- glu ^b	Pn-3- malglu ^b
Red/Blue	Ethiopia Blue R2	CS13	n/a	n/a	n/a	n/a	n/a	n/a
	Lfy Blue R1 x	WE11	39.3	ND	60.7	ND	ND	ND
	Red Ear R4	CS13	29.8	ND	50.9	5.5	ND	5.6
	Red Hybrid Ear R3 x	CS11	43.9	ND	56.1	ND	ND	ND
	Ethiopia Blue2 R9	CS13	42.1	ND	57.9	ND	ND	ND
	Wenwei1 R5 x	WE11	58.4	ND	41.6	ND	ND	ND
	Ethiopia Blue R2	CS13	31.0	6.4	54.9	3.8	ND	3.9
	Wenwei2 R6 x	WE11	26.9	2.3	51.8	3.6	7.8	3.0
	Lfy Blue R1	CS13	35.2	ND	64.8	ND	ND	ND
	Red Ear R4 x	WE11	40.4	ND	59.6	ND	ND	ND
	Ethiopia Blue2 R9	CS13	42.4	ND	57.6	ND	ND	ND
	Ethiopia Blue2 R9 x	WE11	46.7	ND	53.3	ND	ND	ND
	Red Ear R4	CS13	29.4	ND	63.9	ND	ND	6.6
	Ethiopia Blue R2 x	WE11	28.1	ND	49.7	3.8	5.8	5.2
	Red Ear R4	CS13	28.3	5.1	49.9	ND	ND	7.0

^aAll data expressed on a dry weight basis. ^bexpressed as cyanidin equivalents. ^cexpressed as pelargonidin equivalents. n/a = sample not available. ND = not detected. WE11 = Weslaco 2011. CS13 = College Station 2013. CS11 = College Station 2011.

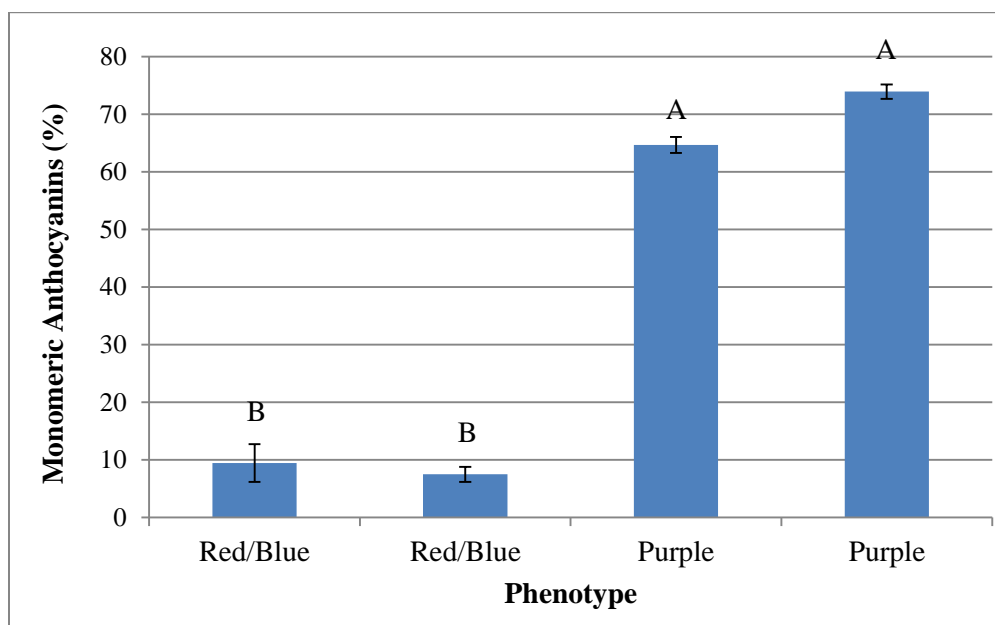


Figure 7. Percent monomeric anthocyanins of representative red/blue vs. purple samples as determined by UV-*vis* spectrometry. Red/blue phenotype contains significantly lower proportion of monomeric anthocyanins than the purple phenotype. Data are expressed as cyanidin-3-glucoside equivalents \pm SD (n=3). Columns with different letters are significantly different (p<0.05).

Table 5. Comparison of total anthocyanin content, total monomeric anthocyanin content, and proportion of monomeric anthocyanins of raw purple and red/blue corn hybrids as determined by UV-vis spectrometry (Mean \pm SD, n=3).

Hybrid variety	Phenotype	Total anthocyanin content (expressed as Cy-3-glu equivalents, $\mu\text{g/g}$, db)		Total monomeric anthocyanin content (expressed as Cy-3-glu equivalents, $\mu\text{g/g}$, db)		Proportion of monomeric anthocyanins (%)	
		Weslaco 2011	College Station 2013	Weslaco 2011	College Station 2013	Weslaco 2011	College Station 2013
Wenwei2 R6 x Maize Morado R11	Purple	1015 \pm 36.9 ^D	3271 \pm 46.6 ^A	592 \pm 78.3 ^D	2406 \pm 103 ^A	58.2 \pm 5.7 ^{B,C}	73.5 \pm 2.2 ^{A,B}
Wenwei1 R5 x Maize Morado R11	Purple	1380 \pm 9.9 ^C	2474 \pm 103.9 ^C	843 \pm 22.7 ^C	1822 \pm 52.2 ^B	61.1 \pm 2.0 ^{A,B}	73.7 \pm 1.2 ^A
LH195 R7 x Maize Morado R11	Purple	2257 \pm 22.8 ^B	1882 \pm 186.0 ^D	1579 \pm 100 ^B	1393 \pm 160 ^C	69.9 \pm 3.8 ^A	73.9 \pm 1.3 ^A
Red Ear R4 x Maize Morado R11	Purple	2940 \pm 141 ^A	2734 \pm 86.8 ^B	1914 \pm 116 ^A	1881 \pm 36.8 ^B	65.1 \pm 1.5 ^{A,B}	68.8 \pm 0.9 ^{A,B}
Red Hybrid Ear R3 x Maize Morado R11	Purple	891 \pm 44.7 ^D	1104 \pm 37.3 ^E	449 \pm 45.7 ^D	714 \pm 27.4 ^D	50.5 \pm 5.1 ^C	64.7 \pm 1.4 ^B
Lfy Blue R1 x Maize Morado R11	Purple	2949 \pm 15.2 ^A	3312 \pm 23.8 ^A	1961 \pm 52.3 ^A	2298 \pm 29.7 ^A	66.5 \pm 2.1 ^{A,B}	69.4 \pm 0.4 ^{A,B}
Wenwei1 R5 x Ethiopia Blue R2	Red/Blue	n/a	63.0 \pm 1.2 ^F	n/a	4.7 \pm 0.9 ^E	n/a	7.5 \pm 1.3 ^C
Ethiopia Blue R2 x Red Ear R4	Red/Blue	n/a	58.5 \pm 1.5 ^F	n/a	5.5 \pm 4.9 ^E	n/a	4.8 \pm 3.3 ^C

Values within the same column with no letters in common are significantly different ($p < 0.05$). n/a = sample not available.

Identification of Major Co-pigments

There were three major co-pigments identified in the different corn phenotypes based on UPLC/MS data; these compounds were present in all phenotypes but varied in content. When possible, the identities of compounds were confirmed through the comparison of HPLC retention times and UV-*vis* characteristics of the sample compounds with those of pure standards (ferulic acid, caffeic acid, and p-coumaric acid). The representative molecular ions and MS/MS fragment ions of the major co-pigments in a red/blue corn extract (Wenwei2 R6 x Lfy Blue R1) are summarized in Table 6.

Table 6. Identification of major co-pigments in a red/blue corn extract (Wenwei2 R6 x Lfy Blue R1) based on UPLC retention time (t_R), UV-*vis* spectroscopic characteristics (λ_{max}), MS and MS/MS spectroscopic pattern. ESI was performed in the negative mode. Peak numbers are referenced to Figure 9. The proposed structure and fragmentation pattern of peak 1 is in Figure 8.

Peak no.	t_R (min)	λ_{max} (nm)	[M-H] ⁻ (m/z)	MS/MS fragments (m/z)	Proposed identification
1	7.704	298/308	436	316, 290, 145, 119	N'-N'-Dicoumaroylspermidine
2	10.982	310	409	289, 259, 149, 135, 119	Unidentified coumaroyl derivative
3	11.209	318	439	289, 149, 135	Unidentified feruloyl derivative

The co-pigment peaks of a red/blue corn sample (Wenwei2 R6 x Lfy Blue R1) are shown in a UPLC chromatogram in Figure 9. **Peak 1** ($t_R = 7.704$, $\lambda_{max} = 298/308$) had a [M-H]⁻ at m/z 436. The MS/MS fragmentation pattern included an ion at m/z 316 (47% intensity, M-120), which signified the loss of a fragment of a coumaroyl unit

without the carboxylic group, and an ion at m/z 290 (M-146), which signifies the loss of a coumaroyl moiety, leaving spermidine and one coumaroyl moiety. Also, the dominant ion (100% intensity) was at m/z 119, which corresponds to a fragment of p-coumaric acid (p-coumaric – C=O) (Svensson et al., 2010). Considering the UV-*vis* characteristics of this peak, the λ_{\max} at 310 nm corresponds to a coumaroyl derivative. The fact that the $[M-H]^-$ has an even m/z value indicates the presence of nitrogen. Spermidine derivatives are nitrogen-containing plant metabolites (polyamines) commonly found in corn, including p-coumarylspermidine and diferuloylspermidine (Naczek and Shahidi, 2006). Based on the molecular weight, fragmentation pattern, and known presence of spermidine derivatives in corn, **peak 1** was presumed to be N'-N'-dicoumaroylspermidine. A proposed structure and fragmentation for the N'-N'-dicoumaroylspermidine compound of **peak 1** is provided in Figure 8. **Peak 2** ($t_R = 10.982$, $\lambda_{\max} = 310$) had a $[M-H]^-$ at m/z 409. The MS/MS fragments included ions at m/z 289, 259, 149, 135, and 119. The fragment ion at m/z 119 corresponds to a fragment of p-coumaric acid, and the UV-*vis* characteristics of this peak (λ_{\max} at 310 nm) are also indicative of a coumaroyl derivative (Svensson et al., 2010). However, there was not enough information to fully identify the structure. **Peak 3** ($t_R = 11.209$, $\lambda_{\max} = 318$) had a $[M-H]^-$ at m/z 439. The MS/MS fragments included ions at m/z 289, 149, and 135. Based on comparison of the UV-*vis* characteristics of this peak with that of a ferulic acid standard ($\lambda_{\max} = 318$) and the ion at m/z 149, which corresponds to a ferulic acid fragment, it was presumed to be a feruloyl derivative (Sun et al., 2007), but complete structure could not be determined. As p-coumaric acid and ferulic acid are the major

phenolic acids detected in plant cell walls, they are highly abundant in grains (Naczki and Shahidi, 2006). In a study identifying and quantifying phenolics in purple corn, similar unknown structures consisting of ferulic or p-coumaric acid were reported (Pedreschi and Cisneros-Zevallos, 2007). Additional data is needed to fully determine the structures of the two unknown compounds of **peaks 2 and 3**, given that they were major compounds in all varieties.

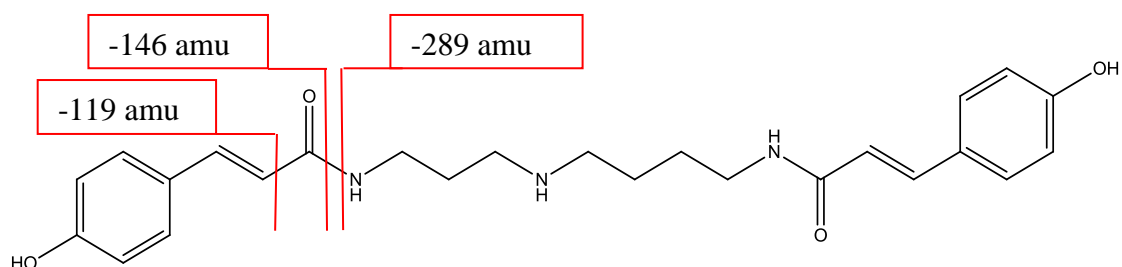


Figure 8. Proposed chemical structure and MS/MS fragmentation of N'-N'-dicoumaroylspermidine (**Peak 1**) detected in pigmented corn extracts.

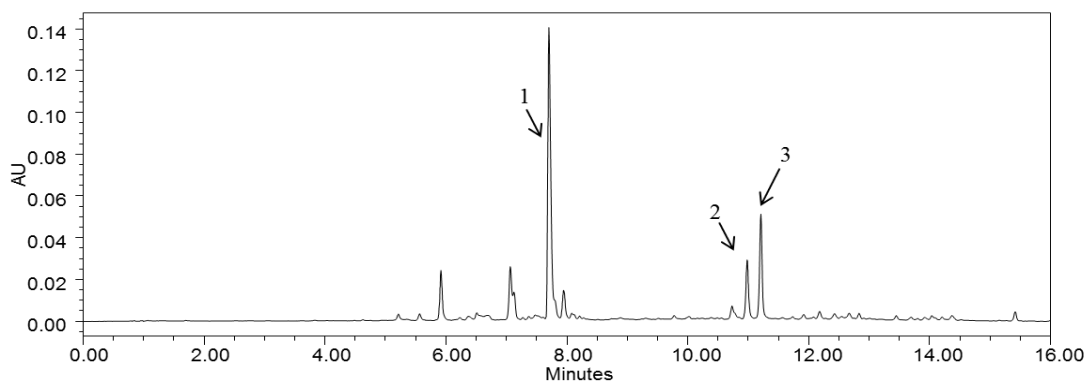


Figure 9. Representative UPLC chromatogram of major co-pigments found in the methanolic extracts from a red/blue corn sample (Wenwei2 R6 x Lfy Blue R1) monitored at 320 nm. See Table 6 for peak identities.

Minor co-pigments were also identified in the pigmented corn, but were more variable among phenotypes than were the major co-pigments. However, patterns among phenotypes were apparent. The general trend was that the purple varieties contained flavone derivatives and the red/blue varieties were higher in phenolic acid derivatives (Figures 10 and 11).

Figure 10 shows the representative UPLC chromatogram of the minor co-pigment peaks of a red/blue corn sample (Wenwei2 R6 x Lfy Blue R1). **Peak 4** ($t_R = 5.568$, $\lambda_{max} = 323$) had a $[M-H]^-$ at m/z 179. The MS/MS fragmentation produced only one dominant ion at m/z 135 (M-44), which signifies the loss of a carboxyl unit, matching the pattern for caffeic acid. Therefore, **peak 4** was identified as caffeic acid, which is commonly reported in yellow corn (Sun et al., 2007; Pedreschi and Cisneros-Zevallos, 2007). **Peak 7** ($t_R = 12.178$, $\lambda_{max} = 315$) had a $[M-H]^-$ at m/z 399. The MS/MS fragmentation pattern included ions at m/z 253 and m/z 163, matching characteristic

fragments of caffeoylglycerol and coumaroyl units, respectively. Thus, **peak 7** was identified as p-coumaroyl-caffeoylglycerol. The presence of p-coumaroyl-caffeoylglycerol has been reported in red sorghum by Svensson et al. (2010). **Peak 8** ($t_R = 12.429$, $\lambda_{max} = 327/294$) had a $[M-H]^-$ at m/z 429. The MS/MS fragments of **peak 8** included ions at m/z 253 and m/z 193, matching characteristic fragments of caffeoylglycerol and feruloyl units, respectively. Thus, **peak 8** was identified as feruloyl-caffeoylglycerol. The presence of feruloyl-caffeoylglycerol has not been reported in corn to our knowledge, but has been identified by Ma et al. (2007) in *Ananas comosus* L. leaves. These three compounds were phenolic acid derivatives detected solely in the red/blue phenotype.

