Developing and characterizing antioxidants and proteins from corn and its co-coproducts

by

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B.S., Henan University of Technology, 2016
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
College of Agriculture

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Abstract

Corn is one of the most cultivated crops worldwide and is an important source of food, feed, and biofuel in the U.S. It is considered an important source of protein and calories for millions of people in the world, especially people in Latin America, Africa, and Asia. Protein content in corn kernels varies among varieties and can be around 8-15%. Among the major cereals, corn is plentiful, cheap, and easily available, and has high phenolic content. It contains up to three times more phenolics compared to wheat, rice, and oats, and exerts the highest antioxidant activity. With the increase in ethanol production in the United States, more corn products are generated each year. Corn dried distillers' grains (e.g., DDGS) are one of the major protein-rich co-products from corn dry milling processing, which are potential sources to produce high-value phenolic compounds and bioactive protein hydrolysates. However, limited information is available on the production and antioxidant performance of corn and corn DDGS antioxidants. The objectives of this dissertation were to produce both phenolic and protein hydrolysates from different varieties of corn and corn DDGS via different treatments and characterize their antioxidant performances with chemical assays and in different model systems.

In the first study, seventeen different varieties of corn were screened and evaluated for their antioxidant compositions and properties. Those corn kernels had different colors and belonged to three different types including field corn (also called dent corn), popcorn, and sweetcorn. Both free and bound phenolics were extracted. Various assays were used to assess their antioxidant potentials. UPLC-DAD-ESI-Q-TOF-MS/MS was used for the characterization and quantification of the phenolic compounds. This section provides detailed understanding on the phenolic content and composition of different varieties of corn, as well as antioxidant activity using advanced instruments and comprehensive analysis.

Based on the first study, four varieties of corn (Hickory king white, Reids yellow dent, Jimmy red, and Ohio blue) were further selected for distilled spirit production with four yeast strains from the species of *Saccharomyces cerevisiae*, including Safspirit GR-2 (GR-2), Red Star Distillers' active dry yeast (DADY), SafSpirit HG-1 (HG-1), and SafSpirit USW-6 (USW-6). Phenolic compounds were extracted from both the original corn kernels and DDGS and thoroughly characterized. Results showed that both corn variety and yeast strains significantly ($p < 0.05$) influenced the total phenolic content (TPC), and DDGS possessed three to four times higher TPC than the unfermented corn. The GR-2 and HG-1 showed better improvement on TPC than other yeasts, and DDGS from blue corn indicated the highest free phenolic content up to 2078.82 mg/g GAE. Moreover, the phenolic profile was changed after fermentation. The DPPH+ and ABTS•+ scavenging activities of phenolic compounds were also improved after fermentation.

In the third study, a sequential preparation procedure of phenolic antioxidants and protein hydrolysate antioxidants was developed. The antioxidant potential of the antioxidants was determined using different chemical assays, and the antioxidant performance of selected antioxidants in o/w emulsions was also evaluated. First, five GRAS organic solvents at 50% (v/v) were used to extract phenolic compounds from DDGS including acetone, ethanol, methanol, 1-proponal, and 2-propnal. The extracted phenolics were named Phenolic-A, Phenolic-E, Phenolic-M, Phenolic-1P, and Phenolic-2P, respectively. The residues were then hydrolyzed using 0.1 AU/g of Alcalase, and the prepared protein hydrolysates were named Hydrolysate-A, Hydrolysate-E, Hydrolysate-M, Hydrolysate-1P, and Hydrolysate-2P, respectively. The phenolic antioxidants showed significantly higher TPC than hydrolysate antioxidants, and the highest was observed on Phenolic-A (67.54 mg GAE/g). According to the results of composition and

phenolic profile determined using the UPLC-DAD-ESI-Q-TOF-MS/MS, extraction solvent was critical to the phenolic acid yield and composition. The hydrolysate antioxidants showed a degree of hydrolysis in the range of 6.39-7.60%, and no significant difference was observed in the peptide content (501.58 -532.69 mg/g) as well as molecular weight distribution. Both phenolic and hydrolysate antioxidants had high antioxidant capacity against DPPH free radical scavenging and ferrous ion chelating. Considering the yield and antioxidant activities, 50% (v/v) of acetone and 50% (v/v) ethanol were the most efficient in producing antioxidants with promising antioxidant capacity. Phenolic-A, Phenolic-E, Hydrolysate-A, and Hydrolysate-E were added into oil in water (o/w) emulsions at both 1.0 and 2.5 mg/mL to evaluate their antioxidant performance. The selected phenolic and hydrolysate showed high efficiency in the prevention of lipid oxidation, and higher dosage showed relatively better prevention which was even better than 1 mg/mL rosemary extract. The results provide evidence that corn DDGS is a potential source of natural antioxidants, and sequential extraction would be a potential way to produce both corn phenolic and peptide antioxidants.

The last study was focused on the preparation and characterization of corn protein concentrate from corn flour and DDGS. This study was conducted with a sequential extraction procedure combined with wet milling followed by physical treatments (sonication and homogenization), and then enzyme hydrolysis (α-amylase and cellulase) to produce concentrated proteins from corn flour and corn DDGS. The extracted proteins possessed a much higher protein content compared to the starting material with high protein recovery. Due to higher protein content in proteins produced from wet-milled corn flour with centrifuge or starch tabling and then followed by other treatments (protein-MC and protein-MT), more rupture of proteins was caused during future extractions inducing unfolding of protein structures and the consequent

exposure of hydrophobic groups and regions buried inside. Therefore, protein-MC and protein-MT showed a relatively higher content of random coil (40.84% and 37.53%, respectively), higher SDS binding capacity (41.45 and 39.58 μg/mg, respectively), and higher free SH groups (3.04 and 3.14 μmol/g, respectively) and bond SS (14.71 and 15.41 μmol/g, respectively) among all prepared proetins. Protein extracted from corn flour (Protein-flour) showed the highest water holding capacity (WHC) with a value of 4.70 g/g, Protein-MC had the second lowest WHC which was 3.17 g/g, and protein-MT had the lowest WHC of 2.91 g/g. The highest oil holding capacity (OHC) was observed on protein-MC, protein-MT, and protein-CD which were 3.52, 3.36, and 3.55 g/g, respectively. However, no significant difference was observed in the in vitro protein digestibility (83.13-85.03%).

Overall, this study generates useful knowledge for producing antioxidative phenolics and protein hydrolysate/protein concentrate from corn and corn DDGS. It revealed that phenolic compounds and bioactive hydrolysates from corn could inhibit lipid oxidation through scavenging free radicals as well as chelating metal ions. Those novel applications could add value to the co-products from corn processing industries and provide alternative naturally derived antioxidant options for food, pet food, and animal feed uses.

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Dedication

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Chapter 1 - Literature review

1.1 Introduction of corn and corn co-products

1.1.1 Production and importance

Cereals are the most important source of food which provides energy for the world population (Food and Agricultural Organization, 2018). Wheat, corn, and rice are the top three production cereal crops worldwide. Of these cereals, the greatest land area by far is devoted to wheat. However, due to its lower average yield per unit of land area, the total production of wheat is less than that of corn or rice. Of these main cereals, wheat, and rice are primarily food crops, whereas corn is primarily used as feed and fuel bioethanol crop but is a principal source of food in developing countries (Ufaz & Galili, 2008).

Corn is the most extensively cultivated and consumed cereal in the world. The global production of corn is more than 115.14 million metric tons as of 2022. The highest production is 34.88 million metric tons provided by the USA, followed by 27.72 million metric tons provided by China (Table 1.1) United States Department of Agriculture, 2022). The American continent produces nearly 55% of the world's total production, followed by Asia, Europe, and Oceania. The U.S. Com Belt produces about 30% of the world's total, followed by China (24.07%), Brazil (10.85%), European Union (4.71%), Argentina (4.08%), India (2.78%), and Mexico (2.40%).

1.1.2 Types of corn

Corn is classified on kernel size and consumption of the endosperm, and the general types including dent (field), flint, flour, sweet, waxy, and pod corn (Ranum et al., 2014). Dent

corn is the predominant corn grown in the USA and also is called field corn. It gets the name because of the dent that forms on the top of each kernel as it dries. The kernels contain a hard form of starch at the sides and a soft type in the center. Dent corn is primarily used for feed, processed foods, and bioethanol production (Serna-Saldivar, 2023). Corn can also be classified based on its sweetness or the amount of sugar in the kernel (Brown & Darrah, 1985; Gibson & Benson, 2002). Sweet corn is mainly used as human food due to its high sugar content and good flavor. It can be eaten free or in a variety of processed forms, such as cooked, frozen, canned, or juiced (Revilla et al., 2021). Flint corn usually has a tough outer layer and a small and soft endosperm. Popcorn is a special kind of flint corn. The high proportions of translucent (vitreous endosperm) in popcorn gives it a large expansion rate when heated (Serna-Saldivar, 2023).

Various types of maize are grown throughout the world - one important difference is color. For example, most of the corn grown in the USA is yellow, while white corn is more popular in Africa, Central America, and the southern United States. There are also colored corns, ranging from red, orange, blue, and purple. These colored genotypes indicate higher nutritional and antioxidant properties than the yellow and white corns (Hallauer & Hastings, 2000).

1.1.3 General use

In contrast to wheat and rice, corn is mostly utilized for animal feeding and bioethanol production. The U.S. corn use from 2010-2020 is shown in Fig. 1.2 and indicates a trend for increased corn production from 2010-2020, as well as an increase in alcohol production. During

the year 2020, 38%, 34%, and 19% of total U.S. corn was used for feed, alcohol for fuel, and alcohol for beverage production, respectively (2021, https://afdc.energy.gov/data).

Corn is widely consumed in numerous traditional forms in Latin America, Asia, Africa, and the Balkans. White dent maize is generally preferred because its favors the final color of processed foods, but yellow types are used in China, Brazil, Argentina, Italy, and several others (Serna-Saldivar, 2023). The use of mature maize on the cob is consumed worldwide. Besides direct consumption, corn products are used widely. Corn can be used as canned and frozen vegetables, or processed into tortillas, arepas, couscous, polenta, porridges, and gruel. Processed corn products are obtained from dry milling, wet milling, and nixtamalization. Corn starch is an important food ingredient usually produced from wet-milling, and it plays a critical role in the food texture. Corn flour is another product generated from dry-milling that can be used for baking, breakfast cereals, and other extrusion snacks (Orhum, 2013). Nixtamalization (or lime-cooking) was widely used to produce tortillas and derived foods.

There is only a small proportion of corn used for human consumption. Most of the corn is used for fuel bioethanol production. According to the report of U.S. Department of Energy (https://afdc.energy.gov/data), the production of ethanol from 2000 to 2021 increases about 8 times from 1,622 to 15,016 million (Fig. 1.3). Dry milling and wet milling processing are two commercial processes to convert corn into ethanol. Dry milling separates the bran and germ by gradual abrasive technique to obtain dry-milled corn flour. About 90% of corn bioethanol is produced through dry milling process (B. Anderson & Almeida, 2019; Belyea et al., 2006; Kelzer et al., 2010). For dry milling process, the ethanol is separated from the water and non-

fermentable residues (including proteins, fibers, oils, and minerals) by distillation. Downstream dewatering, separation, evaporation, mixing, and drying are then used to remove water from the solid residues and to produce a variety of co-product streams in wet or dry, with or without the addition of condensed soluble (Sharma 2016; Yan et al., 2012). Distillers dried grains with soluble (DDGS) is the most popular co-product. Corn DDGS contains all the nutrients from the corn grain in a concentrated form (Babcock et al., 2008). Therefore, it can be a rich source of protein, minerals, and other nutrients. DDGS contains about 24-35% of crude protein. The study of Spiehs et al. (2018) reported that DDGS had a high concentration of lysine (0.85%) and methionine (0.55%) and was limiting in tryptophan and arginine. Moreover, DDGS has been reported to contain high levels of pigments. Corn contains about 30 ppm of xanthophyll and retained in DDGS (rangomg from 4.64-16.97 ppm; Salim et al., 2010). Despite this, the average concentration of carotene is 36.72 ppm (Salim et al., 2010). Wet milling releases starch granules by fractionation of the different components of corn kernels with minimal mechanical damage. This process can produce high purity starch and more than 85% of corn starch is produced. The corn kernels are steeped in a solution of sulfurous acid before grinding which makes the germ, bran, and protein easier to separate during grinding (Abu-Ghannam & Balboa, 2018). Corn gluten meal (CGM) is the major co-product generated from wet-milled corn with a higher protein content than DDGS ranging from 60-75% (Leeson, 2008; G. Li et al., 2019). CGM is high in amino acids, such as Glu, Leu, Pro, Ala, Phe, and Asp (Jiao et al., 2022). It is also high in zeaxanthin concentration of 0.20-0.37 mg/g along with some dietary fiber (Quackenbush et al., 1970).

Traditionally, these co-products are mainly used as animal feed materials or otherwise discarded due to low water solubility and imbalanced amino acid composition. In recent years, there was a growing interest in the modification of corn proteins and an intention to add value to these protein-rich corn co-products. Previous studies showed that hydrolysis could be a feasible approach to increase bioactivity of proteins from some corn co-products (Wang et al., 2014; Yang et al., 2007; Zhou et al., 2015; Zhu et al., 2019). These hydrolyzed proteins and peptides showed an improvement in functional properties and enhanced biological values.

1.2 Corn proteins

1.2.1 Corn protein classification

The corn kernel consists of three major distinct biological structures, which are the bran, the endosperm, and the germ. The storage proteins are the dominant type of proteins and mainly found in the endosperm (75%), and the remainder is in either germ or bran. Corn proteins can be classified into four fractions based on their different solubility. Prolamins (also called zein) and glutelin are the main seed proteins which account for about 68% and 28% of total corn proteins, respectively (Delcour et al., 2010). Zein is insoluble in water but exhibits good solubility in ethanol, and glutelin has good solubility in dilute acid or alkali solutions (Zheng et al., 2015). Albumins are the only type of corn protein that is hydrosoluble, and globulins are soluble in salt solutions (Zheng et al., 2015). Previous research reported that albumins, globulins, and glutelins are higher in lysine (5–6%) compared to zeins (0.1%). The methionine content of glutelins (2.2%) is somewhat higher than that of albumins, globulins, and zeins. Glutamine, proline, leucine, and alanine account for nearly 65% of the total amino acids in the zein fraction (Wilson,

1987; Lawton and Wilson, 2003), and this fraction is much higher in glutamine/glutamic acid (21%) than the other proteins.

