# Dry milling characteristics and polyphenolic contents of adapted colored corn varieties and polyphenol extraction by combining deep eutectic solvents and ultrasound

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by

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The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

**Dry milling characteristics and polyphenolic contents of adapted colored corn varieties and polyphenol extraction by combining deep eutectic solvents and ultrasound**

presented by Ravinder Kumar,

a candidate for the degree of Master of Science,

and hereby certify that, in their opinion, it is worthy of acceptance.

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## Dedication

I dedicate this thesis, which represents the culmination of my efforts during my master's degree, to the divine presence of God, who has served as a guiding light throughout my entire life.

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## Table of contents

## List of abbreviations

LG: Large grits

MG: Medium grits

SG: Small grits

G: Germ

P: Pericarp

F: Fines

EtOH: Ethanol

DES: Deep eutectic solvent

ChCl: Choline chloride

EE: Epicatechin equivalent

ACN: Anthocyanins

C3G: Cyanidin-3-glucoside

GAE: Gallic acid equivalent

CT: Condensed tannins

TFC: Total flavonoids content

TPC: Total phenolics content

CE: Catechin equivalent

TE: Trolox equivalent

## List of figures

## List of tables

**Dry milling characteristics and polyphenolic contents of adapted colored corn varieties and polyphenol extraction by combining deep eutectic solvents and ultrasound**

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## Abstract

Although conventional yellow dent corn (*Zea mays* L.) has been extensively grown and utilized in the US for food, feed, and numerous industrial applications, the degree of associated value-addition is rather modest. Colored corn varieties have recently drawn increased attention due to their relatively higher concentrations of diverse phytochemicals, such as anthocyanins, proanthocyanins, and flavonoids, which may be utilized for a variety of agro-industrial applications. Corn pericarp is a low value cellulosic coproduct of the processing industry. However, in case of colored corn varieties, most of the polyphenolic compounds such as anthocyanins, flavonoids, flavanols, tannins and phenolic acids are concentrated in corn pericarp which can be separated and selectively processed for their economic recovery. The corn endosperm, containing most of the kernel starch and proteins and of prime interest to the processing industry remains unaffected and can be utilized further. Due to the diverse applications of recovered value-added phytochemicals, colored corn varieties can supplement the lower economic value of yellow dent corn. However, polyphenol-rich colored corn varieties need to be adapted to the midwestern US climatic conditions for their economic exploitation.

In this study, dry milling characteristics and polyphenolic contents (anthocyanins, total phenols, and condensed tannins) of fourteen conventionally bred and midwestern US grown colored varieties developed by the USDA-ARS colored corn breeding program at the

University of Missouri and their milling coproducts were ascertained and compared with a commercial purple corn. With mean large grits yield of 22.98% (db), colored varieties had a dominant softer endosperm composition and were found to be more suitable for wet milling and dry grind industry, processing most of the corn grown in the US. The mean starch content of colored varieties was lower than conventional yellow dent corn (~9.91%, db), and the mean protein content was higher (~1.70%, db). Although commercial purple corn contained the highest amounts, four experimental varieties were identified with high concentrations of polyphenols. Through HPLC analyses, a diverse mix of various anthocyanin forms, phenolic acids, and flavonoids were identified. The antioxidant potential of polyphenolic extracts from five selected varieties were evaluated using four different assays, and significant antioxidant activities were observed. Histological analysis was conducted on polyphenol-rich varieties, and most of the pigments were found to be concentrated in pericarp. The pericarp thickness was greatest for the variety containing the maximum polyphenolic compounds. Adapted colored corn varieties grown in the midwestern US can be valorized to recover value-added polyphenolic compounds in line with the circular bioeconomic paradigm.

Polyphenolic compounds can be extracted from plant matrices using a wide array of solvents. However, conventional extraction methods involve the use of organic solvents, which are of concern due to their adverse environmental impacts and generation of waste streams. Therefore, there is a growing demand for green solvents that are renewable, non-toxic, biodegradable, and economically viable. In second study, a combination of deep eutectic solvents (DESs) and ultrasound-assisted extraction were investigated for extracting anthocyanins, condensed tannins, and flavonoids from purple corn pericarp. Initially,

nineteen DESs with different constitutes were prepared and characterized. It was observed that the DESs formed strong intermolecular bonds, their viscosities ranging from 0.0209 to 0.7463 Pa.s at a constant temperature of 50 °C and shear rate of 10 $s^{-1}$. During NIR analysis, dominant stretching of O-H and C-H bonds was observed. The highest and lowest polarities ranged between 50.63 (DES 4) and 46.80 $kcal.mol^{-1}$ (DES 5), respectively, and all the solvent mixes were acidic in nature. In a comparatively shorter extraction time (10 min), significant amounts of anthocyanins (27.41 mg cyanidin-3-glucoside C3G equivalent/g pericarp), condensed tannins (249.33 mg epicatechin EE equivalent/g pericarp), and total flavonoids (36.87 mg catechin CE equivalent/g pericarp) were recovered. Ultrasound-assisted extraction in DESs was found to enhance the extraction efficiency compared to the conventional methods utilizing ethanol and water. This improved extraction efficiency could be attributed to a greater solvent penetration into the plant cell matrix. The highest CUPRAC antioxidant capacity of 392.27 mg Trolox equivalent (TE)/g of pericarp, DPPH free radical scavenging activity of 82.70 mg TE/g of pericarp, and ABTS activity of 34.87 mg TE/g of pericarp, respectively, were observed. C3G, delphinidin, cyanidin chloride, and peonidin as high as 20.56, 9.62, 11.91, and 2.90 mg/g of pericarp, respectively, were detected during anthocyanin profiling of the extracts. Phenolic acids profiling identified caffeic acid, ferulic acid, gallic acid, hesperidin, and chlorogenic acid, the highest values being 3.29, 1.72, 0.63, 15.18, and 5.23 mg/g pericarp, respectively. Finally, epicatechin, naringin and kaempferol concentrations of 102.73, 19.93, and 1.55 mg/g pericarp, respectively, were eluted during flavonoids profiling by HPLC. This work demonstrated a highly efficient and green extraction of bioactive compounds from colored corn pericarp by combing DESs and ultrasound.

# Chapter 1

## Introduction

Corn is the most important cereal crop grown in the US. According to the USDA NASS (2023), more than 92 million acres of farmland will be planted with corn. Corn processing is a major contributor to the economy due to its utilization in a number of industries, yielding hundreds of diverse products including ethanol for gasoline blending, distillers dried grains with solubles (DDGS) for animal feed, starch for food and industrial applications, grits and flour for human consumption, and corn germ oil. In 2022, 79.2 million acres of cultivable land was dedicated to corn with an estimated harvest of 173.3 bushels per acre. The US Department of Energy (2022) estimated that 15.07 billion bushels of corn was produced in 2022 and almost 5.33 billion bushels or 35.4% of the total was utilized by corn dry grind industry for producing fuel ethanol. According to the Renewable Fuels Association (2021), the US alone produced 55% of the world's fuel ethanol (15.01 billion gallons) in 2021 followed by Brazil accounting for another 27% (7.32 billion gallons) while the European Union contributed about 5% to the total (1.35 billion gallons).

Almost all the corn-based ethanol in the US is produced by the corn dry grind (~95%) and corn wet milling industry. The corn dry grind industry requires relatively lower investment compared to the more sophisticated wet milling industry and is optimized for ethanol production. In addition to ethanol, the dry grind industry generates relatively lower value byproducts such as DDGS, corn germ oil and $CO_2$. On the contrary, the corn wet milling process involves complex chemical fractionation of corn kernels into relatively pure starch, protein, germ and fiber fractions (Somavat, 2017). Depending upon the market situation, purified coproducts can be used to produce hundreds of industrially valuable products. When

the ethanol prices are higher, purified starch from the wet milling industry can be fermented to produce ethanol. Due to the abovementioned mentioned reasons, although the corn dry grind process is preferred for ethanol production, the byproducts other than ethanol have relatively lower economic values. Despite several research endeavors to generate value-added byproducts from corn dry grind industry, none have resulted in economically viable products. Therefore, there is a longstanding need for value-addition when it comes to the corn processing industry.

The federal government incentivized the US farmers to grow more corn by enacting The Energy Independence and Security Act of 2007 wherein blending of 10% ethanol in gasoline was mandated by law. This not only reduced the overall gasoline demand but also replaced methyl *tert*-butyl ether (MTBE), a controversial fuel oxygenate (United States. Energy Policy Act, 2005). As a result, the net consumption of ethanol in the US increased from 1.65 billion gallons in 2000 to 14.59 billion gallons in the year 2019. However, at the peak of COVID-19 pandemic in 2020, the ethanol demand came down to 12.68 billion gallons which had subsequently rebounded back to 13.99 billion gallons in 2021 as the pandemic ebbed (U.S. Energy Information Administration, 2022). However, the corn ethanol industry has faced an increased scrutiny in recent times on account of its detrimental environmental effects and due to increased adoption of hybrid electric vehicles (HEVs) and electric vehicles (EVs). Currently, the passenger vehicles account for 27% of the global gasoline consumption which is estimated to decrease with ever increasing number of EVs and HEVs. Manufacturers are working on building heavy trucks and semis with the capability to run on electric batteries and some of them have already started replacing gasoline fueled semis. Accelerating growth in the EVs and HEVs segment can be understood from the following report by the

Alternative Fuel Data Center (U.S Department of Energy, 2023) which identified an increase of E85 fuel stations in the US from 154 in the year 2001 to 4,426 stations in 2022, whereas, the number of electric charging stations increased from 486 in the year 1998 to a total of 53,492 in the year 2022. Similarly, the number of HEVs sold in 2000 was 9,350 which increased to 400,746 HEVs sold in 2019. Hence, with an increased adoption of electric vehicles, the overall demand for ethanol will progressively reduce, resulting in unforeseen challenges for the corn farmers as well as the processing industry.

Pigmented corns, also known as colored corns, are mutations that have purple, red, blue, or black pigments present in pericarp or aleurone layers. These colors are mainly due to the presence of secondary metabolites such as anthocyanins, tannins, phenolic acids and/or flavonoids. Colored corn varieties have been traditionally grown in the Andean region of South America and used for food and beverage applications, and coloration of traditional textiles. Scientific investigators have reported that these colors developed due to the accumulation of polyphenols in certain genotypes of corn at particular geographic locations, with additional effects of environmental factors such as temperature, altitude, and abiotic stress, along with minute effects of other factors such as germination conditions and cultivation practices (Colombo et al., 2021).

High concentrations of phytochemicals such as anthocyanins, phenolics, flavonoids and tannins have been reported in colored corn varieties. Many research groups have identified cyanidin-3-glucoside, cyanidin-3,5-diglucoside, pelargonidin-3-glucoside, cyanidin-3,6-malonylglucoside, peonidin-3-glucoside, delphinidin-3-glucoside, cyanidin-3-rutinoside, pelargonodin-3-glucoside, malvidin, quercetin, naringin, kaempferol, chlorogenic acid, caffeic acid, ferulic acid, p-coumaric acid, o-coumaric acid, hesperidin, and their derivatives

in various colored corn cultivars (Hu et al., 2020; Paulsmeyer et al., 2017; Žilić et al., 2012; Lao & Giusti, 2016). In addition, vanillic acid, syringic acid, various carotenoids (α, β, γ-carotene, lutein) (Naqvi et al., 2009; Taleon et al., 2017), phytosterols such as stigmasterol, campesterol, sitosterol, and sitostanol (Giordano et al., 2016; Moreau et al., 2009), phospholipids such as phosphatidylinositol, phosphatidylcholine, and phosphatidylglycerol (Harrabi et al., 2010), and policosanols (dotriacontanol C32, triacontanol C30, and tetracosanol C24) (Harrabi et al., 2009) have been detected in colored corn varieties in varying amounts. Phenolics include compounds having an aromatic ring with one or more hydroxyl substituents and are present naturally in plants. There are more than 8000 phenolic compounds whose structures have been described in literature. Phenolics are considered as secondary plant metabolites and play an important role in the survival of plants. However, they do not directly help the plants in photosynthesis or with their respiratory mechanisms. These are classified in three major groups, namely, flavonoids, terpenoids, and nitrogen or sulfur containing alkaloids. Further classification of flavonoids is based on their structure which is made up of two aromatic rings (termed A and B rings) which in turn are attached by a bridge of three carbons forming a heterocyclic ring (termed as C rings). Based on different configurations, flavonoids are classified as flavanols (kaempferol, myricetin, quercetin, isorhamnetin, rutin), flavones (luteonin, apigenin), flavanones (eriodictyol, hesperidin, naringenin), flavanols (catechin, epicatechin, epigallocatechin, epigallocatechingallate, epigallocatechin gallate, gallocatechin, theaflavin, theaflavin gallate), isoflavones, flavanonols, and anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin) (Vuolo et al., 2019). Among these phytochemicals, anthocyanins are drawing an increased attention of the scientific community due to their purported health benefits, and are

mainly responsible for the purple, red, blue, and black coloration of plant matter. Colored corn varieties are rich sources of anthocyanins with purple corn reportedly containing many folds higher anthocyanins compared to the blue and red corn varieties (Li et al., 2017). Because of their significant antioxidant, anti-inflammatory, anticarcinogenic and antimicrobial properties, colored corn phytochemicals are increasingly seen as value-added ingredients and finding interesting applications such as natural food colorants, cosmeceuticals, and pharmaceuticals (González-Manzano et al., 2008a; Pedreschi & Cisneros-Zevallos, 2007; Somavat et al., 2016; Sosulski et al., 1982). Antimicrobial capacity of colored corn phytochemical extracts on *Salmonella enteritidis, Staphylococcus aureus*, and *Candida albicans* was evaluated by Zhao et al. (2009), whereas Ranilla et al. (2017) studied their inhibitory effects on *Lactobacillus helveticus, Bifidobacterium longum* and *Helicobacter pylori*. The authors of the first study reported positive inhibitory effects on *S. enteritidis, S. aureus,* and *C. albicans* but no such effects were reported in the second study. Corn silk extract was evaluated against *Aspergillus niger* and *Aspergillus brasiliensis* in another study and was found to be effective in inhibiting growth of these microorganisms along with demonstrating nitric oxide inhibition and amylase inhibition activity (Abirami et al., 2021). These studies demonstrated the potential of corn phenolics for inhibiting unwanted microbial growth.

Colored corn extracts have been extensively tested for their antioxidant properties using various assays whether it be a free radical scavenging assay, or evaluation of their metal chelating activities. Žilić et al. (2012) evaluated colored corns and reported DPPH and ABTS radical scavenging activities in their kernels. Black sweet corn's oxygen radical scavenging capacity (ORAC) and cellular antioxidant bioactivity (CAA) was tracked along the

development of kernels from day 0 to day 35 and the activity was reported to be increasing along the kernel growth (Hu et al., 2020). Boateng et al. (2023) compared DPPH, ABTS and cupric ion reducing antioxidant capacity (CUPRAC) of purple corn pericarp extract and reported significantly higher antioxidant activities in extracts recovered using ultrasonic and microwave-assisted extractions. Corn silks also demonstrated $IC_{50}$ values in DPPH radical scavenging assays and the effects were found to be comparable to many well-known herbs such as *Ginkgo biloba, Thymus serpyllum, Melissa officinalis* and *Salvia officinalis.* Corn cob extracts showed significant DPPH radical scavenging activity when tested against similar concentrations of α-tocopherol and even higher ability to reduce $Fe^{3+}$ to $Fe^{2+}$ when tested against ascorbic acid as positive control (Zilic et al., 2016). Ranilla et al., (2019) evaluated Peruvian colored corns and identified their functional properties in obesity prevention and hyperglycemia. Ferron et al. (2020) evaluated a purple corn variety called moradyn and reported it to be a suitable food for people with the risk of type 2 diabetes. Purple corn has been used for treating urinary infections, cystitis, weight loss and anti-obesity effects of purple corn silk have been reported in a recent study (Chaiittianan et al., 2017). In addition, colored corn polyphenols have been reported to have neuroprotective and antiproliferative effects, and express significant cardioprotective activity (Colombo et al., 2021). Therefore, colored corn polyphenols can be utilized in several agro-industrial applications.

Many fruits, vegetables, flowers, and cereals contain varying amounts of anthocyanin pigments. However, berries are known have their highest concentrations. Bilberry has been reported to contain cyanidin-3-glucoside, delphinidin-3-glucoside, peonidin-3-glucoside, petunidin-3-glucoside, malvidin-3-glucoside and other anthocyanin derivatives totaling 36.8 mg anthocyanins/g of material, blackberry contained cyanidin derivatives equivalent to 10.1

mg anthocyanins/g of extract. Blackcurrant sample had 21.2 mg anthocyanins/g of material containing cyanidin, delphinidin, petunidin and peonidin compounds whereas blueberry anthocyanins profile was made of cyanidin, delphinidin, peonidin, petunidin, malvidin and peonidin compounds totaling 10.3 mg anthocyanins/g of extract. Strawberries contained 50.7 mg of anthocyanins/g of material with cyanidin-3-glucoside and pelargonidin-3-glucoside being the most dominant forms (Tsuda, 2012). Some of the other sources include eggplant skin, elderberries, and red cabbages with approximate anthocyanin contents of 750.0 mg, 13.7 mg and 2.0 mg anthocyanins/g of material, respectively (Nurtiana, 2019; Somavat, 2017). In recent times, colored corn varieties have elicited increased attention due to their higher concentrations of anthocyanins (Ramos-Escudero et al., 2012; Yang et al., 2009), flavanol-anthocyanins (González-Manzano et al., 2008b), phenolic acids (Pedreschi & Cisneros-Zevallos, 2007), and flavanols (Sosulski et al., 1982). Purple corn, also called maiz morado, has been reported to contain ~5.0 g anthocyanins/kg of corn (Li et al., 2017, Yang et al., 2009). After micro image analysis of purple corn kernels, Li et al. (2017) identified that most of the anthocyanin pigments present in corn kernels were concentrated in corn pericarp, a low value cellulosic byproduct of the corn processing industry. Polyphenol-rich purple corn pericarp was recovered at the front end of the process and 26.8 mg anthocyanins/g of pericarp were quantified after multiple extractions (Li et al., 2017). In a recent study, Boateng et al. (2023) used ultrasonic-assisted extraction and quantified 34.9 mg anthocyanins/g of purple corn pericarp. Therefore, on account of their higher polyphenolic contents and due to the concentration of these compounds in pericarp, colored corn varieties present an exciting economic opportunity. Locally adapted colored corn varieties can be processed to recover polyphenol-rich pericarp at the front end and unaffected endosperm,

containing most of the kernel starch and proteins, can be utilized further by the corn processing industry. Value-added polyphenolic compounds with diverse agro-industrial applications can be selectively extracted from pericarp, yielding significant process economic advantages.

Another important area of related research involves identification of most efficient methods for the extraction of polyphenolic compounds from plant matrices. Traditionally, aqueous, or organic solvents have been extensively used in the industry for extracting polyphenolic compounds. Water is considered to be a nonrenewable source and replenishing water sources once depleted, is not possible except for natural changes. In addition, organic solvents such as ethanol, acetone, hexane, chloroform, and ethyl acetate are widely used in the industry. These solvents offer a wide range of polarities which comes handy in extracting a diverse variety of water soluble and non-soluble compounds. However, the large-scale use of organic solvents is a source of great concern. Organic chemicals pollute the environment when they are discarded after utilization and huge amounts of usable water is also wasted in extraction processes (Dai et al., 2013).

Therefore, there is a great interest in exploring environmentally friendly alternative extraction methods such as the use of ionic solvents, subcritical fluid extraction, and more recently in the application of solvent combinations known as deep eutectic solvents (DES) (Sahin, 2019). DES formulation is based on the concept of hydrogen bond formation between a hydrogen bond donor and a hydrogen bond acceptor molecule, together forming a eutectic mix with a lower melting point than the individual constituents. These solvents are generally made using different molar concentrations of salts and are liquid at room temperature. An interesting fact is that their formulation does not involve any defined method or constituents.

These solvents can be prepared using any kind of salts at varying ratios and can be used as per their suitability with the targeted matrices (El Achkar et al., 2021). DES were first studied by Abbott et al., (2001) and the authors reported preparing a eutectic mix using choline chloride and zinc chloride. Subsequently, several other researchers started studying DES and classified them into hydrophobic DES, hydrophilic DES, natural DES and others. DES are considered biodegradable, and possess characteristics of ideal solvents such as non-volatile, non-flammable, recyclable and reusable (Palos-Hernández et al., 2022). These solvents have been extensively used to extract phytochemicals from plant matrices and reported to be reusable after successful separation of compounds. Researchers have reported the use of DES to extract polyphenols from grapes skin, blueberries, jaboticaba pomace, mulberry, wine lees (Palos-Hernández et al., 2022), pigeon pea, green tea, sophora flower, marsh horsetail, tea leaves, and red sage (Ruesgas-Ramón et al., 2017). However, as per the best of our knowledge, none of the researchers have reported the use of DES for the extraction of polyphenols from purple corn pericarp.

Based on above discussion, this study was divided into two main objectives. The first objective involved evaluation of dry milling characteristics and polyphenolic contents (anthocyanins, total phenols, and condensed tannins) of fourteen conventionally bred and midwestern US grown colored varieties developed by the USDA-ARS colored corn breeding program at the University of Missouri and their milling coproducts. A commercially available purple corn variety was used as a control. The overarching aim was to identify whether some of the locally grown experimental varieties contained significant amounts of polyphenolic compounds and whether they could be successfully grown and commercially exploited for value-added phytochemical recovery in the midwestern US. In the second

objective, 16 different deep eutectic solvent combinations were formulated, characterized, and used in conjunction with ultrasound-assisted extraction technique for the recovery of anthocyanins, tannins, and flavonoids from purple corn pericarp. The main aim was to explore whether a combination of DES and ultrasound can be efficiently used to extract polyphenolic compounds from colored corn pericarp, at the same time, minimizing the detrimental environmental effects of the underlying extraction process.

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# Chapter 2

## Evaluation of dry milling characteristics and polyphenolic contents of fourteen conventionally bred colored corn varieties for value-added coproducts recovery

### 2.1 Introduction

Yellow dent corn (*Zea mays* L.) is a major cereal crop in the United States (US) and a vital contributor to the economy due to its utilization in several food/feed, biofuel, and biotechnology industries. In 2021, US corn farmers harvested 15.1 billion bushels of corn from 93 million acres of farmland with an average yield of 175.4 bushels per acre (USDA NASS, 2022). Out of the total, 5.1 billion bushels or approximately 30% of the harvested corn was converted to ethanol using the corn dry grind process contributing $52.1 billion to the national Gross Domestic Product (Renewable Fuels Association, 2022). Ethanol, the prime product of dry grind process is utilized mainly for gasoline blending while other coproducts include dried distillers' grains with solubles (DDGS), corn germ oil and carbon dioxide. However, all these coproducts have a relatively lower economic value. Therefore, the overall process profitability depends solely on ethanol prices. They in turn depend on gasoline prices which have undergone dramatic swings in recent years. Significant research efforts have been directed towards identifying newer, value-added coproducts from the corn dry grind industry including bioactive protein hydrolysates from condensed corn distillers solubles (Sharma et al., 2021, 2020), protein and glycerol (Liu and Barrows, 2013), biodegradable plastics (Bothast and Schlicher, 2005), corn fiber oil (Moreau et al., 1996), furfural (Xiang and Runge, 2014), and biodegradable cat litter (Vaughn et al., 2011). However, none of these coproducts have achieved any commercial breakthroughs.