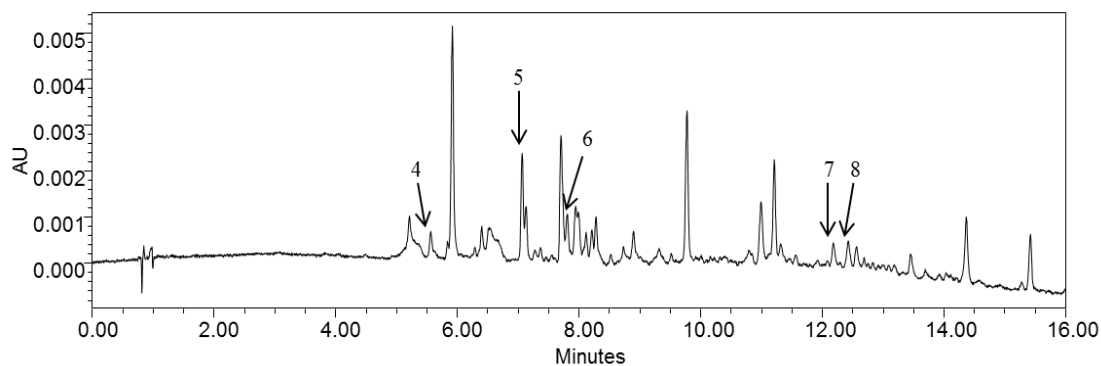


Figure 10. Representative UPLC chromatogram of minor co-pigments found in the methanolic extract from a red/blue corn sample (Wenwei2 R6 x Lfy Blue R1) monitored at 360 nm. See Table 7 for peak identities.

In Figure 11, the UPLC chromatogram of the minor co-pigment peaks of a purple corn sample (Wenwei1 R5 x Maize Morado R11) are shown. **Peak 9** ($t_R = 8.737$, $\lambda_{max} = 351$) had a $[M-H]^-$ at m/z 593. The MS/MS fragmentation produced a single dominant ion (100% intensity) at m/z 285 (loss of 308 amu = rutinose moiety – H₂O), which along with the UV-*vis* characteristics, matched the MS/MS fragmentation for luteolin-7-*O*-rutinoside as reported by Plazonic et al. (2009) as identified in an extract of burr parsley (*Caucalis platycarpos* L.). Thus, **peak 9** was identified as luteolin-7-*O*-rutinoside. **Peak 10** ($t_R = 13.162$, $\lambda_{max} = 352$) had a $[M-H]^-$ at m/z 299. The MS/MS fragmentation produced a dominant ion (100% intensity) at m/z 284 (M-15), which signifies the loss of a methyl group from the luteolin aglycone (Plazonic et al., 2009). Thus, **peak 10** was identified as O-methyl-luteolin. These 2 flavone derivatives have not been previously identified in corn to our knowledge and were only identified in the purple phenotype.

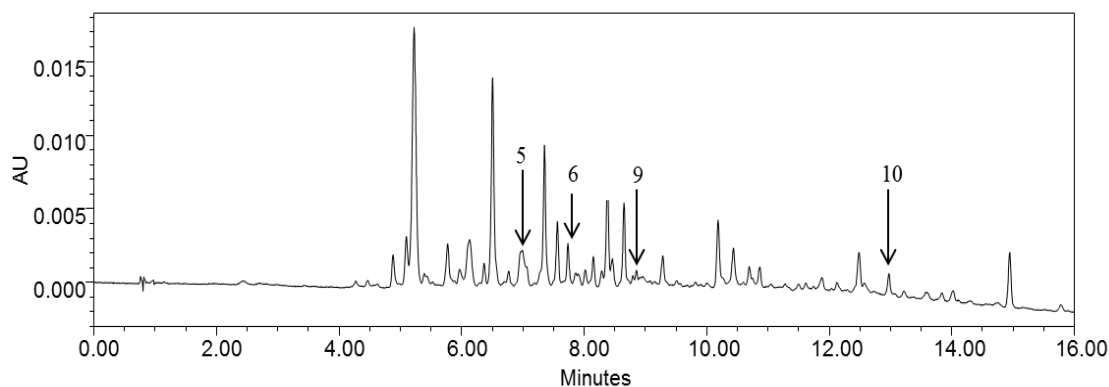


Figure 11. Representative UPLC chromatogram of minor co-pigments found in the methanolic extract from a purple corn sample (Wenwei1 R5 x Maize Morado R11) monitored at 360 nm. See Table 7 for peak identities.

Although the aforementioned pattern was of flavone-type compounds occurring in the purple phenotype and phenolic acid derivatives in the red/blue phenotype, a couple of minor co-pigments (phenolic acids) were present and identified in all four phenotypes. These compounds are identified in both chromatograms of Figures 10 and 11. **Peak 5** ($t_R = 7.055$, $\lambda_{max} = 312$) had a $[M-H]^-$ at m/z 237. The MS/MS fragments of **peak 5** included ions at m/z 163 and m/z 145. The fragment at m/z 163 matches the characteristic fragment of a coumaroyl unit, and the ion at m/z 145 (M-92) corresponds to the loss of glycerol. Thus, **peak 5** was identified as coumaroyl-glycerol, which has been reported in red sorghum by Svensson et al. (2010). **Peak 6** ($t_R = 7.793$, $\lambda_{max} = 318$) had a $[M-H]^-$ at m/z 193. The MS/MS fragments of **peak 6** included ions at m/z 178 and m/z 134. The dominant ion (100% intensity) at m/z 134 (M-59, signifying the loss of C-COOH fragment). Therefore, **peak 6** was identified as ferulic acid, which is the most

common phenolic acid in corn (Sen et al., 1994). The molecular ions and MS/MS fragment ions of the minor co-pigments in the red/blue corn extract (Wenwei2 R6 x Lfy Blue R1) and the purple corn sample (Wenwei1 R5 x Maize Morado R11) are summarized in Table 7.

Table 7. Summary of minor co-pigments identified in a red/blue corn extract (Wenwei2 R6 x Lfy Blue R1) and a purple corn sample (Wenwei1 R5 x Maize Morado R11), based on UPLC retention time (t_R), UV-*vis* spectroscopic characteristics (λ_{max}), MS and MS/MS spectroscopic pattern. ESI was performed in the negative mode. Peak numbers are referenced to Figure 9 and Figure 10.

Peak no.	t_R (min)	λ_{max} (nm)	[M-H] ⁻ (m/z)	MS/MS fragments (m/z)	Proposed identification	Purple	Red/Blue
4	5.568	323	179	135	Caffeic acid		X
7	12.178	315	399	253, 235, 163, 161, 145, 119	p-Coumaroyl-caffeoylglycerol		X
8	12.429	294/327	429	253, 193, 175, 161, 134	Feruloyl-caffeoylglycerol		X
9	8.737	351	593	285, 284	Luteolin-7- <i>O</i> -rutinoside	X	
10	13.162	352	299	284, 271, 256, 255	Methoxy-luteolin	X	
5	7.055	312	237	163, 145, 119	Coumaroyl-glycerol	X	X
6	7.793	318	193	178, 134	Ferulic acid	X	X

X-indicates presence of compound

Quantification of Major Co-Pigments

Major co-pigment content varied significantly among samples, and it was difficult to identify meaningful patterns among phenotypes. The red and red/blue samples generally contained higher amounts of the unidentified feruloyl derivative, with a WE11 red/blue sample (Lfy Blue R1 x Red Ear R4) containing the highest content, $3.14 \pm 0.02 \mu\text{g/g}$. The purple phenotype overall possessed the lowest concentrations of the unidentified coumaroyl derivative, ranging from 0.02-0.07 $\mu\text{g/g}$. While no significant pattern of major co-pigments existed based on this data alone, if samples that contain similar pigment content and profile behave very differently from one another during thermo-alkaline processing, the differences in major co-pigment content of the samples could provide a potential explanation. Additionally, there could be potential health benefits worth investigating in the pigmented corn lines with higher contents of these phenolic compounds. Major co-pigment contents of all samples from both growing years/locations are listed in Table 8.

Conclusions

Seven anthocyanins, three major co-pigments, and several minor co-pigments were identified and quantified in these pigmented corn hybrids using UPLC/MS/ESI data in conjunction with HPLC data. Variation in total pigment content was observed both within and among hybrid varieties as determined by UV-*vis* spectrometry. The individual hybrid variety did not seem to have much effect on anthocyanin profile, as evidenced by almost identical anthocyanin profiles within each phenotype. The red/blue

and blue phenotypes were shown to possess a pigment profile with a high proportion of cyanidin-3-(6''-malonylglucoside) (more stable, acylated anthocyanin), so it is possible that these two phenotypes would retain more color during thermo-alkaline processing. More color was retained by samples of the red/blue phenotype in the pH differential method at pH 4.5 (the pH at which anthocyanins are most susceptible to degradation to their colorless form), supporting the possible stability of this phenotype (Lee et al., 2005).

The findings of this study demonstrate that phenotype has a major effect on both the anthocyanin content and composition of pigmented corn. This information could be used by plant breeders to selectively breed pigmented corn varieties for increased contents of bioactive anthocyanins and other phenolics and/or for better performance of pigments extracted from these corns for use as natural food colorants. Additionally, the differences observed between pigmented corn phenotypes, in both anthocyanin and co-pigment composition, will likely affect color stability through thermo-alkaline processing of this raw corn into tortilla chips.

Table 8. Major co-pigment content of pigmented corn hybrids as determined by HPLC-UV-vis^a

Genetic hybrid variety	Phenotype	N'-N'-dicoumaroylspermidine content (expressed as p-coumaric acid equivalents, µg/g, db)		Unidentified coumaroyl derivative content (expressed as p-coumaric acid equivalents, µg/g, db)		Unidentified feruloyl derivative content (expressed as ferulic acid equivalents, µg/g, db)	
		Weslaco 2011	College Station 2013	Weslaco 2011	College Station 2013	Weslaco 2011	College Station 2013
LAMA Red R10 x Red Hybrid Ear R3	Red	0.25 ± 0.06 ^{J,K}	0.79 ± 0.00 ^{G,H,I,J}	0.05 ± 0.02 ^{F,G}	0.09 ± 0.00 ^{I,J,K}	0.42 ± 0.12 ^{F,G,H,I,J}	0.63 ± 0.02 ^{I,J}
Red Ear R4 x LAMA Red R10	Red	0.48 ± 0.02 ^{H,I}	0.45 ± 0.01 ^K	0.13 ± 0.00 ^{E,F}	0.19 ± 0.00 ^{F,G}	0.81 ± 0.01 ^{D,E,F}	1.3 ± 0.01 ^{D,E}
Wenwei1 R5 x Red Hybrid Ear R3	Red	0.40 ± 0.17 ^{LJ}	0.85 ± 0.04 ^{F,G,H,I}	0.13 ± 0.06 ^{E,F}	0.20 ± 0.00 ^{E,F,G}	0.76 ± 0.33 ^{D,E,F,G}	1.3 ± 0.01 ^{D,E}
LH287 R8 x Wenwei1 R5	Red	0.21 ± 0.03 ^{†,K}	0.41 ± 0.03 ^K	0.55 ± 0.08 ^{†,B}	0.64 ± 0.01 ^A	1.8 ± 0.28 ^{†,B}	2.2 ± 0.04 ^A
Wenwei2 R6 x Red Hybrid Ear R3	Red	0.51 ± 0.00 ^{†,G,H,I}	0.72 ± 0.01 ^{LJ}	0.11 ± 0.00 ^{†,E,F,G}	0.39 ± 0.00 ^B	0.58 ± 0.00 ^{†,D,E,F,G,H}	1.8 ± 0.02 ^C
Red Ear R4 x Wenwei1 R5	Red	0.78 ± 0.02 ^{C,D,E}	0.63 ± 0.09 ^J	0.18 ± 0.01 ^{D,E}	0.16 ± 0.05 ^{G,H}	0.85 ± 0.01 ^{C,D,E}	1.0 ± 0.26 ^{E,F}
Wenwei2 R6 x LH287 R8	Red	0.51 ± 0.01 ^{G,H,I}	0.35 ± 0.00 ^K	0.77 ± 0.06 ^A	0.61 ± 0.00 ^A	1.8 ± 0.14 ^B	2.0 ± 0.00 ^{B,C}
LH195 R7 x Red Hybrid Ear R3	Red	0.67 ± 0.04 ^{†,E,F,G}	1.1 ± 0.14 ^{C,D}	0.46 ± 0.00 ^{†,B,C}	0.31 ± 0.01 ^C	2.8 ± 0.08 ^{†,A}	2.1 ± 0.12 ^{A,B}
Wenwei2 R6 x Maize Morado R11	Purple	0.60 ± 0.01 ^{E,F,G,H}	0.89 ± 0.02 ^{E,F,G,H}	0.03 ± 0.00 ^{F,G}	0.04 ± 0.00 ^{K,L}	0.27 ± 0.00 ^{H,I,J}	0.36 ± 0.02 ^{K,L}
Wenwei1 R5 x Maize Morado R11	Purple	0.87 ± 0.01 ^{C,D}	1.2 ± 0.02 ^{C,D}	0.02 ± 0.00 ^G	0.07 ± 0.00 ^{I,J,K,L}	0.13 ± 0.00 ^{LJ}	0.52 ± 0.01 ^{I,J,K}
LH195 R7 x Maize Morado R11	Purple	1.2 ± 0.01 ^B	1.0 ± 0.06 ^{D,E,F}	0.05 ± 0.00 ^{F,G}	0.06 ± 0.01 ^{J,K,L}	0.53 ± 0.01 ^{E,F,G,H,I}	0.39 ± 0.02 ^{J,K,L}
Red Ear R4 x Maize Morado R11	Purple	0.74 ± 0.00 ^{C,D,E,F}	0.94 ± 0.02 ^{E,F,G}	0.06 ± 0.00 ^{F,G}	0.04 ± 0.01 ^{K,L}	0.58 ± 0.01 ^{D,E,F,G,H}	0.42 ± 0.00 ^{J,K,L}
Red Hybrid Ear R3 x Maize Morado R11	Purple	0.74 ± 0.01 ^{C,D,E,F}	1.0 ± 0.01 ^{D,E}	0.03 ± 0.00 ^{F,G}	0.04 ± 0.00 ^{K,L}	0.25 ± 0.01 ^{H,I,J}	0.35 ± 0.00 ^{K,L}