1.2.2 Extraction of corn proteins from corn and corn co-coproducts

T.B. Osborne was one of the first scientists to describe the properties of corn kernel proteins (Osborne, 1897). In his research, the corn kernel proteins were extracted based on solubility. After grinding into fine powder, proteins were sequentially extracted with water (albumins), 5% saline (globulins), alcoholic solution (zeins), and 5% NaOH (glutelins). Compared to the whole corn kernel, corn co-products such as DDGS and CGM have higher fiber and oil content. This may make it more difficult to extract protein from those materials. There have been a good number of experimental results on the extraction of proteins from corn, corn co-products, or some other cereal co-products such as sorghum and wheat.

The extraction approaches can be divided into two groups: using pretreatment before extraction and using different extraction solvents or equipment. All these treatments focus on two goals, either movement of non-protein components or getting high protein extraction yield and concentration directly, or both. Typical pretreatments are mostly selected for removing nonprotein components, which would most likely be a remedy for the low yield and purity of the extracted proteins. The fat from corn or corn co-products can be removed using chloroform, petroleum ether, and hexane by simply mixing those solvents with samples and stirring at room temperature following a filtration or centrifugation process (Lawton, 2006; Russin et al., 2011; Schober et al., 2010, 2011). Fiber can be removed using enzymatic treatment, elusive process, and air aspiration (Anderson et al., 2012; Xu et al., 2009). Srinivasan et al. (year) found a

combination of sieving and elutriation processes was effective in separating the fiber from two commercial samples of DDGS and increased the value of DDGS by increasing protein content and producing a new coproduct with higher fiber content. Relative to the original DDGS, the crude protein content of the enhanced DDGS was higher by 8.0% for DDGS from the flash drying process and by 6.3% for DDGS from the ring-drying process (Srinivasan et al., 2008). Using air aspiration to remove fiber from DDGS may not be as efficient as sieving and elutriation. A study found that fiber content was only slightly enriched in the aspirated DDGS fraction compared to the residual DDGS fractions (Singh et al., 2002). Enzymatic hydrolysis is another efficient way to remove fiber fractions from corn DDGS. Xylanase, cellulase, hemicellulose, and pectinase exhibited the potential to remove fiber from DDGS (Anderson, 2011; Anderson et al., 2012; Pasangulapati et al., 2012; Xu et al., 2009).

Besides pretreatments, the selection of solvents and extraction processes have also been studied. Previous sections have discussed the four major protein fractions in corn kernel which have different solubilities but limited research on the fractionation of protein in corn coproducts. One published research gave insight into the composition of the protein fractions of DDGS and materials at the base of still after corn ethanol distillation (Wu et al., 1981). Four consistent extraction methods followed by two different treatments of extraction with the reducing agent were employed and compared to determine protein solubility. The four consistent extraction procedures were water extraction, sodium chloride extraction, 70% ethanol extraction, and 70% ethanol with dithiothreitol (DTT) extraction. These first four extractions used for both methods extracted 14% of the total protein. The first reducing method utilized borate, SDS, and DTT extracted only 30% of the corn DDGS protein while 51% of the total protein was left in residue.

The second method with NaOH and DTT at pH 11.9 extracted 28% of the protein, and the next step using NaOH with SDS and DTT extracted an additional 26% protein. Extractions using methods one and two could not extract more than 45 or 70% of the total DDGS protein, respectively. The zein protein was just fractionated and no attempt was made at the characterization of zein proteins. The reasoning provided for the low protein yield in comparison to corn was attributed to the denaturation of the protein during alcohol distillation.

Further work on DDGS extractions with reducing agents was done by Wolf and Lawton (Wolf & Lawton, 1997). Nine different materials were extracted and compared in this work; among them were corn flour, CGM collected in a centrifuge and air-dried, CGM commercially dried in a drum dryer, whole stillage from the dry-grind ethanol process, a DDGS sample of each dry and wet material, all wet samples of whole stillage that had been freeze-dried, and three other DDGS materials that were commercially dried. The yields of crude zein from extractions were 3.2–6.6%, but their protein contents were only 37–57% due to lipids and pigments in the extracts. The authors concluded that because of the low protein purity of the samples, integration of the zein from DDGS into biodegradable materials was still far off. This extraction showed that extracting zein from DDGS was possible, although the yields were low, even with a reducing agent.

Extraction of zein from defatted DDGS under acidic and basic conditions with the reducing agent was done by Xu et al. (2009). Zein was extracted from DDGS using 70% ethanol and 0.25% sodium sulfite with the pH altered using HCl or NaOH. The optimal yield of zein solid obtained was about 90% protein and a recovery of 44% of the protein in DDGS was

attained at pH 2. The high purity of the protein was promising, and the method also extracted a higher yield. The use of a reducing agent may also liberate glutelin proteins.

Furthermore, the production of protein hydrolysates is another pathway for protein extraction. Alcalase, protamex, Flavorzyme, Neutrase, Papain, Ficin, and Bromelain have been reported to produce corn protein hydrolysates from corn or corn coproducts with high bioactivities (Hu et al., 2022; Li et al., 2007; Kim et al., 2004; Wang et al., 2014).

Based on published research, extraction of proteins from corn co-products still faces some challenges. There is still a need to improve the extraction process for production of high yield and functional proteins from those co-products.

1.2.3 Corn protein bioactivities

Previous research reported that corn proteins contained abundant bioactive peptides and structural domains which performed critical functions in human body, including antihypertension, anti-obesity, anticancer, anti-inflammatory, antimicrobial and antioxidation.

Lipid oxidation is a major cause of quality deterioration during processing, handling, and storage of high fat/oil foods or ingredients (Mussinan & Morello, 1998). The formation of off-flavor and various oxidation products, such as peroxides, hydroperoxides, aldehydes, and ketones results in the loss of food texture, aroma, taste, nutrient, shelf stability as well as causing food safety concerns (Amaral et al., 2018; Saiga et al., 2003; Zhou et al., 2013). To retard lipid oxidation, antioxidants have been widely used in these products. Synthetic antioxidants, such as

butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG), and ethoxyquin (EQ) are commonly used in various food and feed products. However, it has been reported that such antioxidants possibly increase health risks due to their toxicity and carcinogenicity (Oliveria et al., 2014; Shahidi & Zhong, 2008). Table 1.1 summarized the identified peptides from different corn co-products and their antioxidant activities against different assays. The peptide AY from CGM hydrolyzed by protamex showed high antioxidant activities against DPPH and superoxide radical scavenging activities (Zhou et al., 2015). Another three peptide, LPF, LLPF, and FLPL were also reported high radical scavenging activities (DPPH, ABTS, superoxide, and hydroxyl) and high metal chelating capacities (Zhuang et al., 2013). Protein hydrolysate (mixture) from DDGS showed high DPPH radical scavenging activity, and it efficiently retard the lipid oxidation in several food model systems (Hu et al., 2020) (Hu et al., 2020). The study of Sharma et al. (2023) identified nine peptides from DDGS proteins (APLA, PLFP, LFLP, LPPYL, PLYPLP, NDWHTGPL, LPPYLPS, GSPFLGQ, and SWQQPIVGG), and all of them showed promising angiotensin-I converting enzyme inhibitory activity and dipeptidyl peptidase4 inhibitory activity.

Several peptides produced from CGM showed the potential of ACE inhibitory activity (Huang et al., 2011; Miguel & Aleixandre, 2006). The ACE (dipeptidyl carboxypeptidase, EC 3.4.15.1) is a key enzyme in the renin–angiotensin system. It is important in the regulation of blood pressure and fluid and electrolyte balance (Martı´nez-Maqueda et al., 2012; Suh et al., 2003). A previous study identified an ACE inhibitory dipeptide, Ala-Tyr, from CGM (Yang et al., 2007). This dipeptide could be considered as a true ACE inhibitor because its IC50 value was unchanged by preincubation with ACE. The Ala-Tyr was found to exhibit *in vivo*

antihypertensive activity after oral administration to spontaneously hypertensive rats. A maximum reduction of systolic blood pressure of 9.5 mmHg was observed after 2h oral administration of 50 mg/kg of Ala-Tyr.

The hepatoprotective effect of corn oligopeptides has been confirmed by some studies. According to the study of Yu et al. (2012), the peptide isolated from corn germ meal exerted a hepatoprotective effect on liver damage induced by carbon tetrachloride. Lv et al. (2013) report that corn peptides also showed a hepatoprotective effect against thioacetamide-induced liver fibrosis. Several bioactive peptides have been reported to exhibit protective effects against the liver injury induced by D-galactosamine or acetaminophen (Zhang et al., 2012). In addition, Zhang et al. (2012) reported that corn oligopeptides derived from CGM by enzymatic hydrolysis have a protective effect on early alcoholic liver injury in rats (Yu et al., 2017).

1.2.4 Utilization and application of corn kernel proteins

The main advantage of corn protein over other plant proteins, such as soy, is not a common allergen. Corn protein also shows a minimal solubility loss after heat treatment, which is beneficial when trying to eliminate antinutritive factors. Corn protein has low aqueous solubility and is deficient in certain essential amino acids such as Lys and Trp, which greatly limits its application in the food industry (Li et al., 2019). If the protein has low solubility, it may not form an adequate gel or stable foam. Only small-scale attempts to improve the nutritional value of foods, such as cookies, bread, and muffins through the incorporation of corn gem proteins (Inglett & Blessin, 1979). Some other studies showed the potential food applications of zein. Although zein is one of the few cereal proteins that is industrially produced in a relatively

pure form, it is rarely used directly for humans. The major applications of zein are as a polymer material for film, coatings, and plastics (Lawton, 2002). Zein nano-breads or zein nano-particles have been used as edible carries for flavor compounds (Guo et al., 2005; Torres-Giner et al., 2008). In addition, food colorants can be encapsulated using zein-based nano-materials to control optical properties and rate of color releasing in food stuffs (Patel & Velikov, 2014). Studies reported corn protein hydrolysates could be a potential source of functional food ingredients with antihypertensive, antioxidative, and hepatoprotective functions (Huang et al., 2011; Wang et al., 2015; Yu et al., 2017). In summary, subjecting corn protein to modification may result in targeted improvement in nutritional value and functional properties. This requires further investigation.

1.3 Corn phytochemicals

1.3.1 Simple phenolics

Phenolics are the most widely distributed secondary metabolites present in corn (Acosta-Estrada et al., 2019). These phytochemicals can be divided into three major categories: simple phenolics, flavonoids, and tannins. Simple phenolics are usually derived from benzoic or cinnamic acids, whereas flavonoids including anthocyanins are built from two units: a C6-C3 component from cinnamic and a C6 fragment from malonyl-CoA (Fig. 1.4) (Acosta-Estrada et al., 2014).

Previous studies have investigated that the soluble conjugates (glycosides) and insoluble are the most common phenolic forms in cereal grains, which are covalently bound to sugar moieties or cell wall structural components (Acosta-Estrada et al., 2019). Corn was reported to

contain up to three times more phenolics compared to wheat, rice, and oats (Adom & Liu, 2002). Most corn phenolic compounds are associated with cell walls in the pericarp and the monolayered aleurone, and about 85% of those phenolics are in the insoluble-bound form (Acosta-Estrada et al., 2019). Lopez-Martinez et al. (2009) reported that the total phenolic levels of eighteen Mexican maize ranged from 17-34 mg/g, and the highest was observed on the purple corn. Zavala-López et al. (2018) extracted both free bound phenolics from 22 modern types of corn, and they found the total free phenolic compounds ranged from 2.01 to 4.47 mg gallic acid equivalent (GAE) per gram of flour (dry weigh basis). The total bound phenolic content was much higher than free fraction with value ranging from 13.17 to 30.27 GAE/g.

Phenolic acids are the most common simple phenolics found in whole grains. The typical phenolic acids in corn kernel include caffeic, coumaric, hydroxybenzoic, protocatechuic, syringic, vainillic, sinapic, syringic, gallic, and ferulic acids (Ndolo & Beta, 2014; Nile & Park, 2014). Ferulic acid is the most predominant phenolic acid found in corn kernel which represents about 70% of the total (Ndolo & Beta, 2014). It is a hydroxycinnamic acid bounded with oligosaccharides by esterification linkages associated to cell walls of pericarp (Acosta-Estrada et al., 2019; Ishii, 1997). Most phenolics are considered high in antioxidant potential, as well as anti-inflammation, anti-cancer, anti-microbial and preventing neuron degeneration properties (Tan et al., 2011). Kim et al. (2012) reported that hydroxycinnamic acid extracted from corn bran showed anti-inflammatory activity in lipopolysaccharide-stimulated Raw 264.7 macrophages. In another study, ferulic acid was also found to prevent chronic neuroinflammation and induce neural progenitor cell proliferation in vitro and in vivo (Wenk et al., 2004; Yabe et al., 2010). Besides, phenolic acids also possess antidiabetic effects by decreasing blood glucose levels,

glucose-6-phosphatase and phosphoenolpyruvate carboxin activities, and glucose and lipid peroxidation thereby increasing glycogen and insulin.

1.3.2 Anthocyanins (pigmented flavonoids)

There are significant amounts of flavonoids in the kernel of corn, such as flavanols, flavanones, flavones, and anthocyanins. Colored corn is one of the richest sources of anthocyanins since these anthocyanins are the pigments responsible for the kernel colors such as red, purple, orange and red (Lao & Giusti, 2016; Nile & Park, 2014). For example, red corn was reported to have 51 mg/kg of anthocyanins, and blue corn had 368 mg/kg. Purple corn contains much higher numbers of anthocyanins with values up to 1,300 to 16,400 mg/kg (De la Parra et al., 2007; Lao and Giusti, 2016; Li et al., 2017). Cyanidin and peonidin glycosides are the main anthocyanins in corn. Cyanidin 3-glucoside is the most abundant in blue and purple corns with concentrations varying from 15.4 to 1,135.4 mg/kg (Abdel Aal et al., 2006; Pedreschi and Cisneros-Zevallos, 2007; Li et al., 2017). Other relevant anthocyanins are cyanidin-3-(60-malonylglucoside), cyanidin-3-O-glucoside-2-malonylglucoside, peonidin-3-O-glucoside, peonidin-3-(60-malonylglucoside), pelargonidin-3-(600-malonylglucoside), and peonidin-3-(dimalonylglucoside) (Li et al., 2017). Previous publications have shown that anthocyanins possess anticancer, antiobesity, and antiinflammation effects (Cone, 2007).