Over the years, federal policies have encouraged the US farmers to grow corn as they became eligible for government payments supporting or protecting their incomes. The driving force behind the enactment of The Energy Independence and Security Act of 2007 was energy security. Corn ethanol not only displaced 10% of the total gasoline demand, but it also acted as an environmentally friendly substitute to methyl *tert*-butyl ether (MTBE), a controversial fuel oxygenate. However, mainly due to the advances in fracking, the US field production of crude oil has increased from 1.85 billion barrels in 2007 to almost 4.48 billion barrels in 2019, making it the largest oil producing country in the world (Energy Information Administration, 2022). Although not bereft of controversy, several researchers have reported that many of the environmental justifications which initially prompted the Renewable Fuels Standard (RFS) to mandate the use of corn ethanol in the US are detrimental to the environment (Hill, 2022; Lark et al., 2022; Wardle, 2018). Of particular concern has been the *food-to-fuel cycle,* wherein extensive energy, and resources in the form of fuel to drive heavy agricultural machinery, which impacts soil characteristics and causes erosion, the use of nitrogenous fertilizers which pollute water, and the application of pesticides and weedicides that adversely affect the soil health, are used to grow corn to be eventually converted into fuel.

In addition, the industry is facing other emerging challenges. During the peak of the COVID-19 pandemic, the overall demand for ethanol in the year 2020/21 was significantly reduced, adversely affecting the corn prices and biofuel production profitability (Farm Bureau, 2020). The annual ethanol production in the US in 2020 was at a seven year low and almost 2 billion gallons less than the previous year (Renewable Fuels Association, 2022). Although gasoline demand and prices have recovered ever since, the industry is facing emerging challenges

mainly due to technological innovations associated with electric vehicles and battery technology. The passenger vehicle segment accounted for ~27% of the total gasoline consumption in 2016. Due to government incentives for all electric and plug-in hybrid vehicles, more and more electric vehicles will replace conventional cars/trucks in coming years and as a result, the global oil demand in the passenger vehicles segment will continue to decrease (Kah, 2018). It has been reported that already more than one million barrels of oil per day is displaced by electric vehicles of all types (BloombergNEF, 2022). Therefore, there is a longstanding need for diversification and value-addition when it comes to the US corn farming and its utilization by the processing industry.

The primary focus of US corn breeders and farmers has been on high yielding and higher starch containing yellow dent varieties and as a result the excellent commercial potential associated with colored corn varieties has been overlooked. Even though the starch content of colored corn varieties can be 8.6 to 6.7% less than the conventional yellow dent corn cultivars (Somavat et al., 2016), these varieties contain very high concentrations of diverse bioactive phytochemicals with potential industrial applications. Various researchers have reported that colored corn varieties are rich sources of anthocyanins (Ramos-Escudero et al., 2012; Yang et al., 2009), flavanol-anthocyanins (González-Manzano et al., 2008), phenolic acids (Pedreschi and Cisneros-Zevallos, 2007) and flavanols (Sosulski et al., 1982). Greater consumption of phytochemicals present in colored fruits and vegetables is often associated with potential health benefits in humans. The generally accepted *modus operandi* with respect to their intervention in human therapeutic targets is based on their free radical scavenging and antioxidant capabilities. In addition, polyphenolic compounds have been reported to be of help in preventing cardiovascular disease (Andriambeloson et al., 1998;

Folts, 1998), demonstrate anti-carcinogenic and tumor inhibiting properties (Hou, 2003; Meiers et al., 2001) and assist in fighting obesity, diabetes, and inflammation (Liu, 2004; Rossi et al., 2003; Tsuda et al., 2003).

In our earlier study, we found that purple corn contained ~5.0 g anthocyanins/kg of corn, and most of the anthocyanins and polyphenols were observed in corn pericarp, a low-value processing coproduct (Li et al., 2017). The corn milling process was adapted to recover the polyphenol-rich pericarp at the front end of the process, facilitating the economic recovery of these value-added compounds (Somavat, 2017). We studied the pest-deterrent properties of polyphenol-rich purple corn pericarp extract on tobacco hornworm (*Manduca sexta* L.) caterpillars. The insect food mixed with pericarp extract was found to have negatively affected the egg hatching rate and larval mass gain, and prolonged their developmental time (Tayal et al., 2020a). Furthermore, these adverse effects were found to be transgenerational, demonstrating the potential of polyphenol-rich purple corn pericarp as a natural pest deterrent in the organic crop sector (Tayal et al., 2020b). However, for their commercial exploitation, these colored varieties need to be adapted to local growing conditions. To this end, we are using conventional breeding practices for adapting some of the colored corn varieties to the midwestern growing conditions and evaluating them.

The aim of this study was to evaluate and identify the experimental colored corn varieties with higher polyphenolic contents and desired milling characteristics for subsequent conventional breeding cycles and their adaptation to the local growing conditions. As discussed at length, the conventional corn ethanol industry has failed to come up with any economically viable value-added coproducts and colored corn varieties have the potential to supplement the lower economic value associated with the conventional yellow dent corn. The

dry milling characteristics of fourteen conventionally bred and midwestern US colored corn varieties were evaluated and compared with a commercially available purple corn variety using a 100 g lab scale dry milling protocol. The phytochemical contents (total monomeric anthocyanins, total polyphenols, and condensed tannins) of whole corn kernels and six dry milling coproducts (pericarp, germ, large grits, medium grits, small grits, and fines) were quantified with an aim of ascertaining the polyphenol rich varieties, and for identifying specific coproducts with relatively higher concentrations of these compounds. In addition, HPLC analyses were performed to identify and quantify the individual anthocyanins, phenolics and flavonoids present in five selected corn varieties. The antioxidant properties of polyphenolic extracts from pericarp of five select corn varieties were evaluated using four different antioxidant assays. Finally, histological analysis was utilized to better understand the location of pigments and polyphenolic compounds in corn kernels from different colored varieties. The overarching aim was to identify the most promising experimental colored corn cultivars for subsequent conventional breeding and adaptation cycles.

**2.2 Materials and methods**

Conventionally bred experimental varieties were grown in 2020 and 2021 at Genetics Farm near Columbia, MO (Latitude 38.9517° N and Longitude 92.3341° W). The 14 experimental varieties included Apache purple (AP), Apache red (AR), Bloody butcher (BB), Hopi/Cherry pericarp (CR), Hopi/Cherry pericarp x Cordoba (HC), Hopi/Cherry pericarp x Maiz morado from 2020 (HM20), Hopi/Cherry pericarp x Maiz morado from 2021 (HM21), Hopi/Cherry pericarp x Maiz negro (HN), Hopi/Cherry pericarp x Oaxaca 128 (HO), Hopi/Cherry pericarp x Sehsapsing (HS), Jimmy red (JR), Oaxaca 128 (O128), Purple butcher purple (PBP), and Purple butcher red (PBR). Grain samples were evaluated for their composition, dry milling

characteristics and polyphenolic contents. A commercially available variety of purple corn called maiz morado (MM) bought from Woodland Foods (Waukegan, IL) was used as a control.

Broken corn and foreign material (BCFM) were removed by using a 12/64" sieve (4.8 mm) and cleaned kernels were stored at 4 °C until further analysis. Visual comparison of evaluated varieties is presented in Fig. 2.1. A Dickey-john GAC® 2500-UGMA (Dickey-john Corporation, Auburn, IL) grain moisture tester was used to measure the moisture of corn kernels which ranged from 6.17% to 12.36%. Unless otherwise stated, all the chemicals and standards used for the spectrophotometric, HPLC and antioxidant analyses were purchased from Sigma-Aldrich (St. Louis, MO). The deionized water (DO) used for the experiments was from Milli-Q $H_2O$ purification system (Millipore®, MA, USA) while the other reagents and solvents used were of analytical grade.


Fig. 2.1. Visual comparison of kernels of different colored corn varieties evaluated in this study
Note: AP: Apache purple, AR: Apache red, BB: Bloody butcher, CR: Hopi/Cherry pericarp, HC: Hopi/Cherry pericarp x Cordoba, HM20: Hopi/Cherry pericarp x Maiz morado 2020, HM21: Hopi/Cherry pericarp x Maiz morado 2021, HN: Hopi/Cherry pericarp x Maiz negro, HO: Hopi/Cherry pericarp x Oaxaca 128, HS: Hopi/Cherry pericarp x Sehsapsing, JR: Jimmy red, MM: Maiz morado, O128: Oaxaca 128, PBP: Purple butcher purple, PBR: Purple butcher red

### 2.2.1 Lab scale 100 g dry milling procedure

A 100 g lab scale dry milling protocol was used for determining the milling characteristics of corn (Somavat et al., 2017). After determining the initial kernel moisture content, requisite amount of water required to raise the sample moisture content to 14% was added, and the kernels mixed with water were allowed to sit overnight in sealed plastic bags. After the samples had gained the required moisture overnight, they were further tempered to a processing moisture content of 23.5% in a custom-made tempering set-up containing 4 L plastic containers rotating horizontally at 0.5 rpm for 20 min. The tempered kernels were degermed using a custom-made small-scale drum degerminator. The degermed corn fractions were conditioned in an air oven at 49 °C for 1 h. After conditioning, a sub-sample for moisture measurement was taken and rest of material was passed through a 5-mesh sieve (4 mm opening) using a lab scale sieve sifter (Model: Table Top, Great Western Manufacturing, Leavenworth, KS) for 1 minute. Tails (+5 fraction, collected on top of the sieve) and throughs (-5 faction, that passed through the sieve) were separated and weighed. The tail fraction was poured in an aluminum pan for separating germ manually from the mixture of endosperm and pericarp fractions. The throughs (-5 fraction) were again passed through a 10-mesh sieve (1.68 mm opening) for 1 minute using the sieve shaker. The +10 fraction or the tails were collected for aspiration and the throughs (-10 fraction) were further sifted using a 25-mesh screen (0.707 mm opening) for 1 minute. The tail fraction (+25) from this separation were termed small grits and weighed. After weighing, a moisture sub sample was taken and the same was done for the -25 fraction (throughs) called fines. The +5 fraction, after germ separation was passed with a slow and even flow through an aspirator (Model: 6DT4-1, Kice Metal Products, Wichita, KS) at 5 mm $H_2O$ negative pressure. The pericarp

fraction in the upper vent was collected in a separate boat and the large grits were collected in a catch pan. The +10 fractions were also passed through the aspirator under same conditions and the collected pericarp fractions were added to the already separated pericarp. The endosperm fractions collected in the catch pan were labeled medium grits. Moisture sub-samples were taken from large grits, medium grits, and pericarp after weighing them. The moisture sub-samples of all six coproducts were kept in an air oven at 135 °C for 2 h and then weighed to calculate the moisture content (Approved Method 44-19, American Association of Cereal Chemists International, 2010).

### 2.2.2 Polyphenol extraction from corn kernels and milling coproducts

Whole corn kernels and corn dry milling coproducts (large grits, medium grits, small grits, germ, pericarp, and fines) were ground using a coffee grinder (Hamilton Beach, VA) for 30 seconds and passed through a 35-mesh sieve (0.5 mm opening). Glass beakers (50 mL) were labelled, and 0.5 grams of ground samples that passed through 34-mesh sieve were suspended in 20 mL (40:1 liquid-to-solid ratio) of 2% aqueous formic acid for the first extraction and stirred at room temperature for 2 h. The suspension was filtered using a Whatman® #4 filter paper and the filtrate was used to measure total monomeric anthocyanins, condensed tannins, and total phenols. A second sequential extraction was done on residues of the first filtration collected from the top of the filter paper by suspending it in a 20 mL solution of 2% formic acid and 25% ethanol for the complete extraction of polyphenols.

### 2.2.3 Measurement of total monomeric anthocyanin concentration

A pH differential was used to measure the total monomeric anthocyanins (Lee et al., 2005). Briefly, 500 µL of two buffer solutions (pH 1.0, 0.25 M KCl buffer and pH 4.5, 0.40 M sodium acetate buffer) were used to dilute 500 µL of polyphenolic extract and 200 µL of the

diluted solution was picked thrice for each extract from first and second sequential extraction and transferred to 96-well plate. The absorbance was read at 520 and 700 nm using a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Scientific, USA). The total monomeric anthocyanins concentration was calculated as g of cyanidin-3-glucoside (C3G) equivalents per kg coproduct using the formula given below:

Total monomeric anthocyanins = A * MW * D * 1000 / (Ɛ * PL * 0.45)

where: A = [(A520 – A700) at pH 1.0 – (A520 – A700) at pH 4.5]; MW = 449.2 g/mol for C3G; D = dilution factor; PL = constant path length 1 cm; Ɛ = 26,900 L/mol.cm, which is the molar extinction coefficient for C3G, 1000 as a conversion factor from grams to milligrams and 0.45 as the conversion factor from the established method to the plate reader method. Results were then expressed as g of cyanidin-3-glucoside (C3G) equivalents per kilogram of coproduct.

**2.2.4 Measurement of total condensed tannins**

Total condensed tannins were measured using an improved butanol-HCl assay (Shay et al., 2017). One mL of reagent 1 (51.5% acetone, 43% butanol, 5% 12N HCl & 0.5% $H_2O$) was added to 29 µL of reagent 2 (2% $NH_4Fe(SO_4)_2$ in 2N HCl) and 78 µL of epicatechin extract. This solution was vortexed for 5 seconds. One part of the solution was kept in dark at room temperature for 2 h and the other part was placed in a water bath at 70 °C for 2.5 h. Next, 200 µL of samples were transferred to 96-well plates and absorbance was read at 550 nm using a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Scientific, USA). Using a standard curve, the difference between the absorbance values of unheated and heated samples was used to express proanthocyanins content in g epicatechin equivalent (EE)/kg of coproduct using a standard curve.

### 2.2.5 Measurement of total polyphenol content

Folin-Ciocalteu's method adapted to a microassay described by Boateng & Yang (2021) was used to measure the total phenolic content (TPC). Briefly, 80 µL of gallic acid standard or extracts were mixed with 400 µL of Folin-Ciocalteu's reagent (10% v/v) in the dark. After 5 min, 320 µL of $Na_2CO_3$ (75 mg/mL) was added and made to react for 30 min in the dark. Afterwards, the 200 µL of solution was transferred to a 96-well plate in triplicates, and absorbance was read at 765 nm using a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Scientific, USA). Total polyphenol content was expressed as g gallic acid equivalent (GAE)/kg of coproduct.

### 2.2.6 Compositional analysis

The crude protein content of corn kernels was analyzed using combustion analysis (LECO) Official Method 990.03 (Association of Official Analytical Chemists, 2005), crude fiber analysis was carried out using Official method 978.10 (Association of Official Analytical Chemists, 2005), crude fat content was measured following Official Method 920.39, (Association of Official Analytical Chemists, 2005), and starch content evaluation was performed using Sigma starch assay kit, Product code STA-20.

### 2.2.7 HPLC analysis

The HPLC analysis for anthocyanins, polyphenols, and flavonoids quantification was carried out using an Agilent 1200 series instrument coupled with a DAD and a C18 column (5 µm, 250 mm × 4.6 mm, Avantor®, USA). Ground whole kernels were passed through a US 35-mesh sieve (0.5 mm opening) and extracted using methanol acidified with 0.1% HCl with a solid to liquid ratio of 1:20. The extract mixture was centrifuged at 12,000 rpm for 10 min at 4 °C and filtered using a 0.22 µm syringe filter for further analyses.

#### 2.2.7.1 Anthocyanin profile

The polyphenolic extracts from corn varieties and standards were eluted using conditions described by Oh et al. (2008) with slight modifications. Mobile phase A (5% formic acid in water) and B (5% formic acid in acetonitrile) were run at a gradient of 5% B (5 min); 100% B (35 min); 100% B (40 min); 5% B (45 min) with a flow rate of 1 mL/min and column temperature of 35 $^{\circ}$C. Different anthocyanins were baseline separated at a wavelength of 520 nm (Fig. 2.2) and the results were expressed in g/kg of whole kernel based on the standard curve for six compounds that were run as standards: cyanidin 3-glucoside chloride, delphinidin chloride, cyanidin chloride, peonidin chloride, malvidin chloride, pelargonidin chloride and with retention times of 3.40, 14.14, 16.10, 17.04, 17.39, and 17.71 min, respectively.

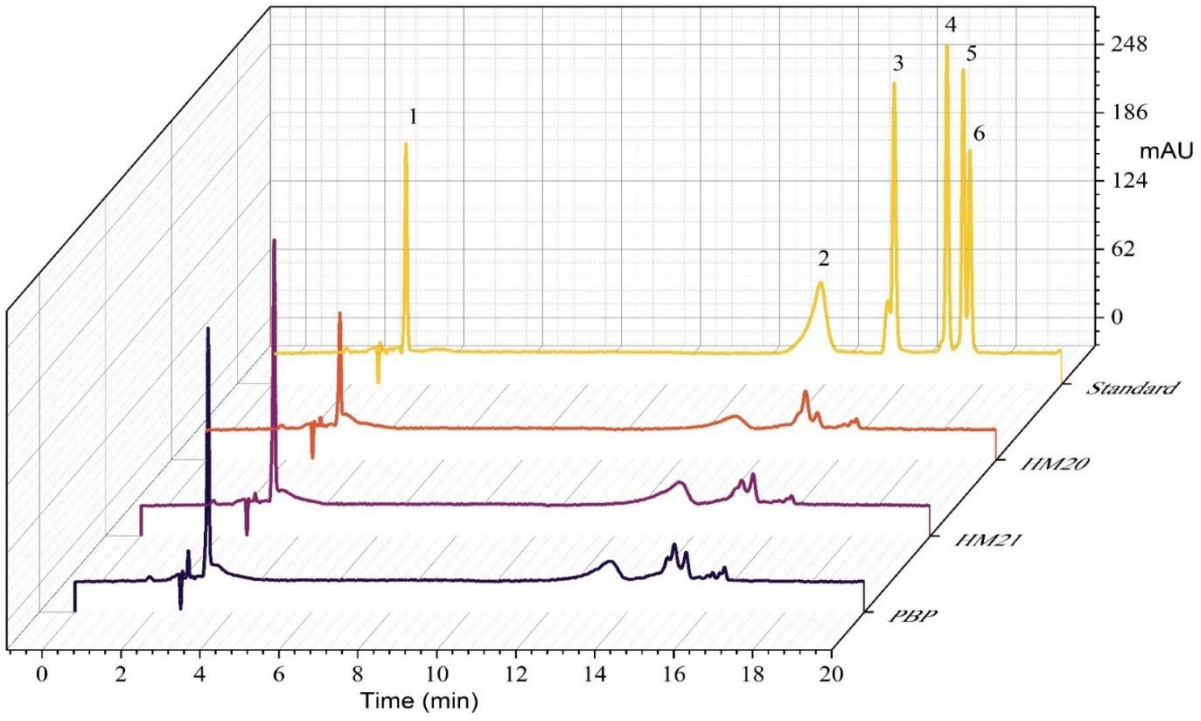


Fig. 2.2. Anthocyanins profile of selected corn varieties identified through HPLC analyses
Note: (1) cyanidin-3-glucoside, (2) delphinidin, (3) cyanidin chloride, (4) peonidin, (5) malvidin, and (6) pelargonidin chloride
HM20: Hopi/Cherry pericarp x Maiz morado 2020, HM21: Hopi/Cherry pericarp x Maiz morado 2021, PBP: Purple butcher purple

**2.2.7.2 Phenolics profile**

The standards and extracts were eluted using conditions described by Kandil et al. (2012). Briefly, the mobile phases A (0.1% acetic acid in water) and B (0.1% acetic acid in methanol) were used as follows: 5% B (0 min); 20% B (15 min); 40% B (35 min); 65% B (42 min); 80% B (50 min); 5% B (52 min); and 5% B (60 min) with a flow rate of 1.0 mL/min and injection volume of 10 µL at a column temperature of 25 °C. Wavelengths of 254, 280 and 360 nm were used and the compounds in various corn extracts were baseline separated as shown in Fig. 2.3. Results were expressed in g of compound per kg of whole corn kernel and were calculated based on the standard curve.

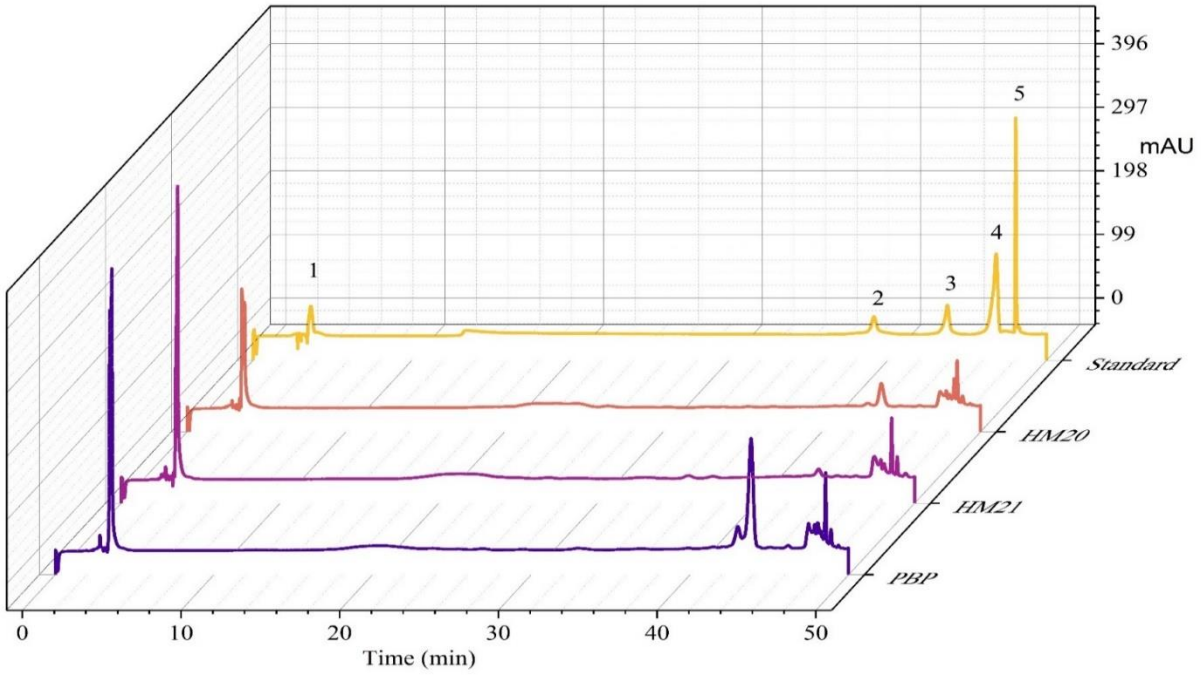


Fig. 2.3. Flavonoids profile of selected corn varieties identified through HPLC analyses
Note: (1) epicatechin, (2) morin, (3) naringin, (4) quercetin, and (5) kaempferol
HM20: Hopi/Cherry pericarp x Maiz morado 2020, HM21: Hopi/Cherry pericarp x Maiz morado 2021, PBP: Purple butcher purple

### 2.2.7.3 Flavonoid profile

The analysis for flavonoids quantification was performed using method described by Das and Singh (2016). Sample injection volume of 20 µL with a flow rate of 1 mL/min was used, while the column was at a temperature of 25 °C. Water with a pH of 2.8 adjusted using acetic acid was mobile phase A while the mobile phase B consisted of 100% acetonitrile. The gradient for elution was as follows: 10% B (5 min); 23% B (31 min); and 35% B (43 min). Finally, the column was washed (100% B, 4 min) and equilibrated (100% A, 3 min). As shown in Fig. 2.4, different flavonoids and kernel content were baseline separated at multiple

detection wavelengths of 261, 280, 320 and 360 nm. The final content was calculated based on standard curve and presented as g/kg of whole corn kernel.