Table 8-Continued

Genetic hybrid variety	Phenotype	N'-N'-dicoumaroylspermidine content (expressed as p-coumaric acid equivalents, µg/g, db)		Unidentified coumaroyl derivative content (expressed as p-coumaric acid equivalents, µg/g, db)		Unidentified feruloyl derivative content (expressed as ferulic acid equivalents, µg/g, db)	
		Weslaco 2011	College Station 2013	Weslaco 2011	College Station 2013	Weslaco 2011	College Station 2013
Lfy Blue R1 x Maize Morado R11	Purple	0.88 ± 0.02 ^C	1.3 ± 0.01 ^B	0.04 ± 0.00 ^{F,G}	0.03 ± 0.02 ^L	0.31 ± 0.08 ^{H,I,J}	0.22 ± 0.04 ^L
Lfy Blue R1 x Ethiopia Blue R2	Blue	1.1 ± 0.01 ^B	1.3 ± 0.03 ^{B,C}	0.20 ± 0.01 ^{D,E}	0.10 ± 0.00 ^{L,J}	0.62 ± 0.03 ^{D,E,F,G,H}	0.37 ± 0.00 ^{J,K,L}
Lfy Blue R1 x Ethiopia Blue2 R9	Blue	0.70 ± 0.01 ^{D,E,F}	0.95 ± 0.02 ^{E,F,G}	0.43 ± 0.01 ^C	0.28 ± 0.01 ^{C,D}	1.2 ± 0.03 ^C	0.99 ± 0.02 ^{F,G}
Ethiopia Blue2 R9 x Ethiopia Blue R2	Blue	1.1 ± 0.01 ^B	n/a	0.13 ± 0.00 ^{E,F}	n/a	0.27 ± 0.01 ^{H,I,J}	n/a
Lfy Blue R1 x Red Ear R4	Red/Blue	0.57 ± 0.01 ^{F,G,H,I}	0.85 ± 0.03 ^{F,G,H,I}	0.39 ± 0.01 ^C	0.16 ± 0.01 ^{G,H}	3.1 ± 0.02 ^A	1.3 ± 0.04 ^D
Red Hybrid Ear R3 x Ethiopia Blue2 R9	Red/Blue	0.77 ± 0.01 ^{†,C,D,E}	1.1 ± 0.00 ^{C,D}	0.04 ± 0.00 ^{†,F,G}	0.24 ± 0.00 ^{D,E,F}	0.25 ± 0.00 ^{†,H,I,J}	0.95 ± 0.01 ^{F,G,H}
Wenwei1 R5 x Ethiopia Blue R2	Red/Blue	0.48 ± 0.06 ^{H,I}	1.0 ± 0.01 ^{D,E,F}	0.13 ± 0.02 ^{E,F}	0.17 ± 0.00 ^{G,H}	0.39 ± 0.05 ^{G,H,I,J}	0.70 ± 0.00 ^{H,I}
Wenwei2 R6 x Lfy Blue R1	Red/Blue	2.4 ± 0.02 ^A	0.86 ± 0.01 ^{F,G,H,I}	0.07 ± 0.00 ^{F,G}	0.25 ± 0.00 ^{C,D,E}	0.09 ± 0.00 ^J	1.2 ± 0.01 ^{D,E,F}
Red Ear R4 x Ethiopia Blue2 R9	Red/Blue	0.50 ± 0.02 ^{G,H,I}	0.75 ± 0.02 ^{H,I,J}	0.18 ± 0.01 ^{D,E}	0.05 ± 0.00 ^{J,K,L}	0.96 ± 0.03 ^{C,D}	0.30 ± 0.01 ^{K,L}
Ethiopia Blue2 R9 x Red Ear R4	Red/Blue	0.48 ± 0.04 ^{H,I}	0.67 ± 0.02 ^J	0.19 ± 0.01 ^{D,E}	0.09 ± 0.00 ^{L,I,K}	0.91 ± 0.02 ^{C,D,E}	0.40 ± 0.01 ^{J,K,L}
Ethiopia Blue R2 x Red Ear R4	Red/Blue	0.91 ± 0.03 ^C	1.5 ± 0.01 ^A	0.27 ± 0.01 ^D	0.12 ± 0.00 ^{H,I}	2.2 ± 0.08 ^B	0.75 ± 0.03 ^{G,H,I}

^a All data expressed as mean ± SD of duplicates on a dry weight basis. Values within the same column with no letters in common are significantly different (p<0.05). n/a-sample not available. †Sample from College Station 2011.

CHAPTER IV

EFFECT OF PIGMENT AND CO-PIGMENT COMPOSITION OF COLORED CORN HYBRIDS ON STABILITY TO THERMO-ALKALINE PROCESSING

Introduction

The anthocyanins that are responsible for the different colors of pigmented corn are generally not stable to the conditions encountered in the nixtamalization process for the production of tortilla chips, especially the high pH. The alkaline pH transforms the vibrantly colored raw corn into a product with a dull, unattractive appearance. As color plays a significant role in influencing consumer opinion of food quality, it is important to lessen this undesirable color change during thermo-alkaline processing of corn into tortilla chips. It is well known that anthocyanins are stable at acidic pH but are generally degraded at neutral and alkaline pHs (Brouillard, 1982). However, some acylated anthocyanins possess greater stability to pH increases due to the phenomenon of intramolecular co-pigmentation and are better able to retain their color (Dangles et al., 1993). In order to obtain nixtamalized products, such as corn tortilla chips, that are naturally colored, the anthocyanins present in the raw pigmented corn need to be partially maintained through the thermo-alkaline processing.

Thus, it is necessary to select pigmented corns that conserve their color during processing of corn into tortilla chips. In the previous chapter, we have shown that the blue and red/blue pigmented corn phenotypes have similar pigment profiles and contain higher proportions of the more stable, acylated anthocyanins than the purple and red

phenotypes. We hypothesize that the red/blue and blue phenotypes will be more stable through processing and lose less of their inherent color. The aim of this study is to determine how the pigment and co-pigment profiles affect color stability of pigmented corn hybrids processed into tortilla chips. This information would lead to the ability to selectively breed for pigmented corn varieties with greater color stability during food processing applications. Color stability was determined based on measurement of visual color parameters, such as chroma, hue, lightness, and total color change, as well as by the quantification of major anthocyanins and co-pigments to determine the effect of tortilla chip processing on the retention of these compounds.

Materials and Methods

Materials

Pigmented Corn Kernels. The same set of pigmented corn kernels (See Table 1) were used to carry out the methods of this chapter.

Methods

Measurement of Visual Color Parameters. Visual color parameters were measured using a Minolta Chroma Meter (Model CR-300, Ramsey, NJ). The colorimeter was calibrated prior to each use with a standard white tile. Lightness (L^*), degree of redness (a^*), and

degree of yellowness (b^*) were the parameters directly measured by the instrument. Measurements were taken at least in triplicate. Measurements were taken on uncooked corn kernels, uncooked ground corn flour, nixtamal (cooked corn), masa, and tortilla chips for each sample and treatment. These values were then converted into total color difference (ΔE), Chroma (C^*), and hue angle (h°) as follows:

$$\Delta E = ((\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2)^{1/2}$$

$$C^* = ((a^*)^2 + (b^*)^2)^{1/2}$$

$$h^\circ = \arctan (b^*/a^*), \text{ for } a>0, b>0 (0^\circ - 90^\circ)$$

$$h^\circ = 180 + \arctan (b^*/a^*), \text{ for } a<0, b>0 (90^\circ - 180^\circ) \text{ and } a<0, b<0 (180^\circ - 270^\circ)$$

$$h^\circ = 360 + \arctan (b^*/a^*), \text{ for } a>0, b<0 (270^\circ - 360^\circ)$$

Alkaline Processing of Pigmented Corn. 30 g of corn were added to large glass tubes containing 90 mL of distilled water (1:3 w/w, corn: water) and either 0% (control), 0.5%, or 1.0% lime (weight of lime per weight of corn). The samples were cooked in a medium kettle filled with water on a DuxTop induction cooktop (Secura, Appleton, WI) for 50 min at approximately 100° C. The cooked samples were steeped for 14 h in a covered water bath that was previously heated to 80 °C and shut off prior to sample entry. After steeping, the pH of the cooking liquor (nejayote) was measured and the liquid was then discarded. The cooked corn (nixtamal) was not washed, as is traditionally done after steeping to remove excess lime and partially remove pericarp, due to the fact that the majority of the anthocyanins are present in the pericarp and aleurone layers, and so pigments would be partially lost through this step (Zazueta-

Morales et al., 2001). The nixtamal was then ground into masa using a coffee grinder (Cuisinart, Model DCG-20N series), and the masa was rehydrated based on moisture content to achieve suitable masa consistency for tortilla production. Pieces of masa (14 g) were then pressed into tortillas using a manual press, and these tortillas were then baked on a hot griddle (190°C) for 30 sec on each side for a total of 2 min. The tortillas were left to equilibrate to room temperature and then fried at 198 °C for 50 sec using an electric deep fryer (Presto, “Fry Daddy Elite” Stock No. 05426). The corn tortilla chips were then stored at -20 °C until further analysis.

Post-processing HPLC analysis. De-fatted corn tortilla chip samples were used for post-processing HPLC analysis. The corn tortilla chips were ground and de-fatted using hexanes (1:3, samples: hexanes) for 1.5 h with agitation. This process was repeated 5 times in order to remove all residual oil, with the saturated hexanes being discarded and fresh hexanes added each time. De-fatted samples were stored open in a fume hood overnight until the hexanes had evaporated.

These de-fatted samples were stored at -20°C until extraction. 2.5 g of sample were extracted in 5 mL 1% HCl in methanol with 2 h of shaking at room temperature. The samples were then centrifuged and the supernatants were collected. An aliquot of each supernatant was then passed through a syringe filter with a 0.45 µm nylon membrane before being injected (3 µL) onto the HPLC column. HPLC analysis was carried out using identical methods and parameters to those previously described in the

chapter III methods section in order to determine any changes in anthocyanin and co-pigment profile and composition due to corn tortilla chip processing.

Statistical Analysis. Data analysis was carried out using one-way ANOVA to detect differences among the means, and Fisher's Least Significant Difference was used to separate means with $P < 0.05$. Statistical Analysis was done using JMP Pro (Version 10.0.0, SAS Institute, Cary, NC).

Results and Discussion

Effect of Thermo-Alkaline Processing on Visual Color Parameters

As mentioned previously, acylated anthocyanins have been shown to possess greater pigment stability (Dangles et al., 1993). All four phenotypes were studied in order to determine the effect of phenotype on color stability during processing. Six representative samples were selected from each phenotype (three from each growing location/year, with the exception of the blue phenotype that had only two blue samples grown in the College Station, TX 2013 set, so four blue samples were processed). Processing was replicated on two separate days.

As previously noted, alkaline pH and high temperature are two of the main factors that lead to transformation and degradation of anthocyanins (Markakis, 1982). In the nixtamalization process used in this study, the pH during processing was 4.82 ± 0.34 at 0% lime (control), 9.06 ± 0.52 at 0.5% lime, and 10.74 ± 0.76 at 1% lime (these

measurements were taken on the nejayote after cooking and steeping). Additionally, the samples were lime-cooked in a water bath at approximately 100°C (boiling), so these two harsh conditions are combined in nixtamalization and tortilla chip processing, which promote the degradation of anthocyanins and subsequent color loss/change.

The changes in visual color of the nixtamalized products (nixtamal, masa, and tortilla chip) were monitored by use of a colorimeter to determine such parameters as chroma, hue, lightness, and total color difference. These values are calculated from the output values of the colorimeter, the L*, a*, and b* values (Table 9). These color parameters can assist in relating instrumental data to visual perception. Chroma indicates the strength of chromatic response, essentially the saturation of the color, the true color of the material is provided by the hue, lightness gives the brilliance of the color, and total color difference/change gives the overall magnitude of change of the sample in relation to the control (Gordillo et al., 2012). Changes in these four parameters are discussed in the context of each stage of processing (nixtamal, masa, and chip)².

² A complete summary of the four parameters (hue, chroma, total color difference (TCD), and lightness) for all processed samples can be found in Appendix A.

Table 9. Summary of the L*, a*, b* values of pigmented corn varieties from four different phenotypes (red (Red Ear R4 x Wenwei1 R5), red/blue (Wenwei1 R5 x Ethiopia Blue R2), blue (Lfy Blue R1 x Ethiopia Blue R2), and purple (Red Ear R4 x Maize Morado R11)) at three stages of processing (nixtamal, masa, and chip).

	Lime %	NIXTAMAL			MASA			CHIP		
		L	a	b	L	a	b	L	a	b
Red/Blue (Wenwei1 R5 x Ethiopia Blue R2)	0	39.2±1.3	17.6±0.83	20.9±0.94	63.2±3.6	4.1±0.22	12.2±0.54	39.1±1.1	9.6±0.45	8.8±0.53
	0.5	24.0±0.71	8.8±1.2	7.2±1.3	57.4±4.1	4.9±0.19	13.7±0.82	40.3±4.4	7.7±0.88	10.1±2.4
	1	26.1±0.83	9.4±0.50	9.0±1.0	52.5±2.3	3.7±0.32	12.8±0.26	38.6±3.0	4.7±0.40	8.0±1.0
Blue (Lfy Blue R1 x Ethiopia Blue R2)	0	43.0±2.5	2.2±0.50	8.7±0.65	64.0±0.50	2.6±0.17	5.5±0.61	38.6±1.6	8.4±0.48	3.0±1.1
	0.5	38.3±4.8	3.9±0.77	15.6±3.9	62.2±0.61	2.2±0.14	6.2±0.32	43.5±1.6	5.9±0.92	3.5±0.55
	1	37.7±0.92	3.3±0.43	13.6±1.1	56.7±1.8	0.45±0.12	4.4±1.5	39.6±1.4	1.2±0.71	2.2±0.81
Purple (Red Ear R4 x Maize Morado R11)	0	16.5±0.69	3.7±0.32	-0.80±0.12	44.1±0.25	7.3±0.22	2.0±0.50	24.5±0.20	4.1±0.43	-0.31±0.10
	0.5	16.9±0.54	1.4±0.17	-0.81±0.17	45.5±0.27	6.3±0.49	2.8±0.08	24.7±0.68	3.5±0.52	-0.13±0.23
	1	17.0±0.30	1.1±0.17	-0.84±0.09	48.2±2.4	5.6±0.53	4.2±0.65	25.4±0.50	3.2±0.55	0.41±0.28
Red (Red Ear R4 x Wenwei1 R5)	0	26.1±0.66	22.1±0.77	10.9±0.42	64.9±0.80	1.9±1.4	16.1±0.62	37.4±0.92	10.5±0.93	12.3±1.4
	0.5	19.7±0.37	3.0±0.98	2.5±0.77	58.5±1.5	1.9±1.6	12.9±0.86	34.0±3.1	7.9±0.51	9.0±2.4
	1	19.0±0.39	1.3±0.56	1.4±0.50	52.2±0.34	2.6±1.8	10.7±0.48	33.4±1.3	7.2±0.76	6.9±0.83

Lightness

Lightness plays a substantial role in visual color perception. If a sample is too light or too dark, changes in chroma or hue may be imperceptible.

Nixtamal. The lightness, or brilliance, of nixtamal was expected to decrease with increasing lime concentration, which is the general trend observed both in Figures 12 and 13. This decrease in lightness was expected due to the fact that as the pH goes above neutral, anthocyanins become blue/purple/black in color, which produces a dull appearance (Fossen et al., 1998). No significant changes were observed in the purple sample because the control nixtamal had a low initial lightness. The presence of lime seemed to have a stronger effect than the actual concentration of lime, as there was no significant difference in lightness between 0.5% and 1% lime for any sample. The fact that no significant difference in lightness occurs between samples at 0.5% lime and 1% lime was most likely due to the relatively small difference in pH between these two treatments, approximately pH 9 and 11, respectively, compared to control (approximately pH 5).

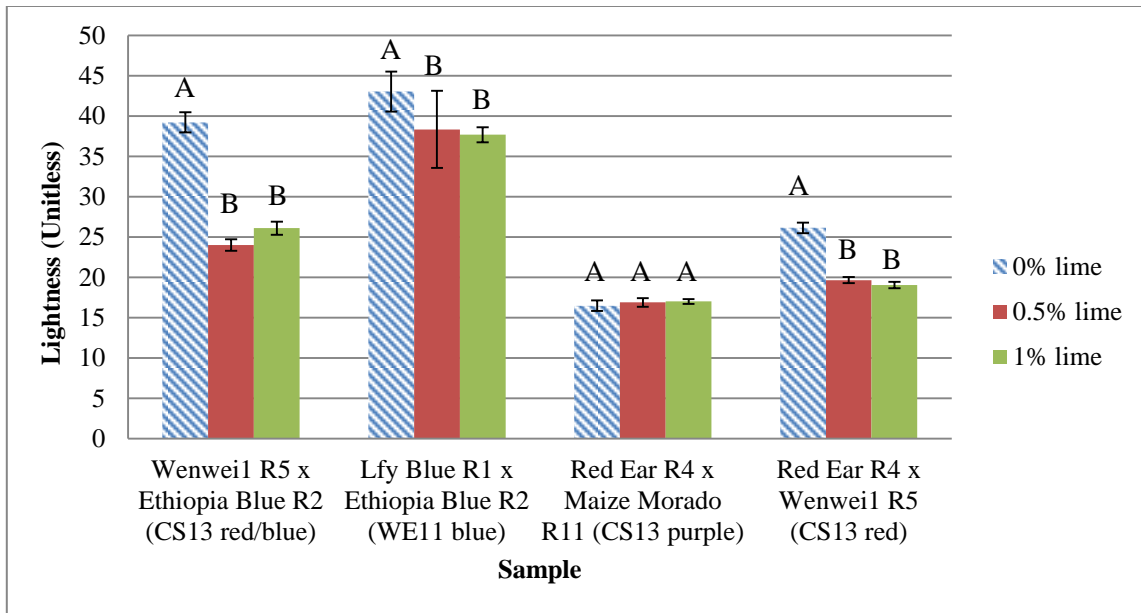


Figure 12. Effect of increasing lime concentration on lightness of red/blue, blue, purple, and red corn samples processed into nixtamal. Data are expressed as mean \pm SD (n = 8). Columns with different letters within sample are significantly different ($p < 0.05$).