1.3.3 Carotenes and xanthophylls

Carotenoids are polyisoprenoids containing 40 carbons. They contribute yellow color to the endosperm of plants. Carotenes and xanthophylls are two types of carotenoids. Yellow corn contains both carotenes and xanthophylls, while white corn only contains xanthophylls. Leeson

& Caston (2004) reported that yellow corn contains 50-80 µg/g, while high-carotenoid corn contained about 95-120 µg/g of carotenoids, which are a good source of lutein, zeaxanthin, and other nutraceuticals. Hence, corn could be used to help to prevent deficiency of vitamin A (Burt et al., 2010; Zhu et al., 2017). Researchers found that unperturbed carotenoids remained stable for a long time but drying at 90°C can cause a loss of 40% of original carotenoids after 18 months' storage (Burt et al., 2010).

1.4 Conclusions and future perspectives

In this review chapter, the general background regarding corn and corn co-products was introduced. The types of corn proteins, types of major phytochemicals, and their bioactivities were summarized. The major corn proteins are zein and glutelins. Corn phytochemicals exist in both soluble and insoluble forms, with ferulic acids as the predominant phenolic compounds in corn kernels. As the demand and nutritional quality of corn products increase, the number of byproducts produced during corn processing also increases. In order to maximize the utilization value of corn, reduce the waste of resources, and fully realize the sustainable development of the corn industry, it is necessary to seek reasonable processing and utilization of corn proteins from corn kernel and co-products. The high-value processing of corn and its co-products has broad market prospects and huge business opportunities, and it is of great practical significance to strengthen the development of corn by-products processing.

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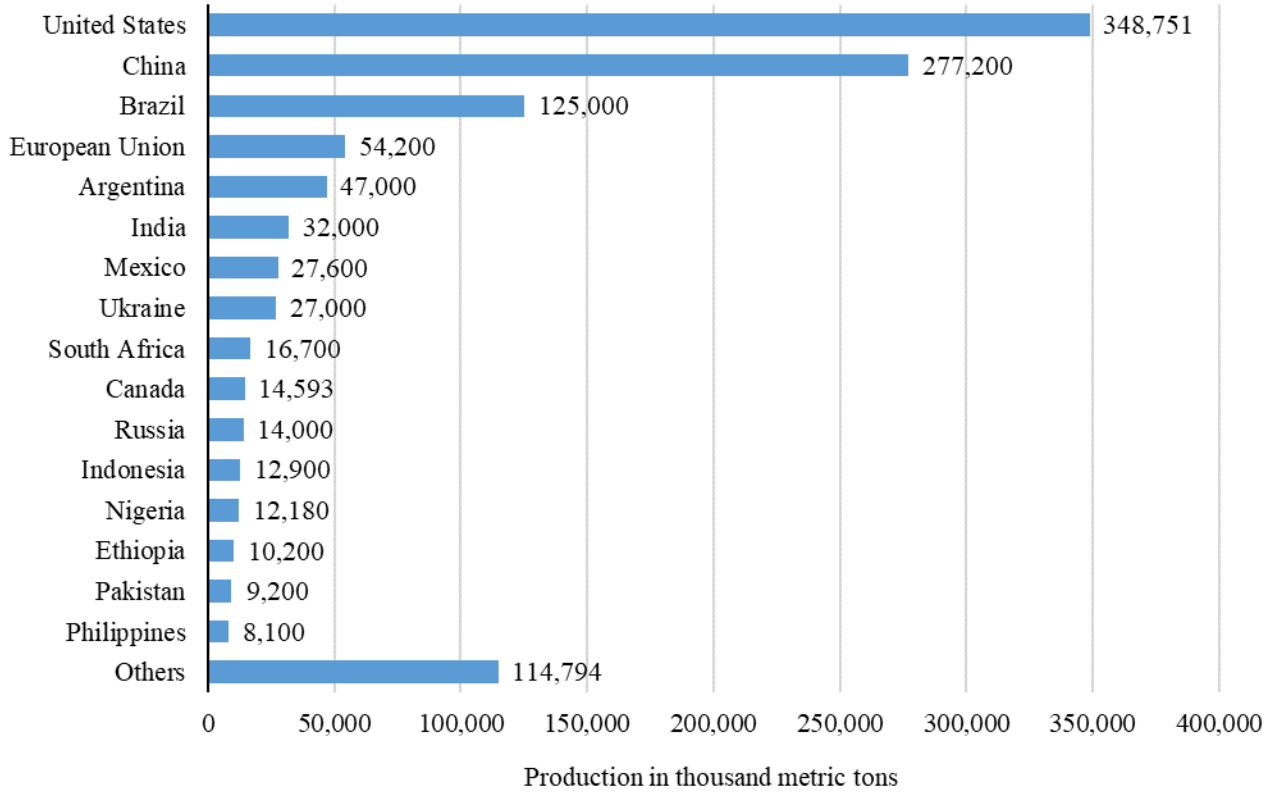

Figure 1. 1 Global corn production in 2022/2023 by country

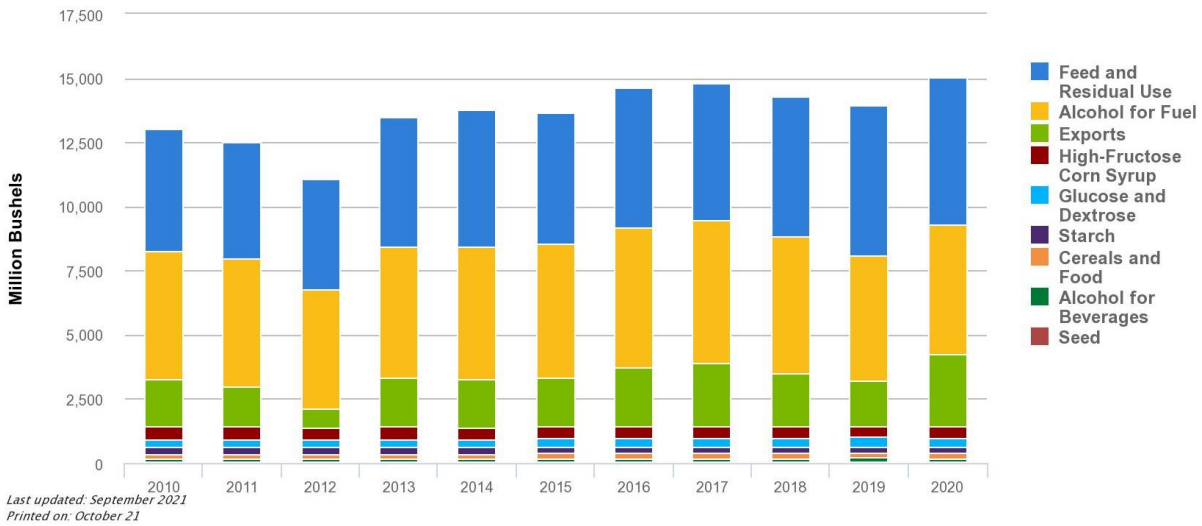

Figure 1.2 U.S. corn use by market year. (https://afdc.energy.gov/data)

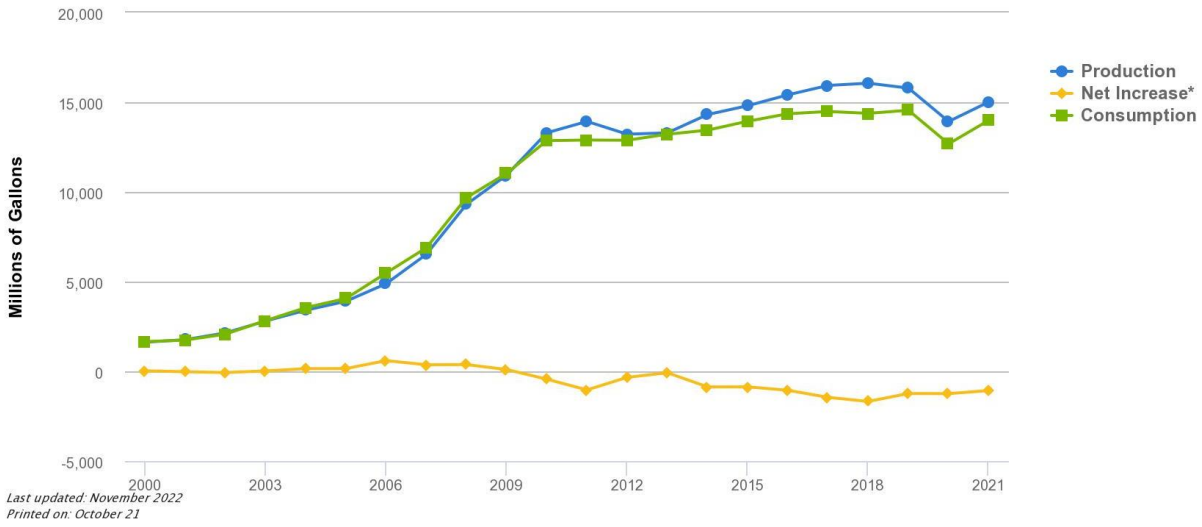

Figure 1.3 U.S. production, consumption, and net increase of ethanol.

(https://afdc.energy.gov/data)

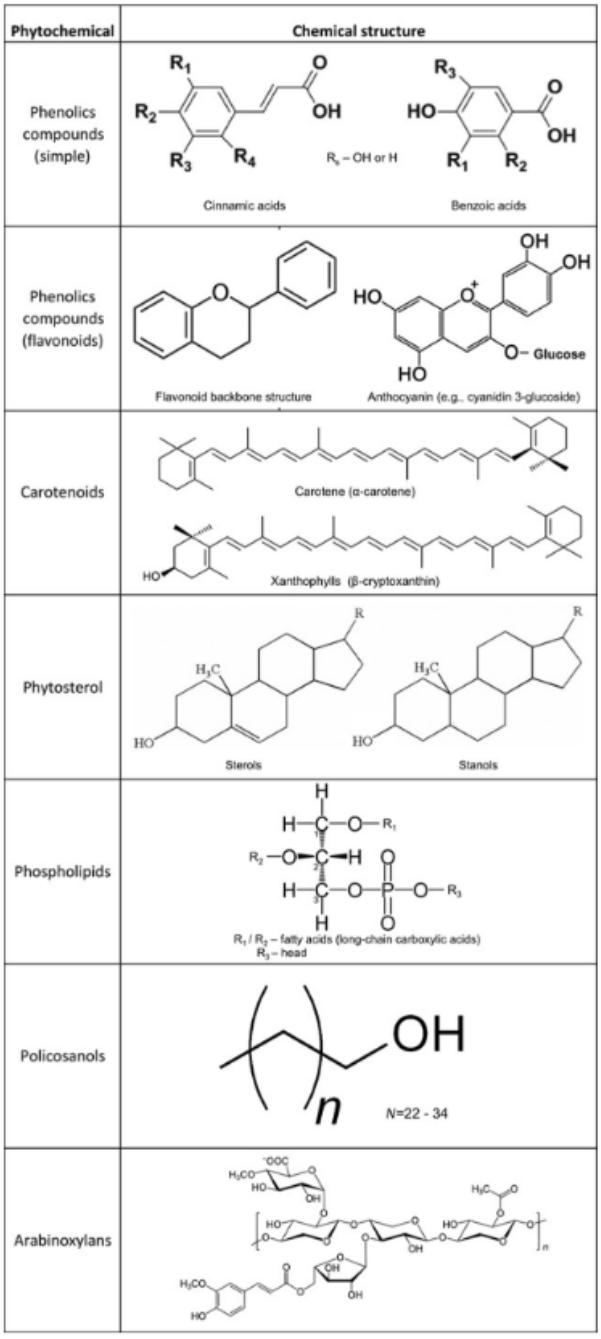

Figure 1.4 Chemical structures of bioactive phytochemicals associated with corn kernels. (Acosta-Estrada et al., 2019)

Table 1.1 Antioxidant peptides derived from corn proteins.

Source of peptides	Characteristics	Activities	Reference
CGM	CSQAPLA, YPKLAPNE, and YPQLLPNE	DPPH, superoxide anion, and hydroxyl radical-scavenging capacity	Jin et al., 2016
CGM	MW of 250–1200 Da	Increasing SOD, GPx activities; reducing MDA concentration	Liu et al., 2015
CGM	AY	DPPH, superoxide, and hydroxyl radicals-scavenging capacity	Zhou et al., 2015
CGM	FPLEMMPF	Antioxidative activity	Zheng et al., 2006
CGM	LPF, LLPF, and FLPF	Hydroxyl, superoxide, ABTS, and DPPH radical-scavenging activity; Fe (II)/Cu (II)-chelating capacity	Zhuang et al., 2013
CGM	MW < 500 Da	ABTS and DPPH radical-scavenging activity	Zhu et al., 2008
CGM	YA and LMCH	Superoxide, ABTS, and DPPH radical-scavenging activity	Tang et al., 2010
CGM	LDYQ	Antioxidative activity	Li et al., 2004
CGM	QQPQPW	Hydroxyl, superoxide, ABTS, and DPPH radical-scavenging activity; Fe (II)-chelating capacity	Wang et al., 2014
CGM	YFCLT	Hydroxyl, superoxide anion, and ABTS radical-scavenging activity; oxygen radical-absorbing capacity (ORAC)	Wang et al., 2015
	GHKPS	DPPH radical-scavenging activity; inhibition of lipid peroxidation; Fe (II)-chelating capacity	Zhuang et al., 2013
Corn germ mill	MGGN, MNN, CAA	radical scavenging, oxygen radical absorbance capacity, cellular antioxidant activity, and intracellular reactive oxygen species scavenging assays	Zhang et al., 2019
Corn germ mill	< 2 kDa	DPPH & ABTS radical scavenging activity; Fe (II)-chelating capacity	Karami et al., 2019
DDGS	Mixture	DPPH radical scavenging activity	Hu et al., 2020

DDGS	APLA, PLFP, LFLP, LPPYL, PLYPLP, NDWHTGPL, LPPYLPS, GSPFLGQ, SWQQPIVGG	angiotensin-I converting enzyme inhibitory activity; dipeptidyl peptidase4 inhibitory activity	Sharma et al., 2023

Chapter 2 - Phenolic composition and their antioxidant activities of seventeen varieties of corn

Abstract

DPPH free radical scavenging activity, metal ion chelating capacity and phenolic content and composition (total phenolic, total flavonoid, and total tannins content) of seventeen corn phenotypes were determined. Total phenolic contents ranged from 1328 to 2379 µg GAE/g of whole grain flour., and corn varieties belongs to sweet corn indicated higher phenolic content. Total flavonoid ranged from 68.23 to 297.09 µg quercin equivalent per gram of whole grain flour, and total tannins range from 216.67 to 3141.67 µg catechin equivalent per gram of whole flour. Most of the phenolics in corn kernels were in the bound form (66-87%), while ferulic acid was the major phenolic compounds. Among the different varieties, bound phenolic extracts of corn appeared to have greater anti-radical and ferrous ion chelating capacity than free phenolic extracts from the same grain samples. The Honey Selected corn and the Silver King corn exhibited relatively higher DPPH scavenging activity, and the Japanese Hulless corn and Robust White corn indicated better ferrous ion chelating capacity. Colored strains were most enriched in total phenolic content as well flavonoid, and tannins. Differences in free-radical scavenging and reducing activities appeared to be dependent on the unique profile of phenolic acid and other phenolics in each variety.