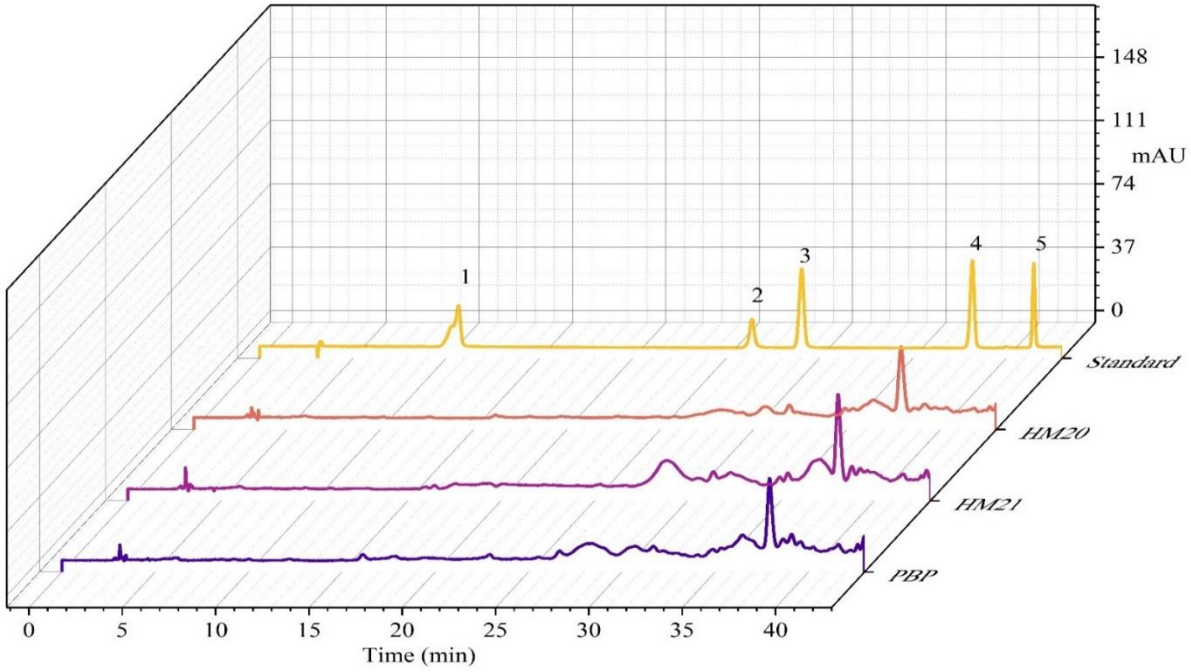


Fig. 2.4. Phenolics profile of selected corn varieties identified through HPLC analyses
Note: (1) gallic acid, (2) chlorogenic acid, (3) caffeic acid, (4) ferulic acid, and (5) hesperidin
HM20: Hopi/Cherry pericarp x Maiz morado 2020, HM21: Hopi/Cherry pericarp x Maiz morado 2021, PBP: Purple butcher purple

### 2.2.8 Antioxidant assays

#### 2.2.8.1 Sample preparation

The pericarp of four conventionally bred polyphenol-rich corn varieties and purple corn (control) was ground and passed through a 35-mesh sieve. The ground pericarp samples were extracted in a solution of water and ethanol (40:60), using a solid-to-liquid ratio of 1:20 in a beaker with continuous magnetic stirring for 2 h. Afterwards, the extract was centrifuged at 10,000 rpm for 10 minutes at 4 °C and vacuum filtered using a Whatman® #4 filter paper.

Residual ethanol was evaporated using a rotavapor (Model R-300, Buchi Corporation, New Castle, DE). The extracts were used for measuring antioxidant capacity.

#### 2.2.8.2 DPPH scavenging assay

The solution for DPPH (1,2-diphenylpicrylhydrazyl) assay was made by mixing DPPH (5 mg) with methanol (100 mL) and the protocol reported by Boateng and Yang (2021) was used. The mixture was incubated in dark for 2 h till the absorbance values in range of 0.702 to 0.715 at 520 nm were obtained. The extract or Trolox samples (100 µL) were mixed with 400 µL of DPPH solution, incubated for 30 min in the dark, and absorbance values were recorded at 520 nm. The capability to scavenge DPPH was expressed as µmol Trolox equivalent (TE)/g of pericarp.

#### 2.2.8.3 ABTS scavenging assay

A previously reported method was used for ABTS scavenging assay (Boateng and Yang, 2021). The stock solution for the assay was prepared by mixing 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) or ABTS (7 mM, 10 mL) and $K_2S_2O_8$ (140 mmol, 166 µL) and the mix was left in the dark overnight. For formulating the ABTS working solution, the stock solution (1.2 mL) was mixed with DI water (60 mL). The absorbance of the solution was adjusted to 0.700 ± 0.004 at 734 nm. After adding 360 µL of the ABTS working solution to Trolox or extract (40 µL), the mixture was left in the dark for 10 min before measuring the absorbance at 734 nm. Trolox was used as a standard and results were expressed as µmol TE/g of pericarp.

#### 2.2.8.4 Cupric ion reducing antioxidant capacity (CUPRAC) assay

Samples of Trolox or extract (20 µL) were added to 800 µL of working solution containing neocuproine (7.5 mM), $H_2O$, $CuCl_2$ (10 mM), and $NH_4Ac$ (1 M) in a ratio of 1:1:1:1 and

incubated at 25 °C for 60 min. Afterwards, the absorbance values were measured at 450 nm (Boateng and Yang 2021). The results were reported as µmol TE/g of pericarp.

#### 2.2.8.5 Reducing power (RP) assay

Following Boateng and Yang (2021), 500 µL sample of potassium ferricyanide (1 g/100 mL) was combined with Trolox or extract (500 µL) and following a further addition of 500 µL of phosphate buffer (0.2 M, pH 6.6), the samples were incubated for 20 min at 50 °C. After an addition of 500 µL of trichloroacetic acid (10 g/100 mL), the mixture was centrifuged for 10 min (10,000 rpm, 4 °C). One mL of the mixture was added to 500 µL of ferric chloride (100 mg/100 mL) and 1 mL of $H_2O$, and absorbance was measured at 700 nm, and results were presented as µmol TE/g of pericarp.

### 2.2.9 Pericarp and aleurone histology

The thickness of cell layers in the pericarp and aleurone layers were analyzed for five mature kernels of six varieties soaked in water for 48 hours and embedded in Tissue Freezing Medium (Leica Biosystems). A cryostat (Leica CM1860) was used to produce 20 µm thick sections from the crown of the kernel. Sections were mounted on glass slides and examined under a Axiovert 200M microscope (Carl Zeiss Light Microscopy) with a 10x objective. The width of the pericarp and aleurone layers was recorded using the Scale bar and Measurements tools in the AxioVision Release 4.8 (Carl Zeiss MicroImaging GmbH) coupled with the microscope and AxioCamMR3 (Carl Zeiss) camera. Data were collected for four regions of the kernel: the two sides (S1 and S2) and the back (B1) of the seed opposite the embryo, as well as at the junction of the endosperm and the embryo (E). Further details about the four regions analyzed for pericarp and aleurone thicknesses are provided in Fig. 2.5. This research was conducted in Dr. Sherry Flint-Garcia's lab.

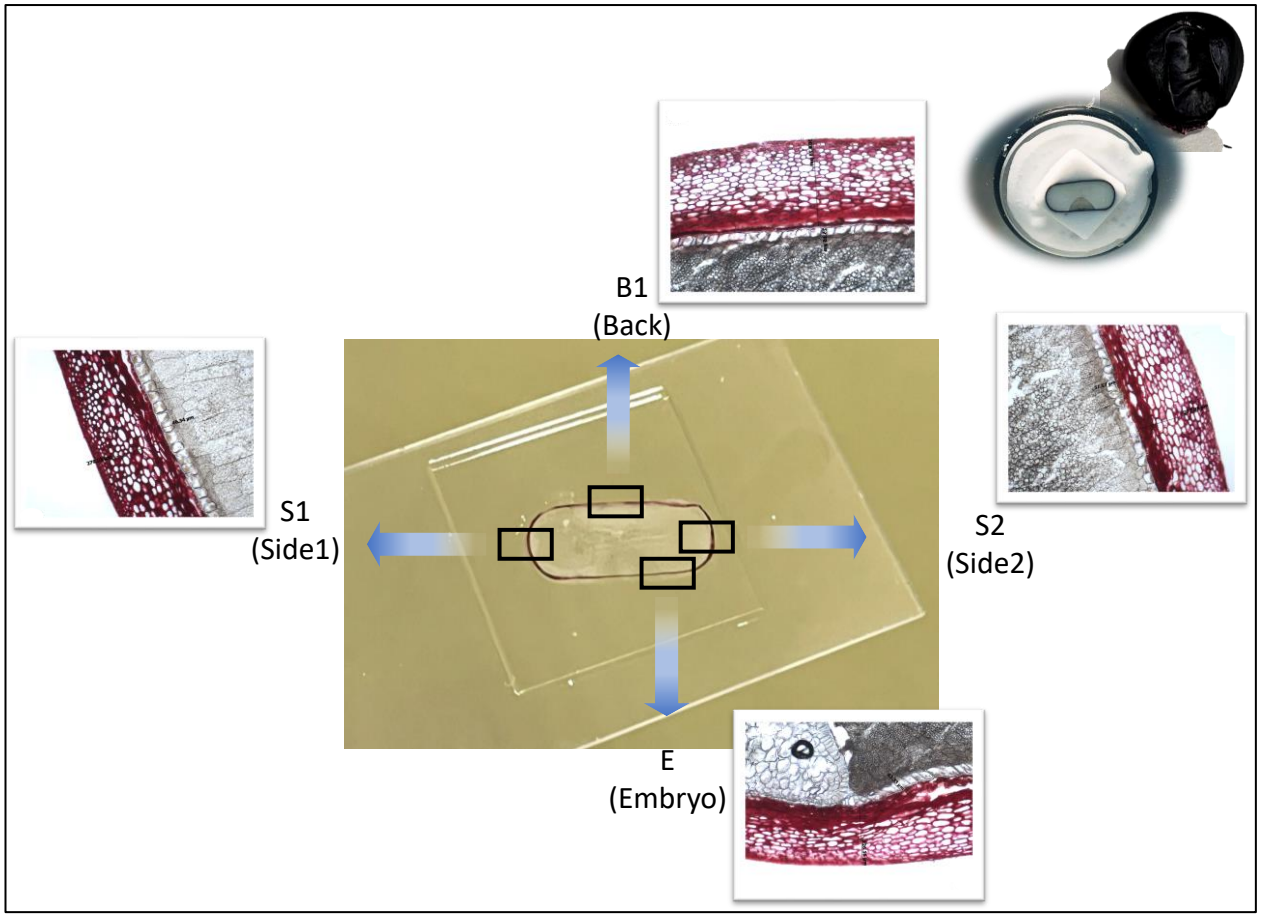


Fig. 2.5. Illustration of four regions (B1, S1, S2, and E) where the pericarp and aleurone thicknesses of kernels from six selected colored corn varieties were evaluated

### 2.2.10 Statistical analysis

All the analyses were conducted in triplicates and results are presented as mean ± standard deviation. One way analysis of variance (ANOVA) with Tukey's range test was conducted to compare the means at $p < 0.05$ using Minitab version 18 (Minitab Inc, State College, Pennsylvania, USA).

## 2.3 Results and Discussion

### 2.3.1 Compositional analysis of corn varieties

The compositional characteristics of corn varieties evaluated are summarized in Table 2.1. Due to their utilization in biofuels, food/feed, and biotechnology industries, starch content is

considered as the most important attribute of a corn variety and most of the breeding efforts aim for increasing the corn starch content (Eckert et al., 2018; Somavat et al., 2016). It was observed that the starch contents of colored corn varieties were statistically different. The compositional variations in corn cultivars have been attributed to various factors including genetic differences, agronomic practices, and environmental conditions among others (Yaqoob et al., 2019). Out of the varieties analyzed, Hopi/Cherry pericarp x Maiz negro (HN) had the highest starch content of 75.55 ± 2.13% (db) which was similar to that present in conventional yellow dent corn (Somavat et al., 2016) and higher compared to the control (MM; 61.63 ± 1.50% (db)), in agreement with the data reported in an earlier study (Somavat et al., 2017). On the other hand, the varieties HC & HM21 had the lowest amount of starch, the values being 59.54 ± 0.22% and 59.55 ± 1.04% (db), respectively. The mean starch content of evaluated colored corn varieties was 65.28% (db) which was lower than the mean starch content of conventional yellow dent corn ~75.19% (db) (Somavat et al., 2017). The crude protein of the colored varieties ranged between 12.16 ± 0.02% (db) for CR to 8.33 ± 0.03% (db) for HM20 with a mean value of 10.46% (db) which was higher compared to the mean protein content of five conventional yellow corn varieties (8.76%, db) reported in an earlier study (Somavat et al., 2017). The protein contents of varieties AP and AR was not different. The highest crude fat content was observed in varieties AP and HS, the values being 5.1 ± 0.17% and 5.14 ± 0.05% (db), respectively. On the other hand, varieties O 128, CR, PBR, HO and HM20 had the lowest crude fat content containing 2.7 ± 0.08%, 2.95 ± 0.1%, 2.65 ± 0.19%, 2.72 ± 0.03%, and 2.87 ± 0.07% (db), respectively. The mean fat content of colored corn varieties 3.80% (db) was comparable to the mean values reported for conventional yellow dent corn (3.73%, db). Finally, the varieties BB and CR had the highest

crude fiber content at 2.16 ± 0.01% and 2.1 ± 0.11% (db), respectively, while the variety AP had the lowest content (1.51 ± 0.04%, db). In general, the colored corn varieties were observed to have a lesser starch content and a greater protein content compared to the conventional yellow dent corn varieties.

**Table 2.1.** Compositional characteristics of different colored corn varieties (%, db)

| Varieties | Crude protein | Crude fat | Crude fiber | Starch |
|---|---|---|---|---|
| **AP** | 11.74 ± 0.05ab | 5.10 ± 0.17a | 1.51 ± 0.04de | 66.92 ± 0.56bc |
| **AR** | 11.84 ± 0.01ab | 3.83 ± 0.08cde | 1.55 ± 0.03cde | 59.68 ± 0.65de |
| **BB** | 10.15 ± 0.13fg | 4.17 ± 0.19bc | 2.16 ± 0.01a | 69.29 ± 0.31b |
| **CR** | 12.16 ± 0.02a | 2.95 ± 0.10f | 2.10 ± 0.11a | 65.55 ± 0.37bcde |
| **HC** | 10.88 ± 0.22cd | 4.65 ± 0.25ab | 1.78 ± 0.03bc | 59.54 ± 0.22e |
| **HM20** | 8.33 ± 0.03j | 2.87 ± 0.07f | 1.63 ± 0.06bcde | 69.38 ± 0.83b |
| **HM21** | 11.41 ± 0.23bc | 3.19 ± 0.04ef | 1.83 ± 0.04b | 59.55 ± 1.04e |
| **HN** | 10.04 ± 0.01fg | 5.18 ± 0.04a | 1.61 ± 0.05bcde | 75.55 ± 2.13a |
| **HO** | 9.80 ± 0.17gh | 2.72 ± 0.03f | 1.75 ± 0.06bcd | 66.47 ± 0.56bc |
| **HS** | 9.34 ± 0.02hi | 5.14 ± 0.05a | 1.70 ± 0.02bcde | 71.02 ± 2.29ab |
| **JR** | 10.22 ± 0.13efg | 4.04 ± 0.29bcd | 1.74 ± 0.01bcd | 62.35 ± 0.72cde |
| **MM** | 8.84 ±0.00ij | 3.39 ± 0.01def | 1.66 ± 0.01bcde | 61.63 ± 1.5cde |
| **O128** | 10.47 ± 0.15def | 2.70 ± 0.08f | 1.71 ± 0.03bcde | 65.75 ± 1.16bcd |
| **PBP** | 10.86 ± 0.03cde | 4.44 ± 0.09abc | 1.72 ± 0.02bcde | 62.90 ± 0.79cde |
| **PBR** | 10.84 ± 0.08cde | 2.65 ± 0.19f | 1.48 ± 0.04e | 65.15 ± 0.56bcde |

Mean ± SD from two replicates. Means followed by the same letter in a column are not different ($p < 0.05$)

Note: AP: Apache purple, AR: Apache red, BB: Bloody butcher, CR: Hopi/Cherry pericerp, HC: Hopi/Cherry pericarp x Cordoba, HM20: Hopi/Cherry pericarp x Maiz morado 2020, HM21: Hopi/Cherry pericarp x Maiz morado 2021, HN: Hopi/Cherry pericarp x Maiz negro, HO: Hopi/Cherry pericarp x Oaxaca 128, HS: Hopi/Cherry pericarp x Sehsapsing, JR: Jimmy red, MM: Maiz morado, O128: Oaxaca 128, PBP: Purple butcher purple, PBR: Purple butcher red

### 2.3.2 Dry milling characteristics

Corn dry milling process involves physical fractionation of corn kernels into starch and protein containing endosperm fractions and aim for the maximum removal of pericarp and germ fractions (Rausch et al., 2009). Depending upon the size of separated endosperm fractions, a variety of products such as flaking grits, cones, meal and flour are generated.

Corn kernels were fractionated into six milling coproducts, namely, large grits, medium grits, small grits, germ, fines, and pericarp, and the respective coproduct yields (%, db) are summarized in Table 2.2. It has been widely reported that the yield of various dry milling coproducts depends on endosperm hardness. Corn kernel hardness in turn depends upon the relative proportion of vitreous to floury endosperm in a variety (Correa et al., 2002). Hard endosperm corn varieties tend to yield a greater fraction of large grits and are preferred for corn dry milling, whereas the varieties with a softer endosperm yield lesser large grits and higher proportion of smaller endosperm fractions (small grits, fines, etc.) (Somavat et al., 2016; Watson, 1987). In corn varieties with a dominant floury endosperm, the protein matrix surrounding the starch molecules is relatively weaker (Correa et al., 2002). Out of the varieties evaluated, O128 yielded the greatest fraction of large grits 34.52% (db), which was followed by the varieties CR, BB, and JR (28.05, 27.53 and 27.27% (db), respectively), implying most of these colored corn varieties had a dominant softer endosperm composition which agreed with an earlier reported study (Somavat et al., 2016). Medium grits yield was highest for PBR (47.06%, db) followed by the varieties AR, PBP, and AP (46.83, 45.45, and 45.86% (db), respectively). The germ content of the varieties ranged from a high of 12.79% (db) (HS) to a low of 7.59% (db) in PBR. Since it has been reported in several earlier studies that the polyphenolic compounds of interest in colored corn varieties are concentrated in corn pericarp, it the coproduct of prime interest in colored corn varieties (Li et al., 2019, 2017). The pericarp yield from variety HM21 (6.45%, db) was observed to be similar to the pericarp yield from control variety MM (6.51%, db) but was lower than the values reported in previous studies (Somavat et al., 2016). This could be attributed to varietal and seasonal differences associated with the processed kernels. The lowest fraction of pericarp was

recovered from HN (3.18%, db). The small grits yield was highest in HM21 (20.99%, db) which was less than control MM (23.61%, db) whereas the lowest yield was observed in CR (11.77%, db). Finally, the variety HM20 yielded 6.58% (db) fines which was greatest among all other varieties except the control MM (10.76%, db) and the value was lowest for AR with 3.03% (db). Overall, the colored corn varieties had a dominant floury endosperm composition and were found to be more suitable for wet milling and dry grind processing.

**Table 2.2.** Dry milling characteristics of processed corn varieties (yield %, db)

| Variety | Large grits | Medium grits | Small grits | Fines | Germ | Pericarp | Total |
|---|---|---|---|---|---|---|---|
| **AP** | 21.85 ± 0.95cde | 45.86 ± 0.72abc | 16.01 ± 0.58de | 3.69 ± 0.12efg | 8.41 ± 0.38ef | 3.98 ± 0.32de | 99.81 ± 0.19ab |
| **AR** | 22.02 ± 1.28cde | 46.83 ± 1.55ab | 14.60 ± 1.27efg | 3.03 ± 0.11g | 8.67 ± 1.07def | 4.91 ± 0.32bc | 100.05 ± 0.57ab |
| **BB** | 27.53 ± 1.99b | 37.02 ± 2.78ef | 14.95 ± 0.65def | 5.85 ± 0.12bc | 9.72 ± 0.65cdef | 4.99 ± 0.16bc | 100.07 ± 0.73ab |
| **CR** | 28.05 ± 2.17b | 38.36 ± 0.58def | 11.77 ± 0.11g | 4.27 ± 0.29ef | 12.23 ± 0.18ab | 5.46 ± 0.18b | 100.13 ± 1.85ab |
| **HC** | 24.12 ± 1.39bcd | 41.90 ± 1.32bcde | 15.71 ± 0.14de | 4.11 ± 0.14efg | 10.99 ± 0.37abcd | 3.87 ± 0.10def | 100.71 ± 0.25a |
| **HM20** | 19.53 ± 1.64de | 40.03 ± 2.34def | 19.75 ± 0.95bc | 6.58 ± 0.22b | 9.97 ± 0.07bcde | 4.11 ± 0.13de | 99.96 ± 0.34ab |
| **HM21** | 13.17 ± 0.67f | 42.95 ± 0.31abcd | 20.99 ± 0.78ab | 5.59 ± 0.10bcd | 11.66 ± 0.57abc | 6.45 ± 0.07a | 100.80 ± 0.72a |
| **HN** | 23.74 ± 0.24bcd | 43.03 ± 0.43abcd | 15.61 ± 0.49de | 3.41 ± 0.16fg | 11.41 ± 0.59abc | 3.18 ± 0.02f | 100.39 ± 0.40ab |
| **HO** | 24.34 ± 0.37bcd | 37.01 ± 0.52ef | 17.07 ± 0.55cde | 4.69 ± 0.16cde | 11.65 ± 0.83abc | 4.53 ± 0.14cd | 99.29 ± 0.52ab |
| **HS** | 20.26 ± 0.80de | 39.9 ± 0.59def | 17.90 ± 0.49cd | 6.03 ± 0.17b | 12.79 ± 0.80a | 3.51 ± 0.07ef | 100.39 ± 0.50ab |
| **JR** | 27.27 ± 1.18bc | 41.46 ± 1.13cde | 11.97 ± 0.28fg | 4.38 ± 0.23ef | 9.86 ± 0.30cdef | 5.01 ± 0.05bc | 99.95 ± 0.04ab |
| **MM** | 16.64 ± 2.68ef | 31.84 ± 1.78g | 23.61 ± 1.89a | 10.76 ± 1.01a | 8.18 ± 1.00ef | 6.51 ± 0.47a | 97.54 ± 1.79b |
| **O128** | 34.52 ± 2.45a | 36.32 ± 1.52fg | 12.08 ± 1.09fg | 3.73 ± 0.33efg | 8.97 ± 0.61def | 3.99 ± 0.21de | 99.62 ± 0.11ab |
| **PBP** | 19.79 ± 0.64de | 45.45 ± 0.88abc | 16.14 ± 0.44de | 4.62 ± 0.22de | 8.99 ± 0.71def | 4.88 ± 0.20bc | 99.86 ± 0.46ab |
| **PBR** | 21.93 ± 0.77cde | 47.06 ± 1.35a | 14.86 ± 0.60ef | 3.99 ± 0.02efg | 7.59 ± 0.59f | 4.44 ± 0.07cd | 99.86 ± 0.40ab |

Mean ± SD from three replicates. Means followed by the same letter in a column are not different ($p < 0.05$)
Note: AP: Apache purple, AR: Apache red, BB: Bloody butcher, CR: Hopi/Cherry pericarp, HC: Hopi/Cherry pericarp x Cordoba, HM20: Hopi/Cherry pericarp x Maiz morado 2020, HM21: Hopi/Cherry pericarp x Maiz morado 2021, HN: Hopi/Cherry pericarp x Maiz negro, HO: Hopi/Cherry pericarp x Oaxaca 128, HS: Hopi/Cherry pericarp x Sehsapsing, JR: Jimmy red, MM: Maiz morado, O128: Oaxaca 128, PBP: Purple butcher purple, PBR: Purple butcher red

### 2.3.3 Polyphenolic contents of whole corn kernels

Since one of the main aims of this project was to identify locally grown colored corn varieties with the highest concentrations of polyphenols for subsequent breeding and adaptation cycles, the varieties were assessed for their polyphenolics. The total monomeric anthocyanin concentration of commercial purple corn variety maiz morado (MM) was observed to be the highest at 4.26 g C3G equivalent/kg corn (Table 2.3) which was followed by the varieties HM21, PBP, AP and HM20 (1.71, 1.59, 1.12 and 0.81 g C3G equivalent/kg corn, respectively). The total phenolic content of MM was observed to be 11.67 g GAE equivalent/kg corn, which was greater than HM21, PBP, and AP, the values being 6.09, 5.3, and 4.95 g GAE equivalent/kg corn, respectively. Similarly, MM variety had the highest condensed tannins content (44.68 g epicatechin equivalent/kg corn) compared to HM21, PBP, and HM20 (24.4, 16.86, and 13.71 g epicatechin equivalent/kg corn, respectively). The amount of polyphenolics quantified in the commercial variety MM agreed with several other studies with purple corn varieties (González-Manzano et al., 2008; Ramos-Escudero et al., 2012; Yang et al., 2009). The commercial control variety MM was found to be far more superior in terms of overall polyphenol content. However, at least four conventionally bred and Missouri-grown colored corn varieties, namely, HM20, HM21, PBP, and AP, were identified to be promising for further breeding efforts. Great variations in concentrations of polyphenolic compounds in evaluated varieties could be attributed to genetic, environmental, and agronomic differences.