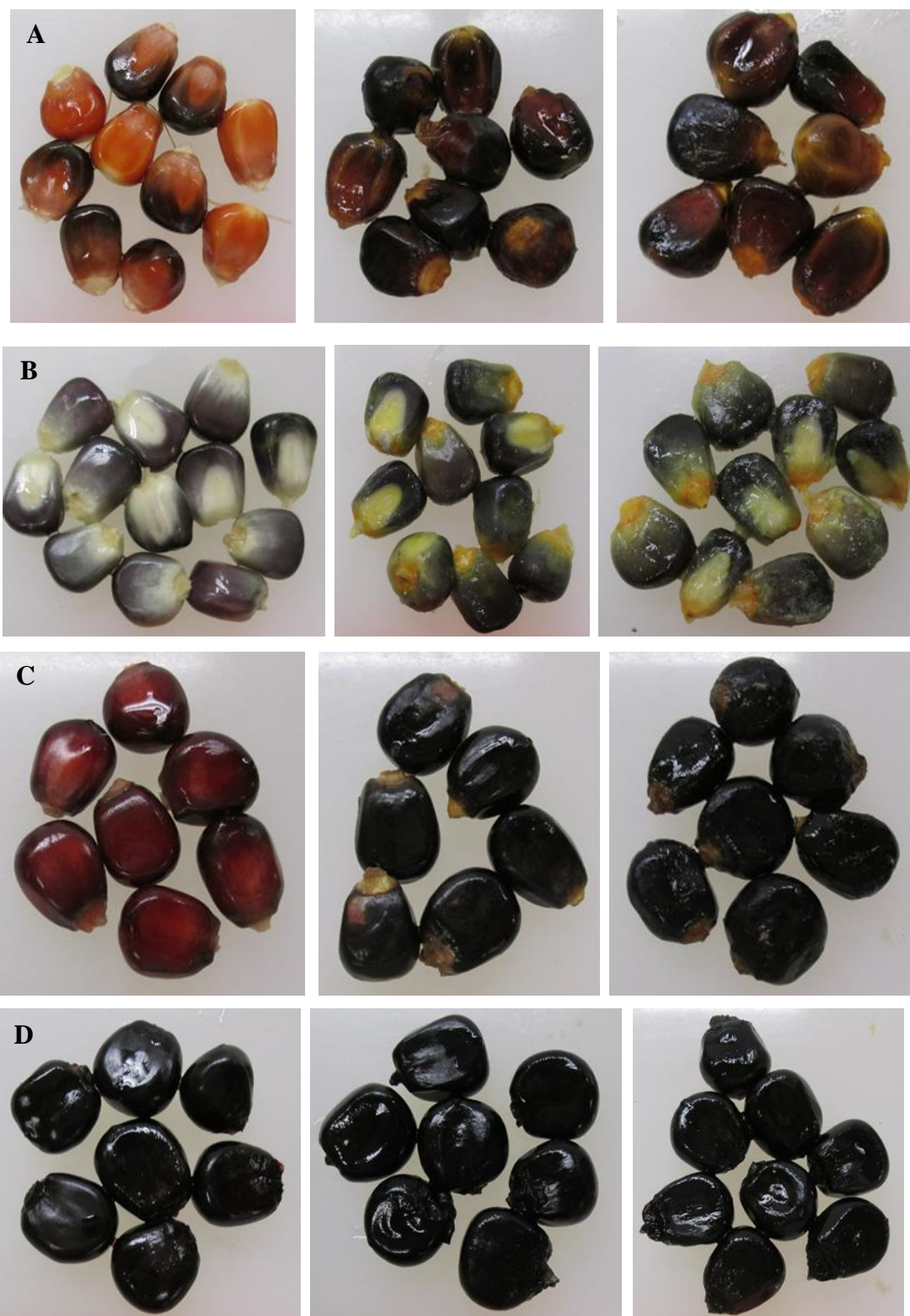


Figure 13. Pictures of nixtamal. A-red/blue sample, B-blue sample, C-red sample, D-purple sample. Each row depicts 0%, 0.5%, and 1% lime concentration from left to right.

Masa. In general, lightness values of masa were higher than nixtamal due to the exposure of the lighter colored endosperm (either yellow or white) from the grinding step. The lightness was expected to again decrease with increase in pH, and this trend was observed in the red/blue, blue, and red samples (Figure 14). The purple sample had very slight increases in lightness, only significant at the 1% lime level ($p < 0.05$), but the lightness was generally the same at all lime concentrations. The purple sample appeared and behaved differently than the samples of the other three phenotypes throughout processing because it was essentially too dark to detect many differences with the colorimeter.

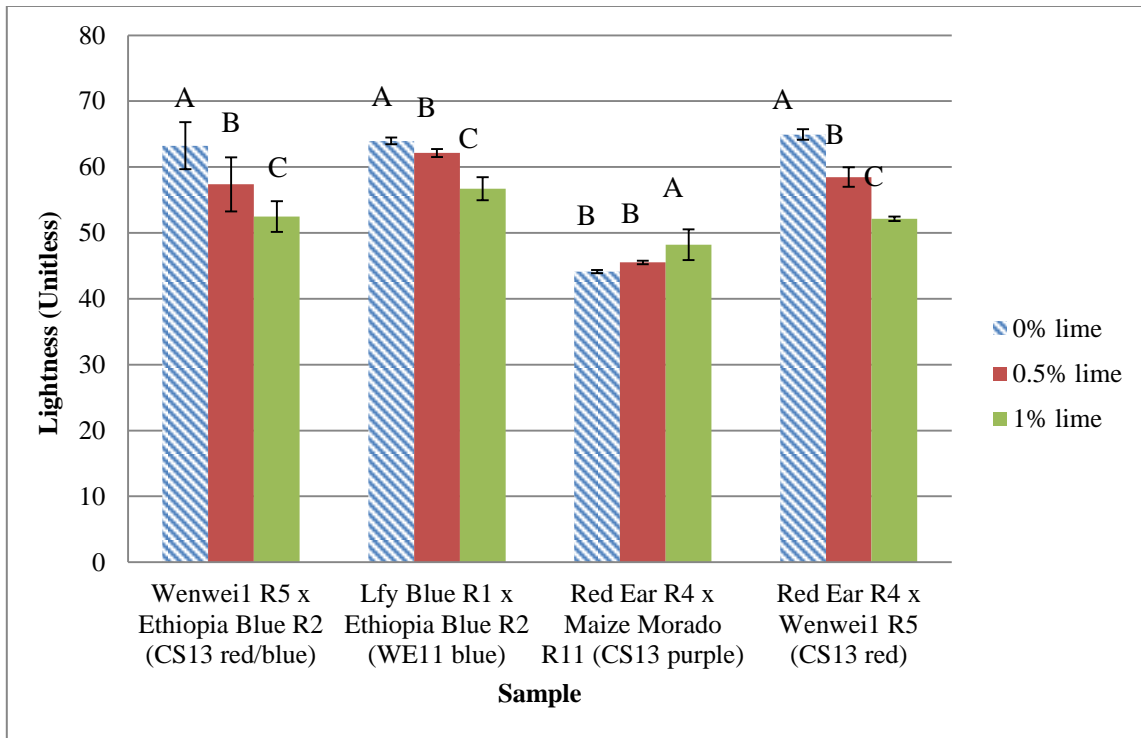


Figure 14. Effect of increasing lime concentration on lightness of red/blue, blue, purple, and red corn samples processed into masa. Data are expressed as mean \pm SD (n = 8). Columns with different letters within sample are significantly different ($p < 0.05$).

Chip. There was no clear trend in lightness value for the chips, as can be seen instrumentally and visually (Figures 15 and 16). Many observed factors affected the lightness of the chip including puffing/pillowing, as well as some toast spots from cooking the tortillas on the griddle. Therefore, it was hard to draw meaningful conclusions from the chip lightness data (Figure 15). These values are still important, however, because they affect how the other parameters, hue and chroma, are perceived.

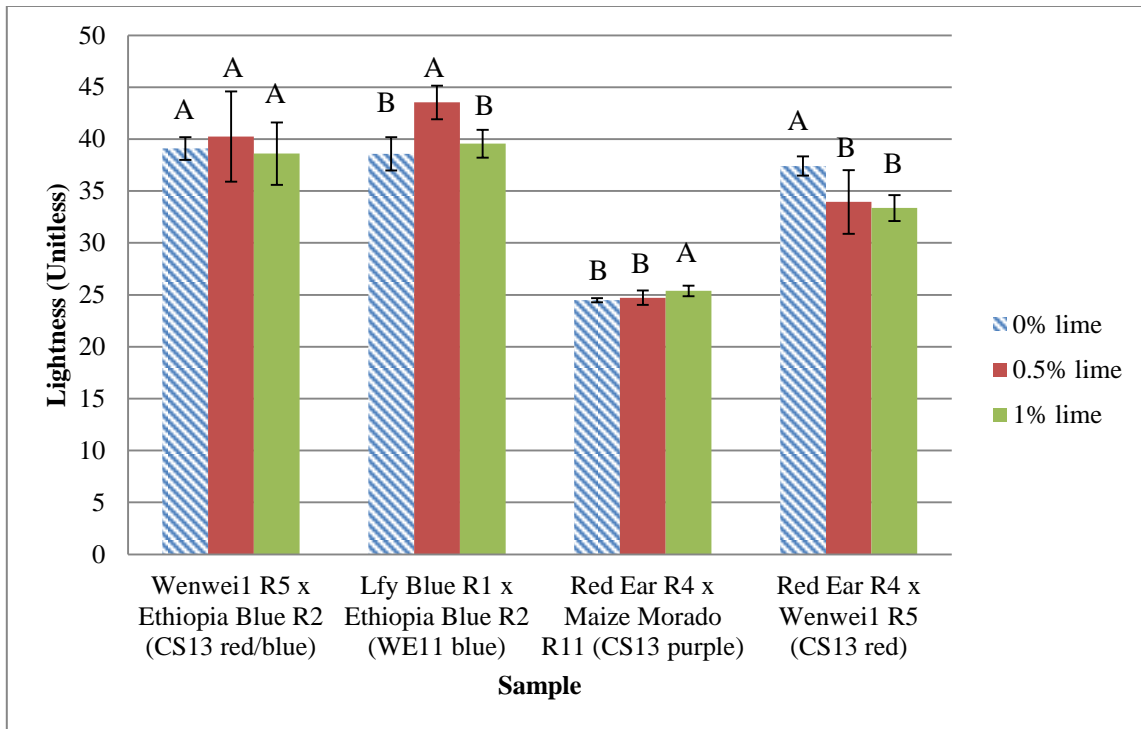


Figure 15. Effect of increasing lime concentration on lightness of red/blue, blue, purple, and red corn samples processed into tortilla chips. Data are expressed as mean \pm SD ($n = 12$). Columns with different letters within sample are significantly different ($p < 0.05$).

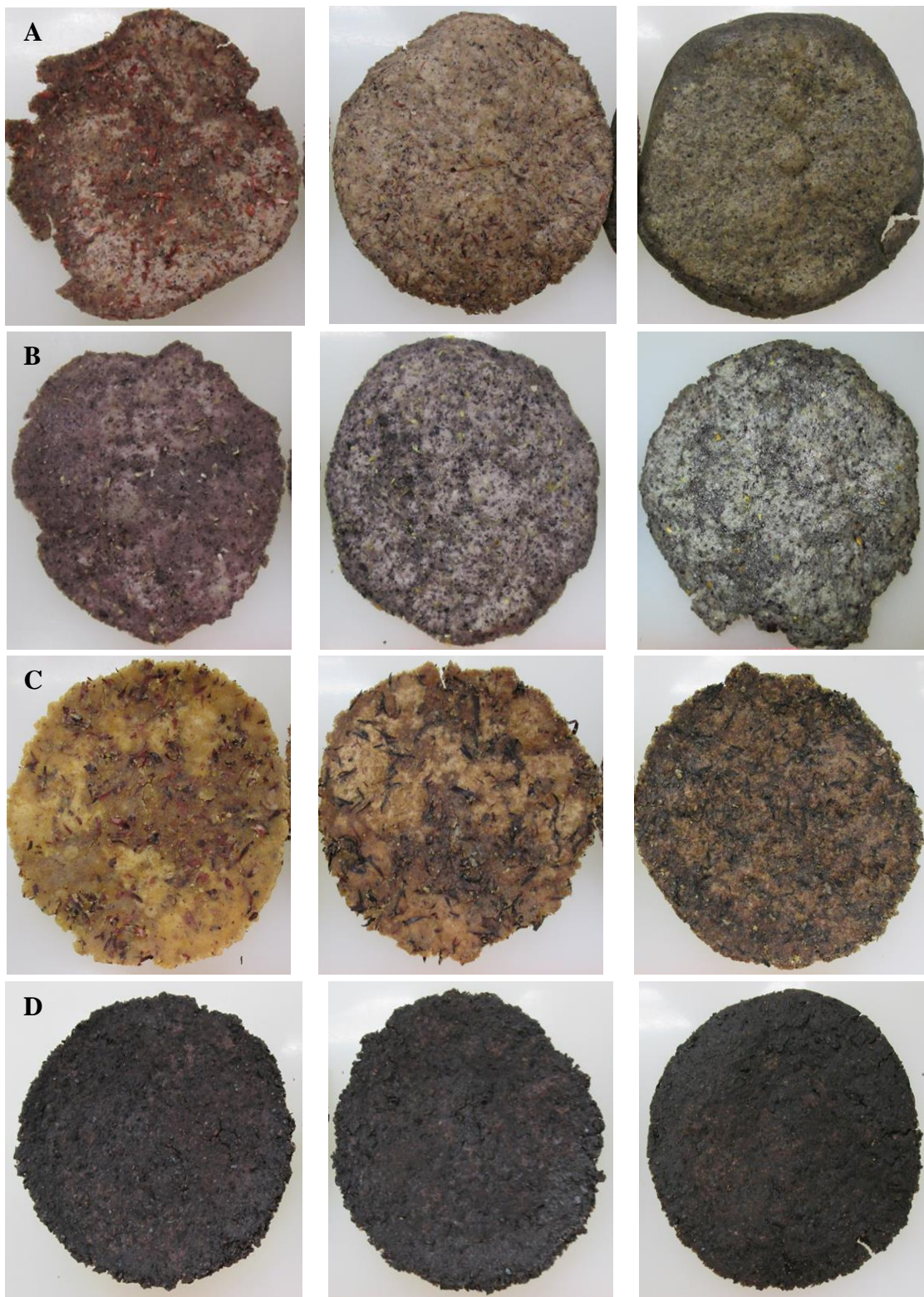


Figure 16. Pictures of tortilla chips. A-red/blue sample, B-blue sample, C-red sample, D-purple sample. Each row depicts 0%, 0.5%, and 1% lime concentration from left to right.

Chroma

The high pH of nixtamalization causes some anthocyanins to become colorless as a result of hydration, which causes bleaching due to formation of the carbinol and chalcone forms. Others become duller as mentioned previously, due to the change in anthocyanin color at alkaline pH to colors of dark blue/purple/black, which are caused by deprotonation that leads to the quinoidal base forms (Castañeda-Ovando et al., 2009; Fossen et al., 1998). As the lime concentration is increased (to 1% lime), the treatment becomes harsher causing more pigments to undergo one of these two changes, contributing to lower chroma values overall.

Nixtamal. In the nixtamal, a decrease in chroma value with increasing lime concentration was expected, and this occurred in the red/blue, purple, and red samples. The blue sample, however, had increased chroma at 0.5% lime and 1% lime (Figure 17). This is likely due to the fact that the control blue nixtamal has a large amount of white on the kernel, but the 0.5% lime and 1% lime nixtamals underwent a yellowing effect as seen in the images in Figure 13. While the blue sample chroma was confounded and subsequently increased by yellowing, the red and red/blue samples just became substantially duller as a result of increasing pH and had the expected decrease in chroma.