2.1 Introduction

Corn (Zea mays L.) is a widely consumed cereal in Central and North America mainly in the form of food products such as tortillas and tortilla chips (De La Parra et al., 2007). Pigmented corn contains anthocyanins or carotenoids, and phenolic compounds which are phytochemicals synthesized in the plant by secondary metabolism; although these compounds are considered nonnutritive, interest in antioxidant and bioactive properties has increased due to their health benefits (Heinonen et al., 1998; Rice-Evans & Miller, 1996; Setchell & Cassidy, 1999). Epidemiological and *in vitro* research suggests an inverse relationship between the consumption of fruits and vegetables and the incidence of various chronic and degenerative diseases that come with aging such as cancer, cardiovascular diseases, cataracts, and brain and immune dysfunction (Ames et al., 1993). The health benefiting properties of these plant metabolites have been related not only to high antioxidant and anti-radical activities, but also to several other biological properties, such as antimutagenic or estrogenic activities, inhibition of enzymes, and induction of detoxification enzymes such as glutathione transferase and quinone reductase.

Corn of different phenotypes has been shown to exhibit different antioxidant activities and profiles. Several studies have reported a range of bioactivities of corn components, the antioxidant and anticarcinogenic effect of white corn polyphenolics such as ferulic and r-coumaric acid along with their respective derivatives (Andreasen et al., 2001; Kroon & Williamson, 1999; Rondini et al., 2002). It has been found that purple, blue, and red pigmented maize inhibits colorectal carcinogenesis in male rats (Hagiwara et al., 2001), and possesses antimutagenic (Yoshimoto et al., 1999) and radical scavenging activities (Oki et al., 2002). The antioxidant activity, phenolic acid, and anthocyanin contents of some Mexican maize phenotypes

have been reported (Del Pozo-Insfran et al., 2006; Pozo-Insfran et al., 2007). However, a broad survey of the antioxidant capacity of phenotypes that represent the diversity of corn types and varieties has not been conducted extensively. Free-radical scavenging and ferrous ion are generally accepted mechanisms for antioxidant activity related to the inhibition of lipid peroxidation (Arnao, 2000; Brand-Williams et al., 1995; D. Huang et al., 2005).

The objective of this study was to provide a fundamental understanding of phenolic compounds in 17 different corn varieties (some are colored) grown in the United States by determining the levels of phenolic compounds and their antioxidant capacities.

2.2 Materials and methods

2.2.1 Chemicals and reagents

A total of 17 different varieties of corn were purchased from Hoss Tools (Norman Park, GA, USA). Those corn belongs to three different types including dent corn (Jimmy red, Hickory king white, Indian flour, Ohio blue, Reids yellow dent, and Truckers favorite yellow), popcorn (Japanese hulless, Robust white, Robust yellow, South American, and Strawberry), and sweet corn (Avalon, Honey selected, Kandy korn, Peaches and cream, Providence, and Silver king). All other chemicals, solvents, and reagents used were at least analytical grade and purchased from Sigma-Aldrich (St. Louis, Mo, USA) or Fisher Scientific (Fairlawn, NJ, USA).

2.2.2 Extraction of free and bound phenolic compounds

Free and bound phenolic compounds were extracted according to the method of Tian & Li (2018) with some modifications. To extract free phenolic fraction, 2g of ground corn flour

was mixed with 80% (v/v) acetone (1:10, w/v) and shaken at 250 rpm for 10 min using a platform shaker. The mixture was centrifuged at 3500 *g* for 15 min. The supernatant was collected, and the residue was re-extracted one more time as mentioned above. The collected supernatant was combined and dried using a rotary evaporator and reconstituted with UPLC grade methanol (1:1, w/v). The solution was then filtered through a 0.2-µm filter and kept at -20 C until further analysis. To extract bound phenolic fraction, 2 g of ground corn flour was extracted with 80% acetone to remove free phenolic compounds as described above. The residue was then mixed with 20 mL 6 M NaOH and shaken at 250 rpm for 3 h in the dark. The mixture was then acidified to pH 2.0 with 12 M HCl and centrifuged at 8000 xg for 15 min. The supernatant was collected and extracted with ethyl acetate. The supernatant was collected, combined, and dried using a rotary evaporator, and then reconstituted with 2 mL of UPLC grade methanol. Prepared phenolic extracts were stored at -20 ºC in the dark until further analysis.

2.2.3 Determination of total phenolic content (TPC)

The TPC of samples were quantified using the method described in Tian et al. (2022). Gallic acid was used to prepare the standard curve, and TPC was shown as µg gallic acid equivalents per gram of flour (µg GAE/g).

2.2.4 Determination of total flavonoid content (TFC)

The TFC of samples were determined according to the method of Peng et al. (2019) with minor modification. In brief, 50 µL of samples was mixed with 50 µL of 2% (w/v) aluminum chloride and 100 µL of 5% (w/v) sodium acetate in a 96-well plate. The mixture was incubated for 30 min in the dark and then the absorbance was measured at 440 nm. Quercetin (0-50

μmol/mL) was used as standard and TFC was expressed in μg quercetin equivalents per gram of sample (μg QE/g).

2.2.5 Determination of total tannin content (TTC)

The TTC of samples were determined following a previous publication (Lyu et al., 2023). Briefly, 150 μL of sample, 25 μL of 4% (w/v) vanillin solution, and 25 μL of 32% (v/v) sulphuric acid were added to a 96-well plate. The mixture was shaken in the dark for 30 min at 25 ˚C, and the absorbance was measured at 517 nm. Catechin (0-1000 μg/mL) was used as a standard. The TTC was expressed in μg catechin equivalents per gram of sample (μg CE/g)

2.2.6 DPPH free radical scavenging activity

The DPPH radical scavenging activity was measured by modifying the method of Subbiah et al. (2020). DPPH radical solution was prepared by dissolving 4 mg of DPPH in 100 mL methanol. 20 μL sample, 20 μL methanol and 200 μL DPPH solution was added into a 96-well plate. The mixture was incubated for 30 min in the dark and then the absorbance was measured at 517 nm. The blank was 20 μL distilled water with 20 μL methanol and 200 μL DPPH solution. The scavenging activity was calculated using following equation:

$$DPPH\ scavenging\ rate\ (\%) = [\frac{A_{blank} - A_{sample}}{A_{blank}}] \times 100$$

Where A_{blank} was the absorbance of blank, and A_{sample} was the absorbance of sample.

2.2.7 Metal chelating capacity

The chelating capacity of Fe^{2+} was determined following the method of Tian et al. (2019). The chelating capacity was calculated using following equation:

$$Fe^{2+}\ chelating\ capacity\ (\%) = \left[\frac{A_{blank} - A_{sample}}{A_{blank}}\right] \times 100$$

2.2.8 Phenolic composition determined by UPLC-DAD chromatography

Phenolic content and composition were analyzed using a ultra-high performance liquid chromatography coupled with diode-array detection (UPLC-DAD, Waters Corporation, Milford, MA, USA). The setting of parameters was according to our previous publication (Tian et al., 2019). Six phenolic acid standards were used to for quantification including 4-hydrobenzoic acid, vanillic acid, syringic acid, p-coumaric acid, ferulic acid, and sinapic acid.

2.2.9 Statistical analysis

All experiments were completed in triplicates, and results were presented as mean ± standard deviation. Data were analyzed with SAS Studio software 2023 (SAS Institute, Cary, NC, USA). One-way analysis of variance (ANOVA) was performed with Tukey's post-hoc test to determine significant differences between the means ($p < 0.05$).

2.3 Results and discussion

2.3.1 Total phenolic content (TPC)

The ranges of TPC levels observed among the 17 corn varieties were in the range of 1328 to 2379 µg GAE/g (Table 1). Both corn type and color had significant effects on the TPC ($p < 0.0001$) (Table 2). The sweet corn showed a significantly higher TPC than the other type of corn ($p < 0.05$) due to their softer structure of endosperm compared to field and popcorn, which enhanced their phenolic extraction. Corn with bicolor and blue color indicated relatively higher TPC. Among the groups of pigmented corn phenotypes, the TPC were 1363 µg GAE/g for

Jimmy Red corn (red color), 1328 µg GAE/g for Indian Flour corn (mixed color), 2034 µg GAE/g for Ohio Blue corn (blue color), and 1753 µg GAE/g for Strawberry corn (red). The Ohio Blue corn exhibited the greatest phenolic levels among those colored varieties, while the Honey Selected corn (yellow color) appeared to have the greatest phenolic levels among all the varieties. The free phenolic levels ranged from 232 to 595 µg GAE/g of whole corn kernels (Table 1). The highest free phenolic content was observed on the Ohio Blue corn (blue color) and the Strawberry corn (red color) with a value of 390 and 330 µg GAE/g ($p < 0.05$), respectively. The bound phenolic levels ranged from 973 to 2019 µg GAE/g of whole kernels (Table 1). Among all varieties, the Avalon corn (white color) and Honey Selected corn (yellow color) were the strains with significantly higher bound phenolic content, which were 1959 and 2019 µg GAE/g ($p < 0.05$), respectively.

Previous studies have investigated that the soluble conjugates (glycosides) and insoluble are the most common phenolic forms in cereal grains, which are covalently bound to sugar moieties or cell wall structural components (Acosta-Estrada et al., 2014). Corn was reported to contains up to three times more phenolics compared to wheat, rice, and oats (Adom & Liu, 2002). Most corn phenolic compounds are associated with cell walls in the pericarp and the monolayered aleurone, and about 85% of those phenolics are in the insoluble-bound form (Acosta-Estrada et al., 2019; Gutiérrez-Uribe et al., 2010). The data in Table 1 clearly shows that the majority of phenolic compounds in selected corn varieties were in the bound fraction with a contribution of 66-87% to the total phenolics in the different varieties, which is in agreement with previous studies (De La Parra et al., 2007; Del Pozo-Insfran et al., 2006). Lopez-Martinez et al. (2009) reported that the total phenolic levels of eighteen Mexican maize ranged from 17-34

mg/g, and the highest was observed on the purple corn. Our results were lower than theirs which may be due to the different extraction corn varieties and different extraction protocols. Studies reported the TPC results were affected by many factors, such as pretreatments of raw materials (sonication, microwave, enzyme hydrolysis, et al.), solvents used for extraction, extraction time, and extraction temperature (Annegowda et al., 2012; Paucar-Menacho et al., 2017; Proestos & Komaitis, 2008; Swain & Hillis, 1959; Zhou & Yu, 2004).

2.3.2 Total flavonoid content (TFC)

Flavonoids are another important phenolic compound in corn. The total flavonoid content (TFC) was expressed as µg quercetin equivalents per gram of sample (Table 1). Both corn type and color affected the TFC ($p < 0.0001$; Table 2). The sweet corn also had relatively higher TFC, but the corn with observed red color had higher TFC than the other corns ($p < 0.05$). The TFC of all corn varieties was in the range of 68.23-297.09 µg QE/g. The Strawberry corn (red color) showed the highest TFC (297.09 µg QE/g), followed by the Jimmy Red corn (red color) with the second highest TFC (139.66 µg QE/g) ($p < 0.05$), and the lowest TFC was observed on the Japanese Hulless corn (white color) with a value as low as 68.23 µg QE/g. Kurilich & Juvik, (1999) and Lopez-Martinez et al. (2009) have reported that white, yellow, and orange corn had lower anthocyanin content than other colored corn varieties. The major pigment type in those phenotypes were carotenoids, particularly in the form of lutein and zeaxanthin (Kurilich & Juvik, 1999). Thus, corn varieties that were most abundant in phenolics were also most abundant in anthocyanins, which contribute to the total phenolic levels. Moreover, it has been found that polyphenolic compounds are one of the most effective antioxidant constituents in plant foods,

including fruits, vegetables, and grains. Hence, the quantification of polyphenolic contents and assessing their contribution to antioxidant activity is important.

2.3.3 Total tannin content (TTC)

Tannins are polyphenolic secondary plant metabolites which are capable of binding and precipitating alkaloids, saccharides, proteins, and other macromolecules (Adegbusi et al., 2022). From our results (Table 1), the TTC of the eighteen strains of corn was in the range of 216.67-3141.67 μg CE/g, and the Ohio Blue corn (blue color) indicated the highest TTC, followed by the Jimmy Red corn (red color) ($p < 0.05$). Corn type and color significantly affected the TTC (Table 2). The field corn had relatively TTC than other types, and TTC of blue corn was significantly higher than that in others ($p < 0.05$). The TTC of selected corn varieties was in the similar range of previous publications. There was little information on tannins in corn since it was generally considered as low tannins cereal. One of the previous studies showed that the TTC of yellow maize flour was 0.213% (tannin acid equiv./dw) (Adegbusi et al., 2022). Another publication reported that the TTC of three orange maize were 1.79, 2.17, and 2.72 % (tannin acid equiv./dw), respectively (Alamu et al., 2020). The TTC value may be related to the varieties, as well as extraction procedure and testing protocols.