**Table 2.3.** Quantification of anthocyanins, total phenols, and condensed tannins in whole kernels of colored corn varieties. Anthocyanin content: g cyanidin 3-glucoside equivalent/kg corn; Total polyphenols: g gallic acid equivalent/kg corn; and condensed tannins: g epicatechin equivalent/kg corn

| Variety | Anthocyanins | Total phenols | Condensed tannins |
|---|---|---|---|
| **AP** | 1.12 ± 0.01c | 4.95 ± 0.03d | 6.38 ± 0.54efgh |
| **AR** | 0.02 ± 0.01h | 3.24 ± 0.04g | 2.08 ± 0.38h |
| **BB** | 0.09 ± 0.01h | 2.31 ± 0.02i | 1.47 ± 0.72h |
| **CR** | 0.59 ± 0.01ef | 2.93 ± 0.02h | 9.32 ± 3.11de |
| **HC** | 0.44 ± 0.00fg | 2.97 ± 0.03h | 6.11 ± 0.72efgh |
| **HM20** | 0.81 ± 0.01d | 4.34 ± 0.02e | 13.71 ± 0.59cd |
| **HM21** | 1.71 ± 0.01b | 6.09 ± 0.04b | 24.4 ± 2.22b |
| **HN** | 0.31 ± 0.01g | 3.59 ± 0.09f | 5.45 ± 0.39efgh |
| **HO** | 0.40 ± 0.01fg | 2.27 ± 0.02i | 7.70 ± 1.02efg |
| **HS** | 0.67 ± 0.01de | 3.56 ± 0.08f | 8.08 ± 1.60ef |
| **JR** | 0.04 ± 0.01h | 1.85 ± 0.02j | 2.48 ± 0.38gh |
| **MM** | 4.26 ± 0.21a | 11.67 ± 0.02a | 44.68 ± 1.77a |
| **O128** | 0.28 ± 0.01h | 2.36 ± 0.01i | 3.41 ± 1.58fgh |
| **PBP** | 1.59 ± 0.00b | 5.30 ± 0.01c | 16.86 ± 2.56c |
| **PBR** | 0.02 ± 0.01h | 2.30 ± 0.01i | 4.03 ± 0.70efgh |

Mean ± SD from three replicates. Means followed by the same letter in a column are not different ($p < 0.05$)

Note: AP: Apache purple, AR: Apache red, BB: Bloody butcher, CR: Hopi/Cherry pericarp, HC: Hopi/Cherry pericarp x Cordoba, HM20: Hopi/Cherry pericarp x Maiz morado 2020, HM21: Hopi/Cherry pericarp x Maiz morado 2021, HN: Hopi/Cherry pericarp x Maiz negro, HO: Hopi/Cherry pericarp x Oaxaca 128, HS: Hopi/Cherry pericarp x Sehsapsing, JR: Jimmy red, MM: Maiz morado, O128: Oaxaca 128, PBP: Purple butcher purple, PBR: Purple butcher red

### 2.3.4 Total monomeric anthocyanin contents of milling coproducts

One of the main advantages associated with the colored corn varieties is the fact that their pericarp contains disproportionately higher concentration of bioactive compounds. Once the pericarp is separated at the front end, it can be selectively processed to economically recover value-added bioactive compounds with diverse agro-industrial applications. The corn endosperm containing most of the starch and protein, remains unaffected and can be utilized further.

Dry milling coproducts from all the corn varieties were evaluated for total monomeric anthocyanins (ACNs), and the results are summarized in Table 2.4. According to Moreno et al. (2005), total anthocyanin content can be predicted visually according to the color of corn kernels. It was observed that the darker the color of the corn kernels, the higher was the ANCs content. Purple-colored corn varieties had a higher ACNs whereas compared to red-colored ones. As expected, the ACNs content was generally observed to be highest in corn pericarp compared to other coproducts. Among the experimental varieties, the highest content was 23.81 g C3G/kg pericarp in HM21. In comparison, the lowest was 0.1 g C3G/kg pericarp in AR, the anthocyanin content of HM21 was similar to the control (24.14 g C3G/kg pericarp). For the variety O128, the pigments were located in the aleurone layer rather than in the pericarp (0.25 g C3G/kg pericarp). Besides these, the varieties PBP, HM20, and AP also had higher anthocyanin contents in their pericarp, the values being 23.16, 12.01 and 7.90 C3G/kg pericarp, respectively.

Among the rest of the coproducts, large grits were observed to be fraction with the highest ACNs for most varieties except AR, HN, JR, O128, and PBR. Large grits from MM had 3.24 g C3G/kg coproduct followed by HM21, which had 1.13 g C3G/kg ACNs in large grits. The ACN content of medium grits of all the varieties did not exceed 1 g/kg except that of the control variety MM (1.4 g C3G/kg). The remaining coproducts had a relatively lower ACNs content.

**Table 2.4.** Quantification of anthocyanins in dry milling coproducts from different colored corn varieties (g cyanidin 3-glucoside equivalent/kg coproduct)

| | Coproducts | | | | | |
|---|---|---|---|---|---|---|
| **Variety** | **Pericarp** | **Germ** | **Large grits** | **Medium grits** | **Small grits** | **Fines** |
| **AP** | 7.90 ± 0.03d | 0.52 ± 0.02e | 0.82 ± 0.01d | 0.77 ± 0.00b | 0.68 ± 0.00d | 0.81 ± 0.01a |
| **AR** | 0.10 ± 0.00h | 0.01 ± 0.00h | 0.00 ± 0.00j | 0.01 ± 0.00g | 0.02 ± 0.00i | 0.07 ± 0.01i |
| **BB** | 0.24 ± 0.01h | 0.05 ± 0.00h | 0.10 ± 0.01i | 0.11 ± 0.00f | 0.13 ± 0.00h | 0.17 ± 0.02h |
| **CR** | 4.72 ± 0.02e | 0.77 ± 0.02c | 0.58 ± 0.00f | 0.44 ± 0.00c | 0.65 ± 0.01d | 0.42 ± 0.01de |
| **HC** | 5.11 ± 0.01e | 0.29 ± 0.01g | 0.49 ± 0.01g | 0.30 ± 0.01e | 0.37 ± 0.01g | 0.32 ± 0.01g |
| **HM20** | 12.01 ± 0.02c | 0.52 ± 0.01e | 0.70 ± 0.01e | 0.34 ± 0.00d | 0.53 ± 0.03e | 0.29 ± 0.01g |
| **HM21** | 23.81 ± 0.19ab | 0.62 ± 0.01d | 1.13 ± 0.02b | 0.38 ± 0.00d | 1.08 ± 0.01b | 0.64 ± 0.01c |
| **HN** | 2.36 ± 0.03g | 0.27 ± 0.02g | 0.33 ± 0.02h | 0.28 ± 0.02e | 0.35 ± 0.01g | 0.38 ± 0.02ef |
| **HO128** | 3.63 ± 0.04f | 0.37 ± 0.01f | 0.58 ± 0.01f | 0.35 ± 0.01d | 0.37 ± 0.02g | 0.29 ± 0.01g |
| **HS** | 5.54 ± 0.78e | 0.54 ± 0.01e | 0.80 ± 0.01d | 0.77 ± 0.02b | 0.49 ± 0.01f | 0.44 ± 0.01d |
| **JR** | 0.14 ± 0.01h | 0.05 ± 0.01h | 0.06 ± 0.00ij | 0.09 ± 0.00f | 0.13 ± 0.00h | 0.17 ± 0.01h |
| **MM** | 24.14 ± 0.48a | 1.32 ± 0.01a | 3.24 ± 0.07a | 1.40 ± 0.03a | 1.48 ± 0.00a | 0.76 ± 0.02b |
| **O128** | 0.25 ± 0.00h | 0.28 ± 0.01g | 0.25 ± 0.01h | 0.29 ± 0.00e | 0.38 ± 0.01g | 0.37 ± 0.00f |
| **PBP** | 23.16 ± 0.13b | 1.11 ± 0.02b | 0.91 ± 0.01c | 0.47 ± 0.01c | 0.92 ± 0.00c | 0.68 ± 0.01c |
| **PBR** | 0.14 ± 0.01h | 0.04 ± 0.00h | 0.04 ± 0.00ij | 0.04 ± 0.01g | 0.05 ± 0.01i | 0.07 ± 0.00i |

Mean ± SD from three replicates. Means followed by the same letter in a column are not different ($p < 0.05$)
Note: AP: Apache purple, AR: Apache red, BB: Bloody butcher, CR: Hopi/Cherry pericarp, HC: Hopi/Cherry pericarp x Cordoba, HM20: Hopi/Cherry pericarp x Maiz morado 2020, HM21: Hopi/Cherry pericarp x Maiz morado 2021, HN: Hopi/Cherry pericarp x Maiz negro, HO: Hopi/Cherry pericarp x Oaxaca 128, HS: Hopi/Cherry pericarp x Sehsapsing, JR: Jimmy red, MM: Maiz morado, O128: Oaxaca 128, PBP: Purple butcher purple, PBR: Purple butcher red

### 2.3.5 Total phenolic content (TPC) of milling coproducts

Phenolics help in protecting plants from biotic and abiotic stresses, regulate hormones and numerous other biochemical reactions, and are crucial for cell division (Tayal et al., 2020). Free, esterified, and insoluble-bound phenolic compounds are found in corn (Méndez-Lagunas et al., 2020). TPC was assessed in milling coproducts of colored corns varieties (Table 2.5), and the pericarp from control variety MM was found to contain the highest amount of TPC (62.52 ± 0.15 g GAE/kg pericarp). These results agreed with Trehan et al. (2018), wherein high TPC were reported in colored corn. The pericarp from variety PBP, HM21, and HM20 also contained high amounts of TPC, the values being 53.40 ± 0.25, 41.75 ± 0.01 and 18.76 ± 0.09 g GAE/kg pericarp, respectively. The control variety MM was observed to contain higher TPC in large, medium, and small grits with 9.48 ± 0.05, 4.78 ± 0.07, and 5.13 ± 0.02 g GAE/kg coproduct, respectively. These amounts were found to be higher compared to an earlier study with purple corn (Li et al., 2017). Overall, the pericarp fraction of colored corn varieties contained the highest amounts of TPC.

**Table 2.5.** Quantification of total polyphenols in dry milling coproducts from different colored corn varieties (g gallic acid equivalent/kg coproduct)

| | Coproducts | | | | | |
|---|---|---|---|---|---|---|
| **Variety** | **Pericarp** | **Germ** | **Large grits** | **Medium grits** | **Small grits** | **Fines** |
| **AP** | 12.76 ± 0.15g | 6.86 ± 0.02a | 5.29 ± 0.04b | 4.54 ± 0.02a | 4.41 ± 0.01b | 4.98 ± 0.04a |
| **AR** | 13.34 ± 0.03f | 5.05 ± 0.02cd | 3.44 ± 0.02d | 2.47 ± 0.04cd | 3.22 ± 0.03de | 3.74 ± 0.04bc |
| **BB** | 10.1 ± 0.02i | 3.81 ± 0.03f | 2.11 ± 0.08ij | 1.93 ± 0.01f | 2.58 ± 0.08i | 3.16 ± 0.03cde |
| **CR** | 10.5 ± 0.06i | 4.77 ± 0.01de | 2.74 ± 0.03f | 2.86 ± 0.05c | 3.11 ± 0.04ef | 2.84 ± 0.04efg |
| **HC** | 10.19 ± 0.09i | 3.86 ± 0.01f | 2.51 ± 0.02fg | 2.58 ± 0.01cd | 2.70 ± 0.01hi | 2.42 ± 0.02fg |
| **HM20** | 18.76 ± 0.09d | 4.45 ± 0.05e | 3.33 ± 0.05de | 2.46 ± 0.03de | 3.37 ± 0.03d | 3.15 ± 0.02cde |
| **HM21** | 41.75 ± 0.01c | 4.46 ± 0.05e | 4.28 ± 0.04c | 2.64 ± 0.01cd | 4.12 ± 0.01c | 3.18 ± 0.02cde |
| **HN** | 6.76 ± 0.20l | 4.96 ± 0.33cd | 2.40 ± 0.24gh | 2.51 ± 0.36cd | 2.78 ± 0.23ghi | 3.66 ± 0.68bcd |
| **HO** | 8.72 ± 0.04k | 3.77 ± 0.01f | 3.52 ± 0.03d | 2.52 ± 0.02cd | 3.21 ± 0.00de | 2.33 ± 0.02g |
| **HS** | 12.03 ± 0.01h | 4.73 ± 0.25de | 3.13 ± 0.04e | 3.45 ± 0.03b | 2.96 ± 0.02fg | 3.02 ± 0.03def |
| **JR** | 9.39 ± 0.06j | 3.04 ± 0.03g | 1.74 ± 0.02k | 1.87 ± 0.17f | 2.27 ± 0.02j | 2.33 ± 0.01g |
| **MM** | 62.52 ± 0.15a | 5.34 ± 0.01c | 9.48 ± 0.05a | 4.78 ± 0.07a | 5.13 ± 0.02a | 3.08 ± 0.02def |
| **O128** | 3.06 ± 0.10m | 3.91 ± 0.07f | 1.88 ± 0.02jk | 2.07 ± 0.02ef | 2.88 ± 0.03fgh | 2.81 ± 0.03efg |
| **PBP** | 53.40 ± 0.25b | 5.84 ± 0.02b | 4.22 ± 0.03c | 2.65 ± 0.01cd | 4.24 ± 0.02bc | 3.86 ± 0.02b |
| **PBR** | 15.09 ± 0.07e | 4.48 ± 0.04e | 2.18 ± 0.00hi | 1.84 ± 0.01f | 2.62 ± 0.01i | 2.85 ± 0.01efg |

Mean ± SD from three replicates. Means followed by the same letter in a column are not different ($p < 0.05$)
Note: AP: Apache purple, AR: Apache red, BB: Bloody butcher, CR: Hopi/Cherry pericarp, HC: Hopi/Cherry pericarp x Cordoba, HM20: Hopi/Cherry pericarp x Maiz morado 2020, HM21: Hopi/Cherry pericarp x Maiz morado 2021, HN: Hopi/Cherry pericarp x Maiz negro, HO: Hopi/Cherry pericarp x Oaxaca 128, HS: Hopi/Cherry pericarp x Sehsapsing, JR: Jimmy red, MM: Maiz morado, O128: Oaxaca 128, PBP: Purple butcher purple, PBR: Purple butcher red

### 2.3.6 Condensed tannins (CT) content of milling coproducts

CT were the most prevalent phytochemicals detected in colored corn varieties, far exceeding the amounts of anthocyanins and total phenols (Table 2.6). The highest concentration of CT was identified in the pericarp of HM21 (253.12 ± 3.37 g epicatechin/kg pericarp), followed by the varieties MM, PBP, HM20, and HS. These results agreed with several other studies wherein CT were reported to be the most dominant phytochemicals quantified in colored corn varieties such as 1381 ± 69.8 μg catechin/g dry matter in purple corn (Suriano et al., 2021), and 310.6 ± 28.7 mg catechin/g pericarp in red corn (Chen et al., 2017). Medium grits from HM21 also had a higher CT concentration (17.94 ± 5.03 g epicatechin/kg coproduct). Overall, the control variety MM was identified to contain the highest CT with significant amounts identified in germ, large grits, and small grits, the values being 18.42 ± 0 .54 g, 39.73 ± 0.5 g, and 12.95 ± 1.4 g epicatechin/kg coproduct, respectively. The varieties JR, AR, and BB contained the least amount of CT and also had the lowest anthocyanin concentrations.

**Table 2.6.** Quantification of condensed tannins in dry milling coproducts from different colored corn varieties (g epicatechin equivalent/kg coproduct)

| | Coproducts | | | | | |
|---|---|---|---|---|---|---|
| **Varieties** | **Pericarp** | **Germ** | **Large grits** | **Medium grits** | **Small grits** | **Fines** |
| **AP** | 37.85 ± 0.79g | 4.14 ± 0.48efg | 6.71 ± 0.34bc | 5.05 ± 0.46bcd | 2.62 ± 0.58cd | 4.02 ± 0.26bcde |
| **AR** | 4.94 ± 1.54i | 0.25 ± 1.17hi | 0.00 ± 0.00c | 1.37 ± 0.66cd | 0.42 ± 0.21d | 2.75 ± 1.83cde |
| **BB** | 2.81 ± 0.14i | 0.57 ± 0.60hi | 1.3 ± 0.18c | 1.85 ± 0.11cd | 1.14 ± 0.53d | 0.45 ± 0.70e |
| **CR** | 51.53 ± 3.93f | 7.61 ± 0.37cd | 7.37 ± 1.76bc | 6.37 ± 0.41bcd | 6.44 ± 0.24b | 3.58 ± 0.26bcde |
| **HC** | 57.76 ± 2.81f | 4.15 ± 0.49efg | 7.75 ± 0.14bc | 5.17 ± 2.29bcd | 7.54 ± 0.21b | 6.11 ± 1.61abcd |
| **HM20** | 125.98 ± 2.91d | 9.57 ± 0.59bc | 6.50 ± 1.21bc | 6.10 ± 0.69bcd | 7.11 ± 0.70b | 1.09 ± 1.07de |
| **HM21** | 253.12 ± 3.37a | 9.41 ± 1.24bc | 6.33 ± 2.39bc | 17.94 ± 5.03a | 5.53 ± 2.00bc | 8.88 ± 3.79ab |
| **HN** | 20.27 ± 2.26h | 3.36 ± 0.52fgh | 5.81 ± 1.26bc | 6.95 ± 3.30bcd | 7.18 ± 0.85b | 2.56 ± 0.54cde |
| **HO** | 38.90 ± 0.76g | 6.94 ± 2.10cde | 10.14 1± 5.66b | 8.88 ± 1.49b | 6.17 ± 0.88b | 4.49 ± 2.56abcde |
| **HS** | 74.35 ± 4.47e | 2.08 ± 0.65ghi | 10.56 ± 1.57b | 7.75 ± 1.36bc | 6.48 ± 0.81b | 8.09 ± 0.40abc |
| **JR** | 0.24 ± 1.00i | 0.00 ± 0.00i | 0.35 ± 0.64c | 0.55 ± 1.14d | 0.00 ± 0.00d | 1.87 ± 0.27de |
| **MM** | 193.37 ± 6.34b | 18.42 ± 0.54a | 39.73 ± 0.50a | 10.75 ± 0.73b | 12.95 ± 1.40a | 9.69 ± 2.12a |
| **O128** | 4.56 ± 1.09i | 5.70 ± 0.33def | 10.50 ± 1.35b | 5.02 ± 0.69bcd | 7.87 ± 0.15b | 8.61 ± 0.39ab |
| **PBP** | 163.27 ± 1.26c | 11.36 ± 0.67b | 9.49 ± 2.40b | 6.78 ± 1.03bcd | 4.95 ± 0.30bc | 9.67 ± 0.87a |
| **PBR** | 8.21 ± 0.96i | 2.77 ± 0.99fghi | 7.29 ± 3.49bc | 0.27 ± 0.35d | 1.92 ± 0.78d | 3.78 ± 0.22bcde |

Mean ± SD from three replicates. Means followed by the same letter in a column are not different ($p < 0.05$)
Note: AP: Apache purple, AR: Apache red, BB: Bloody butcher, CR: Hopi/Cherry pericarp, HC: Hopi/Cherry pericarp x Cordoba, HM20: Hopi/Cherry pericarp x Maiz morado 2020, HM21: Hopi/Cherry pericarp x Maiz morado 2021, HN: Hopi/Cherry pericarp x Maiz negro, HO: Hopi/Cherry pericarp x Oaxaca 128, HS: Hopi/Cherry pericarp x Sehsapsing, JR: Jimmy red, MM: Maiz morado, O128: Oaxaca 128, PBP: Purple butcher purple, PBR: Purple butcher red

### 2.3.7 HPLC analysis

#### 2.3.7.1 Anthocyanins evaluation using HPLC analysis

Standards of six different anthocyanins were used and only three of those, namely, cyanidin 3-glucoside, cyanidin chloride, and delphinidin were identified in evaluated corn varieties (Fig. 2.6). Cyanidin 3-glucoside and cyanidin chloride were observed to be the most abundant forms followed by delphinidin. The control variety MM had the highest total anthocyanin content (5.28 g anthocyanins/kg corn) followed by HM21, PBP, and HM20 (3.92, 3.03 and 2.39 kg anthocyanins/kg corn, respectively). The varieties HM20 and HM21 had a relatively higher delphinidin content. No anthocyanins were detected in the varieties AR, BB, JR, and PBR varieties. These results were consistent with other studies where cyanidin and their derivatives were reported to be the significant anthocyanins in colored corn (Chatham et al., 2018; Lao and Giusti, 2016; Li et al., 2017; Peniche-Pavía and Tiessen, 2020).

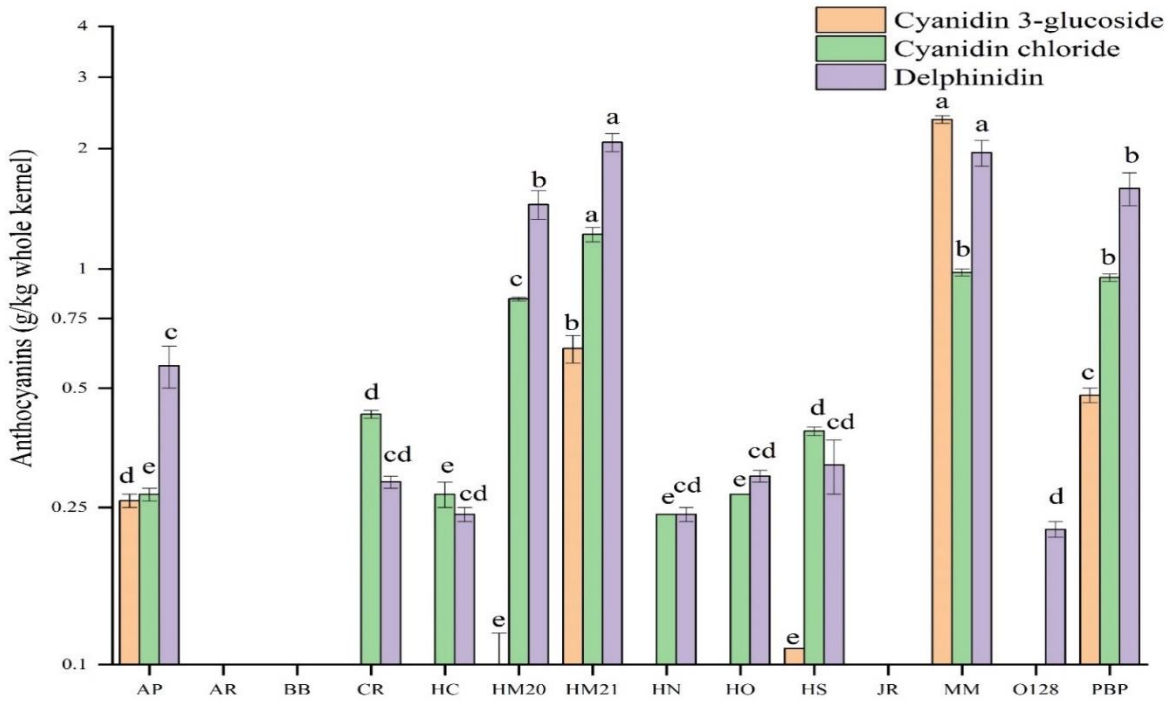


**Fig. 2.6.** Identification and quantification of different anthocyanins present in colored corn varieties using HPLC
Note: AP: Apache purple, AR: Apache red, BB: Bloody butcher, CR: Hopi/Cherry pericarp, HC: Hopi/Cherry pericarp x Cordoba, HM20: Hopi/Cherry pericarp x Maiz morado 2020, HM21: Hopi/Cherry pericarp x Maiz morado 2021, HN: Hopi/Cherry pericarp x Maiz negro, HO: Hopi/Cherry pericarp x Oaxaca 128, HS: Hopi/Cherry pericarp x Sehsapsing, JR: Jimmy red, MM: Maiz morado, O128: Oaxaca 128, PBP: Purple butcher purple, PBR: Purple butcher red

### 2.3.7.2 Phenolics evaluation using HPLC analysis

The phenolic acid profile of colored corn varieties has been assessed in earlier studies wherein ferulic acid was observed to be the most abundant phenol, and observations from the current study summarized in Fig. 2.7 are consistent with those findings (Cuevas Montilla et al., 2011; Ranilla et al., 2017). In addition to ferulic acid, hesperidin, and caffeic acid were the other identified phenols at 38.4, 41.4, and 29.1 min retention times, respectively. Other researchers have reported the presence of *p*-coumaric acid, chlorogenic acid, and vanillic acid along with their derivatives in colored corns but they were not identified in our analyses

(Cristianini and Guillén Sánchez, 2020; Lao et al., 2017; Ranilla et al., 2021). The control variety (MM) had the highest concentration of caffeic acid (6.32 ± 0.19 g/kg corn) followed by HM21 and PBP (2.30 ± 0.04 and 1.35 ± 0.02 g/kg corn, respectively). Ferulic acid was present in all the evaluated varieties ranging from a high of 3.96 ± 0.01 g/kg corn in HS to a low of 0.75 ± 0.03 g/kg corn in PBR. Hesperidin concentration was highest in AR (10.40 ± 0.35 g/kg corn). It has been reported that only a small portion of total phenolic acids can be extracted without hydrolysis treatment of plant matter, as most of them exist in bound forms (Lao et al., 2017). Overall, the variety AR had the highest total phenolics content, followed by the control variety (12.58 ± 0.35 and 9.37 ± 0.46 g/kg corn, respectively).