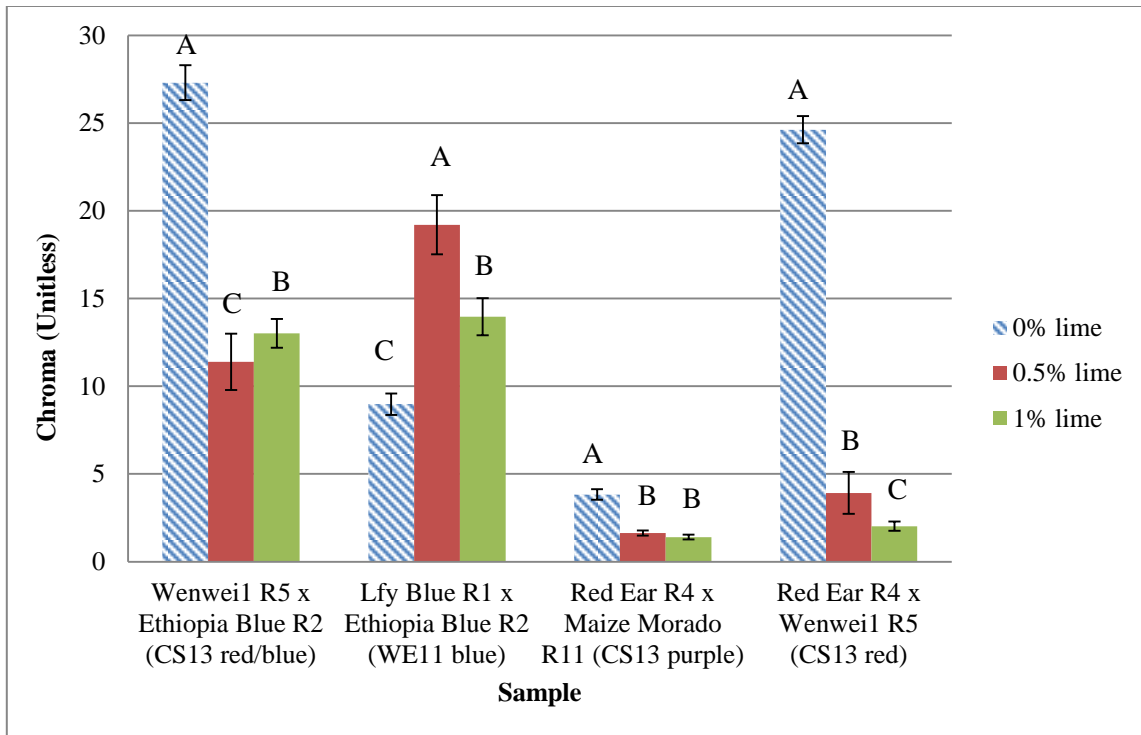


Figure 17. Effect of increasing lime concentration on chroma of red/blue, blue, purple, and red corn samples processed into nixtamal. Data are expressed as mean \pm SD (n = 8). Columns with different letters within sample are significantly different ($p < 0.05$).

Masa. A clear trend of decreasing chroma was observed in the red masa sample, but the other masa samples experienced only modest changes in chroma at either lime concentration (Figure 18). Due to the slightly observable yellowing effect in the nixtamal, the 0.5% and 1% masa of the red/blue sample showed small increases in chroma value. There is no chemical change between nixtamal and masa, only a physical grinding step, so the slight increases in chroma for the red/blue and blue samples observed in Figure 18 can again likely be attributed to yellowing.

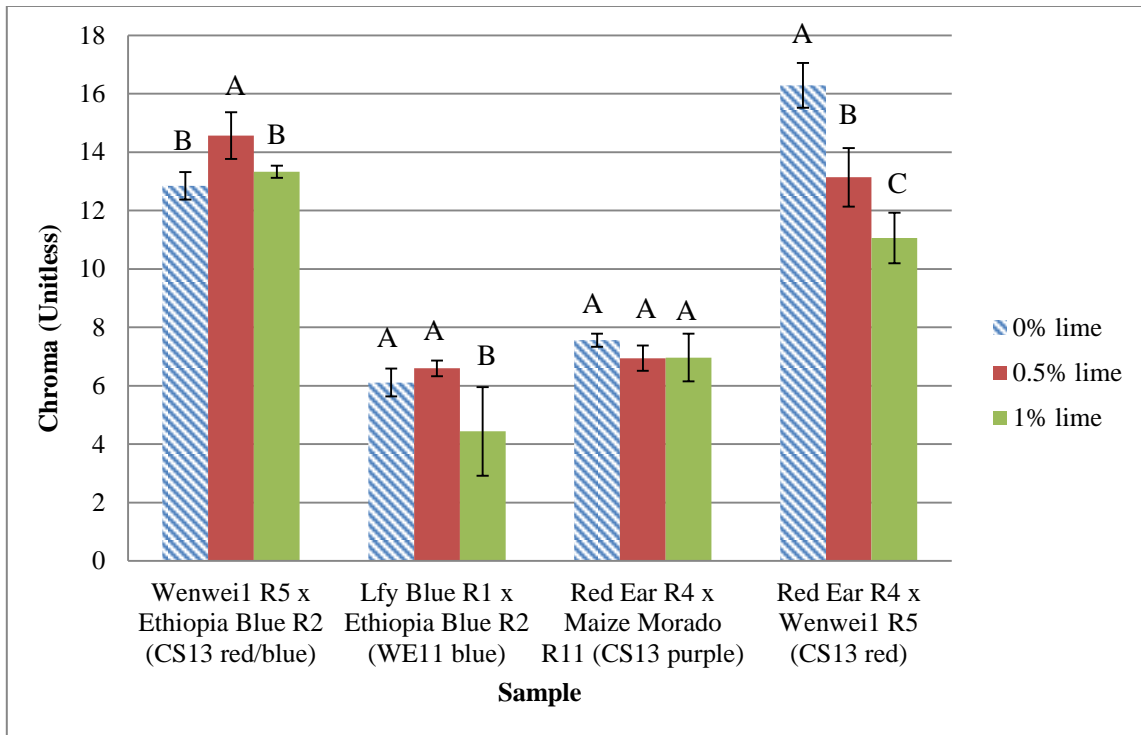


Figure 18. Effect of increasing lime concentration on chroma of red/blue, blue, purple, and red corn samples processed into masa. Data are expressed as mean \pm SD (n = 8). Columns with different letters within sample are significantly different ($p < 0.05$)

Chip. In the chips, the expected trend of decreasing chroma with increasing lime concentration was more easily observed (Figure 19). All samples show a significant difference in chroma when comparing the control chip to the 1% lime treatment. The red/blue sample had an insignificant ($p > 0.05$) decrease in chroma at the 0.5% lime level. The purple sample had minor measured changes in chroma, and in observing the images of purple chips in Figure 16D, this fact is likely due to the darkness of the chips at all lime concentrations, which made differences not easily distinguishable instrumentally or even visually. Due to the fact that the red/blue and red chip samples

had the highest chroma values at all lime concentrations, these samples produced better chips than the blue and purple samples because they were less dull and therefore more visually appealing (Figure 19). However, as is apparent in Figure 16C, the red chip chroma value is confounded by the yellow endosperm, which caused the chroma value to be high, giving a false indication of superior performance by the red chip anthocyanins.

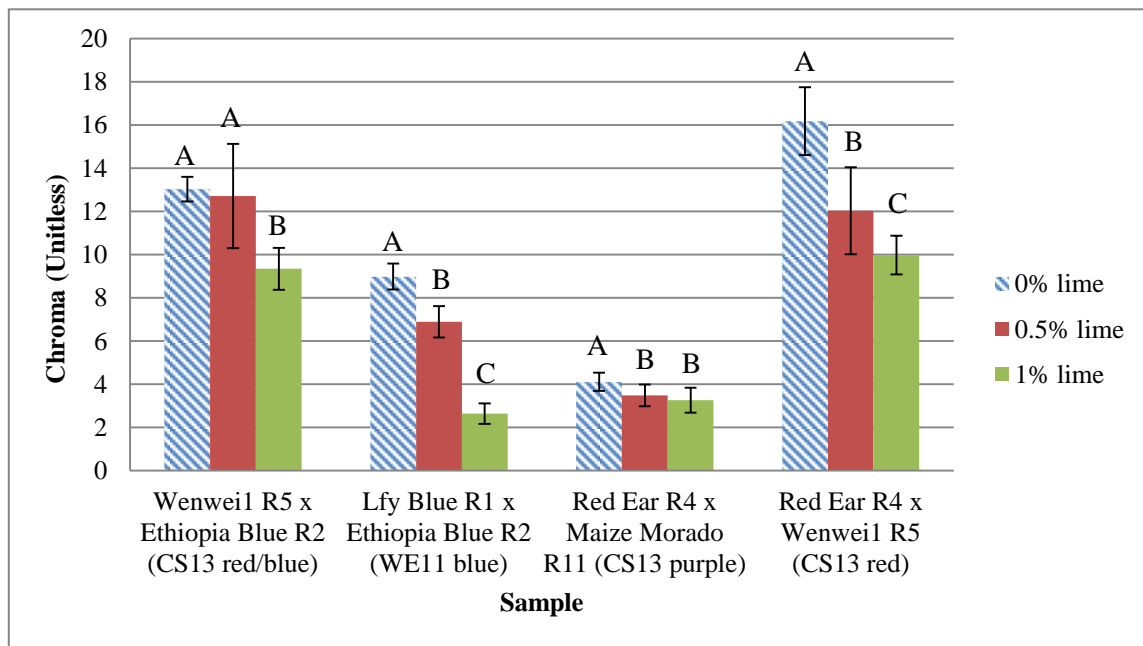


Figure 19. Effect of increasing lime concentration on chroma of red/blue, blue, purple, and red corn samples processed into tortilla chips. Data are expressed as mean \pm SD (n = 12). Columns with different letters within sample are significantly different (p < 0.05).

Hue

As mentioned previously, lightness has a large impact on the hue that is perceived by the eye, and such it is difficult to consider hue as a completely independent visual parameter.

Nixtamal. Although an instrumental change in hue value was observed for the purple nixtamal, the chroma and lightness values were so low that an instrumental change in hue between the control and lime treated nixtamals was not apparent (Figure 13D). The red nixtamal sample underwent a shift from red to orange/yellow with increasing lime concentration (Figure 20). Observing the red/blue nixtamal in the picture in Figure 13A, the hue seems fairly consistent with mainly a decrease in lightness, and this trend is supported by colorimeter data in Figure 20. The blue nixtamal shows no significant changes in hue instrumentally, although in the picture (Figure 13B), yellowness seems to intensify with increasing lime concentration. Perhaps the yellowness is present in the control blue nixtamal but with a very low chroma and high lightness value, which could allow the control nixtamal to appear less yellow to the eye.

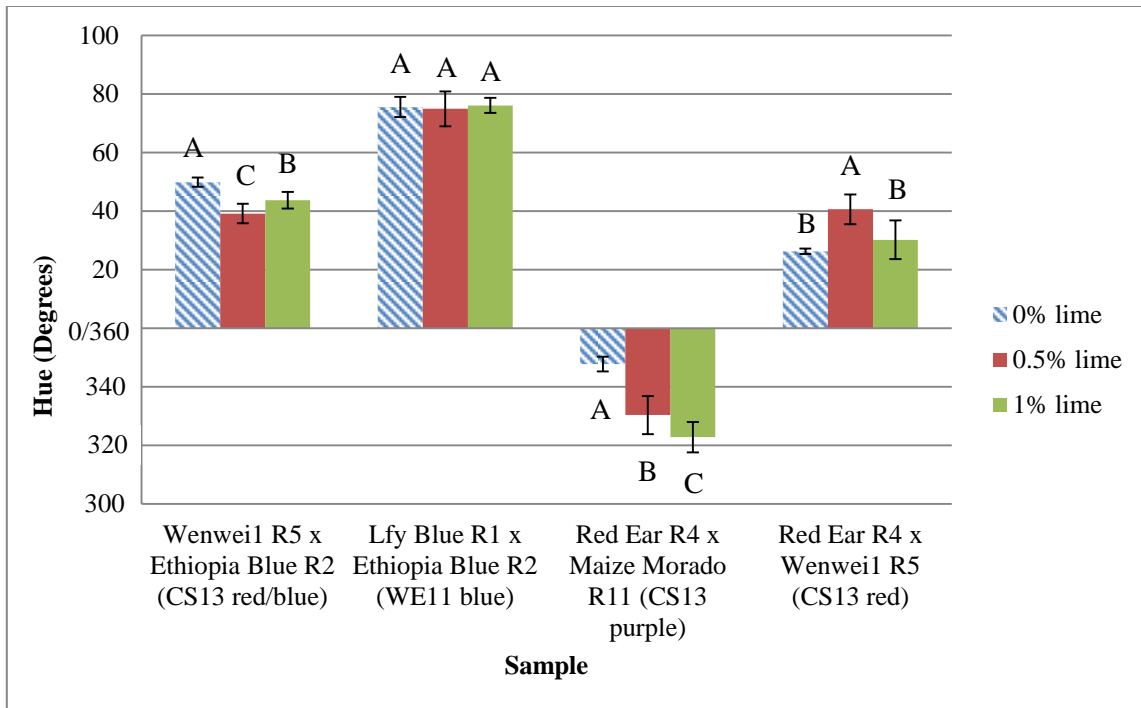


Figure 20. Effect of increasing lime concentration on hue of red/blue, blue, purple, and red corn samples processed into nixtamal. Data are expressed as mean \pm SD (n = 8). Columns with different letters within sample are significantly different ($p < 0.05$).

Masa. The hue values for the masa are primarily based upon endosperm color, with pieces of pericarp and aleurone making small contributions. The red/blue, blue, and red samples had hue values in the range of approximately 65-85 degrees, indicating general yellowness. The purple sample, which had a white endosperm, had hue values indicative of a shift from red to orange with increasing lime concentration (Figure 21). The hue of the purple masa sample was therefore mainly driven by color changes in the pericarp. The visual changes in Figure 22D, are minimal due to the low chroma values of the

purple samples. When referencing these images, the shift to orange as indicated by the colorimeter data is not apparent because the samples are predominantly gray.

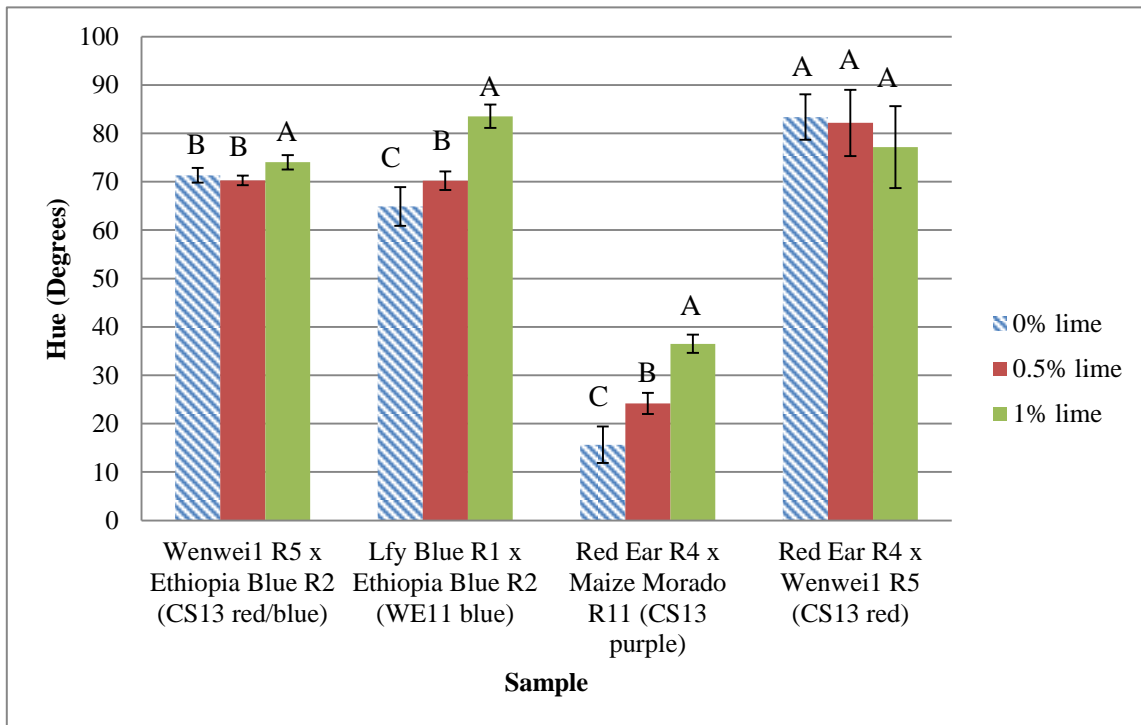


Figure 21. Effect of increasing lime concentration on hue of red/blue, blue, purple, and red corn samples processed into masa. Data are expressed as mean \pm SD (n = 8). Columns with different letters within sample are significantly different ($p < 0.05$).