2.3.4 Phenolic acid composition

In this study, 4-hydroxybenzoic acid, vanillic acid, syringic acid, ρ-coumaric acid, ferulic acid, and sinapic acid were detected in the extracts (Table 4). The results had confirmed that bound phenolics were the major phenolic acid fraction in corn kernels. Sosulski et al. (1982) reported that cis and trans-ferulic, ρ-courmaric, and syringic acids were the predominant

phenolic acids in corn flour which agreed with our study. Considering the extraction procedure, the phenolic acids were extracted after alkaline processing which was alkaline delignification process. Solubilization affects the ether and ester bonds of phenolic acids linked to lignin (Méndez-Lagunas et al., 2020; Sun et al., 2003). Al Arni et al. (2007) found that ρ-coumaric acid, ferulic acid, syringic acid, and some traces of other compounds were the main products extracted after the solubilization of lignin by alkaline hydrolysis of maize. According to Table 4, our results agreed with the precious publications. Ferulic acid was the major phenolic acid in corn kernel, and its bound fraction accounted for more than 90% of total ferulic acid. The ferulic acid is mainly associate with arabinoxylans and other polysaccharides present within cell walls of the aleuronic layer (Yadav et al., 2007). It has the ability to form complexes with pentosans and proteins and it is important in the formation of the dough texture with its semi-elastic properties (Klepacka & Fornal, 2006). Žilić et al. (2012) extracted phenolic compounds from 10 different colored maize kernels, and they found that about 76- 91% of the total ferulic and ρ-coumaric acid in maize kernels is in the bound form. Lopez-Martinez et al. (2009) analyzed phenolic compounds from 17 strains of Mexican maize. They reported that about 94-98% of total ferulic acids were in the bound form in maize kernels. Among selected corn varieties, the Robust Yellow contained the highest total ferulic acid content (1834.73 µg/g) followed by the South American (1484.82 µg/g), Avalon (1479.65 µg/g), and Honey Selected (1452.30 µg/g). The Robust Yellow, South American and Honey selected are yellow corn, and the Avalon corn is white corn. Žilić et al (2012) reported the ferulic acid content in white and yellow corn were 2777.97 and 2859.81 µg/g, respectively. Our results were lower than Žilić's but higher than that reported by Woo et al. (2018) (12.75 µg/g, yellow dent). The difference in ferulic content

between our study and other publications may be caused by different types and varieties, extraction procedures, and analytical procedures.

Syringic acid also present in high amount in cereals, such as maize, wheat, sorghum, and rice (Srinivasulu et al., 2018). Our results showed syringic acid present in both free and bound fraction. The Silver King showed the highest syringic acid content (608.82 µg/g), following with the Ohio blue (405.34 µg/g). However, the majority of syringic acid was in free form in Silver King, but for Ohio Blue was opposite. That indicated the forms of extracted phenolic was highly related on the varieties and types. Méndez-Lagunas et al. (2020) reported that blue corn contained 3.21 µg/g of syringic acid in free form and 0.86 µg/g in bound form. Sosulski et al. (1982) reported 1.1 ppm of syringic acid in free fraction and 10.4 ppm in bound fraction. The ρ -coumaric acid is a hydroxyl derivative of cinnamic acid, and it is also an important phenolic acid play a role in cell wall cross linking (Kiliç & Yeşiloğlu, 2013). The highest total ρ-coumaric acid content was observed on the Robust White (143.59 µg/g), followed by Avalon (119.98 µg/g) and Honey Selected (122.38 µg/g). Chiremba et al. (2012) demonstrated that ρ -coumaric acid content in hard cultivars of white dent corn were in the range of 232-488 µg/g, and in soft cultivars were in the range of 85-175 µg/g. Sosulski et al. (1982) got slightly different results with 18.9 µg/g for yellow dent corn, 1.3 µg/g for Mexican blue, and 6.6 µg/g for white corn. Sinapic acid was reported that presenting in significant amounts in cereal grains (Y. Li et al., 2018). Table 4 shows that it was mainly found in free form ranged from 8.13-745.09 µg/g. The Strawberry corn had the highest sinapic content with 737.50 µg/g for free fraction and 7.59 µg/g in bound fraction.

The 4-hydrozybenzoic acid and vanillic acid content were relatively lower than other phenolic acids. There was no 4-hydrozybenzoic acid detected in the free phenolic fraction of all varieties belonging to popcorn and two field corn (Jimmy Red and Hickory King). Moreover, sweet corn contained significantly higher 4-hydrozybenzoic acid than other types of corn. The highest content was observed in two sweet corn, Peaches and Cream (38.09 μg/g) and Providence (36.11 μg/g). Vanillic acid was found in both free and bound forms. The Silver King corn showed the highest vanillic acid content (58.97 μg/g), followed by Ohio Blue (51.45 μg/g) and Kandy Korn (53.95 μg/g).

Comparing the three types of corn, sweet corn indicated relatively higher total content of phenolic compounds, as well as content of each phenolic acids. According to the study of Chiremba et al. (2012), the phenolic acid content was associated with hardness of corn kernel, and the maize bran ferulic acid content was strongly negatively correlated to kernel hardness. It may be due to the chemical bonding of phenolic content and other compounds within the plant cell walls. For example, ferulic acid and its oligomers are the most prevalent in forming linkages with cell walls. They simultaneously form ester–ether linkages between the non-starch polysaccharide arabinoxylan and lignin (Tsuda et al., 2018).

2.3.5 DPPH free radical scavenging activity

The DPPH assay has been widely used to determine the antioxidant capacity of extracted phenolics. This method is based on the formation of the non-radical form DPPH-H due to the reaction of DPPH free radicals and a hydrogen-donating antioxidant (Mishra et al., 2012). According to Fig.1, the DPPH scavenging activity of bound fraction (56.94-78.86%) was much

higher than free fraction (30.07-66.66%). De La Parra et al. (2007) concluded that bound phenolics were the primary contributors to antioxidant activity. It may be due to their higher phenolic content compared to the free fraction. However, the study of Méndez-Lagunas et al. (2020) got an opposite conclusion. The DPPH value of free phenolic extracted from blue corn was 1.65 ascorbic acid equivalent /100 ml, but the value was 0.18 for bound fraction. That may be caused by the different extraction procedures. Méndez-Lagunas et al. (2020) used pure ethanol to extract free phenolics and 4 M NaOH , while De La Parra et al. (2007) 80% chilled ethanol for extraction of free phenolics and 2 M NaOH for bound fraction.

The difference in the DPPH scavenging activity was not obviously different between each type. Among all free fraction, the free phenolic extracted from Indian Flour, Strwabery, and Silver King were the most promising antioxidative. Among all bound fraction, the bound phenolic extracted from three sweet varieties (Honey Selected, Providence, and Avalon), and two popcorn varieties (Robust Yellow and South American) existed the highest DPPH scavenging activity. Ferulic acid and sinapic acid significantly affected the DPPH scavenging activity of free phenolic compounds, with a correlation of 0.542 and 0.583 ($p < 0.05$) (Table 3). However, DPPH scavenging activity of bound phenolic fraction significantly correlated with total phenolic content ($r = 0.657$, $p < 0.01$), the content of ρ-coumaric acid ($r = 0.516$, $p < 0.05$), and the content of ferulic acid ($r = 0.672$, $p < 0.01$).

2.3.6 Metal chelating activity

Although DPPH assay has been widely used, it is necessary to use multiple assays for a comprehensive evaluation of antioxidant potential. The chelating capacity of metal ions is

another mechanism responsible for the antioxidant capacity. The ferrous ion chelating capacity ranged from 8.87 to 25.75% for free phenolics, and 34.53 to 73.83% for bound phenolics (Fig. 2). The same as DPPH scavenging activity, the bound fraction showed significantly higher chelating capacity than the free fraction. The relatively higher chelating activity was found in the bound fraction extracted from three varieties of popcorn (Japanese Hullness, South American, and Robust White), one sweet corn variety (Avalon), and one field corn (Jimmy Red), with ferrous ion chelating capacity more than 65%. There was no significant correlation of ferrous ion chelating activity to free phenolics, but it was highly correlation to vanillic acid ($r = 0.672$, $p < 0.01$) and syringic acid ($r = 0.727$, $p < 0.01$) in the bound fraction. Moreover, ferrous ion chelating capacity was negatively correlated to the total tannins content ($r = 0.656$, $p < 0.01$). Polyphenols have the ability to chelating metal ion mainly because of the presence of ortho-dihydroxy polyphenols, such as molecules bearing catechol or galloyl groups (Adegbusi et al., 2022; Shahidi et al., 1992). Although there was no correlation between ρ-coumaric acid and metal chelating ability, a published research demonstrated that ρ-coumaric acid had high ferrous ions chelating capacity with a 78.3% chelation of ferrous ion at 45μg/mL (Kiliç & Yeşiloğlu, 2013).

2.4 Conclusions

A broad range of traditional and colored corn varieties were assessed for various anti-radical activities. Although the specific profiles of phenolic compounds in these samples are undoubtedly related to the antioxidant activities observed, there appeared to be a general relationship between the enrichment of phenolic content and the relative level of antioxidant activity since only DPPH scavenging activity was highly correlated with the phenol content. The

metal chelating capacity may be more related to some specific phenolic compound in the corn kernel. The most phenolic-rich strains were the Honey Selected corn and the Avalon corn, and these samples consistently exhibited great antioxidant effects. In addition, extracts of the strain were also among the most effective anti-radical isolates among the 17 strains examined. The colored corn indicated relatively higher total phenolic, flavonoid, and tannin content, but it highly depended on the corn type. Ferulic acid was among the groups of phenolic compounds that are prevalent in corn kernel and was strongly correlated to antioxidant activities. This study screened different types and varieties of corn and analyzed their phenolic content and composition, which may give useful information to help understand the relationship between those phenolic and antioxidant capacities.

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Table 2.1 Total phenolic content, total flavonoid content, and total tannin content of free and bound phenolic fractions of 17 corn varieties.

Type	Sample	Color	Total phenolic content (µg GAE/g)			Total flavonoid content (µg GE/g	Total tannins content (µg CE/g)
			Free	Bound	Total		
Field Corn	Jimmy Red	Red	553.92 ± 26.61^{c}	973.35 ± 60.47^{j}	1363.02 ± 71.51^{i}	139.66 ± 7.91^{b}	1258.33 ± 104.08^{b}
	Hickory King White	White	270.42 ± 6.25^{ghi}	1106.10 ± 23.11^{ghi}	1376.52 ± 25.38^{i}	75.65 ± 0.66^{hi}	216.67 ± 14.43^{g}
	Indian Flour	Mixed	311.07 ± 17.23^{def}	1016.85 ± 42.73^{ij}	1327.92 ± 59.04^{i}	81.8 ± 2.03^{hi}	608.33 ± 52.04^{c}
	Ohio Blue	Blue	389.67 ± 19.04^{a}	1479.60 ± 83.23^{e}	2033.52 ± 106.72^{ef}	93.51 ± 4.29^{efgh}	3141.67 ± 208.17^{a}
	Reids Yellow Dent	Yellow	262.02 ± 13.37^{ghi}	1119.60 ± 20.27^{gh}	1381.62 ± 32.17^{i}	100.95 ± 4.23^{de}	491.67 ± 14.43^{cdef}
	Truckers Favorite Yellow	Yellow	291.42 ± 19.94^{efg}	1070.85 ± 23.03^{hi}	1362.27 ± 20.88^{i}	113.23 ± 5.95^{cd}	450.00 ± 0.00^{cdef}
Popcorn	Japanese Hullless	White	248.22 ± 11.83^{hi}	1535.85 ± 19.65^{e}	1784.07 ± 18.38^{g}	68.23 ± 2.26^{j}	408.33 ± 14.43^{def}
	Robust White	White	326.37 ± 19.46^{de}	1328.10 ± 21.21^{f}	1654.47 ± 31.36^{h}	85.8 ± 3.86^{fghi}	466.67 ± 28.87^{cdef}
	Robust Yellow	Yellow	594.57 ± 41.92^{de}	1791.60 ± 23.69^{bc}	2121.72 ± 24.54^{de}	98.8 ± 2.16^{ef}	583.33 ± 14.43^{cd}
	South American	Yellow	232.47 ± 7.16^{i}	1590.60 ± 24.68^{d}	1823.07 ± 28.58^{g}	90.09 ± 1.86^{efgh}	358.33 ± 28.87^{fg}
	Strawberry	Red	330.12 ± 14.59^{a}	1158.60 ± 29.24^{gh}	1753.17 ± 22.07^{gh}	297.09 ± 7.42^{a}	600.00 ± 25.00^{c}
Sweet Corn	Avalon	White	342.72 ± 22.27^{cde}	1958.85 ± 36.61^{a}	2301.57 ± 45.78^{ab}	82.37 ± 2.81^{ghi}	441.67 ± 14.43^{cdef}
	Honey Selected	Yellow	464.52 ± 21.31^{cd}	2018.85 ± 34.248^{a}	2379.42 ± 20.65^{a}	120.51 ± 5.67^{c}	566.67 ± 14.43^{cde}
	Kandy Korn	Yellow	360.57 ± 24.39^{b}	1191.6 ± 18.08^{g}	1656.12 ± 37.34^{h}	119.94 ± 1.93^{ij}	491.67 ± 14.43^{cdef}
	Peaches and Cream	Bicolor	360.27 ± 27.71^{cd}	1596.60 ± 18.08^{d}	1956.87 ± 26.92^{f}	95.23 ± 5.14^{efg}	558.33 ± 38.19^{cde}
	Providence	Bicolor	442.92 ± 16.44^{b}	1820.10 ± 19.44^{b}	2263.02 ± 19.73^{bc}	98.66 ± 4.45^{ef}	466.67 ± 14.43^{cdef}
	Silver King	White	451.62 ± 11.14^{b}	1714.35 ± 33.80^{c}	2165.97 ± 30.85^{cd}	100.37 ± 3.66^{de}	383.33 ± 14.43^{efg}

For each column, values with different letters are significantly different ($p < 0.05$).