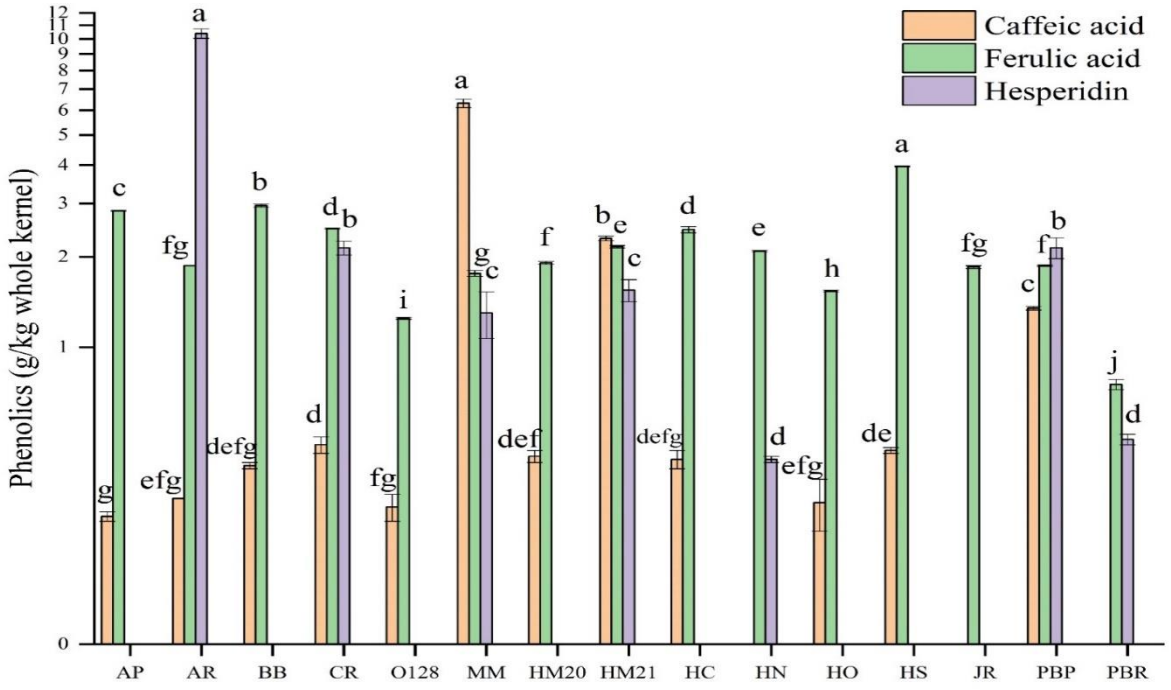


**Fig. 2.7.** Identification and quantification of different phenols present in colored corn varieties using HPLC
Note: AP: Apache purple, AR: Apache red, BB: Bloody butcher, CR: Hopi/Cherry pericarp, HC: Hopi/Cherry pericarp x Cordoba, HM20: Hopi/Cherry pericarp x Maiz morado 2020, HM21: Hopi/Cherry pericarp x Maiz morado 2021, HN: Hopi/Cherry pericarp x Maiz negro, HO: Hopi/Cherry pericarp x Oaxaca 128, HS: Hopi/Cherry pericarp x Sehsapsing, JR: Jimmy red, MM: Maiz morado, O128: Oaxaca 128, PBP: Purple butcher purple, PBR: Purple butcher red

### 2.3.7.3 Flavonoids evaluation using HPLC analysis

Organic solvents are highly efficient and preferred for the extraction of flavonoids from plant matrices (Lao et al., 2017). The standards of epicatechin, morin, quercetin, naringenin, and kaempferol were run and eluted at retention times of 3.64, 39.02, 43.73, 46.9 and 48.09 min, respectively. As summarized in Fig. 2.8, epicatechin was the most abundant flavonoid identified in all the evaluated varieties followed by quercetin. On the other hand, naringenin and kaempferol were present in relatively lower quantities. These results were in accordance with other studies on purple corn varieties wherein the authors reported 17.62 ± 0.92 mg catechin/g of purple corn (Trehan et al., 2020), and 0.23 ± 0.01 g of catechin equivalent/g of purple corn, respectively (Ramos-Escudero et al., 2012).

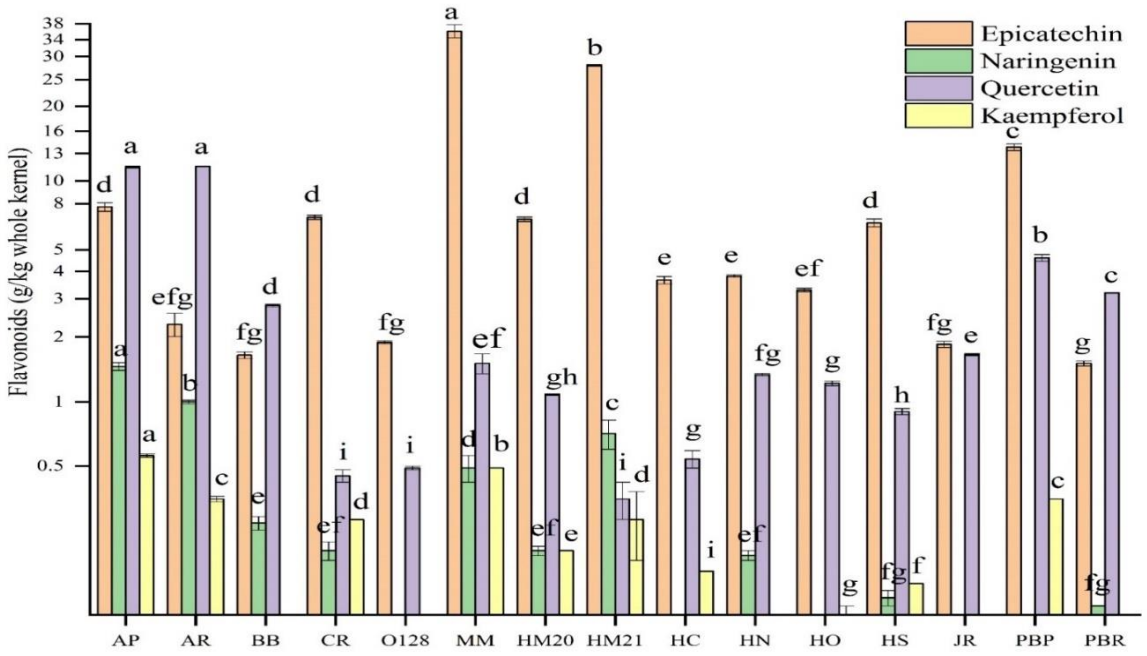


**Fig. 2.8.** Identification and quantification of different flavonoids present in colored corn varieties using HPLC
Note: AP: Apache purple, AR: Apache red, BB: Bloody butcher, CR: Hopi/Cherry pericarp, HC: Hopi/Cherry pericarp x Cordoba, HM20: Hopi/Cherry pericarp x Maiz morado 2020, HM21: Hopi/Cherry pericarp x Maiz morado 2021, HN: Hopi/Cherry pericarp x Maiz negro, HO: Hopi/Cherry pericarp x Oaxaca 128, HS: Hopi/Cherry pericarp x Sehsapsing, JR: Jimmy red, MM: Maiz morado, O128: Oaxaca 128, PBP: Purple butcher purple, PBR: Purple butcher red

These differences can be attributed to variations in cultivars and underlying agronomic conditions. It has been reported that catechin or epicatechin is a significant nutritional component of total flavonoids (Birt and Jeffery, 2013). The control variety (MM) had the highest amount of total flavonoids followed by HM21, AP, and PBP with 38.57 ± 0.30, 29.38 ± 0.30, 21.21 ± 0.09, and 18.77 ± 0.08 g/kg corn, respectively. Although epicatechin was most the prominent flavonoid present in most of the corn varieties evaluated, the varieties PBR, BB, AR and AP had higher concentrations of quercetin. Kaempferol was present in minute amounts in all the varieties except O128 and PBR. Similarly, naringenin was not identified in O128, JR, and PBR and present in small amounts in others. Overall, the colored corn varieties were identified to be rich sources of flavonoids. Fig. 2.9 contains the chromatographs with standard peaks and the peaks of the individual anthocyanins, flavonoids, and phenolics identified in the control variety MM.

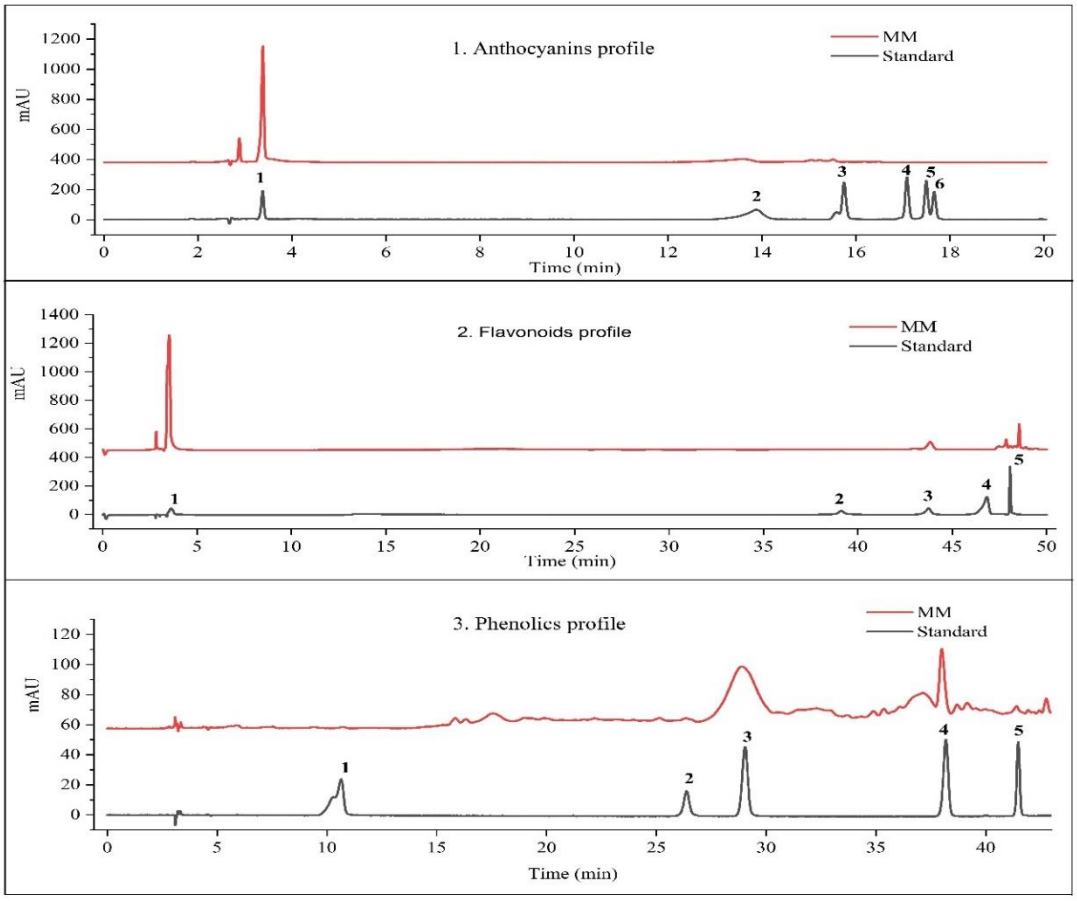


**Fig. 2.9.** Chromatographs with standard peaks and individual anthocyanins, flavonoids, and phenolics detected in Maiz morado (MM)
Note: In anthocyanins profile, the peaks correspond to: (1) cyanidin-3-glucoside, (2) delphinidin, (3) cyanidin chloride, (4) peonidin, (5) malvidin, and (6) pelargonidin chloride
In flavonoids profile, the peaks correspond to: (1) epicatechin, (2) morin, (3) naringin, (4) quercetin, and (5) kaempferol
In phenolics profile, the peaks correspond to: (1) gallic acid, (2) chlorogenic acid, (3) caffeic acid, (4) ferulic acid, and (5) hesperidin

### 2.3.8 Antioxidant properties

Various researchers have reported a positive correlation between antioxidant-rich diets and better health outcomes in humans. An important mechanism by which antioxidants present in plants confer health benefits is by neutralizing free radicals, which promote oxidative processes harmful to biological cells. The methods utilized for quantifying antioxidant

activities can be distinctly classified into spectrometric, electrochemical, and chromatographic assays. Among the vast array of assays available, each one has associated advantages and disadvantages. For example, ABTS and CUPRAC assays can quantify both hydrophilic and lipophilic antioxidants, whereas the DPPH assay can only quantify hydrophobic compounds (Munteanu and Apetrei, 2021). Keeping these underlying differences in mind, four distinct antioxidant assays were used in this study to evaluate five corn varieties. The selected assays were as follows: i) 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, ii) 2-2's-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS·+) radical inhibition antioxidant capacity assay, iii) Cupric ion reducing antioxidant capacity (CUPRAC) assay, and iv) Reducing power assay (RP) (Añibarro-Ortega et al., 2020; Boateng et al., 2021). The corn varieties selected for antioxidant assays were HM21, HM20, PBP, AP, and control MM, and this selection was based on relatively higher concentrations of polyphenols in them. The total phenolic content of a biological material correlates with its antioxidant activity. Among the evaluated varieties, HM20 was found to be having the highest antioxidant capacity in two of the assays (DPPH, ABTS) and second highest activities behind MM for the other two protocols (CUPRAC, and RP), the values being 69.32 ± 0.04, 29.87 ± 0.07, 515.1 ± 10.74, and 693.24 ± 2.43 μmol TE/g of pericarp, respectively (Table 2.7.). The antioxidant activities in the DPPH assay varied between a high of 69.32 ± 0.04 for HM20 and a low of 63.58 ± 0.11 μmol TE/g of pericarp, respectively for the variety HM21. Corresponding antioxidant activities for blueberry, blackberry, and raspberry have been reported to be 14.56 ± 2.57, 22.10 ± 9.7, and 21.50 ± 8.0 μmol TE/g fresh fruits, respectively (Ruiz-Torralba et al., 2018).

Similar trends were observed in the ABTS assay where the antioxidant activities ranged between a high (PBP) and low (AP) of 30 ± 0.06 and 28.54 ± 0.37 μmol TE/g of pericarp,

respectively. In a similar assay conducted on 12 Peruvian corn accessions by Ranilla et al. (2019), the DPPH scavenging activity ranged between 67.63 to 18.79 μmol TE/g dry weight, respectively. In another study by Hosu et al., (2016), the antioxidant activity of cornelian cherries was reported to be 36.13 ± 1.18 μmol TE/g of fresh weight. However, greater variability was observed in the OH radical production capability of pericarp extracts from the evaluated varieties while performing the CUPRAC and reducing power assays. In the CUPRAC assay, the variety HM20 demonstrated almost twice the antioxidant activity compared to PBP, the values being 515.11 ± 21.49 and 201.62 ± 17.57 μmol TE/g of pericarp, respectively. The control variety MM was identified to contain twice as much antioxidant activity as present in AP (938.15 ± 30.21 and 403.93 ± 55.68 μmol TE/g of pericarp, respectively). These antioxidant activities were found to be higher compared to other polyphenol-rich fruits and vegetables such as blueberry (56.80-172.40 μmol TE/g dry weight), blackberry (157.90-257.50 μmol TE/g dry weight), cherry (65.20-135.80 μmol TE/g dry weight), whereas the antioxidant activity of MM were observed to be comparable to green tea extracts (772.20-1326.00 μmol TE/g dry weight) as reported by George et al. (2022).

The ferric-reducing power assay measures the capacity of electron donation by evaluating the effective reduction of ferric ion to their ferrous form. The variety MM had the highest antioxidant activity whereas the variety AP had the lowest, the values being 796.09 ± 16.08 and 272.34 ± 2.72 μmol TE/g of pericarp, respectively. The varieties PBP (623.67 ± 11.07 μmol TE/g of pericarp) and HM21 (486.06 ± 5.78 μmol TE/g of pericarp) also demonstrated higher antioxidant activities. In a study by Karaaslan et al. (2018), the antioxidant properties of strawberry fruit extracts using five different solvents were evaluated using the RP assay.

The antioxidant activities reportedly ranged from 545.20 ± 12.50 to 286.50 ± 19.30 mg TE/100 g strawberry extract, respectively. Overall, the colored corn pericarp extracts demonstrated significant antioxidant activities compared to other polyphenol-rich fruits and vegetables for all the four assays. However, the observed trends were inconsistent as the total polyphenolic content of varieties did not directly correlate with the observed antioxidant activities. However, this anomaly could be attributed to the fact that spectrophotometric protocols provide an approximation of phytochemical contents as well as corresponding antioxidant activities. Therefore, it can be inferred that colored corn varieties can be valorized to recover value-added bioactive compounds with significant antioxidant activities.

**Table 2.7**. Antioxidant activities of pericarp extract from selected corn varieties

| Varieties | DPPH (µmol TE/g pericarp) | ABTS (µmol TE/g of pericarp) | CUPRAC (µmol TE/g of pericarp) | Reducing power (µmol TE/g of pericarp) |
|---|---|---|---|---|
| **HM21** | 63.58 ± 0.11d | 29.34 ± 0.07bc | 276.78 ± 7.45d | 486.06 ± 5.78d |
| **AP** | 67.14 ± 0.10b | 28.54 ± 0.37d | 403.93 ± 55.68c | 272.34 ± 2.72e |
| **PBP** | 66.77 ± 0.35b | 30.00 ± 0.06a | 201.62 ± 17.57d | 623.67 ± 11.07c |
| **MM** | 64.81 ± 0.54c | 29.08 ± 0.06cd | 938.15 ± 30.21a | 796.09 ± 16.08a |
| **HM20** | 69.32 ± 0.04a | 29.87 ± 0.07ab | 515.11 ± 10.74b | 693.24 ± 2.43b |

Mean ± SD from three replicates. Means followed by the same letter in a column are not different ($p < 0.05$)
Note: AP: Apache purple, HM20: Hopi/Cherry pericarp x Maiz morado 2020, HM21: Hopi/Cherry pericarp x Maiz morado 2021, MM: Maiz morado, PBP: Purple butcher purple

### 2.3.9 Histological analysis

The commercial purple corn variety MM had the thickest pericarp (213.3 µm, averaged across the S1 and S2 sides of the kernels), followed by HM21 (176.8 µm), PBP (157.4 µm), HM20 (125.1 µm), and AP (117.3 µm) (Fig. 2.10A). The variety MM contained the highest amount of kernel anthocyanins, total phenols and condensed tannins, the values being 4.26 ± 0.21 C3G, 11.67 ± 0.02 GAE and 44.68 ± 1.77 EE/kg kernels, respectively (Table 2.3). It

was observed that in all the polyphenol-rich corn varieties, anthocyanin pigments were mainly concentrated in pericarp. Furthermore, the pericarp thickness was greatest for the variety MM which also contained the highest polyphenolic compounds, an important observation which could help in further breeding efforts (Fig 2.10 and 2.11). The mean dry milling yield of corn pericarp was highest for the variety MM at 6.51%, db (Table 2.2), which could be explained based on histological observations. The pericarp of variety O128 did not contain any pigments, but they were identified in aleurone layer just beneath the pericarp (Fig. 2.10); this is consistent with previous reports of clear pericarp/pigmented aleurone varieties such as O128 (Li et al., 2017).