Figure 22. Pictures of masa. A-red/blue sample, B-blue sample, C-red sample, D-purple sample. Each row depicts 0%, 0.5%, and 1% lime concentration from left to right.

Chip. Again for the purple chip sample, extremely low chroma and lightness values made changes in hue imperceptible to visual observation (Figure 16D). The color of the red/blue chip sample changed from orange to yellow with increasing lime concentration, which is observable in the picture in Figure 16A. The blue chips appeared predominantly gray at all lime concentrations, with the control appearing redder and shifting toward yellow with increasing lime concentration (Figures 23 and 16B).

The red chips, both visually and instrumentally (Figures 23 and 16C) showed a shift from more yellow to more orange. Although it appeared that the chip became darker and less yellow (Figure 16C), some of this can be attributed to the decrease in chroma with increasing lime concentration. Additionally, in determining the hue for the red chip, there were some confounding variables that are visually apparent (Figure 16C). At all lime concentrations for the red chip, the pericarp appeared tougher than the other three samples and it wasn't solubilized as much as the pericarps of the other three phenotypes, and therefore little of the red pigments bled from the pericarp into the remainder of the chip. Another confounding variable here in determining the hue was that the majority of the visible color was yellow due to the color of the endosperm, and the lack of solubilization of the pericarp which contains the anthocyanins. This could explain why the hue data for the red chip does not numerically show that the red chip is "red."

Overall, the red/blue chip sample performed best based on the hue data because it maintained a desirable color after thermo-alkaline processing.

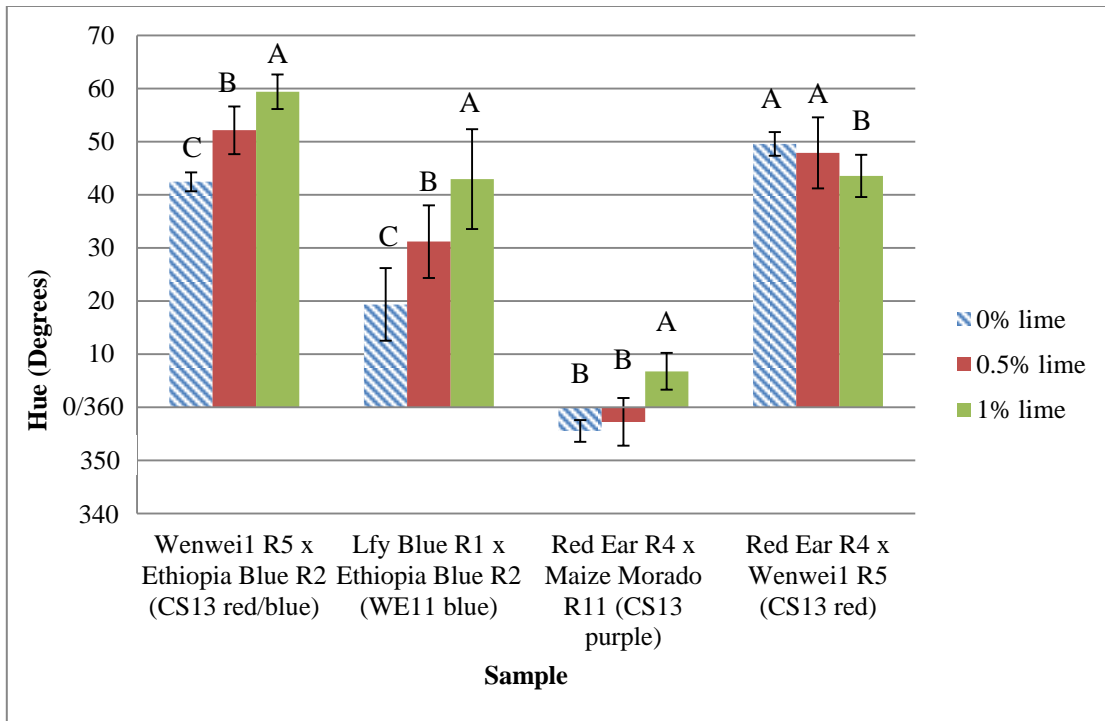


Figure 23. Effect of increasing lime concentration on hue of red/blue, blue, purple, and red corn samples processed into tortilla chips. Data are expressed as mean \pm SD (n = 12). Columns with different letters within sample are significantly different (p < 0.05)

Total Color Difference

Total color difference essentially combines the previous three parameters and in this way acts as a quick tool to relate visual and instrumental data.

Nixtamal. (Figure 24) The purple nixtamal had the least overall change, which as previously mentioned can be attributed to the initial darkness and low chroma of the

control. The red/blue and red nixtamal samples had the greatest color difference overall, indicating a large visual change, which is supported by the images in Figure 13.

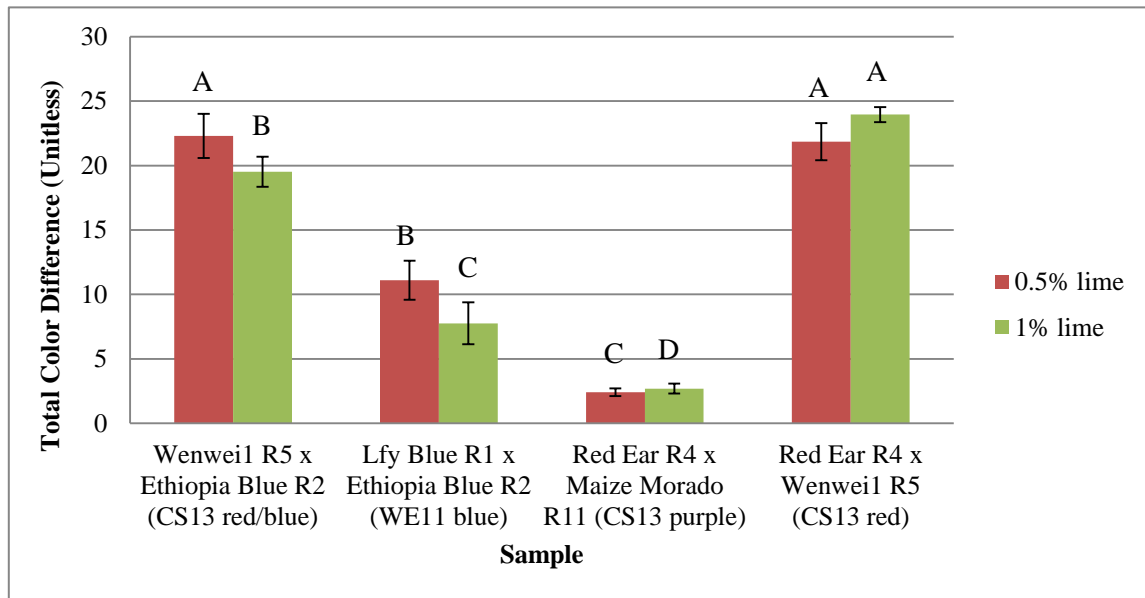


Figure 24. Effect of increasing lime concentration on the total color difference of red/blue, blue, purple, and red corn samples processed into nixtamal. Data are expressed as mean \pm SD (n = 8). Columns with different letters within treatment are significantly different ($p < 0.05$).

Masa. (Figure 25) For all masa samples the total change at the 1% lime concentration was significantly greater than at the 0.5% lime level ($p < 0.05$). The blue and purple samples were least different from control at the 0.5% lime concentration.

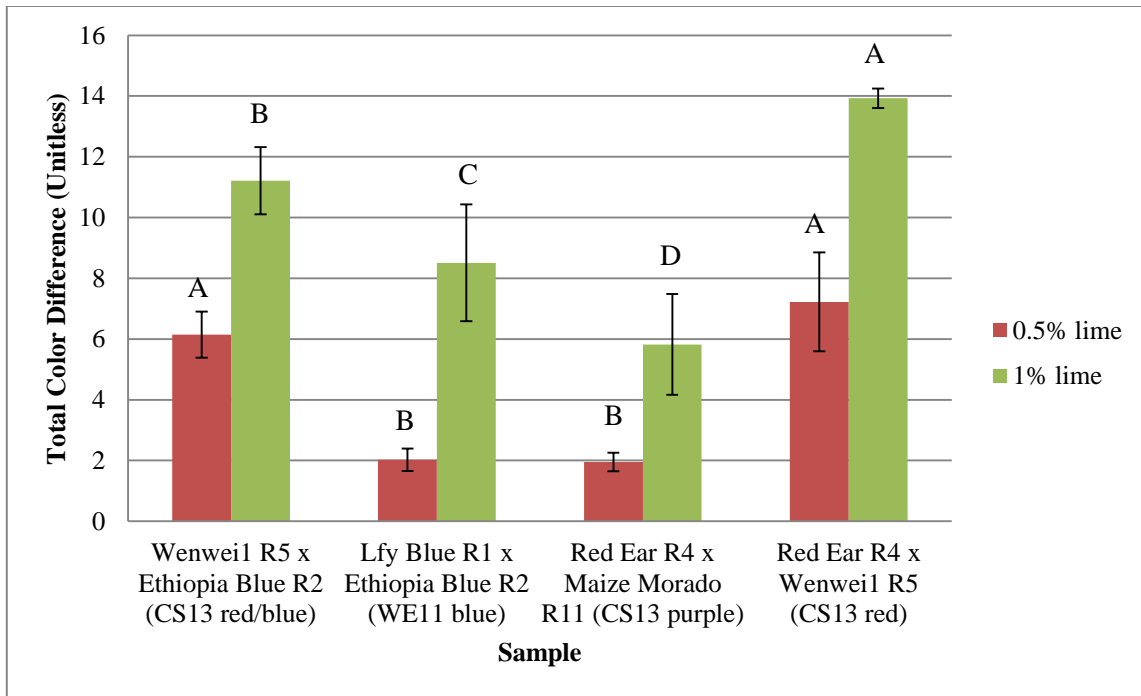


Figure 25. Effect of increasing lime concentration on the total color difference of red/blue, blue, purple, and red corn samples processed into masa. Data are expressed as mean \pm SD (n = 8). Columns with different letters within treatment are significantly different ($p < 0.05$).

Chip. (Figure 26) Total color difference is an important parameter when measuring how similar the visual color properties of two objects are, and is therefore highly useful in color comparison. In this application, however, total color change is not as useful as an indicator of success because it is dependent on the original color content, meaning that objects with low initial lightness and chroma will appear to be more stable because the lightness and chroma cannot decrease much further. This observation can be seen in the purple chip, where the low lightness in each stage of processing relates to a low total color change. For this reason, it is important to pair total color change with other

observable parameters to reveal more useful information. For example, in the red and blue samples, the total color change is high, which is reflected in the visual observation, however in the red/blue chip sample the total color change is lower, representing a more stable color, which supports our hypothesis that the red/blue phenotype would be more stable through thermo-alkaline processing.

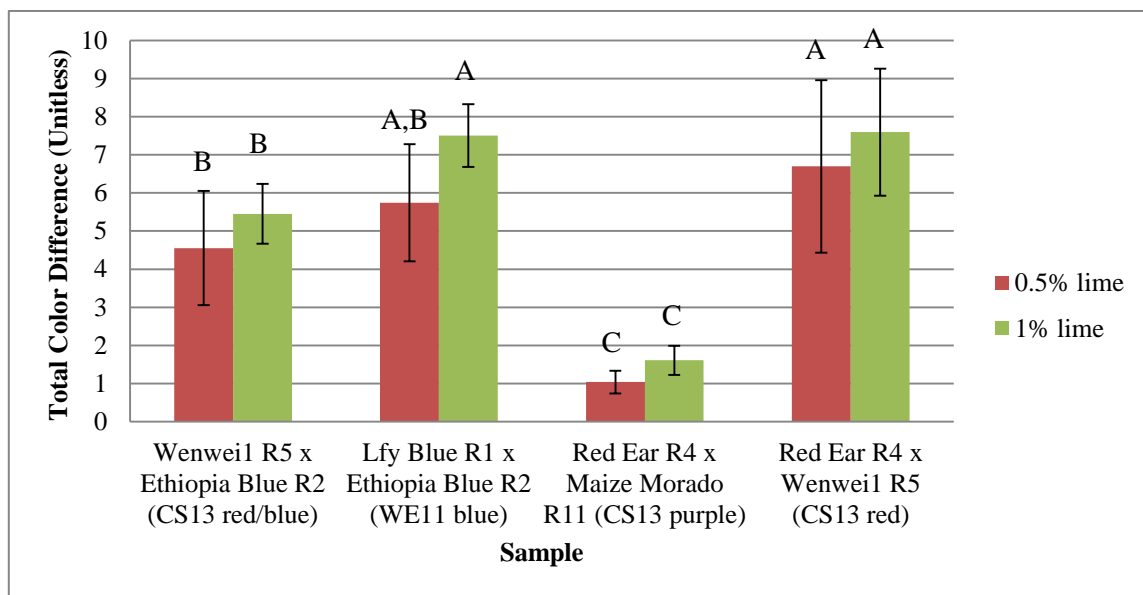


Figure 26. Effect of increasing lime concentration on the total color difference of red/blue, blue, purple, and red corn samples processed into tortilla chips. Data are expressed as mean \pm SD (n = 12). Columns with different letters within treatment are significantly different ($p < 0.05$).

Overall, the visual and instrumental outcomes of thermo-alkaline processing were similar to expectations. For instance, for the purple samples, the initial darkness of

the raw kernels made it seem that variability in visual parameters would be hard to observe, and this was the case at all processing steps. The low lightness and chroma values of this sample indicate that samples from the purple phenotype may not be suitable for thermo-alkaline processing into tortilla chips.

The non-purple samples behaved fairly similarly at the chip stage, with the main difference occurring in the chroma measurements. Although the trends in chroma of the red/blue, blue, and red samples were similar, the red/blue and red samples held up much better to increased lime concentration and maintained a higher chroma throughout the process. However, as can be seen visually and in the hue data instrumentally (Figures 16C and 23) there were confounding variables affecting the red sample readings. A major problem encountered in processing of the red sample was its tough pericarp, which did not solubilize and break apart as easily as the other three samples to allow the red pigment to be released. Due to this fact, the yellow endosperm influenced the visual color parameters, making some of the interpretations difficult for this sample.

While the blue sample had lower chroma than the red/blue and red samples, it had lightness values comparable to the red/blue sample, which is an important factor in visual appeal. Based on the data and observations in this portion of the study, the red/blue and blue phenotypes seemed to perform better overall through processing into tortilla chips than the other two phenotypes. This matched our earlier hypothesis based on our knowledge of their pigment profiles.

Effect of Thermo-Alkaline Processing on Anthocyanin and Major Co-pigment Content

Samples were selected for HPLC analysis and subsequent quantification of anthocyanins and major co-pigments based on their total color difference values of the tortilla chips after thermo-alkaline processing. Based on this data, one sample from each phenotype was selected to observe the changes in anthocyanin and co-pigment content through processing as a result of different lime concentrations during nixtamalization. In addition to the quantification of the individual anthocyanins and major co-pigments in the processed samples by HPLC analysis, total pigment content was determined on the processed samples as well, using a spectrophotometer.