Table 2.2 Analysis of variance for the effects of corn type, color, and their interaction on TPC, TTC, and TFC

Source of variance	TPC			TFC			TTC		
	df	*F-value*	*P-value*	*df*	*F-value*	*P-value*	*df*	*F-value*	*P-value*
Corn type	2	90.81	<.0001	2	74.48	<.0001	2	293.27	<.0001
Color	5	11.04	<.0001	5	357.01	<.0001	5	590.38	<.0001
Interaction	3	5.45	0.0023	3	314.13	<.0001	3	39.91	<.0001

Table 2.3 Correlation analysis of total phenolic content, total flavonoid content, total tannin content, and antioxidant activities

	TPC (free)	TPC (bound)	DPPH (free)	DPPH (bound)	Metal chelating (free)	Metal chelating (bound)
TPC-free	/	/	0.473	-.524*	0.056	0.056
TPC-bound	/	/	0.286	0.657**	0.106	-0.026
TFC	0.514*	-0.062	0.318	-0.319	-0.194	-0.031
TTC	-0.247	0.636**	-0.168	-0.476	0.098	-0.656**
4-hydroxybenzoic (free)	0.301	0.184	0.150	-0.213	-0.006	-0.064
4-hydroxybenzoic (bound)	0.566*	0.654**	0.428	0.010	-0.064	-0.353
vanillic acid (free)	0.458	0.408	0.454	-0.143	0.122	-0.162
vanillic acid (bound)	0.568*	0.025	-0.140	-0.444	0.028	-0.672**
syringic acid (free)	0.304	0.268	0.452	0.098	-0.090	0.191
syringic acid (bound)	0.572*	0.067	-0.134	-0.453	-0.027	-0.727**
ρ-coumaric acid (free)	0.258	-0.216	0.176	-0.069	-0.110	0.387
ρ-coumaric acid (bound)	-0.164	0.587*	0.147	0.516*	-0.320	0.209
Ferulic acid (free)	-0.658**	-0.016	0.542*	-0.188	-0.066	0.106
Ferulic acid (bound)	-0.083	0.899**	0.111	0.672**	-0.419	0.158
Sinapic acid (free)	0.403	-0.319	0.583*	-0.180	0.025	-0.086
Sinapic acid (bound)	0.247	0.011	0.173	-0.153	-0.226	-0.010

* Indicated significantly different with $p < 0.05$., and ** indicated significantly different with $p < 0.01$.

Table 2.4 Phenolic acid composition of corn form different varieties

Type	Variety	Color	Free (µg/g)	Bound (µg/g)	Total (µg/g)
			4-Hydroxybenzoic acid		
Field Corn	Jimmy Red	Red	ND	2.033 ± 0.104^{i}	2.033 ± 0.104^{ij}
	Hickory King White	White	ND	1.543 ± 0.173^{i}	1.543 ± 0.173^{j}
	Indian Flour	Mixed	2.988 ± 0.139^{e}	1.421 ± 0.208^{i}	4.409 ± 0.346^{gh}
	Ohio Blue	Blue	7.936 ± 0.485^{c}	2.735 ± 1.074^{b}	31.671 ± 0.589^{b}
	Reids Yellow Dent	Yellow	ND	1.764 ± 0.069^{i}	1.764 ± 0.069^{j}
	Truckers Favorite Yellow	Yellow	ND	5.389 ± 0.277^{fgh}	5.389 ± 0.277^{fg}
Popcorn	Japanese Hullless	White	ND	4.360 ± 0.139^{gh}	4.360 ± 0.139^{gh}
	Robust White	White	ND	8.891 ± 0.450^{e}	8.891 ± 0.450^{e}
	Robust Yellow	Yellow	ND	7.201 ± 0.485^{ef}	7.201 ± 0.485^{ef}
	South American	Yellow	ND	3.405 ± 0.381^{hi}	3.405 ± 0.380^{ghi}
	Strawberry	Red	ND	5.928 ± 0.346^{fg}	5.928 ± 0.346^{fg}
Sweet Corn	Avalon	White	5.364 ± 0.381^{d}	20.281 ± 0.069^{c}	25.646 ± 0.312^{c}
	Honey Selected	Yellow	1.935 ± 0.242^{f}	18.101 ± 0.312^{cd}	20.036 ± 0.069^{d}
	Kandy Korn	Yellow	13.432 ± 0.554^{b}	19.620 ± 1.005^{cd}	33.043 ± 1.559^{b}
	Peaches and Cream	Bicolor	20.698 ± 0.520^{a}	17.391 ± 0.831^{d}	38.089 ± 1.351^{a}
	Providence	Bicolor	8.304 ± 0.242^{c}	27.801 ± 1.351^{a}	36.105 ± 1.108^{a}
	Silver King	White	ND	25.082 ± 0.139^{b}	25.082 ± 0.139^{c}
			Vanillic acid		
Field Corn	Jimmy Red	Red	4.282 ± 0.331^{i}	3.473 ± 0.271^{cdef}	7.755 ± 0.241^{jk}
	Hickory King White	White	4.261 ± 0.121^{i}	1.981 ± 0.211^{h}	6.242 ± 0.090^{k}
	Indian Flour	Mixed	5.454 ± 0.060^{hi}	2.876 ± 0.090^{fgh}	8.330 ± 0.151^{jk}
	Ohio Blue	Blue	22.839 ± 0.482^{e}	28.613 ± 0.572^{a}	51.452 ± 0.090^{b}
	Reids Yellow Dent	Yellow	3.622 ± 0.422^{i}	3.750 ± 0.121^{cde}	7.372 ± 0.301^{jk}
	Truckers Favorite Yellow	Yellow	4.922 ± 0.331^{hi}	3.260 ± 0.271^{cdefg}	8.181 ± 0.603^{jk}
Popcorn	Japanese Hullless	White	11.675 ± 0.060^{fg}	3.643 ± 0.211^{cde}	15.318 ± 0.271^{gh}
	Robust White	White	8.607 ± 0.241^{gh}	3.707 ± 0.060^{cde}	12.314 ± 0.181^{hi}
	Robust Yellow	Yellow	5.497 ± 0.482^{hi}	2.493 ± 0.090^{fgh}	7.989 ± 0.572^{jk}
	South American	Yellow	7.159 ± 0.362^{hi}	3.899 ± 0.211^{cd}	11.057 ± 0.572^{ij}
	Strawberry	Red	12.208 ± 0.693^{fg}	8.693 ± 0.121^{b}	20.900 ± 0.572^{ef}
Sweet Corn	Avalon	White	35.260 ± 0.572^{c}	3.494 ± 0.241^{cdef}	38.754 ± 0.814^{c}
	Honey Selected	Yellow	27.122 ± 0.392^{d}	4.197 ± 0.512^{c}	31.319 ± 0.904^{d}
	Kandy Korn	Yellow	61.665 ± 0.934^{b}	2.280 ± 0.030^{gh}	53.945 ± 0.964^{b}
	Peaches and Cream	Bicolor	19.089 ± 0.121^{e}	3.558 ± 0.030^{cde}	22.647 ± 0.151^{e}
	Providence	Bicolor	14.424 ± 0.814^{f}	4.112 ± 0.271^{cd}	18.536 ± 1.085^{fg}
	Silver King	White	55.862 ± 3.495^{a}	3.111 ± 0.181^{efg}	58.973 ± 3.314^{a}
			Syringic acid		
Field Corn	Jimmy Red	Red	38.665 ± 2.148^{fg}	32.824 ± 1.487^{h}	71.489 ± 3.634^{ij}
	Hickory King White	White	26.166 ± 1.982^{hi}	31.890 ± 1.817^{h}	57.822 ± 3.800^{kl}
	Indian Flour	Mixed	52.332 ± 1.322^{e}	37.730 ± 1.156^{gh}	90.062 ± 2.478^{g}
	Ohio Blue	Blue	70.204 ± 3.139^{d}	335.132 ± 2.808^{a}	405.336 ± 5.947^{b}
	Reids Yellow Dent	Yellow	36.445 ± 1.322^{fg}	68.335 ± 0.826^{de}	104.780 ± 2.148^{f}
	Truckers Favorite Yellow	Yellow	45.089 ± 2.643^{ef}	76.161 ± 2.643^{c}	121.250 ± 5.286^{e}
Popcorn	Japanese Hullless	White	24.530 ± 0.661^{hi}	38.548 ± 2.313^{gh}	63.078 ± 2.974^{jk}
	Robust White	White	30.2454 ± 0.165^{gh}	44.739 ± 2.808^{g}	74.993 ± 2.643^{hij}
	Robust Yellow	Yellow	36.445 ± 1.982^{fg}	43.454 ± 2.313^{g}	79.899 ± 4.295^{ghi}
	South American	Yellow	ND	45.440 ± 2.148^{g}	45.440 ± 2.148^{l}

	Strawberry	Red	37.613 ± 1.652^{fg}	87.842 ± 1.322^{b}	125.456 ± 0.330^{e}
Sweet Corn	Avalon	White	ND	65.765 ± 1.156^{de}	65.765 ± 1.156^{jk}
	Honey Selected	Yellow	95.201 ± 0.826^{c}	54.317 ± 2.148^{f}	149.519 ± 1.322^{d}
	Kandy Korn	Yellow	24.881 ± 1.156^{hi}	61.676 ± 0.330^{ef}	86.557 ± 1.487^{gh}
	Peaches and Cream	Bicolor	17.288 ± 0.661^{i}	67.867 ± 0.496^{de}	85.156 ± 1.156^{gh}
	Providence	Bicolor	156.878 ± 0.826^{b}	72.890 ± 2.643^{cd}	229.768 ± 3.469^{c}
	Silver King	White	538.618 ± 7.103^{a}	70.204 ± 1.817^{cd}	608.822 ± 5.286^{a}
			ρ-coumaric acid		
Field Corn	Jimmy Red	Red	3.937 ± 0.105^{a}	46.497 ± 0.754^{g}	56.338 ± 1.017^{g}
	Hickory King White	White	1.209 ± 0.040^{efg}	32.483 ± 0.835^{hi}	35.507 ± 0.935^{hi}
	Indian Flour	Mixed	1.356 ± 0.007^{ef}	38.864 ± 0.236^{gh}	42.254 ± 0.218^{h}
	Ohio Blue	Blue	0.783 ± 0.033^{i}	41.573 ± 0.291^{g}	43.531 ± 0.209^{h}
	Reids Yellow Dent	Yellow	0.755 ± 0.015^{i}	71.624 ± 0.354^{e}	73.511 ± 0.318^{ef}
	Truckers Favorite Yellow	Yellow	1.091 ± 0.040^{gh}	64.357 ± 0.626^{e}	67.085 ± 0.527^{f}
Popcorn	Japanese Hullless	White	0.855 ± 0.025^{i}	55.420 ± 0.590^{f}	57.558 ± 0.527^{g}
	Robust White	White	3.377 ± 0.084^{b}	135.255 ± 2.533^{a}	143.594 ± 2.742^{a}
	Robust Yellow	Yellow	1.803 ± 0.076^{d}	95.517 ± 4.294^{c}	99.979 ± 4.485^{c}
	South American	Yellow	1.407 ± 0.051^{e}	83.680 ± 2.878^{d}	87.198 ± 2.751^{d}
	Strawberry	Red	4.088 ± 0.015^{a}	66.199 ± 0.835^{e}	76.419 ± 0.872^{e}
Sweet Corn	Avalon	White	2.054 ± 0.080^{c}	114.840 ± 1.153^{b}	119.976 ± 1.353^{b}
	Honey Selected	Yellow	0.927 ± 0.004^{hi}	120.111 ± 2.233^{b}	122.377 ± 2.224^{b}
	Kandy Korn	Yellow	ND	29.049 ± 2.315^{i}	29.049 ± 2.315^{i}
	Peaches and Cream	Bicolor	1.171 ± 0.007^{fg}	39.269 ± 1.062^{gh}	42.196 ± 1.044^{h}
	Providence	Bicolor	1.112 ± 0.011^{g}	83.070 ± 2.488^{d}	85.849 ± 2.515^{d}
	Silver King	White	1.69 ± 0.069^{d}	72.477 ± 4.503^{e}	76.682 ± 4.331^{e}
			Ferulic acid		
Field Corn	Jimmy Red	Red	10.761 ± 0.338^{bcd}	600.961 ± 2.838^{j}	611.722 ± 3.176^{g}
	Hickory King White	White	3.406 ± 0.245^{h}	589.636 ± 6.827^{j}	593.042 ± 7.072^{g}
	Indian Flour	Mixed	6.541 ± 0.230^{efgh}	682.049 ± 21.140^{i}	688.59 ± 21.370^{f}
	Ohio Blue	Blue	4.317 ± 0.215^{gh}	970.537 ± 10.800^{f}	974.855 ± 11.015^{d}
	Reids Yellow Dent	Yellow	3.753 ± 0.123^{gh}	919.596 ± 7.947^{fg}	923.349 ± 8.069^{d}
	Truckers Favorite Yellow	Yellow	3.536 ± 0.184^{h}	810.108 ± 4.863^{h}	813.644 ± 5.047^{e}
Popcorn	Japanese Hullless	White	8.158 ± 0.245^{def}	1375.705 ± 16.814^{de}	1383.863 ± 16.569^{c}
	Robust White	White	7.377 ± 0.061^{defg}	979.628 ± 3.344^{f}	987.004 ± 3.283^{d}
	Robust Yellow	Yellow	8.743 ± 0.368^{def}	1825.989 ± 25.574^{a}	1834.733 ± 25.942^{a}
	South American	Yellow	5.153 ± 0.322^{fgh}	1479.671 ± 14.482^{b}	1484.824 ± 14.804^{b}
	Strawberry	Red	39.606 ± 3.759^{a}	901.545 ± 14.697^{g}	941.15 ± 18.455^{d}
Sweet Corn	Avalon	White	9.991 ± 0.261^{cde}	1469.659 ± 17.290^{bc}	1479.649 ± 17.550^{b}
	Honey Selected	Yellow	13.625 ± 0.583^{bc}	1438.677 ± 3.728^{bc}	1452.302 ± 4.311^{b}
	Kandy Korn	Yellow	13.961 ± 0.322^{b}	957.292 ± 18.394^{fg}	971.253 ± 18.716^{d}
	Peaches and Cream	Bicolor	8.375 ± 0.276^{def}	1414.258 ± 10.371^{cde}	1422.633 ± 10.647^{bc}
	Providence	Bicolor	8.787 ± 0.092^{def}	1437.473 ± 9.113^{bcd}	1446.26 ± 9.205^{bc}
	Silver King	White	12.79 ± 0.353^{bc}	1372.147 ± 33.781^{e}	1384.937 ± 34.134^{c}
			Sinapic acid		
Field Corn	Jimmy Red	Red	68.954 ± 4.949^{ef}	2.624 ± 0.146^{f}	71.579 ± 4.803^{efg}
	Hickory King White	White	26.681 ± 0.255^{hij}	ND	26.681 ± 0.255^{kl}
	Indian Flour	Mixed	745.194 ± 11.025^{a}	1.647 ± 0.000^{f}	746.840 ± 11.025^{a}
	Ohio Blue	Blue	97.719 ± 1.164^{cd}	2.702 ± 0.109^{f}	100.421 ± 1.274^{de}
	Reids Yellow Dent	Yellow	48.345 ± 2.511^{fgh}	2.830 ± 0.146^{f}	51.175 ± 2.365hij
	Truckers Favorite Yellow	Yellow	56.527 ± 3.821^{fg}	18.088 ± 0.691^{b}	74.615 ± 4.512ef
Popcorn	Japanese Hullless	White	12.196 ± 0.437^{ij}	ND	12.196 ± 0.437l
	Robust White	White	18.371 ± 0.873^{ij}	ND	18.371 ± 0.873kl
	Robust Yellow	Yellow	33.319 ± 1.055^{hi}	6.690 ± 0.218^{e}	40.009 ± 1.274ilk

	South American	Yellow	29.357 ± 1.346^{hij}	ND	29.357 ± 1.346jkl
	Strawberry	Red	737.501 ± 17.174^{a}	7.590 ± 0.327^{e}	745.091 ± 17.502a
Sweet Corn	Avalon	White	42.453 ± 0.582^{gh}	12.607 ± 0.509^{c}	55.060 ± 1.092fgh
	Honey Selected	Yellow	82.616 ± 4.184de	ND	82.616 ± 4.184de
	Kandy Korn	Yellow	27.273 ± 0.364hij	10.317 ± 0.400d	37.590 ± 0.764ijk
	Peaches and Cream	Bicolor	8.130 ± 0.801j	ND	8.130 ± 0.801l
	Providence	Bicolor	113.388 ± 4.985c	ND	113.388 ± 4.985c
	Silver King	White	227.574 ± 3.238b	20.326 ± 0.801a	247.901 ± 4.039b

For each phenolic acid in one fraction across all varieties, values with different letters are significantly different ($p < 0.05$).