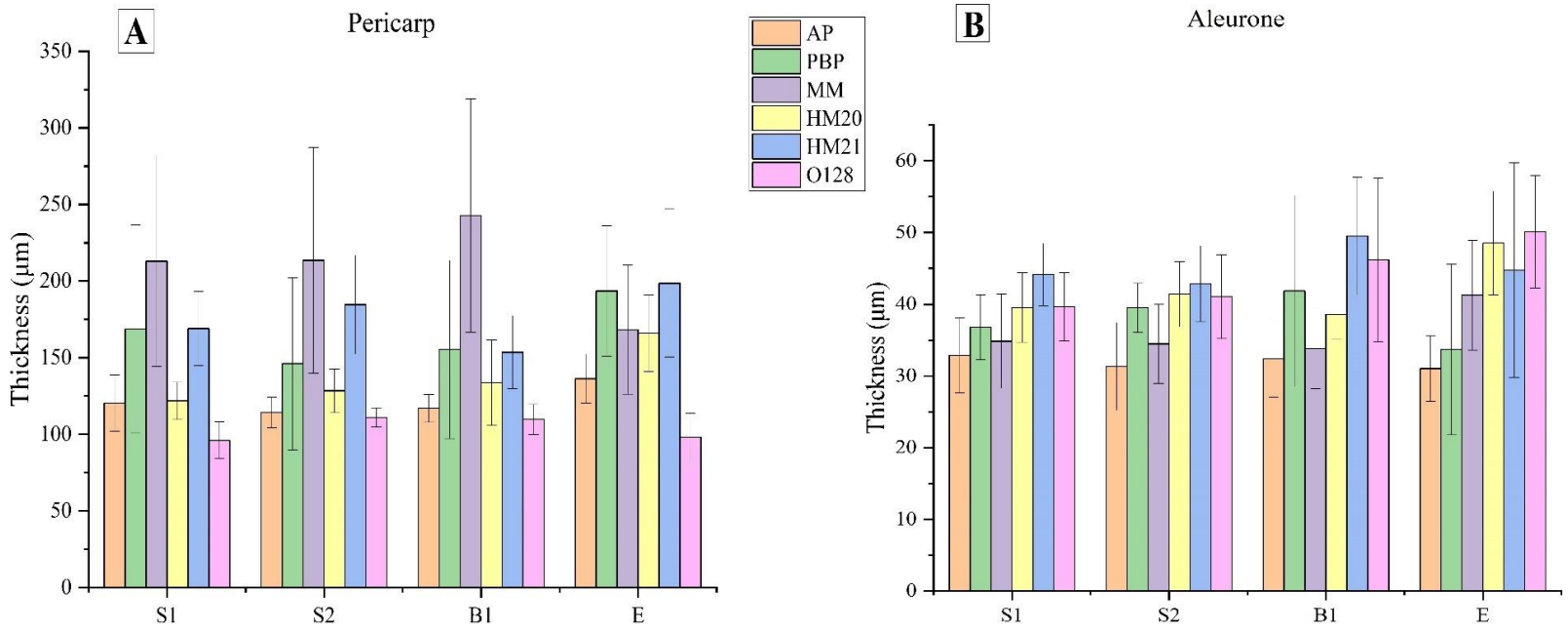


**Fig. 2.10.** Comparisons of pericarp (A) and aleurone (B) thicknesses at four select locations S1 (side 1), S2 (side 2), B1 (back) and E (embryo) of kernels from six colored corn varieties. Presented are the mean values from five replicates
Note: AP: Apache purple, PBP: Purple butcher purple, MM: Maiz morado, HM20: Hopi/Cherry pericarp x Maiz morado 2020, HM21: Hopi/Cherry pericarp x Maiz morado 2021, 0128: Oaxaca 128

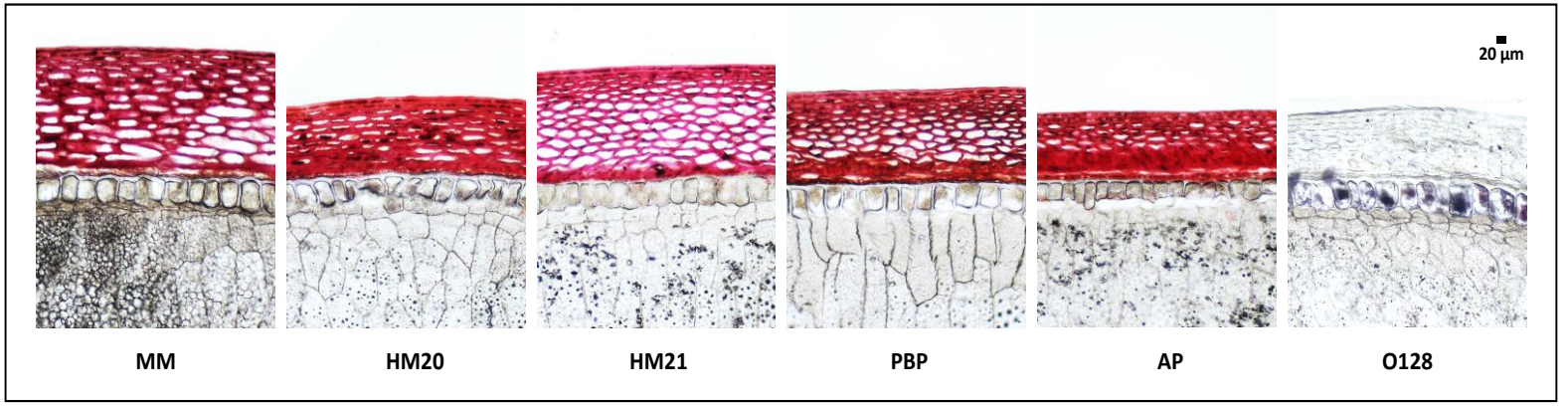


**Fig. 2.11.** Comparison of pericarp thickness in the five varieties with the highest pericarp anthocyanin concentration and a clear pericarp control (O128). Aleurone layer can be observed underneath the pigmented pericarp layer. The variety O128 had a clear pericarp and most of the pigments were identified in aleurone layer. All figures are the same scale; bar = 20 µm

Note: MM: Maiz morado, HM20: Hopi/Cherry pericarp x Maiz morado 2020, HM21: Hopi/Cherry pericarp x Maiz morado 2021, PBP: Purple butcher purple, AP: Apache purple, 0128: Oaxaca 128

## 2.4 Conclusions

The evaluated colored corn varieties had a lower mean starch content (65.28%, db) than conventional yellow dent corn (75.19%, db). However, the mean protein content was ~1.70% (db) higher. Since the conventional corn processing industry is mainly interested in starch, yellow dent varieties have been selected based on their starch contents. In this study, most of the processed colored varieties had a dominant softer endosperm composition with mean large grits yield of 22.98% (db), which was lower compared to the mean yields from hard endosperm varieties (>40.00%, db). Corn varieties with a relatively harder endosperm composition are preferred in the corn dry milling industry as they yield a greater fraction of large grits used in making breakfast cereals. However, softer endosperm varieties are preferred in corn wet milling and corn dry grind industry as it is relatively earlier to free starch molecules from the surrounding protein matrix. Therefore, the evaluated colored corn varieties were found to be suitable for corn wet milling and dry grind industries, accounting for most of the US corn utilization. Although, the control variety MM contained the highest amounts of anthocyanins, total phenols, and condensed tannins (4.26 g C3G, 11.67 g GAE, and 44.68 g epicatechin equivalents/kg corn, respectively), four of the experimental varieties, namely, HM20, HM21, PBP, and AP were identified to be rich sources of polyphenolic compounds. In all the varieties, the greatest concentrations of polyphenolic compounds were identified in corn pericarp, a low-value cellulosic coproduct of the corn processing industry. HPLC analyses were performed on five select varieties for identifying and quantifying the individual anthocyanins, phenols, and flavonoids. Cyanidin-3-glucoside and cyanidin chloride were the most prevalent anthocyanins whereas ferulic and caffeic acids were the predominant phenols. Epicatechin was the most abundant flavonoid, while quercetin,

naringenin and kaempferol were also detected. Significant antioxidant activities were observed in pericarp extracts of five selected varieties in four different antioxidant assays. Histological analysis confirmed the concentration of pigments in corn pericarp and the variety with the highest polyphenolic content was observed to have the thickest pericarp. Conventionally adapted and midwestern-grown colored corn varieties with higher amounts of value-added bioactive compounds in pericarp can be potentially valorized for diverse agro-industrial applications and can supplement the lower economic value of yellow dent corn.

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# Chapter 3

## Ultrasound-assisted extraction of polyphenols from purple corn pericarp in deep eutectic solvents

### 3.1 Introduction

Corn (*Zea mays* L.) domestication evidence dates to 2500 BCE. Initially domesticated in Southern Mexico, corn is now widely grown across the world (García-Lara & Serna-Saldivar, 2018). United States is the largest producer of corn followed by China and Brazil (World Grain, 2023). In 2022, an estimated 89.5 million acres of farmland was planted with corn in the US resulting in an overall production of 13.7 billion bushels. Most of the acreage is under yellow dent corn, which has been traditionally utilized by the corn dry grind, wet milling, and dry milling industries to produce fuel ethanol, animal feed, grits and flour, and hundreds of other industrially important products. However, 35-40% of the annual harvest is converted to ethanol, mainly using the corn dry grind process. Other than ethanol, most of processing byproducts such as distillers dried grains with solubles (DDGS), corn germ oil, and carbon dioxide have relatively lower economic values. Therefore, there have been a longstanding quest for value-addition in the industry. Despite considerable investment and research efforts by the scientific community, there have been few economic breakthroughs.

With the focus of the US corn breeders, growers, and processors directed towards higher yielding and higher starch containing yellow dent corn varieties, the economic potential of polyphenol-rich colored corn cultivars such as purple and blue corn has been largely overlooked. Despite having 8.6 to 6.7% less starch than the standard yellow dent corn (Somavat et al., 2016), colored corns have relatively higher quantities of phytoconstituents and can be economically exploited for their efficient recovery. In addition, due to the well-

documented health benefits associated with anthocyanins and polyphenolic compounds, colored corn cultivars have attracted the interest of scientists in recent years (Lao and Giusti, 2016). For above reasons, breeding practices have been suggested to boost the polyphenolic contents of corn cultivars using different methods, primarily starting with those traditionally grown in South and Central America (Colombo et al., 2021). Purple corn, also known as maiz morado, is a highly pigmented corn mutation grown in lower valleys of Ecuador, Peru, Argentina, and Bolivia (Lao et al., 2017; Lao & Giusti, 2016). Researchers have quantified significant amounts of phytochemicals such as anthocyanins, flavanol-anthocyanins, phenolic acids, and flavanols in colored corn cultivars and their polyphenolic extracts have demonstrated significant biological activities as antioxidant, anti-obesity, anti-cancer, and anti-inflammatory compounds *in-vitro* (Lao and Giusti, 2018; Ramos-Escudero et al., 2012; Somavat et al., 2018).

During the processing of corn kernel, numerous prime and byproducts are produced (Tayal et al., 2020). Pericarp is the outermost coat of the corn kernel and mainly consists of cellulose and hemicellulose. Humans cannot digest pericarp and it is considered a low-value byproduct of the processing industry, often utilized as a ruminant food. However, in case of colored corn varieties, most of the polyphenolic compounds have been identified to be concentrated in corn pericarp (Li et al., 2017; Somavat, 2017). Researchers proposed a novel method involving separation of polyphenol-rich colored corn pericarp at the front end of the process and selective extraction of separated pericarp for the economic recovery of natural red food dye and other value-added phytochemicals. These extracts could then be used as nutraceuticals or starting materials for synthesizing natural food colorants and biologically active food additives (Chen et al., 2017; Li et al., 2017; Li et al., 2019; Somavat, 2017).

Since the starch and protein containing corn endosperm fraction remains unaffected, it can be utilized by the conventional processing industry yielding significant process economic advantages.

Organic solvents are extensively used in industry for the extraction of polyphenolic compounds from various plant matrices due their higher effectiveness. However, the use of potentially harmful organic solvents and huge amounts of water consumed in these extractions is a cause of concern. As a result, there is an increased demand for the identification of environmentally friendly solvents (Ślusarczyk et al., 2023).The most important factor necessitating the development of greener extraction techniques is the fact that the concerned consumers want finished products that have none or minimal potentially hazardous components in them. One approach for increasing the extraction efficiency of polyphenolic compounds such as anthocyanins, tannins, and phenols involves using organic solvents in conjunction with various assisted techniques such as microwave and ultrasound (Mir-Cerdà et al., 2023). The most used extraction solvents are ethanol, methanol, acetone, and hexane. The Code of Federal Regulations (CFR), Title 21 specifies the types of solvents that can used in food and drugs, the amount of maximum solvent residues the finished products can have and assigns the enforcement duties to the Food and Drug Administration (FDA). Included in the list are 1,3-butylene glycol, ethyl acetate, ethylene dichloride, isopropyl alcohol, methyl alcohol, methylene chloride and others. It is very difficult to extract diverse phytochemicals from highly complex plant matrices without using solvents since they accelerate the diffusion rate, help with bond breakage, and have higher specificity for targeted compounds. However, due to their potentially harmful effects on environment and human health, there is an increasing demand of novel, green solvents that are renewable,

have very little or no toxicity, biodegradable, recyclable, possess increased dissolving capacity, have desired specificity, and are economical to use. The quest for ideal solvents that meet all the above criteria is still ongoing.

Water is a green solvent used for extraction, but it is challenging to recycle after use, and pollution of water bodies after discharge is a significant hindrance. Also, some methods using water for extraction require special conditions such as subcritical water extraction requires higher temperature and pressure conditions, increasing the operational costs. Among different green solvents currently researched are ionic liquids and deep eutectic solvents (DES) (Chemat et al., 2019). Ionic liquids are formed when anions or cations are mixed, and they stay in a liquid form at room temperature (Choi & Verpoorte, 2019). These are considered desirable green solvents because of their non-volatile nature, low vapor pressure, lubricating and better hydraulic properties, low viscosity, extended range of solubility, and due to the relative ease of adjusting their acidity levels (Singh & Savoy, 2020). Another important class of solvents gaining popularity are DES. First discovered by Abbott et al., (2001), during their research on finding the reasons behind some salts existing in a liquid state at room temperatures and in their quest for modify the properties of new moisture-stable, Lewis-acidic ionic liquids formed using various metal ions. They discovered that choline chloride (ChCl), when mixed in a set molar ratio with different compounds turned into liquids and the liquid mix had a lower freezing point than the individual freezing points of the constituents. This kickstarted increased research interest in DES. These solvents are defined as binary, ternary, and quaternary salts that form a molten mix at temperatures below their individual melting points when mixed at set molar ratios. The underlying phenomenon involves change in charge by hydrogen bonding between a hydrogen-donating atom and a

hydrogen-accepting atom (Abbott et al., 2004). Most DESs are constituted from nontoxic, biodegradable compounds; thus, they make up excellent green solvents that can be used for research and industry (Choi & Verpoorte, 2019).

Various researchers have reported using DESs for efficiently extracting polyphenolic compounds. For example, 42.4 mg of cyanidin-3,5-diglucoside equivalents/g of grape skin, 149.5 mg, 5.9 mg, and 17.8 mg of quercetin, kaempferol and isorhamnetin equivalent/g of *Flos sophorae*, and 37.0 mg of gallic acid equivalent/g of purple corn cob, respectively, were extracted using DES (Barba et al., 2022; Jeong et al., 2015; Nam et al., 2015). In addition to environmentally friendly solvents, there is an increased scientific interest in novel extraction techniques, that may reduce the amount of solvents used as well as lower the extraction times. To this effect, ultrasound or microwave-based extraction methods are extensively researched as they vastly increase the solvent penetration rate in complex plant matrices. The high-frequency vibrations or cavitations formed by ultrasound causes one or a mix of effects including fragmentation, increased absorption, increased shear force, localized erosion, and increased swelling index, which are responsible for increased extraction of bioactive compounds (Kumar et al., 2021). The ultrasound effect can be variably applied using multiple factors such as wave frequency, ultrasound power, time, temperature, and the ratio of solute to solvent (Dzah et al., 2020).

There is a rich abundance of anthocyanins and other polyphenolic compounds in purple corn. Most of these phytochemicals have been identified in corn pericarp, which can be selectively processed for their economical extraction. To the best of our knowledge, none of the researchers have investigated the use of DESs for extracting purple corn pericarp phenolics.

Furthermore, there is no reported study on the synergistic effects of DESs and ultrasound-assisted extraction for recovering phenolics (anthocyanins, flavonoids, and condensed tannins) from purple corn pericarp. Therefore, the aim of this research was to formulate, and characterize various DES combinations and to evaluate them in conjunction with ultrasound-assisted extraction technique for their potential to extract bioactive compounds from purple corn pericarp.

## 3.2 Materials and methods

### 3.2.1 Materials

Purple corn was procured from Woodland Foods (Waukegan, IL). Corn was stored in a freezer at -4 °C and pericarp was separated from the kernels using a lab scale dry milling protocol proposed by Rausch et al., (2009). Separated pericarp was powdered using a Hamilton Beach coffee grinder, sieved through a 500 μm pore size mesh for homogeneity, packed in low density polythene bags and stored in a freezer at -4 °C until analyzed. Choline chloride >98%, 1,4-butanediol 99%, 1,3-butanediol 99%, lactic acid 90%, DL-malic acid, glycerol 99+%, sodium acetate, ethylene glycol 99+%, citric acid 99%, levulinic acid 98%, and glucose 99.5+% were purchased from Sigma-Aldrich (St. Louis, MO). All the standards for anthocyanins (cyanidin-3-glucoside, cyanidin chloride, delphinidin, malvidin and peonidin), flavonoids (epicatechin, kaempferol, morin, naringin and quercetin), phenolic acids (caffeic acid, chlorogenic acid, ferulic acid, gallic acid, and hesperidin) and other chemicals used for HPLC, spectrophotometric, and antioxidant analysis were bought from Sigma-Aldrich (St. Louis, MO). The deionized water used for all other experiments was sourced from Milli-Q H2O purification system (Millipore®, MA, USA). Other reagents and

solvents used in the experiments were of analytical grades and did not undergo any purification prior to usage.

### 3.2.2 Preparation of DES

A total of 19 different DES combinations were prepared by using a modified heating and stirring method described by Fu et al., (2021). Different constituents of DES combinations and the ratios of individual components is depicted in Table 3.1. Constituents combined at specific ratios were put in a sealed glass tube with a stir bar and placed in a heated oil bath set at 80 °C and the mix was stirred continuously. The time taken by various DES mixtures to convert to a transparent liquid state varied from 30 minutes to an hour. Upon attaining a transparent liquid state, the DES solvents were considered to be formulated and stored at 4 °C until further use. The first three solvent combinations containing 1,3-butanediol and 1,4-butanediol were found to be unstable. Although they attained a clear liquid state after heating, however with time, the constituents precipitated and thus those combinations were excluded from the later study. Choline chloride has a quaternary structure and is considered safe due to its presence in plants and animals, was the most prevalent constituent of prepared DES combinations. It can accept two hydrogen atoms and acts as a hydrogen bond acceptor (HBA) in DES. The heating and stirring method was found to be well suited to the constituents in this study as they were in a liquid state (lactic acid, glycerol, ethylene glycol, 1,3-butanediol), and a temperature of 80 °C was chosen to melt the constituents as described by Ali et al., (2019). Various research groups have reported using similar combinations for the extraction of anthocyanins and polyphenols from different plants and plant parts (Alam et al., 2021; Bajkacz & Adamek, 2018; Hammond et al., 2017; Pal & Jadeja, 2020; Wang et al., 2019; L. H. Xu et al., 2021; Zannou et al., 2020).

**Table 3.1.** Chemical composition and molar ratios of different DES combinations evaluated in this study

| Solvent | HBD | HBA | Component | Ratio | References |
|---|---|---|---|---|---|
| DES 1 | 1,3-Butanediol | ChCl | | 2:1 | (Wang et al., 2019) |
| DES 2 | 1,3-Butanediol | ChCl | | 3:1 | - |
| DES 3 | 1,4-Butanediol | ChCl | | 2:1 | - |
| DES 4 | 1,4-Butanediol | ChCl | | 3:1 | - |
| DES 5 | Lactic acid | Glucose | Water | 5:1:3 | (Xu et al., 2021) |
| DES 6 | Lactic acid | ChCl | | 1:1 | (Bajkacz & Adamek, 2018) |
| DES 7 | Lactic acid | Sodium acetate | Water | 3:1:4 | (Pal & Jadeja, 2020) |
| DES 8 | Malic acid | ChCl | Water | 1:1:2 | (Hammond et al., 2017) |
| DES 9 | Glycerol | ChCl | | 2:1 | (Silva et al., 2020) |
| DES 10 | Glycerol | ChCl | | 3:1 | - |
| DES 11 | Ethylene glycol | ChCl | | 2:1 | (Zannou & Koca, 2022) |
| DES 12 | Glycerol | ChCl | | 3:2 | (Silva et al., 2020) |
| DES 13 | Glycerol | Lactic acid | | 2:1 | (Velásquez et al., 2021) |
| DES 14 | Glycerol | Citric acid | | 4:1 | (Silva et al., 2020) |
| DES 15 | Levulinic acid | ChCl | | 2:1 | (Xu et al., 2019) |
| DES 16 | Levulinic acid | ChCl | | 3:1 | - |
| DES 17 | ChCl | Glycerol | Citric acid | 1:4:1 | (Silva et al., 2020) |
| DES 18 | 1,3-Butanediol | ChCl | | 4:1 | (Alam et al., 2021) |
| DES 19 | 1,4-Butanediol | ChCl | | 4:1 | - |

### 3.2.3 Infrared spectrum measurement

Various DES combinations were analyzed using ATR-FTIR spectrometer (Nicolet 380, Thermo Scientific, USA) for confirming the formulation of desired chemical bonds. The solvents were tested at a spectral resolution of 4 $cm^{-1}$ between 4000 and 400 $cm^{-1}$ at a rate of 32 scans per minute. Following the collection of all the spectra, the data were evaluated utilizing Omnic software (OMNIC™ Series, Thermo Scientific, USA).

### 3.2.4 Solvatochromic parameters measurement

The polarity of the different solvent mixtures was measured using Nile red (Jeong et al., 2017). Nile red was used as a solvatochromic probe, and a stock solution of 0.5 mg $mL^{-1}$ in methanol was prepared and stored at 4 °C. Later the stock solution was mixed with the DES in a ratio of

1:10,000, and the maximum wavelength was noted between a range of 400 to 800 nm using a microplate reading spectrophotometer (Multiskan Sky, Thermo Scientific, USA). The polarity was expressed as molar transition energy ($E_T$ (NR)) according to Eq. (1)

$$E_T\ (\mathrm{NR}) = \mathrm{h} \times \mathrm{c} \times v_{max} \times \mathrm{N}_A = \frac{28591}{\lambda_{max}}$$

Where h denotes the Planck constant, the velocity of light is denoted by c, $v_{max}$ stands for the highest number of wave absorbance, $N_A$ is the Avogadro constant, and $\boldsymbol{\lambda}_{max}$ is the absorption spectra of the dye.

### 3.2.5 Measurement of various physical properties

Viscosity was measured using a Rheometer (Kinexus Pro; NETZZSCH, Selb, Germany) fitted with plate geometry diameter of 20 mm (PU20 SR1740 SS). Each solvent was analyzed on a temperature ramp from 20 °C to 50 °C at a rate of 5 °C $min^{-1}$. Uniform gap of 1 mm was maintained and a constant shear rate of 10 $s^{-1}$ was used (Castro et al., 2018).
The pH of DES was measured using a Thermo Scientific Orion Star A211 pH analyzer (Thermo Scientific, USA) which was calibrated using standard solutions of pH 4.01 and 7.00 (Alrugaibah et al., 2021).

### 3.2.6 Polyphenol extraction

Prepared DES solution was mixed with pericarp powder in a 50 mL centrifuge tube and placed in a circulating water bath set at 50 °C (Thermofisher scientific, USA). Ultrasonic probe (Sonic dismembrator, model FB505, Fisher scientific, USA) with a diameter of 9 mm was inserted in this centrifuge tube such that the tip of the probe was immersed in the liquid. Solid to solvent ratio of 1:50 was used and sonication was performed for 10 minutes with an amplitude of 30% and a pulse duration of 10 seconds on and 2 seconds off. After 10 min, 5

mL of water was added, and the extract was centrifuged at 4500 rpm for 10 min at 4 °C (Allegra X-15R, Beckman Coulter, Inc., USA). Later the extract was vacuum filtered using a Fisher brand grade P4 filter paper and the filtered extract was used for further analysis.

### 3.2.7 Total monomeric anthocyanin content

The total monomeric anthocyanin content of the extracts was measured using a pH differential method (Lee et al., 2005) Two pH buffers, pH 1.0 (0.25 M KCl) and pH 4.5 (0.40 M sodium acetate), were poured into separate centrifuge tubes to dilute the extract in a ratio of 1:20 and then vortexed. Afterwards, 200 μL sample was pipetted into a 96-well plate, and absorbance was read at 520 and 700 nm using a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Scientific, USA). The results were calculated according to the following formula and presented as mg of cyanidin-3-glucoside equivalent per g of pericarp:

Total monomeric anthocyanins (mg/g) = A * MW * D * 1000) / (Ɛ * PL * 0.45)

here: A = (A520 – A700) at pH 1.0 – (A520 – A700) at pH 4.5; MW = 449.2 g/mol for C3G; D = dilution factor; PL = constant path length 1 cm; Ɛ = 26,900 L/mol.cm which is the molar extinction coefficient for C3G, 1000 as a conversion factor from grams to milligrams, and 0.45 as the conversion factor from the established method to the plate reader method.

### 3.2.8 Total condensed tannins

Also known as proanthocyanidins, condensed tannins were calculated according to a previously described method (Boateng & Yang, 2021a) wherein vanillin was condensed onto a phloroglucinol nucleus which was catalyzed by $H_2SO_4$. Firstly, catechin or extract (200 μL) was taken in a centrifuge tube followed by the addition of 1 mL of freshly prepared vanillin in methanol (3% w/v). Afterwards, 1 mL of $H_2SO_4$/methanol (30% v/v) was added, the mixture was vortexed and placed in a water bath for 20 min at 30 °C. After that absorbance

was read at 500 nm in a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Scientific, USA) using 96-well plates. The standard curve was applied to determine the CT and reported as mg of epicatechin (EE) equivalent/g of pericarp.