The red sample (Red Ear R4 x Wenwei1 R5) had such a low measured anthocyanin content after processing, with no peaks detectable or quantifiable at all lime concentrations (0%, 0.5%, and 1%) (Tables 10 and 11). This was likely due to the visibly tougher pericarp causing low extractability of these pigments.

As hypothesized, the samples from the red/blue and blue phenotypes, which contain higher proportions of cyanidin-3-(6''-malonylglucoside), retained substantially more anthocyanins than the purple sample, as determined by HPLC analysis of anthocyanins retained through thermo-alkaline processing (Table 10). After the 0.5% lime treatment, the red/blue and blue samples had retained a majority of their initial anthocyanin content, with both experiencing <10% loss. The sample from the purple phenotype, which contains a higher proportion of monomeric anthocyanins, experienced greater loss in anthocyanin content, with only 26% retention after 0.5% lime treatment and 6% retention after 1% lime treatment.

Table 10. Summary of the percent retention of major anthocyanins of pigmented corn varieties from three different phenotypes (red/blue (Wenwei1 R5 x Ethiopia Blue R2), blue (Lfy Blue R1 x Ethiopia Blue R2), and purple (Red Ear R4 x Maize Morado R11)) after nixtamalization at two lime concentrations (0.5% and 1% lime, with respect to 0% lime control) and processing into tortilla chips as determined by HPLC.

	Cy-3- Glu	Pg-3- Glu	Pn-3- Glu	Cy- malglu1	Cy- malglu2	Pg- malglu	Pn- malglu	Total % retention
Red/blue 0.5%	107.9	ND ^a	ND	ND	76.8	ND	ND	90.2
Red/blue 1%	68.5	ND	ND	ND	46.7	ND	ND	56.1
Blue 0.5%	89.9	ND	ND	ND	100.1	ND	ND	93.4
Blue 1%	58.7	ND	ND	ND	75.4	ND	ND	64.5
Purple 0.5%	22.4	35.7	37.2	ND	39.3	0	0	25.8
Purple 1%	6.5	0	0	ND	12.9	0	0	6.4

^aND=not detectable

Table 11. Summary of the percent retention of major co-pigments of pigmented corn varieties from four different phenotypes (red (Red Ear R4 x Wenwei1 R5) , red/blue (Wenwei1 R5 x Ethiopia Blue R2), blue (Lfy Blue R1 x Ethiopia Blue R2), and purple (Red Ear R4 x Maize Morado R11)) after nixtamalization at two lime concentrations (0.5% and 1% lime, with respect to 0% lime control) and processing into tortilla chips as determined by HPLC.

	N'N'- Dicoumaroyl- spermidine	Unidentified coumaroyl derivative	Unidentified feruloyl derivative	Total % retention
Red 0.5%	97.9	92.5	97.3	87.8
Red 1%	70.8	75	81.9	27.7
Red/blue 0.5%	79.7	103.7	101.9	85.3
Red/blue 1%	75	88.9	98.1	62
Blue 0.5%	80.8	109.8	115.9	106.4
Blue 1%	71.8	100	114	85.8
Purple 0.5%	97.6	128.6	115.7	141.9
Purple 1%	70.7	71.4	74.3	16.4

Observing the effects of thermo-alkaline processing on the major co-pigments in these four samples, an interesting trend was observed (Table 11). For the red/blue, blue, and purple samples (at the 0.5% lime treatment), higher levels of the unidentified coumaroyl derivative and the unidentified feruloyl derivative were observed post-processing. All samples experienced a loss in all three major co-pigments after the 1% lime treatment, with the red and the purple samples retaining substantially less than the red/blue and blue samples. This loss of phenolic compounds during nixtamalization is commonly reported in the literature, and it is mainly due to the alkaline hydrolysis of cell walls, which releases these phytochemicals that were bound in the raw corn (Gutiérrez-Urbe et al., 2010). The increase in phenolic compounds at the 0.5% lime level could thus be due to the fact that these once bound compounds containing ferulic and p-coumaric acid were hydrolyzed and had not yet leached into the wastewater (nejayote). Whereas the decrease in these same phenolic compounds at the 1% lime level likely signifies that these compounds have been hydrolyzed and lost to the nejayote. The recovery of these phenolic compounds lost to the nejayote during nixtamalization from the solubilization/hydrolysis of the pericarp could be a source of bioavailable nutrients that could potentially be used to create value-added health products (Gutiérrez-Urbe et al., 2010).

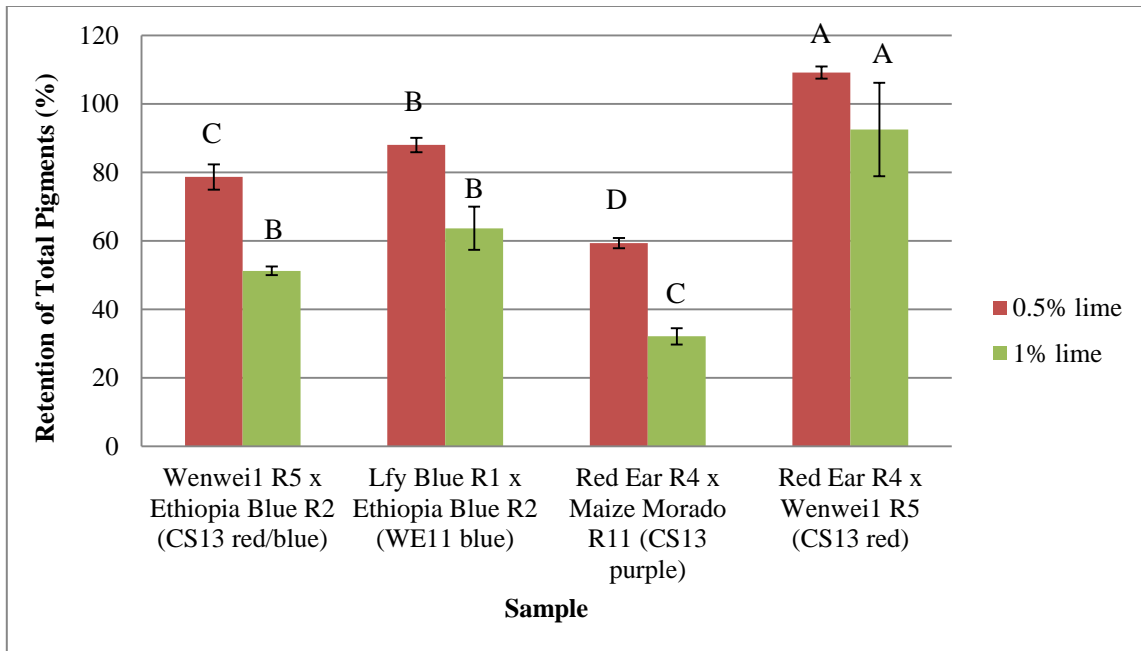


Figure 27. Percent retention of pigments in red/blue, blue, purple, and red corn samples after nixtamalization at two lime concentrations (0.5% and 1% lime, with respect to 0% lime control) and processing into tortilla chips as determined by UV-vis spectroscopy. Data are expressed as mean \pm SD (n = 6). Columns within the same treatment with different letters are significantly different ($p < 0.05$).

Spectroscopy data (Figure 27) also confirmed that the sample from the purple phenotype (Red Ear R4 x Maize Morado R11) lost the most pigments at both the 0.5% and 1% lime treatment levels, retaining only 59.3% and 32.1%, respectively. The blue (Lfy Blue R1 x Ethiopia Blue R2) and red/blue (Wenwei1 R5 x Ethiopia Blue R2) samples displayed similar trends to one another, with pigment retentions of 88.0% and 78.6%, respectively at the 0.5% lime level; and 63.6% and 51.2% retained, respectively, at the 1% lime level. In the red sample (Red Ear R4 x Wenwei1 R5), an increase of 9.1% was observed at the 0.5% lime level, and a loss of only 7.5% (92.5 % retention) at the

1% lime level. The retention of pigments for the red sample was significantly higher than the retention for the other three phenotypes at both lime treatment levels ($p < 0.05$). This is likely due to the fact that the red sample appears to have a tougher pericarp than the other three samples (Figure 16C). This tough pericarp doesn't solubilize as easily and thus protects the pigments better because they aren't as easily released.

In summary, the quantitative information from HPLC and spectroscopic analysis on pigment retention shows a good correlation with the visual and instrumental findings from the previous section (*Effect of Thermo-Alkaline Processing on Visual Color Parameters*). The red sample retained the greatest amount of total pigments through thermo-alkaline processing into tortilla chips (Figure 27). This may be explained by the visibly tougher pericarp of the red sample (Figure 16C), which likely had a protective effect on the pigments it contained because it was not solubilized as easily as the other three phenotypes in the alkaline conditions. The red/blue and blue samples had the next greatest retention of total anthocyanins (Table 10 and Figure 27), which can be attributed to their more stable pigment profiles, which also led to production of visually appealing chips (Figure 16). The purple sample had the greatest loss of total anthocyanins (Table 10 and Figure 27), which was expected due to the high proportion of monomeric anthocyanins in its pigment profile. It is difficult again to relate visual and instrumental data for this sample because changes were essentially imperceptible due to the darkness of this sample.

Conclusions

The purple samples lost significantly greater amounts of anthocyanins than the other phenotypes, and were also too dark for commercial consumer appeal during all stages of thermo-alkaline processing into tortilla chips. Therefore, the purple phenotype does not seem like a viable choice for direct use as an ingredient for colored tortilla chip production. However, the purple phenotype contained the highest anthocyanin content before processing, so this phenotype could have potential for use as a natural colorant in more acidic food applications, giving potent color as well as a high amount of bioactive anthocyanins. Additionally, due to the attractive, vibrant color of the purple corn before thermo-alkaline processing, the use of the highly pigmented pericarp as a post-processing inclusion or in a blend with yellow or white corn masa could create a tortilla chip with a pleasant, healthy appearance.

Based on changes in total pigments, as well as visual appearance, both the red/blue and blue phenotypes seem like suitable corns for tortilla chip processing at the 0.5% lime level. The red/blue phenotype had a slight advantage at the 1% lime level (the lime concentration used in commercial tortilla chip processing); especially when considering visual appearance of all tortilla chips at this level (Figure 16). Greater retention of anthocyanins by the blue phenotype at both lime concentrations could indicate that products made from this corn have greater potential health benefits. As hypothesized in the previous chapter, anthocyanin profile seems to be the greatest predictor of color stability during thermo-alkaline processing. Samples from these two phenotypes (red/blue and blue) contained similar, high proportions of the more stable

cyanidin-3-(6''-malonylglucoside), and these two phenotypes showed the greatest performance at the alkaline pH encountered in tortilla chip processing. Either manipulating lime concentration (to a lower level such as 0.5%) during commercial processing or incorporating a washing step to remove excess lime/alkalinity could lead to improved and desirable color in tortilla chips made from pigmented corn.

CHAPTER V

SUMMARY

This study has shown that there is a clear effect of phenotype on pigment composition of colored corn; phenotype was therefore a better predictor of color stability during thermo-alkaline processing than specific hybrid variety. There was an interaction effect of phenotype and environment on total pigment content. Thermo-alkaline processing caused general decreases in anthocyanin content, as well as changes in visual color attributes of the pigmented corn samples processed into tortilla chips.

Color stability during thermo-alkaline processing seemed mostly related to initial anthocyanin profile. The red/blue and blue pigmented corn lines contained cyanidin-3-(6''-malonylglucoside) in greatest proportion and also had significantly lower proportions of monomeric anthocyanins than the purple samples as determined by the pH differential method. These samples underwent modest losses in total pigment content, retaining 78.6 % and 88.0%, respectively at the 0.5% lime level, and retaining 51.2% and 63.6%, respectively at the 1% lime level. The red/blue chip sample also had a high chroma value, which indicates more vibrancy was retained through processing. While the blue sample had lower chroma than the red/blue sample, it had lightness values comparable to the red/blue sample, which is an important factor in visual appeal. In fact, from this study chroma and lightness values seemed to have the greatest correlation with visual perception.

While the red chip sample had comparable chroma and lightness values to the red/blue chip, this instrumental data was confounded by the fact that the pericarp, which contains most of the pigments, was not solubilized during nixtamalization, and thus the pigment were not very extractable. As such most of the color parameter readings were based upon its yellow endosperm. The purple sample behaved completely differently than the other three phenotypes due to its low chroma and lightness values which made color changes difficult to measure. Additionally, this sample lost significantly higher amounts of total pigments at both lime treatment levels. Due to its overall dark and dull appearance and poor anthocyanin retention, this phenotype seems least suited for thermo-alkaline processing into tortilla chips. However, due to the fact that the purple phenotype had significantly higher total anthocyanin contents than the other three phenotypes, pigmented corn from this phenotype could have potential as a natural food colorant in acidic food applications, providing powerful color and/or high levels of bioactive anthocyanins. Additionally, samples from the purple phenotype were attractively colored before exposure to the alkaline conditions of tortilla chip processing, so using the vibrantly pigmented pericarp as a post-processing inclusion or in a blend with white or yellow corn masa could produce table tortillas or tortilla chips with a healthy and unique appearance.

Based on the observations in this study, the red/blue and blue phenotypes seemed to perform better overall through processing into tortilla chips than the other two phenotypes. However, due to the adverse effect of thermo-alkaline processing on the chroma properties of the blue chip, the red/blue phenotype seems like the best choice for

genetic improvement because of its more favorable visual appearance overall. The information on the effect of phenotype and environment on the pigment profile of pigmented corn and the subsequent behavior during thermo-alkaline processing is useful to selectively breed pigmented corn varieties for greater color stability and retention of potentially bioactive compounds. Additionally, there was a clear effect of environment on total pigment content in the non-purple samples, with the samples grown in Weslaco, TX in 2011 having consistently higher levels of total anthocyanins than the same genetic hybrid varieties grown in College Station, TX in 2013. Further investigation is needed to determine which environments produce pigmented corn with the highest pigment contents because corn with high bioactive properties is desired for use in value-added foods.

Future Studies

Our results show that pigmented corns from the blue and red/blue phenotypes retain a greater portion of their anthocyanins through thermo-alkaline processing and experience less visual color degradation as well. This suggests that these phenotypes could be genetically improved for use in commercial tortilla chip processing. However, additional work needed includes:

- 1) Investigating the effect of growth environment on total pigment content of pigmented corn phenotypes.
- 2) Evaluating the effect of tortilla chip processing on the bioactivity of the anthocyanins and co-pigments in pigmented corn.

- 3) Consideration of alternative processing technologies that may permit greater retention of anthocyanins and co-pigments.