ND means the compound was not detected.

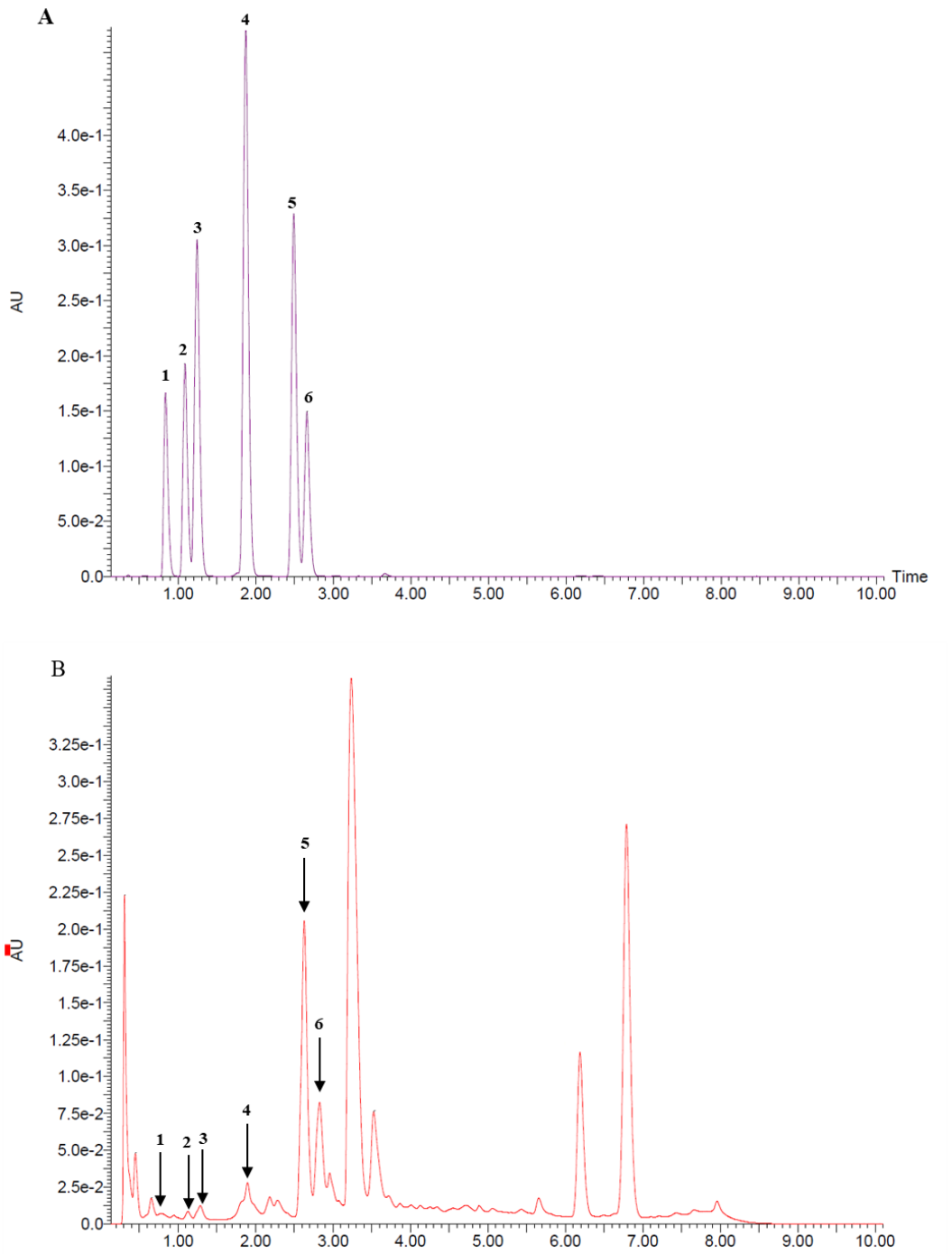

Figure 2.1 UPLC chromatograms of phenolic acid standards (A) and free phenolics extracted from the Strawberry corn (B). The peaks from 1-6 represent for 4-hydroxybenzoi acid, vanillic acid, syringic acid, ρ-coumaric acid, ferulic acid, and sinapic acid, respectively.

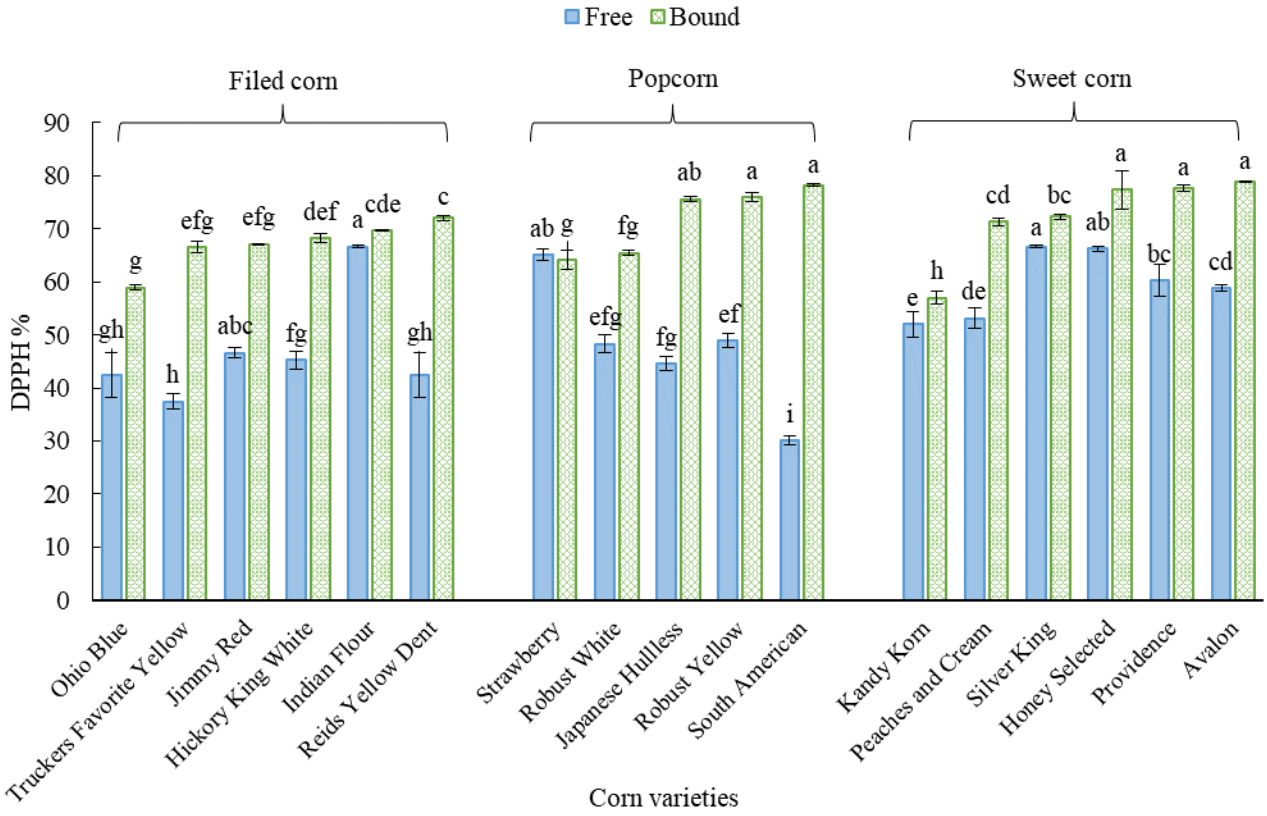

Figure 2.2 DPPH scavenging activities of free and bound phenolic fractions of 17 corn varieties.

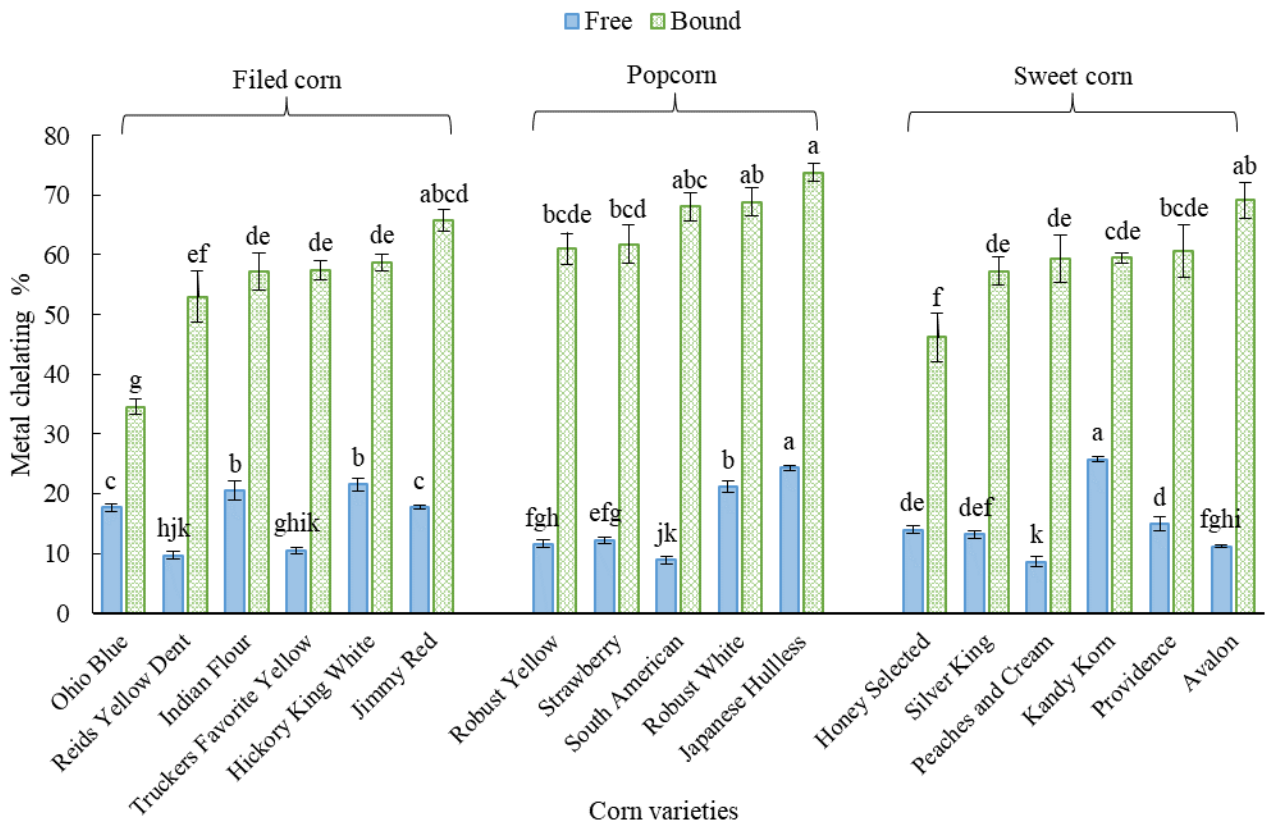

Figure 2.3 Fe^{2+} chelating capacity of free and bound phenolic fractions of 17 corn varieties.

Chapter 3 - Effect of corn variety and yeast strain on the composition and antioxidant activity of phenolic compounds extracted from dried distiller's grains

Abstract

In this study, dried distiller's grains (DDG) were obtained from distilled spirit production using four varieties of corn (Hickory king white, Reids yellow dent, Jimmy red, and Ohio blue) and four yeast strains (GR-2, DADY, HG-1, and USW-6) through liquid fermentation. Phenolic compounds were extracted from both original corn kernel as a control and DDG. Results showed that both corn variety and yeast strain significantly influenced the total phenolic content (TPC) ($p < 0.05$), and the TPC of DDG was three to four times higher than that of the unfermented corn. Yeast GR-2 and HG-1 yielded higher TPC than other yeasts, and DDG from blue corn had the highest free phenolic content up to 2,078.82 μg/g gallic acid equivalent per gram of DDG. Moreover, the phenolic composition was affected by fermentation. Six phenolic acid standards (namely 4-hydrobenzoic acid, vanillic acid, syringic acid, p-coumaric acid, ferulic acid, and sinapic acid) were used for quantification analysis of extracted phenolic compounds. Results showed that the DPPH+ and ABTS•+ scavenging activities of phenolic compounds were improved by fermentation. This study can provide a prospective approach to improve the phenolic content and their antioxidant activities.

3.1 Introduction

The development of nutraceuticals and functional foods has been a new trend in the cereal processing industry. Phenolics are the most widely distributed secondary metabolites present in grains (Li et al., 2017). They have received a great amount of attention in human diet worldwide due to their health benefits for human, including antioxidant property, disease prevention capacity (e.g., anti-carcinogenic, anti-inflammatory, and antidiabetic), and activity against microbial toxins (Cai et al., 2004; Chen et al., 2021; Hitayezu et al., 2015; Vermerris et al., 2006).