### 3.2.9 Total flavonoids content

A modified method reported by Boateng et al., (2021) was used to measure total flavonoids content. Briefly, in a 500 µL sample of extract or catechin standard in a centrifuge tube, 2 mL distilled water was added, which was followed by an addition of 150 µL of $NaNO_2$ (5% w/v). After waiting for 6 min, 150 µL of $AlCl_3.6H_2O$ (10% w/v) was added and the mixture was kept for 6 min at room temperature. Later, 2 mL of NaOH (4% w/v) was added. The total volume was made 5 mL by adding distilled water, and after waiting for 15 min, the mixture was vortexed and 200 µL sample was pipetted in 96-well plates, and absorbance was read at 510 nm using a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Scientific, USA). Total flavonoid content was calculated using the absorbance at 510 nm and expressed as mg catechin (CE) equivalent/g of pericarp.

### 3.2.10 HPLC analysis for anthocyanins, phenolic acids, and flavonoid profile

The HPLC analysis for various polyphenols was performed using an Agilent 1200 series instrument coupled with DAD and a C18 column (5 µm, 250 mm × 4.6 mm, Avantor®, USA). The vacuum-filtered original extract was diluted thrice and then filtered using a 0.22 µm nylon syringe filter.

For anthocyanin quantification, a modified method described by Oh et al., (2008) was applied. Two mobile phases used were A (5% formic acid in water) and B (5% formic acid in acetonitrile), which were run through the stationary phase at a gradient of 5% B (5 min); 100% B (35 min); 100% B (40 min); 5% B (45 min) at an injection volume of 10 µL and a

flow rate of 1 mL/min at a column temperature of 35 °C. Anthocyanin standards of cyanidin-3-glucoside, delphinidin, cyanidin chloride, peonidin chloride, malvidin chloride, and pelargonidin chloride were used, and the peaks were measured at a wavelength of 520 nm. Phenolic acid profile was studied using mobile phase A (0.1% acetic acid in water) and mobile phase B (0.1% acetic acid in methanol), which were run at a variable gradient of 5% B (0 min); 20% B (15 min); 40% B (35 min); 65% B (42 min); 80% B (50 min); 5% B (52 min); and 5% B (60 min) at a constant flow rate of 1 mL/min, constant temperature of 25 °C and autosampler injection volume of 10 µL (Kandil et al., 2012). Extracts were compared against gallic acid, chlorogenic acid, caffeic acid, ferulic acid, and hesperidin standards using multiple wavelengths of 254, 280 and 360 nm.

For flavonoid characterization, epicatechin, morin, quercetin, naringin and kaempferol standards were baseline separated at wavelengths of 261, 280, 320 and 360 nm using mobile phases A (water at pH 2.8) and B (100% acetonitrile) with a gradient flow of mobile phase as 10% B (5 min); 23% B (31 min); and 35% B (43 min). Finally, the column was washed (100% B, 4 min) and equilibrated (100% A, 3 min). This was carried out at a constant flow rate of 1 mL/min, injection volume of 5 µL, at a constant column temperature of 25 °C. All the values were calculated based on the standard curves and reported as mg/g equivalent of the respective compounds in purple corn pericarp.

### 3.2.11 Antioxidant activity measurement

#### 3.2.11.1 DPPH scavenging capacity

DPPH (1,2-diphenylpicrylhydrazyl) working solution was prepared by mixing 5 mg of DPPH in 100 mL methanol. The mix was kept in dark for two hours, and the absorbance value was verified to be within a range of 0.702 to 0.715, as discussed by Boateng & Yang, (2021b).

Later, the extract or Trolox was mixed with DPPH in a ratio of 1:4 and incubated for 30 min in dark. Spectrophotometric evaluation was done at 520 nm. Results were expressed in terms of mg Trolox equivalent (TE)/g of pericarp.

#### 3.2.11.2 ABTS scavenging capacity

A previously reported method was used for ABTS scavenging assay (Boateng and Yang, 2021). The stock solution for the assay was prepared by mixing ABTS or 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (7 mM, 10 mL) and 166 µL of 140 mmol $K_2S_2O_8$ and the solution was let to react overnight in dark. Following the method used by Boateng & Yang, (2021), a working solution was prepared by adding 1.2 mL of this mother solution in 60 mL of deionized water (DI) water and the pH was adjusted to 0.700 ± 0.004. Next, the extract or Trolox were mixed to this working solution in a ratio of 1:9 and the solution was let to react for 10 min in dark followed by absorbance measurements at 734 nm. The ABTS scavenging ability was expressed in terms of mg TE/g of pericarp.

#### 3.2.11.3 Cupric ion-reducing antioxidant capacity (CUPRAC) assay

Following a previous described method, the working solution was prepared by mixing neocuproine (7.5 mM), $H_2O$, $CuCl_2$ (10 mM), and $NH_4Ac$ (1 M) in equal proportions and kept at 25 °C for 60 min (Boateng & Yang, 2021). The working solution (800 µL) was mixed with 20 µL of extract or Trolox and incubated for 60 minutes at 25 °C followed by absorbance measurements at 450 nm. The results were expressed as mg TE/g of pericarp.

### 3.2.12 Statistical analysis

All the experiments were conducted in triplicates and results were presented as mean ± standard deviation. The software used for statistical purposes was Minitab version 18 (Minitab Inc. State College, Pennsylvania, USA). Tukey's test was run to compare means at

$p < 0.05$. Diagrams were prepared using Origin Pro 9.1 software (Origin Lab Inc., Northampton, Massachusetts, USA).

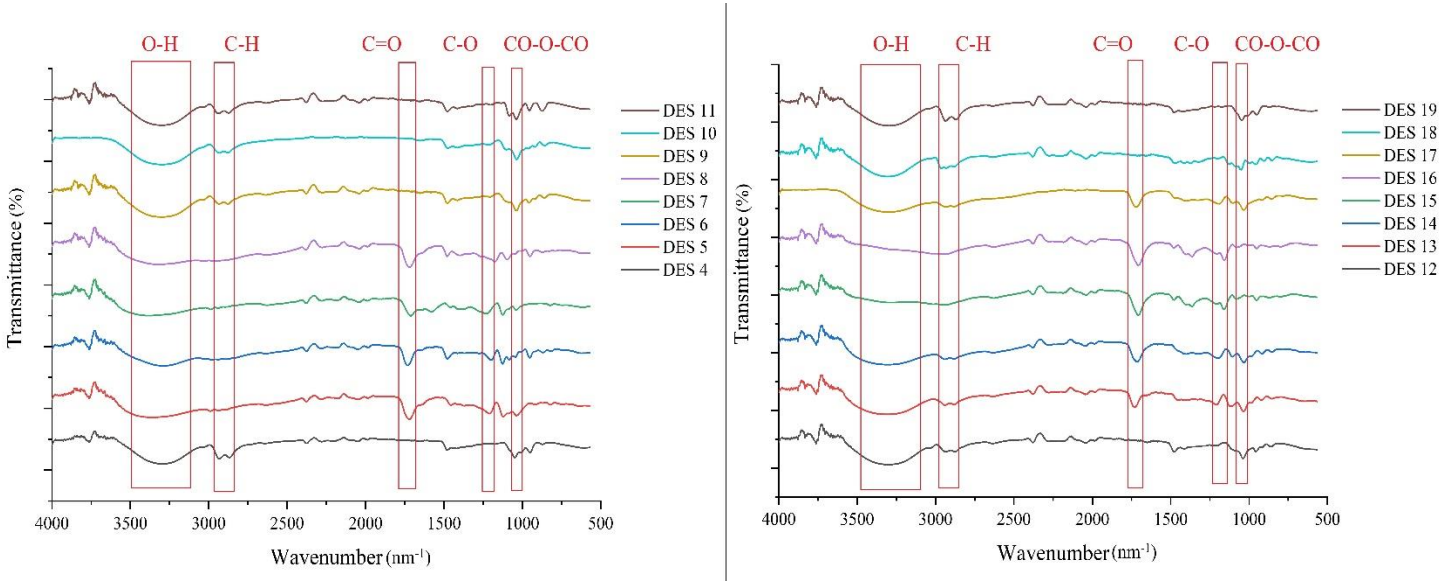
### 3.3 Results and discussion

#### 3.3.1 Characterization of DES

Prepared DESs were scanned using a FT-IR spectrometer, and the collected IR spectra were analyzed to check whether the O-H, C–O, C–C, and other bonds were formed in the solutions and whether there were some underlying differences in the formulations. In general, the spectra collected from most of the DES were similar with significant peaks occurring at 866, 952, 1040, 1120, 1160, 1240, 1370, 1450, 1710, 2870, 2950, and 3310 $cm^{-1}$, respectively (Fig. 3.1). Starting from the functional group region of the IR spectrum (>1500 $cm^{-1}$) in the wavenumber range of 3200–3550 $cm^{-1}$, the distinctively strong O-H stretching, and broad vibrations were observed in solvents which implied the presence of intermolecular O-H bonds, formed between ChCl and the hydrogen donor compounds, that might be an acid or an alcohol. All the solvents had these O-H stretching bonds present; however, some did not have very prominent intermolecular bonds and showed less transmittance. These were mainly the solvents prepared using levulinic acid as a hydrogen bond donor (HBD), which could be due to the protons of the hydroxyl group in ChCl interacted with the carbonyl group of levulinic acid, and the resulting bonds were shifted or were not that strong (Gajardo-Parra et al., 2019). An observation of C-H stretching medium vibrations suggest the presence of alkanes or C-C since these were present in the range of 2840–3000 $cm^{-1}$. Next was the intense peak at 1720 $cm^{-1}$, which corresponds to C=O stretching. These were absent in the DES solutions prepared using alcohols as HBDs and ChCl as HBA. These observations were in agreement with studies by Jurić et al., (2021) and Yue et al., (2022), and the observed IR

spectrums resembled the results reported in their studies. In fingerprint region (<1500 $cm^{-1}$), the spectrums represented C–H bending and O–H bending with small sharp bands followed by medium bands of O–H bending in the 1310–1390 $cm^{-1}$ range. Solvents prepared using lactic acid, citric acid, and levulinic acid, including DES 5, 6, 7, 13, 14, 15, 16, and 17, showed C–O stretching bands at wavenumbers from 1200–1275 $cm^{-1}$. Furthermore, a prominent CO–O–CO stretching band was present at 1040 $cm^{-1}$, and C=C bending absorption bands were also observed in the 790–995 $cm^{-1}$. Following these were alkyl halides with Br and Cl identified in range of 600–400 $cm^{-1}$ (AlOmar, 2016; Smith, 2018; Millipore sigma, 2023).

**Fig. 3.1.** Graph depicting transmittance in DES combinations during FTIR analysis




Note: Major bonds are highlighted in red boxes with the bonds identified on the top.

Various physicochemical characteristics of DES combinations are presented in Table 3.2. Firstly, the solvent viscosities were measured. According to Dai et al., (2016), viscosity plays an important part in an extraction procedure as the lower is the solvent viscosity, the higher is the mass transfer into the particle matrix, and more extraction is possible. According to Abbott, (2004) the correlation of viscosity with extraction process is based on the phenomenon that ions can transfer easily through the holes in the solvent at lower viscosity and vice versa, thus providing better conductivity. Another factor that plays a role is higher degree of hydrogen bonding which results in higher surface tension, making the DES interact strongly with the plant matrix immersed in it and boosting the solubilization of the plant parts, thus increasing the extraction (Huang et al., 2022). The initial viscosities of various DES at 20 $^{\circ}$C ranged from 85 mPa·s to 16800 mPa·s and gradually decreased when the temperature was ramped to 50 $^{\circ}$C at a linear rate (Fig. 3.2). The final viscosities of the solvents at 50 $^{\circ}$C decreased experienced decreases ranging 3.5 times to 26 times, with the lowest and highest values being 7.58 mPa·s (DES 11) and 746.20 mPa·s (DES 14), respectively. Water could be added to lower the viscosities of DES combinations, but that could result in breakage of hydrogen bonds, thereby decreasing the extraction efficiency. Therefore, water was only added to the solvents after extraction so that filtering was easier, and the extracted polyphenols could dissolve properly. In this study, all the extractions were carried out at 50 $^{\circ}$C, therefore the DES viscosities were lower than what it would be at room temperature.

**Fig. 3.2.** Graph for viscosities of different DES combinations with temperature ramp

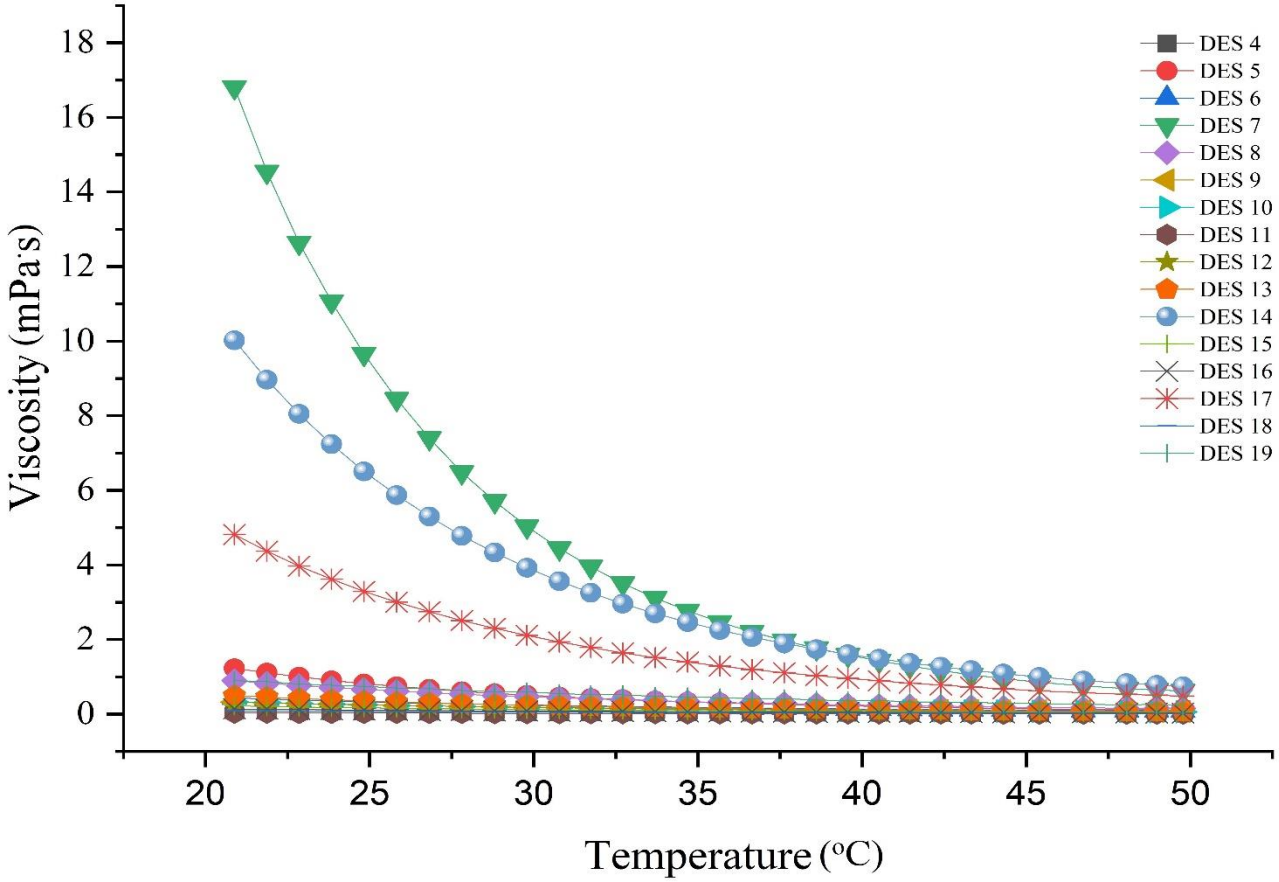


**Table 3.2.** Physicochemical properties of various DES combinations

| Solvent | Polarity (kcal/mol) | pH | Viscosity at 50 °C (Pa.s) |
|---|---|---|---|
| **DES 4** | 50.63 ± 0.08a | 4.84 ± 0.09b | 0.0242 ± 0.00hi |
| **DES 5** | 46.80 ± 1.55f | -0.29 ± 0.04h | 0.1092 ± 0.00ef |
| **DES 6** | 48.21 ± 0.00def | 1.03 ± 0.03e | 0.0868 ± 0.00fg |
| **DES 7** | 49.64 ± 0.00abcd | 5.39 ± 0.04a | 0.6246 ± 0.02b |
| **DES 8** | 47.86 ± 0.04ef | -0.11 ± 0.01gh | 0.1537 ± 0.02e |
| **DES 9** | 49.41 ± 0.04abcd | 4.32 ± 0.04c | 0.0671 ± 0.00fghi |
| **DES 10** | 49.64 ± 0.00abcd | 4.77 ± 0.02b | 0.0627 ± 0.01fghi |
| **DES 11** | 50.31 ± 0.04ab | 5.33 ± 0.07a | 0.0085 ± 0.00i |
| **DES 12** | 49.96 ± 0.04abc | 4.72 ± 0.09b | 0.0674 ± 0.01fgh |
| **DES 13** | 48.40 ± 0.04de | 0.91 ± 0.02e | 0.0630 ± 0.00fghi |
| **DES 14** | 47.78 ± 0.04ef | 0.09 ± 0.02g | 0.7463 ± 0.04a |
| **DES 15** | 48.87 ± 0.00bcde | 1.03 ± 0.01e | 0.0465 ± 0.00ghi |
| **DES 16** | 48.54 ± 0.00cde | 0.56 ± 0.03f | 0.0311 ± 0.00ghi |
| **DES 17** | 47.70 ± 0.08ef | 0.10 ± 0.03g | 0.4860 ± 0.06c |
| **DES 18** | 50.63 ± 0.04a | 4.38 ± 0.05c | 0.0209 ± 0.00hi |
| **DES 19** | 50.37 ± 0.04a | 3.73 ± 0.22d | 0.2352 ± 0.01d |

Different lowercase letters in the same column indicate statistical differences ($p < 0.05$).

According to Craveiro et al., (2016), addition of water breaks the network of hydrogen bonds, causing the overall polarity of DES to decrease; hence the solvation capability of the solvent also decreases. However, Jeong et al., (2017) and Dai et al., (2016) reported that the polarity of the solvent did not have any effect on the extraction of compounds. In prepared DES samples, the Et (NR) values for polarity ranged from 50.63 ± 0.08 to 46.8 ± 1.55 kcal.mol$^{-1}$ with a difference of 3.83 kcal.mol$^{-1}$ (Table 3.2). Comparison of polarities of DES from other studies is difficult since the polarity scales are probe-dependent; thus, different solvatochromic probes may yield different polarity values (El Achkar et al., 2021). Most of the formulated DES combinations were observed to be acidic or highly acidic (Table 3.3). Their pH ranged from -0.11 ± 0.01 (DES 8) at the higher end of the acidic scale to 5.39 ± 0.04 (DES 7), a value closer to neutral. Overall, the pH values of prepared DES were in accordance with those reported by Alrugaibah et al., (2021). A lower pH value translates to

an abundance of $H^+$ ions present in the solvent. Since there was an abundance of flavylium cations in the extracts, anthocyanins and their derivatives were more stable at this lower pH (Deng et al., 2018).

### 3.3.2 Total monomeric anthocyanin content

Researchers have reported that most of the purple corn phenolics including anthocyanins, phenolic acids, flavonoids, and condensed tannins are concentrated in corn pericarp (Li et al., 2017; Somavat, 2017). Approximately 45.9% of the total anthocyanins contained in the whole purple corn kernel were quantified in pericarp fraction separated using lab scale corn dry milling. As far as separated pericarp fraction is concerned, a total anthocyanin content of 26.8 ± 0.06 g C3G equivalent per kg of purple corn pericarp was identified (Li et al., 2017). This amount was reported as a sum of three sequential extractions performed on ground pericarp sample. The first extraction cycle involved addition of 0.5 g pericarp in 20 mL of 2% aqueous formic acid and the supernatant was collected after continuous stirring at room temperature for 2 hr. Following supernatant recovery using a filter paper, the pericarp sample was again extracted in 2% aqueous formic acid for a further 2 hr with stirring. Following the second supernatant recovery, final extraction on pericarp sample was done in an aqueous ethanolic solution (20% v/v), acidified with 2% formic acid (Li et al., 2017). In this study, by using the combination of different DESs and 10 min ultrasound treatment, mean anthocyanin concentration of 22.12 g C3G equivalent per kg of pericarp was quantified. Various DES combinations could extract different amounts of anthocyanins ranging from 8.51 ± 0.88 g for DES 15 to 27.41 ± 0.74 g of C3G equivalent per kg of pericarp for DES 11, respectively, shown in Table 3.3. On the other hand, the combination of DI water and 10 min ultrasound treatment resulted in quantification of 22.79 ± 0.38 g C3G equivalent/kg of pericarp whereas

the combination of ethanol and water (50:50) followed by 10 min ultrasound treatment extracted 17.57 ± 0.89 g of C3G equivalent per kg of pericarp. In general, the solvents having alcohols as HBD were observed to be having higher extraction efficiencies compared to others with acids as HBD. The most efficient DES combinations for anthocyanin extraction contained glycerol, ethylene glycol, and 1,3 and 1,4-butanediol as HBD's. On similar lines, the aqueous ethanolic solvent combination also yielded higher amounts of anthocyanins. The differences were observed to be insignificant among alcohol-based DES combinations.

**Table 3.3.** Total anthocyanin content (TA), Total condensed tannins (CT), Total flavonoids content (TFC), and corresponding antioxidants activities (DPPH, ABTS, and CUPRAC assays) of purple corn pericarp extracts recovered using different DES

| Solvent | TA (mg C3G/g) | CT (mg EE/g) | TFC (mg CE/g) | DPPH (mg TE/g) | ABTS (mg TE/g) | CUPRAC (mg TE/g) |
|---|---|---|---|---|---|---|
| **DES 4** | 24.95 ± 0.47abc | 220.76 ± 20.89ab | 32.27 ± 1.16b | 68.55 ± 2.08h | 34.96 ± 0.07a | 140.38 ± 8.82gh |
| **DES 5** | 26.19 ± 1.73ab | 222.19 ± 47.37ab | 26.95 ± 4.22b | 66.55 ± 0.41h | 30.62 ± 0.06h | 146.86 ± 12.86fgh |
| **DES 6** | 16.61 ± 3.44def | 200.94 ± 23.80abc | 27.13 ± 4.18b | 78.94 ± 0.67bcde | 32.32 ± 0.17f | 197.26 ± 5.89cde |
| **DES 7** | 23.23 ± 3.58abcd | 249.33 ± 28.57a | 25.65 ± 1.81b | 80.45 ± 0.46ab | 34.52 ± 0.06c | 114.43 ± 10.28h |
| **DES 8** | 23.46 ± 3.55abcd | 226.88 ± 4.12ab | 34.55 ± 2.11b | 79.44 ± 0.39bcd | 32.04 ± 0.03fg | 271.73 ± 11.02b |
| **DES 9** | 23.22 ± 0.81abcd | 222.22 ± 17.28ab | 36.87 ± 2.06b | 68.99 ± 0.31h | 34.84 ± 0.03abc | 163.62 ± 12.77efg |
| **DES 10** | 24.03 ± 0.53abcd | 212.51 ± 17.01ab | 34.57 ± 2.29b | 72.12 ± 0.23g | 34.82 ± 0.03abc | 150.91 ± 6.99fgh |
| **DES 11** | 27.41 ± 0.74a | 242.93 ± 25.81a | 34.23 ± 2.11b | 74.31 ± 0.87fg | 34.93 ± 0.15ab | 232.81 ± 17.58bc |
| **DES 12** | 23.08 ± 0.78abcd | 204.30 ± 18.07abc | 35.00 ± 4.24b | 60.85 ± 0.62i | 34.61 ± 0.03bc | 220.38 ± 14.92cd |
| **DES 13** | 27.41 ± 3.22a | 240.37 ± 24.23a | 25.52 ± 2.00b | 77.82 ± 0.27cde | 32.71 ± 0.06e | 392.27 ± 21.80a |
| **DES 14** | 24.94 ± 3.26abc | 224.01 ± 23.78ab | 24.81 ± 2.17b | 76.38 ± 0.32ef | 31.97 ± 0.13g | 140.38 ± 4.26gh |
| **DES 15** | 8.51 ± 0.88f | 146.13 ± 7.57bc | 29.54 ± 1.78b | 77.38 ± 0.09de | 33.27 ± 0.00d | 216.18 ± 5.96cd |
| **DES 16** | 10.91 ± 1.63ef | 162.94 ± 17.71abc | 27.54 ± 7.91b | 76.44 ± 0.18ef | 33.10 ± 0.17d | 165.09 ± 1.01efg |
| **DES 17** | 18.34 ± 3.9bcde | 230.49 ± 35.52ab | 30.06 ± 2.32b | 80.20 ± 0.49abc | 32.94 ± 0.03de | 181.46 ± 3.50def |
| **DES 18** | 25.77 ± 1.71abc | 209.09 ± 34.14ab | 34.20 ± 2.18b | 82.70 ± 0.70a | 34.87 ± 0.00ab | 200.64 ± 6.08cde |
| **DES 19** | 25.88 ± 1.46ab | 214.89 ± 18.70ab | 35.39 ± 5.22b | 81.51 ± 0.62ab | 34.70 ± 0.03abc | 168.34 ± 6.01efg |
| **Water** | 22.79 ± 0.38abcd | 197.84 ± 2.61abc | 54.97 ± 0.32a | 74.56 ± 0.72fg | 34.75 ± 0.12abc | 169.57 ± 12.01efg |
| **EtOH** | 17.57 ± 0.89cde | 120.60 ± 2.81c | 29.17 ± 1.71b | 67.24 ± 0.62h | 34.91 ± 0.14ab | 63.61 ± 2.07i |

Different lowercase letters in the same column indicate statistical differences ($p < 0.05$).