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APPENDIX A

COLOR PARAMETER SUMMARY OF RED/BLUE PHENOTYPE

Summary of CS13 Wenwei1 R5 x Ethiopia Blue R2									
	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	42.4±1.8	52.1±4.5	59.4±3.2	71.3±1.5	70.3±0.97	74.0±1.5	49.8±1.6	39.1±3.3	43.7±2.8
Chroma	13.0±0.57	12.7±2.4	9.3±0.97	12.9±0.47	14.6±0.80	13.3±0.21	27.3±1.0	11.4±1.6	13.0±0.82
TCD	0	4.4±0.49	5.2±0.21	0	6.1±0.50	10.8±1.8	0	22.3±1.9	19.5±0.74
Lightness	39.1±1.1	40.3±4.3	38.6±3.0	63.2±3.6	57.4±4.1	52.5±2.3	39.2±1.2	24.0±0.71	26.1±0.83
Summary of WE11 Lfy Blue R1 x Ethiopia Blue R2									
	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	35.1±4.5	35.2±3.4	38.9±3.2	54.8±8.2	55.4±6.7	63.5±2.5	28.0±3.0	36.2±4.8	35.4±9.1
Chroma	11.8±1.3	10.4±2.0	6.9±0.79	12.6±0.63	11.5±0.57	10.5±0.24	20.0±2.5	6.6±0.90	3.3±0.71
TCD	0	2.6±1.6	5.7±3.0	0	11.2±4.2	19.9±5.7	0	14.5±4.1	18.4±4.1
Lightness	34.1±1.7	31.9±2.1	31.2±1.2	61.2±2.8	50.1±2.0	41.5±2.0	27.5±2.7	22.7±1.4	20.2±0.96
Summary of WE11 Red Ear R4 x Ethiopia Blue2 R9									
	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	33.7±3.4	33.6±3.6	37.9±4.0	52.6±4.4	57.7±0.54	62.1±0.88	31.0±2.3	35.1±6.1	53.1±5.1
Chroma	12.3±0.47	10.1±1.2	8.9±1.8	13.0±0.97	12.0±0.86	10.9±0.27	23.1±3.0	5.1±1.4	4.8±1.3
TCD	0	3.7±2.3	5.5±2.8	0	5.8±3.1	12.6±4.1	0	19.5±5.1	20.2±3.7
Lightness	36.5±1.5	33.6±1.9	32.3±2.1	59.3±3.4	54.3±0.79	47.1±1.2	29.5±2.3	22.0±1.2	22.2±1.1
Summary of CS13 Ethiopia Blue2 R9 x Red Ear R4									
	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	33.1±6.1	36.0±4.2	39.9±2.8	53.8±2.7	60.6±1.9	64.7±1.4	30.5±3.2	54.2±8.2	50.5±6.2

Chroma	12.1±0.78	9.7±1.9	8.6±2.2	12.0±0.60	11.2±0.34	10.2±0.75	21.7±2.0	5.2±1.5	3.3±0.99
TCD	0	3.3±0.77	4.9±2.1	0	4.0±0.00	10.1±2.5	0	18.9±3.6	20.7±4.1
Lightness	34.5±1.3	32.7±1.5	33.0±1.8	56.8±1.8	53.1±2.0	47.1±1.3	29.7±3.1	22.4±1.2	21.1±0.95

Summary of WE11 Ethiopia Blue R2 x Red Ear R4

	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	30.7±2.5	32.5±3.8	35.8±4.8	58.2±1.5	60.6±0.17	65.8±2.2	25.0±1.6	40.8±3.7	49.4±7.6
Chroma	10.8±0.39	7.5±0.38	6.2±1.4	11.9±0.51	9.8±0.88	10.3±0.65	22.1±2.1	5.5±0.90	3.2±1.0
TCD	0	4.1±0.04	5.4±1.8	0	5.0±3.4	8.6±2.8	0	17.5±2.2	20.1±1.7
Lightness	33.0±1.2	30.5±0.87	30.4±1.6	55.0±2.2	50.5±0.68	46.7±0.40	26.2±1.1	21.6±0.51	20.6±0.78

Summary of CS13 Ethiopia Blue R2 x Red Ear R4

	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	27.4±0.96	29.3±2.8	36.7±3.1	48.4±1.4	58.5±1.2	64.4±0.22	29.7±2.0	39.5±10.6	38.8±8.7
Chroma	11.1±0.46	7.1±0.70	6.2±1.1	11.2±0.63	9.8±0.86	10.1±0.62	20.1±0.87	5.7±1.7	3.2±0.51
TCD	0	4.8±0.51	5.7±0.18	0	5.6±0.10	8.2±0.73	0	15.9±2.2	18.6±0.19
Lightness	32.2±1.3	29.7±0.59	29.8±1.3	53.4±0.97	48.3±0.99	45.8±0.61	27.6±1.3	21.5±1.6	20.0±0.60

COLOR PARAMETER SUMMARY OF PURPLE PHENOTYPE

Summary of CS13 Red Ear R4 x Maize Morado R11									
	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	356±2.1	357±4.5	6.8±3.5	15.6±3.8	24.2±2.2	36.5±1.9	348±2.5	330±6.5	323±5.2
Chroma	4.1±0.42	3.5±0.50	3.3±0.58	7.6±0.22	6.9±0.43	7.0±0.81	3.0±0.30	1.6±0.15	1.4±0.14
TCD	0	0.99±0.02	1.5±0.29	0	1.9±0.39	5.2±2.4	0	2.4±0.41	2.7±0.50
Lightness	24.5±0.20	24.7±0.68	25.4±0.50	44.1±0.25	45.5±0.27	48.2±2.3	16.5±0.65	16.9±0.54	17.0±0.30
Summary of WE11 LH195 R7 x Maize Morado R11									
	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	20.8±3.0	26.8±2.2	34.6±6.5	31.1±0.47	55.2±5.3	59.8±1.9	358±0.58	337±5.2	342±7.5
Chroma	6.4±0.75	7.0±0.96	7.1±1.6	6.8±0.08	8.2±0.13	8.7±0.18	6.4±0.31	1.7±0.09	1.5±0.10
TCD	0	2.2±0.50	4.1±2.7	0	36.0±1.9	37.9±2.2	0	4.9±0.17	4.9±0.18
Lightness	28.9±1.2	29.4±0.52	30.4±1.4	53.1±1.3	55.5±1.3	54.5±1.6	17.4±0.68	16.8±0.55	17.6±0.32
Summary of WE11 Red Ear R4 x Maize Morado R11									
	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	356±5.4	13.4±3.9	22.2±4.4	19.7±2.6	42.5±3.4	51.9±11.0	354±2.0	330±4.1	330±6.8
Chroma	3.8±0.58	4.5±0.57	4.0±1.0	6.8±0.21	7.2±0.31	7.2±0.30	5.1±0.50	1.8±0.15	1.2±0.14
TCD	0	2.0±0.90	2.3±0.98	0	2.9±0.19	4.8±0.29	0	3.5±0.66	4.1±0.57
Lightness	26.1±0.47	27.5±0.15	27.4±0.81	51.4±0.40	51.6±0.64	49.0±1.7	17.3±0.52	17.0±0.78	16.6±0.70
Summary of WE11 Red Hybrid Ear R3 x Maize Morado R11									
	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	6.5±1.0	20.0±2.0	26.2±1.4	14.5±1.3	35.0±0.55	42.7±1.8	353±1.1	331±9.1	325±5.7
Chroma	6.8±0.26	6.4±0.56	5.7±0.88	6.5±0.18	6.1±0.40	7.2±0.13	5.2±0.29	1.9±0.12	1.4±0.10
TCD	0	2.0±0.27	2.6±0.36	0	2.5±0.21	4.7±0.01	0	3.6±0.03	4.1±0.14

Lightness	27.6±1.3	28.8±0.48	28.0±0.57	53.3±1.2	53.9±1.7	50.1±1.6	16.7±0.55	17.3±1.1	16.9±0.51
Summary of CS13 LH195 R7 x Maize Morado R11									
	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	9.9±3.5	28.3±6.9	39.3±2.6	30.9±1.9	54.5±0.18	62.6±0.86	357±1.9	329±9.5	331±6.7
Chroma	5.5±0.42	5.5±0.66	6.4±0.47	6.1±0.14	7.4±0.09	8.3±0.32	6.2±1.0	1.6±0.19	1.4±0.18
TCD	0	2.2±1.0	4.0±0.39	0	3.1±0.18	4.8±0.64	0	4.9±0.95	5.0±1.1
Lightness	28.5±0.49	28.9±1.2	30.8±0.40	53.3±1.0	53.8±0.74	54.7±2.4	17.4±0.51	17.0±0.37	17.5±0.32
Summary of CS13 Red Hybrid Ear R3 x Maize Morado R11									
	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	3.6±6.0	10.4±2.4	17.4±4.6	23.2±2.1	37.9±2.2	51.5±1.8	353±1.1	330±6.2	322±3.7
Chroma	5.4±1.5	4.4±0.34	3.5±0.53	6.7±0.18	6.5±0.30	6.4±0.32	4.8±0.29	1.6±0.13	1.4±0.08
TCD	0	1.7±1.1	2.6±1.6	0	2.8±1.5	3.9±1.3	0	3.5±0.03	3.7±0.01
Lightness	26.7±1.1	26.5±0.65	26.1±0.46	54.1±2.1	55.2±0.41	56.1±0.73	16.7±0.62	17.5±0.70	16.9±0.40

COLOR PARAMETER SUMMARY OF RED PHENOTYPE

Summary of CS13 Red Ear R4 x Wenwei1 R5									
	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	49.6±2.2	47.9±6.7	43.5±4.0	83.4±4.7	82.2±6.8	77.1±8.5	26.2±0.93	40.6±5.1	48.8±20.6
Chroma	16.2±1.6	12.0±2.0	10.0±0.9	16.3±0.77	13.1±1.0	11.1±0.86	24.6±0.78	3.9±1.2	2.0±0.27
TCD	0	5.6±3.7	7.6±1.3	0	7.2±0.68	13.9±0.05	0	21.8±1.8	24.0±0.71
Lightness	37.4±0.92	34.0±3.1	33.4±1.3	64.9±0.80	58.5±1.5	52.1±0.34	26.1±0.66	19.7±0.37	19.0±0.39
Summary of WE11 LAMA Red R10 x Red Hybrid R3									
	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	59.9±4.7	49.6±7.4	51.9±1.8	76.8±1.2	79.3±2.9	81.2±4.3	28.5±1.8	48.0±6.2	43.3±4.1
Chroma	19.9±2.6	9.1±0.76	9.7±0.61	17.0±0.38	13.2±0.54	11.7±1.3	28.7±2.8	4.7±0.87	3.1±0.50
TCD	0	14.8±6.6	13.6±4.4	0	7.3±0.45	11.7±2.4	0	26.0±3.0	27.3±2.8
Lightness	41.8±2.9	32.4±1.5	33.1±0.61	63.1±0.75	56.9±0.86	52.8±2.0	29.9±1.2	20.9±0.93	20.9±0.72
Summary of WE11 Wenwei2 R6 x Red Hybrid Ear R3									
	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	54.6±4.8	45.8±3.2	47.2±3.8	77.3±4.8	70.6±0.95	71.4±1.3	31.7±3.2	40.8±5.1	41.7±5.0
Chroma	16.8±0.86	10.7±1.2	6.9±0.82	13.2±1.0	11.3±0.48	9.9±0.92	29.2±4.4	4.3±0.39	3.8±0.59
TCD	0	9.5±0.23	13.3±2.0	0	8.3±5.3	12.1±1.0	0	26.9±5.8	27.4±6.0
Lightness	40.1±1.5	33.3±0.74	31.7±1.3	63.4±3.3	55.5±0.53	51.8±2.8	30.6±2.3	20.7±0.54	20.6±0.71
Summary of WE11 Red Ear R4 x Wenwei1 R5									
	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	57.6±6.6	45.3±7.1	46.8±1.4	83.9±5.1	82.8±6.6	77.7±6.1	27.7±2.3	43.3±7.1	59.2±15.4
Chroma	20.0±2.0	12.0±2.8	11.2±0.48	16.7±0.36	13.5±0.34	11.7±0.28	27.6±1.1	4.4±0.68	2.5±0.49
TCD	0	12.7±0.96	12.7±6.2	0	6.2±0.21	13.7±0.86	0	24.3±1.2	26.5±1.3

Lightness	43.8±4.0	34.5±2.9	35.3±0.99	65.5±0.76	60.3±1.1	52.9±0.54	27.2±0.93	20.6±0.60	20.1±0.48
Summary of CS13 Red Ear R4 x LAMA Red R10									
	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	52.6±4.8	46.3±3.8	37.9±3.1	76.7±8.9	75.2±6.3	72.7±8.6	21.2±2.5	38.7±10.2	48.4±29.4
Chroma	17.7±2.8	10.1±0.42	5.8±1.5	18.5±0.42	14.5±0.38	11.8±0.45	23.4±1.6	4.1±1.1	2.9±0.46
TCD	0	9.9±3.4	15.2±1.1	0	7.4±1.6	14.7±1.9	0	20.0±0.06	21.7±0.81
Lightness	38.2±2.4	32.0±0.36	28.6±1.2	62.5±2.2	56.3±0.92	49.4±0.6	24.7±0.77	20.0±0.58	20.0±0.71
Summary of CS13 Wenwei2 R6 x Red Hybrid Ear R3									
	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	65.3±1.3	57.5±0.70	50.0±2.6	75.7±11.3	79.9±9.9	74.8±12.0	29.7±2.9	33.4±5.9	44.5±25.7
Chroma	20.7±.15	11.2±1.1	8.3±1.7	14.3±0.12	12.0±0.67	10.7±0.17	25.7±0.57	4.1±0.69	2.7±0.74
TCD	0	14.3±4.0	18.4±4.9	0	3.2±1.6	9.5±1.1	0	23.6±0.72	25.2±0.97
Lightness	45.9±0.59	35.5±1.5	32.8±1.9	61.6±2.5	59.9±1.3	52.9±1.7	29.0±0.80	19.7±0.37	19.3±0.64

COLOR PARAMETER SUMMARY OF BLUE PHENOTYPE

Summary of WE11 Lfy Blue R1 x Ethiopia Blue R2									
	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	19.4±6.82	31.2±6.8	42.9±9.4	64.9±4.0	70.2±1.9	83.5±2.4	75.5±3.4	74.9±6.0	76.1±2.5
Chroma	9.0±0.60	6.9±0.72	2.6±0.47	6.1±0.47	6.6±0.27	4.4±1.5	9.0±0.61	19.2±1.7	14.0±1.1
TCD	0	5.7±1.5	7.5±0.82	0	2.0±0.37	8.5±1.9	0	11.1±1.5	7.8±1.6
Lightness	38.6±1.6	43.5±1.6	39.6±1.4	64.0±0.50	62.2±0.61	56.7±1.8	43.0±2.5	38.3±4.8	37.7±0.92
Summary of WE11 Ethiopia Blue2 R9 x Ethiopia Blue R2									
	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	17.7±3.5	26.9±6.3	34.6±11.7	55.4±3.6	50.6±8.7	89.4±1.5	73.5±4.6	76.8±1.8	75.7±1.5
Chroma	7.9±0.62	7.2±1.2	2.8±0.51	5.5±0.38	5.1±0.31	4.6±0.99	9.1±0.33	15.3±2.0	14.1±1.5
TCD	0	5.3±0.91	6.2±0.55	0	3.8±0.99	8.3±0.99	0	7.8±0.73	8.0±0.33
Lightness	35.9±0.49	41.2±1.5	38.3±2.7	63.7±2.2	59.9±1.3	56.0±2.1	43.6±1.2	39.2±2.3	37.5±1.3
Summary of CS13 Lfy Blue R1 x Ethiopia Blue R2									
	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	14.3±1.1	26.9±2.3	39.0±10.1	66.9±16.3	80.9±18.5	89.5±1.1	77.3±3.6	76.1±4.1	78.6±0.86
Chroma	9.2±0.29	6.8±0.75	3.4±0.78	4.2±0.53	4.7±0.83	3.6±0.28	9.3±0.82	17.9±4.1	18.1±1.2
TCD	0	3.8±1.2	6.9±0.11	0	1.3±0.88	3.9±1.4	0	13.5±0.40	10.6±0.46
Lightness	40.3±2.5	42.7±0.97	42.9±1.2	62.1±0.74	61.8±0.79	58.7±1.5	47.8±1.8	38.9±3.4	42.1±0.98
Summary of CS13 Lfy Blue R1 x Ethiopia Blue2 R9									
	CHIP			MASA			NIXTAMAL		
	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime	0% lime	0.5% lime	1% lime
Hue(deg)	31.2±1.8	44.1±2.8	58.4±5.2	75.8±3.6	73.0±1.9	87.0±0.64	67.8±4.4	76.5±1.6	73.5±1.5
Chroma	11.5±0.37	9.5±0.51	6.2±0.86	5.4±0.61	6.2±0.44	5.6±0.64	10.2±0.78	24.9±4.4	21.4±2.4
TCD	0	3.4±0.14	7.2±0.53	0	0.98±0.36	3.6±1.4	0	15.7±5.8	11.3±2.7

Lightness 44.2±0.64 44.2±2.7 46.7±1.9 65.3±0.50 65.4±0.91 62.2±2.2 44.8±2.7 45.1±3.9 44.2±2.4