### 3.3.3 Total Condensed tannins content

Condensed tannins are classified as a distinct group within the broad category of polyphenols and demonstrate significant antimicrobial and antioxidant activities (Peng et al., 2018). Tannins also reportedly help in stabilizing colors by reacting with anthocyanins through co-pigmentation or condensation reactions (Li et al., 2017). Using the combination of DI water and ultrasound, 197.84 ± 2.61 g of epicatechin (EE) equivalent/kg of pericarp were quantified, whereas the ethanol and water blend (50:50) and ultrasound treatment resulted in a lower extraction, the value being 120.60 ± 2.81 g of EE/kg of pericarp (Table 3.3). Under similar extraction conditions, the DES combinations extracted a mean of 214.37 g of EE/kg of pericarp. For the evaluated DES combinations, quantified values of total condensed tannins ranged between 146.12 ± 7.57 g (DES 15) to 249.33 ± 28.57 g (DES 7) of EE/kg of pericarp, respectively. The DES formed using levulinic acid were observed to have extracted the lowest amounts, the values being 146.12 ± 7.57 g of EE/kg of pericarp (DES 15) and 162.94 ± 17.71 g of EE/kg of pericarp (DES 16), respectively. There have been no prior studies concerning the extraction of condensed tannins from colored corn varieties using DES. Boateng et al., (2023) reported an optimized microwave-assisted extraction experiment which yielded almost 279.48 g of EE/kg purple corn pericarp. In general, the purple corn pericarp has been reported to contain very high amounts of condensed tannins and similarly higher amounts of condensed tannins were quantified in current study with the most efficient DES combinations (Li et al., 2017; Li et al., 2019).

### 3.3.4 Total flavonoids content

The flavonoid family consists of a larger group of phenolics that also includes anthocyanins, however, they have been evaluated separately in purple corn varieties due to their relatively

higher anthocyanin concentrations (Lao et al., 2017). The highest extraction of flavonoids using DES was observed in DES 9, a formulation containing glycerol and ChCl, and the quantified amount being 36.87 ± 2.06 g catechin equivalent/kg of pericarp (Table 3.3), while the lowest concentration (24.81 ± 2.17 g CE/kg of pericarp) was quantified in DES 14, which contained glycerol and citric acid. The mean value of total flavonoids quantified in DES combinations was 30.89 g CE/kg of pericarp. No significant difference was observed among the DES combinations. However, the DES combinations were observed to be less efficient in flavonoid extraction compared to the combination of DI water and ultrasound treatment, wherein 54.97 ± 0.32 g CE/kg of pericarp were quantified. On the other hand, the flavonoid yield was similar to those extracted using a combination of ethanol and water under the same conditions (29.17 ± 1.71 g CE/kg of pericarp). A plausible reason could be higher solubility of purple corn flavonoids in water-based solvents compared to other organic solvents or DES. According to Pérez-Gregorio et al., (2010), flavonoids are made up of glucosides and aglycones that are highly soluble in water and alcohol, respectively. In an earlier study by Li et al., (2019), double extraction of purple corn pericarp in 2% aqueous formic acid solution yielded a mean of 23.00 mg rutin equivalent of flavonoids/g pericarp. The DES solutions used in this study were found to have extracted great amounts of flavonoids in comparison.

### 3.3.5 Identification of anthocyanins, phenolics, and flavonoids

As illustrated in Table 3.4, four major anthocyanin compounds were eluted during HPLC analysis of DES extracts at 520 nm against 6 major anthocyanin compounds which were used as standards. The anthocyanin forms detected were cyanidin-3-glucoside (C3G), delphinidin chloride, cyanidin chloride, and peonidin, and these results agreed with an earlier work on purple corn conducted by Chatham & Juvik, (2021), Ferron et al., (2020) and Suriano et al.,

(2021) wherein C3G was reported to be the most dominant anthocyanin form present in the purple corn. Whereas C3G and cyanidin chloride were detected in all the DES combinations, delphinidin was absent in DES 8, 12, 14, and 17, the solvents consisting of malic acid, citric acid, glycerol and ChCl. Peonidin was only identified in DES 4, 10, 12, 17, and 18, most of which were alcohol-based solvents. The highest amount of C3G was present in DES 13 (20.56 ± 0.31 mg C3G equivalent/g of pericarp), whereas the DES 7 had the largest quantity of delphinidin (9.62 ± 0.31 mg/g of pericarp). However, the aqueous ethanolic solvent extracted the greatest amount of delphinidin, the value being 10.14 ± 0.24 mg/g of pericarp. The largest amount of peonidin was quantified in DES 4 (2.90 ± 0.02 mg/g of pericarp), which was different from other solvents, the values ranging between 2.73 ± 0.02 mg/g of pericarp in DES 10 to 2.82 ± 0.01 mg/g of pericarp in DES 12. It was observed that the extent of recovery of individual anthocyanin forms in the extracts was dependent upon the composition of the solvents.

**Table 3.4.** Anthocyanins quantification of pericarp extracts from various DES through HPLC analysis

| | Anthocyanins (mg/g pericarp) | | | |
|---|---|---|---|---|
| **Solvent** | **C3G** | **Delphinidin** | **Cyanidin chloride** | **Peonidin** |
| **DES 4** | 11.48 ± 1.51ef | 4.98 ± 0.19d | 6.72 ± 0.22f | 2.90 ± 0.02a |
| **DES 5** | 18.42 ± 0.15b | 7.37 ± 0.22b | 3.85 ± 0.00h | ND |
| **DES 6** | 6.24 ± 0.17h | 4.83 ± 0.02d | 3.02 ± 0.02i | ND |
| **DES 7** | 14.27 ± 0.06c | 9.62 ± 0.31a | 9.00 ± 0.11c | ND |
| **DES 8** | 10.21 ± 0.34fg | ND | 8.67 ± 0.06cd | ND |
| **DES 9** | 11.28 ± 0.18ef | 4.87 ± 0.08d | 11.91 ± 0.34a | ND |
| **DES 10** | 12.48 ± 0.17de | 4.55 ± 0.08d | 5.45 ± 0.09g | 2.73 ± 0.02c |
| **DES 11** | 14.15 ± 0.50c | 6.23 ± 0.36c | 5.59 ± 0.03g | ND |
| **DES 12** | 11.89 ± 0.28de | ND | 6.71 ± 0.15f | 2.82 ± 0.01b |
| **DES 13** | 20.56 ± 0.31a | 6.77 ± 0.19bc | 3.90 ± 0.08h | ND |
| **DES 14** | 13.50 ± 0.28cd | ND | 8.76 ± 0.07cd | ND |
| **DES 15** | 2.84 ± 0.10jk | 3.03 ± 0.10e | 7.89 ± 0.08e | ND |
| **DES 16** | 4.10 ± 0.02ij | 3.30 ± 0.01e | 7.97 ± 0.07e | ND |
| **DES 17** | 5.21 ± 0.03hi | ND | 8.17 ± 0.06de | 2.78 ± 0.03bc |
| **DES 18** | 5.31 ± 0.06hi | 4.58 ± 0.12d | 10.41 ± 0.14b | 2.73 ± 0.04c |
| **DES 19** | 6.63 ± 0.30h | 4.40 ± 0.15d | 10.9 ± 0.16b | ND |
| **Water** | 9.39 ± 0.44g | ND | 8.96 ± 0.30c | ND |
| **EtOH** | 1.37 ± 0.16k | 10.14 ± 0.24a | 8.81 ± 0.21c | ND |

Different lowercase letters in the same column indicate statistical differences ($p < 0.05$).

The results from HPLC analysis for phenolics identification and quantification are presented in Table 3.5. Hesperidin and caffeic acid were the phenolic acids detected in most of the solvent combinations. The highest amount of hesperidin eluted was 15.18 ± 1.20 mg/g of pericarp in DES 17, and the greatest amount of caffeic acid was quantified in DES 8 (3.29 ± 0.34 mg/g of pericarp). Relatively smaller amounts of gallic and ferulic acid were observed in only a few DES combinations. Chlorogenic acid was detected only in DES 18, the quantified amount being 5.23 ± 0.10 mg chlorogenic acid/g of the pericarp. These results were in agreement with those reported for colored corn varieties by Harakotr et al., (2014), Hernández et al., (2018), and Zhang et al., (2019), wherein the caffeic and ferulic acids were

reported to be the major phenolic compounds. Solvent composition dependent extraction patterns for individual phenolics were observed.

**Table 3.5.** Phenolics quantification of pericarp extracts from various DES combinations through HPLC analysis

| | Phenolics (mg/g pericarp) | | | | |
|---|---|---|---|---|---|
| **Solvent** | **Caffeic acid** | **Ferulic acid** | **Gallic acid** | **Hesperidin** | **Chlorogenic acid** |
| **DES 4** | ND | 1.72 ± 0.04a | ND | ND | ND |
| **DES 5** | ND | 1.28 ± 0.23b | 0.54 ± 0.06b | ND | ND |
| **DES 6** | 1.39 ± 0.07cd | ND | ND | 3.46 ± 0.51cde | ND |
| **DES 7** | ND | 1.43 ± 0.21ab | ND | ND | ND |
| **DES 8** | 3.29 ± 0.34a | ND | ND | 3.82 ± 0.71cde | ND |
| **DES 9** | ND | ND | ND | 3.74 ± 1.63cde | ND |
| **DES 10** | 1.32 ± 0.08cd | ND | ND | 6.42 ± 0.1bc | ND |
| **DES 11** | ND | ND | ND | ND | ND |
| **DES 12** | ND | ND | 0.63 ± 0.04a | 2.25 ± 1.97de | ND |
| **DES 13** | ND | ND | 0.61 ± 0.02a | 2.42 ± 0.38de | ND |
| **DES 14** | ND | ND | ND | 4.45 ± 0.69cd | ND |
| **DES 15** | 1.11 ± 0.10d | ND | ND | ND | ND |
| **DES 16** | 1.21 ± 0.07d | ND | ND | ND | ND |
| **DES 17** | 1.47 ± 0.46cd | ND | ND | 15.18 ± 1.20a | ND |
| **DES 18** | 2.05 ± 0.08bc | ND | ND | 6.80 ± 0.43bc | 5.23 ± 0.10a |
| **DES 19** | 1.51 ± 0.46cd | ND | ND | 10.16 ± 1.21b | ND |
| **Water** | ND | ND | ND | ND | ND |
| **EtOH** | 2.61 ± 0.02ab | 0.59 ± 0.43c | ND | 6.12 ± 3.23bcd | ND |

Different lowercase letters in the same column indicate statistical differences ($p < 0.05$).

The results from identification and quantification of flavonoids in various solvent combinations using HPLC are summarized in Table 3.6. From their research on colored corn cultivars, Chatham et al., (2018) and González-Manzano et al., (2008), reported catechin or epicatechin as the most dominant flavonoids present in the corn. In addition, naringin and kaempferol contents ranging from 19.93 ± 0.82 mg/g to 5.09 ± 0.09 mg/g of pericarp, and 1.55 ± 0.06 mg/g to 0.82 ± 0.06 mg/g of pericarp, respectively, were reported. In current study, epicatechin was found to be the most dominant and widely identified flavonoid in

evaluated solvents. DES 8 (100.16 ± 2.67 mg/g pericarp) contained the greatest amount of epicatechin whereas the lowest identified amount (23.05 ± 0.92 mg/g pericarp) was in DES 12, while none were detected in DES 15, 16, and aqueous ethanolic solvents. Naringenin was the second most abundant flavonoid identified, with the quantified values ranging between 19.93 ± 0.82 mg/g (DES 5) to 5.09 ± 0.09 mg/g pericarp (EtOH), respectively. Relatively smaller amounts of kaempferol were also identified, with DES 15 (1.55 ± 0.07 mg/g pericarp) having the highest and DES 12 (0.82 ± 0.06 mg/g pericarp) the lowest quantified values, respectively.

**Table 3.6.** Flavonoids quantification of pericarp extracts from various DES through HPLC analysis

| | **Flavonoids (mg/g pericarp)** | | |
|---|---|---|---|
| **Solvent** | **Epicatechin** | **Naringin** | **Kaempferol** |
| **DES 4** | 52.17 ± 2.97c | 5.40 ± 0.20cd | 1.32 ± 0.03ab |
| **DES 5** | 65.13 ± 3.96b | 19.93 ± 0.82a | 1.12 ± 0.01abc |
| **DES 6** | 102.73 ± 1.66a | 5.67 ± 0.08cd | 1.14 ± 0.02abc |
| **DES 7** | 30.12 ± 2.74fg | ND | 1.07 ± 0.02bc |
| **DES 8** | 100.16 ± 2.67a | ND | 0.93 ± 0.01bc |
| **DES 9** | 25.54 ± 0.60fg | ND | ND |
| **DES 10** | 29.30 ± 2.27fg | ND | 0.86 ± 0.05bc |
| **DES 11** | 33.76 ± 0.30ef | ND | 1.21 ± 0.02abc |
| **DES 12** | 23.05 ± 0.92g | ND | 0.82 ± 0.06c |
| **DES 13** | 32.35 ± 0.65ef | ND | 1.12 ± 0.08abc |
| **DES 14** | 40.47 ± 2.90de | ND | 1.05 ± 0.07bc |
| **DES 15** | ND | 6.08 ± 0.16bc | 1.55 ± 0.06a |
| **DES 16** | ND | 6.47 ± 0.08b | 1.30 ± 0.51ab |
| **DES 17** | 52.32 ± 4.68c | 5.95 ± 0.01bc | ND |
| **DES 18** | 43.32 ± 1.27d | 5.37 ± 0.03cd | 0.93 ± 0.02bc |
| **DES 19** | 40.63 ± 0.95de | 5.62 ± 0.09cd | ND |
| **Water** | 8.52 ± 0.40h | ND | ND |
| **EtOH** | ND | 5.09 ± 0.09d | ND |

Different lowercase letters in the same column indicate statistical differences ($p < 0.05$).

In general, solvent dependent extraction of individual anthocyanins, phenolics and flavonoids forms were observed, implying a possibility of utilizing specifically constituted solvent compositions if individual phytochemical recovery is desired. However, an in-depth enquiry into the underlying chemical interactions was beyond the scope of this work.

### 3.3.6 Antioxidant activity of extracts

Polyphenolic compounds possess significant antioxidant activities, and the presence of very high concentrations of these compounds in purple corn pericarp warranted the evaluation of antioxidant activities of extracts. Among various phytochemicals, anthocyanins have been identified to have significant antioxidant activities (Velásquez et al., 2021). Due to their inherent antioxidant activities, phytochemicals have been reported to positively contribute towards a food products' texture, flavor, and color during storage (Gulcin, 2020). In order to evaluate the antioxidant activities of the extracts, DPPH radical scavenging activity, ABTS scavenging activity, and CUPRAC assays were performed, and promising results were obtained, as presented in the Table 3.3. According to Gulcin, (2020), it is not reliable to conclude the antioxidant potential of a biological specimen based on only one test assay. Such assays are generally based either on hydrogen atom transfer (HAT) or single electron transfer (SET) method. The ABTS assay entails both these mechanisms, whereas DPPH and CUPRAC are based on the SET mechanism (Xiao et al., 2020).

The extract from DES 18 (82.7 ± 0.7 mg TE/g of pericarp) demonstrated the highest antioxidant activity in scavenging DPPH free radicals, followed by DES 19, DES 7, and DES 17, the latter falling in the same statistical group with antioxidant activities of 81.51 ± 0.62 mg TE/g, 80.45 ± 0.46 mg TE/g, and 80.2 ± 0.49 mg TE/g of pericarp, respectively (Table 3.3). The lowest DPPH scavenging activity was observed in DES 12 with a Trolox equivalent

of 60.85 ± 0.62 mg/g of pericarp. The DPPH activity observed in water-based and ethanol/water-based solvents was lower compared to their DES counter parts. The overall DPPH radical scavenging ability of purple corn pericarp was found to be similar to those reported in studies on colored corn by Deng et al., (2015) and Polthum et al., (2014). In case of ABTS scavenging activity assay, DES 4 showed the highest antioxidant activity (34.96 ± 0.07 g TE/g pericarp), however, the activity of water and ethanol/water based solvents was similar, and so was the case for DES 9, 10, 11, 18, and 19. The range of antioxidant activity was not great among the solvent combinations; with the highest and lowest activities ranging between 34.96 mg/g and 30.62 mg TE/g of pericarp, respectively. The CUPRAC assay is applicable to both lipophilic and hydrophilic antioxidants, and there are no reactions involving simple sugars and citric acid constituents (Gulcin, 2020). The greatest antioxidant activity in the CUPRAC assay was observed in DES 13 (392.27 ± 21.8 mg TE/g of pericarp) followed by DES 8 (271.73 ± 11.02 mg TE/g of pericarp) while the lowest activities were observed in DES 4 and DES 14 (140.38 ± 8.82 and 140.38 ± 4.26 mg TE/g pericarp, respectively). As was the case in DPPH assay, the water and aqueous ethanolic solvents demonstrated lower CUPRAC activities compared to the DES-based solvents. In general, the purple corn pericarp extracts recovered using DES combinations demonstrated significant antioxidant activities.

### 3.4 Conclusions

In this study, different DES combinations were prepared, characterized, and used in conjunction with ultrasound-assisted extraction technique for extracting polyphenolic compounds from purple corn pericarp. A DI water (100%) and an aqueous ethanolic (50:50) solvent combination was used for comparison purposes. Generally regarded as green

solvents, the DES combinations had relatively higher polarities and demonstrated variable pH, but they were observed to be in acidic ranges that are amenable for polyphenol extraction. At a temperature of 50 ˚C, they displayed lower viscosities, making them suitable for polyphenolic extractions. The hydrogen bond formation in DES combinations were confirmed using FT-IR analysis. The DES combinations, in conjunction with the ultrasound-assisted extraction technique, were able to extract significant amounts of polyphenolic compounds from purple corn pericarp in a relatively lower extraction time of 10 min. The highest amounts of total monomeric anthocyanins, condensed tannins and total flavonoids quantified were 27.41 mg C3G/g (DES 11), 249.33 mg EE/g (DES 7), and 35.39 mg CE/g pericarp (DI water), respectively. During HPLC quantification, various individual forms of anthocyanins, tannins, and flavonoids including C3G, cyanidin chloride, delphinidin, peonidin, caffeic acid, ferulic acid, gallic acid, hesperidin, chlorogenic acid, epicatechin, naringin, and kaempferol were detected. Overall, a solvent dependent extraction of individual anthocyanins, phenolics and flavonoids forms were observed. The polyphenolic extracts demonstrated significant antioxidant activities. In conclusion, DES combinations in conjunction with ultrasound-assisted technique can be used to efficiently extract polyphenols from purple corn pericarp. However, further efforts are required for extraction optimization, identification of methodologies for efficient separation of individual phytochemicals, and in exploring the reuse potential of DESs. Also warranted are extensive toxicological studies of these extracts before their utilization for food applications.

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# Chapter 4

## Conclusions and future recommendations

### 4.1 Conclusions

Corn processing industry in the United States, especially the corn dry grind industry, which processes ~35% of the harvested corn suffers from a lack of value-added processing byproducts. Reduced consumption of gasoline due to the adoption of electric vehicles will progressively decrease corn-ethanol demand for blending. Colored corn varieties contain significant amounts of value-added polyphenolic compounds and may supplement the lower economic value of yellow dent corn. This research work was focused on evaluating the valorization potential of conventionally bred and Missouri grown pigmented corn varieties. Fourteen experimental varieties were dry milled and polyphenolic contents of the kernels and dry milled coproducts were evaluated and compared against a commercially available purple corn variety. Overall aim was to identify polyphenol-rich experimental varieties with desired milling characteristics for subsequent breeding and adaptation cycles. Furthermore, we wanted to test whether environmentally friendly deep eutectic solvents (DES) in conjunction with ultrasound-assisted extraction technique could be used to efficiently extract phytochemicals from purple corn pericarp. The main conclusions of our research are as follows:

1. The evaluated colored corn varieties had a lower average starch content compared to the conventional yellow dent corn, while their mean protein content was higher. Most of the colored varieties had a softer endosperm composition, resulting in lower yields of large grits. Among most of the varieties analyzed, the highest concentrations of polyphenols were identified in corn pericarp, a low-value cellulosic coproduct of the

processing industry. Different anthocyanin, phenol, and flavonoid forms were identified through HPLC analysis, and significant antioxidant activities of colored corn phytochemicals were observed. Midwestern grown and conventionally bred colored corn varieties have an excellent potential for the valorization of value-added phytochemicals with diverse agro-industrial applications.

2. Different deep eutectic solvent (DES) combinations were formulated, characterized, and used in conjunction with ultrasound-assisted extraction technique to extract polyphenolic compounds from purple corn pericarp. The DES combinations exhibited higher polarities and acidic pH ranges, implying their suitability for polyphenol extraction. These formulations displayed lower viscosities at 50 °C, a desirable characteristic for extraction processes. The DES combinations, along with ultrasound-assisted extraction, extracted significant amounts of polyphenolic compounds from purple corn pericarp within a short extraction time. The extracted compounds contained diverse forms of individual anthocyanins, tannins, and flavonoids, which were quantified during HPLC analysis. A solvent dependent extraction of various individual phytochemicals was observed. The recovered polyphenolic extracts demonstrated significant antioxidant activities in three assays.

**4.2 Future recommendations**

Based on our inferences, the following are some of the suggestions for future research in this area:

1. The breeding and adaptation of polyphenol-rich colored corn varieties should be continued with an aim of developing higher yielding and polyphenol-rich colored corn varieties adapted to the midwestern climatic conditions.

2. Significant pigmentation was observed in cellulosic plant parts such as cobs and tassels, they must also be evaluated for their valorization potential.
3. Investigation of health promoting properties of colored corn phytochemicals *in-vitro* and *in-vivo* will help with their utilization as functional food ingredients.
4. Further optimization of solvent composition for the efficient extraction of individual phytochemical forms is required, and so is the exploration of DES reuse potential.
5. Lastly, extensive toxicological studies of DES-based polyphenolic extracts are warranted before their use for food applications.