Among major cereals, corn is plentiful, cheap, easily available, and rich in phenolic content. Corn contains up to three times more phenolics compared to wheat, rice, and oats, and has the highest antioxidant activity among those cereal grains (Adom & Liu, 2002). Phenolics in corn exist mainly in soluble conjugates (glycosides) and insoluble forms, which are covalently bound to sugar moieties or cell wall structural components (Acosta-Estrada et al., 2014). Most corn phenolic compounds are associated with cell walls in pericarp and monolayered aleurone, and about 85% of phenolics are in the insoluble-bound form (Acosta-Estrada et al., 2019; Gutiérrez-Uribe et al., 2010). Ferulic acid was reported to be the major phenolic compound representing about 70% of the total phenolics in corn (Ndolo & Beta, 2014). The insoluble-bound ferulic acids (e.g., diferulic, trifeluric, and pentaferulic) are covalently linked to polysaccharides to form crosslinks and strengthen cell walls. Therefore, efficient extraction of corn phenolic compounds is mainly focused on the insoluble-bound fraction. Some previous studies used physical or chemical treatments to extract phenolic compounds from cereal grains or plants, such as alkaline hydrolysis, acid hydrolysis, enzymatic hydrolysis, microwave,

ultrasound, and heat treatment (Herrera & Luque De Castro, 2004; Jokić et al., 2012; Stalikas, 2007; L. Wang et al., 2019; H. Zheng et al., 2009). However, those treatments are not sufficient in the extraction of insoluble phenolic compounds because most of them are conjugated to cellulose, hemicellulose, lignin, pectin, and/or rod-shaped structural proteins (Ayoub et al., 2016; Wong, 2006; Yeo & Shahidi, 2017). Recently, there has been increasing interest in utilizing microbial fermentation to improve the extraction of phenolic compounds from cereal grains. Microorganism communities, such as Bacillus subtilis, Monascus anka, and Saccharomyces cerevisiae, are widely used in the fermentation industries because of their high efficiency and their positive effect on the release of the bioactive phenolic compounds in cereals and grains (Hur et al., 2014; Huynh et al., 2014; Lee et al., 2008). However, little information is available about the potential of fermentation in enhancing the phenolic content and bioactivity in corn kernels, especially in dried distiller's grains generated from corn spirit fermentation.

Therefore, this study aimed to investigate the effects of corn variety and yeast strain on the content, composition, and antioxidant activity of phenolic acids extracted from DDG. Phenolic content, composition, and antioxidant activities were analyzed among four corn varieties (including blue corn, red corn, white corn, and yellow corn) and four yeast strain from the species of Saccharomyces cerevisiae. This work provides a potential of fermentation process in developing functional ingredients from corn, which would be widely used in food industry in the future.

3.2 Materials and Methods

3.2.1 Raw materials and chemicals

Four different varieties of corn, including Hickory king white corn (white corn), Reids yellow dent corn (yellow corn), Jimmy red corn (red corn), and Ohio blue corn (blue corn) were purchased from Hoss Tools (Norman Park, GA, USA). Four yeast strains are all from the species of Saccharomyces cerevisiae, including Safspirit GR-2 (GR-2) (Fermentis Co., Marcq-en Baroeul, France), Red Star Distillers' active dry yeast (DADY), SafSpirit HG-1 (HG-1), and SafSpirit USW-6 (USW-6) (Lesaffre, Milwaukee, WI, USA). All other chemicals, solvents, and reagents used were at least analytical grade and purchased from Sigma-Aldrich (St. Louis, Mo, USA) or Fisher Scientific (Fairlawn, NJ, USA).

3.2.2 Preparation of corn flour and DDG

Corn kernels were milled into flour with particle size less than 1 mm using a UYD Cyclone Sample Mill (UDY Corporation, Fort Collins, CO, USA), and the unfermented flour was considered as control. Corn DDG was prepared using a liquid fermentation procedure (Weiss et al., 2023). A total of 16 corn DDG samples were prepared with four corn varieties and four yeast strains. The samples were then dried and stored at -20°C until further analysis. Corn flour without fermentation was used as a control.

3.2.3 Extraction of phenolic compounds

Free and bound phenolic compounds from corn flour and DDG were extracted according to the method of Tian et al. (2022) with some modifications. Briefly, the sample was extracted twice with acetone (80%, v/v) at 1:5 (w/v) under shaking in the dark for 20 min each time to extract free phenolic compounds. After extraction, the supernatants were combined, dried, and reconstituted to a final volume of 5 mL using LC-MS grade methanol. The precipitate was

subjected to extraction of bound phenolic compounds where 4 M NaOH was mixed with the precipitate at 1:10 (w/v) and shaken at 300 rpm in the dark for 3 h. At the end of extraction, concentrate HCl (12 N) was added to acidify the mixture to pH 2.0 and centrifuged (8000 rpm, 20 ºC, 15 min). The supernatant was collected and extracted with ethyl acetate 3 times at 1:5 (w/v). The organic phase was combined, dried, and then reconstituted to a final volume of 5 mL using LC-MS grade methanol. Both reconstituted free and bound phenolic compound solutions were filtered through 0.2-μm filters, and the filtrates were stored at -20 °C until further analysis.

3.2.4 Determination of total phenolic content (TPC)

The TPC was determined using Folin-Ciocalteu method with some modifications (Tian & Li, 2018). Briefly, 0.1 mL phenolic extracts were mixed with 7.9 mL DI water and 0.5 mL Folin-Ciocalteu reagent in a 15-mL tube. After 10 min, 1.5 mL Na_2CO_3 solution (20%, v/w) was added to the mixture and reacted for 2 h in the dark. The absorbance was measured at 765 nm using a UV1600-PC spectrophotometer (Radnor, VWR, PA, USA). Gallic acid was used as a standard, and the TPC was expressed as mg gallic acid equivalent per gram of sample (mg GAE/g).

3.2.5 Determination of free radical scavenging activity

The 1.1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity of phenolic compounds was measured by following the method of Hu et al. (2021) with some modifications. Briefly, 20 μL sample and 200 μL DPPH solution (0.1 mM in methanol) were added to a well of a 96-well plate. The mixture was incubated in the dark for 30 min and then the absorbance was measured at 517 nm. Trolox was used as a standard, and the results were expressed as mM Trolox equivalents per gram of sample (mmol TE/g).

The 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) free radical scavenging activity of phenolic compounds was determined according to a previous method reported by Thaipong et al. (2006) with some modifications. In brief, stock solution was prepared by mixing 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution in equal quantities and reacted for 12 h in the dark. The ABTS reactive solution was prepared by diluting the stock solution with methanol until the absorbance reached 1.1±0.02 at 734 nm. To determine the ABTS activity, 50 μL sample was mixed with 3.9 mL ABTS reactive solution and reacted for 10 min in the dark followed by the measurement of absorbance at 734 nm. Trolox was used as a standard, and the results were expressed as mM Trolox equivalents per gram of sample (mmol TE/g).

3.2.6 Identification of phenolic compounds

Both quantitative and qualitative analyses of extracted phenolic compounds were accomplished on an ultra-high performance liquid chromatography coupled with diode-array detection and electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-DAD-ESI-Q-TOF-MS/MS) (Model, Waters Corporation, Milford, MA, USA). The flow rate and gradients of mobile phase, and MS parameters were the same as a previous study of Tian et al. (2019). Six phenolic acid standards were used for quantification, including 4-hydrobenzoic acid, vanillic acid, syringic acid, p-coumaric acid, ferulic acid, and sinapic acid.

3.2.7 Statistical analysis

The experiments in this study were carried out in triplicate, unless specified, otherwise. The data were reported as mean ± standard deviation. Significant difference among means ($P <$

0.05) were analyzed using the least significant difference (LSD) method. Data were subjected to either one-way or two-way analysis of variance (ANOVA), and Tukey's post-hoc test was done using SAS software (OnDemand for Academics, SAS Institute, Cary, NC, USA).

3.3 Results and Discussion

3.3.1 Effects of yeast strain and corn variety on TPC

The TPC of each corn and DDGS is listed in Table 1. Regarding the effect of yeast strain, GR-2 and HG-1 achieved higher TPC after fermentation. Their free TPC were the highest (1,828.35 and 1,861.72 µg GAE/g, respectively). Overall, DDGS had three to four times higher TPC than the unfermented corn for both free and bound phenolic compounds. One possible reason is that the consumption of starch during fermentation concentrated the phenolic compounds in DDGS. Another reason could be that microbial fermentation enhanced the extraction of phenolic compounds from DDGS. Most phenolic compounds in corn are associated to cell walls in the pericarp and the monolayered aleurone (Acosta-Estrada et al., 2019). The majority of phenolic compounds in cereals occur as soluble conjugates (glycosides) and insoluble forms, and the bound fraction are usually bound to sugar moieties or cell wall structural components through covalent bonds (Acosta-Estrada et al., 2014). For corn, about 85% of phenolic compounds in corn kernel are present in as insoluble form (Adom & Liu, 2002; Gutiérrez-Uribe et al., 2010). The microorganisms and enzymes could destroy cell walls and the crystalline structure of starch to weaken the linkages between the phenolic compounds and the cell walls (L. Liu et al., 2017). According to Luo et al. (2021), the mixed culture fermentation with Monoscus anka, S. cerevisiae, and B. subtilis significantly increased the TPC by 23 folds compared to the unfermented control. Similar results were also reported by Chen et al. (2021).

Their study demonstrated that TPC increased by 18 folds after co-microbiological fermentation using Monoscus anka, S. cerevisiae, and B. subtilis. Regarding corn variety, the original blue corn had the highest free TPC (852.45 μg/g) and the highest average free TPC value (2,078.82 μg/g), followed by the original red corn with 620.2 μg/g free phenolics and 1,540.37 μg/g average free phenolics. The TPC of bound phenolic compounds was in the range of 1,923.53 to 2,030.40 μg/g with no significant difference among corn varieties. Previous studies reported that colored corn, such as blue, purple, and red corn, usually contained higher phenolic compounds and carotenoids than other types of corn, especially in anthocyanins, lutein, and β-carotene (Lao et al., 2017; Lopez-Martinez et al., 2009; Žilić et al., 2012). The statistical analysis showed that both corn variety and yeast strain had significant effects on both free and bound TPC with $p < 0.0001$ (Table 2).

3.3.2 Effect of yeast fermentation on the variation of phenolic compositions

The UPLC system coupled with a PDA detector was used to investigate the shift of phenolic compositions of corn before and after fermentation. The corn phenolics were quantified using six standards, including 4-hydroxybenzoic acid, vanillic acid, syringic acid, p-coumaric acid, ferulic acid, and sinapic acid. The content of each phenolic acid was significantly increased after fermentation, except for the vanillic acid and sinapic acid in the bound fraction, and ρ-coumaric acid in the free fraction (Table 3). The white corn DDGS fermented using the GR-2 strain had significantly lower vanillic content (2.11 μg/g) in the bound fraction compared to the unfermented white corn (4.53 μg/g). The blue corn DDGS fermented using the HG-1 strain had significantly lower sinapic content (14.23 μg/g) in the bound fraction compared to the unfermented blue corn (25.86 μg/g). Ferulic acid was the major bound phenolic compound in

both unfermented corn and DDGS. For all corn varieties, only red corn had undetected 4-hydrobenzoic acid in the bound fraction. However, all DDGS showed two to three times higher content of 4-hydrobenzoic acid in the bound phenolic extracts compared to the unfermented corn. Different yeast strains had effects on single phenolic acid during the fermentation. However, the reasons behind this result remained unsure and need to be studied in the future.

3.3.3 Effects of yeast strain and corn variety on the antioxidant activities

Besides the phenolic compositions, fermentation could also alter the antioxidant activity of the extracted phenolic compounds. The effects of corn varieties and yeast stains on the antioxidant activity were evaluated using DPPH (Table 4) and ABTS assays (Table 5). Both corn variety and yeast stain affected the DPPH+ scavenging activity of free phenolic extracts, while only yeast strain had a significant effect on the DPPH+ scavenging activity of bound phenolic extracts.

Upon the effect of corn variety, unfermented blue and red corn had significantly higher DPPH+ scavenging capacity than unfermented white and yellow corn. The free and bound phenolic compounds extracted from the unfermented blue and red corn were much higher than those extracted from white and yellow corn, and the same trend was also observed on the average DPPH values. It might be due to the higher TPC of blue and red corn as shown in Table 1. Previous studies demonstrated a positive linear correlation between antioxidant capacity and TPC (Cai et al., 2004; Djeridane et al., 2006; Katalinic et al., 2006; Wojdylo et al., 2007). For free phenolics, the blue corn had the highest DPPH+ scavenging activity (2.03 mmol TE/g for the control, and 3.41 mmol TE/g for the average) followed by red corn (1.51 mmol TE/g for the

control, and 2.70 mmol TE/g for the average). For bound phenolics, the red corn showed a relatively higher DPPH+ scavenging activity with a value of 3.30 mmol TE/g for the unfermented corn, but it had the highest average DPPH value (4.40 mmol TE/g). Compared with the unfermented corn, the DPPH+ scavenging capacity of all DDGS was increased about 1.5 to 2 times after fermentation. The highest average DPPH+ scavenging activity of free phenolics was observed with the DDGS from GR-2 fermentation (2.79 mmol TE/g). For bound phenolics, the DDGS from DADY (4.39 mmol TE/g) and GR-2 (4.26 mmol TE/g) fermentations showed the highest DPPH+ scavenging capacity of the extracted phenolics. However, the radical scavenging activity of ABTS•+ had slightly different trends. As shown in Table 2, ABTS•+ scavenging activity of free phenolic compounds was significantly affected by both corn variety and yeast strain, while ABTS•+ scavenging activity of bound phenolics was only significantly affected by yeast strains. Of the unfermented corn, the highest scavenging activity was observed with the blue corn (7.72 mmol TE/g) followed by red corn (5.58 mmol TE/g). Both corn varieties also had the first (blue corn, 12.31 mmol TE/g) and second highest (red corn, 8.61 mmol TE/g) on the average value of ABTS•+ scavenging activity, respectively. No significant difference was observed in the control yellow and white corn. The antioxidant activity of bound phenolic compounds was only affected by yeast strain; hence, no significant difference was observed regarding the bound phenolic compounds among corn varieties. Moreover, yeast strain had a great impact on the ABTS•+ scavenging capacity of phenolic compounds. GR-2 was better to improve the ABTS•+ scavenging activity with the highest average value for free phenolics (9.82 mmol TE/g, $p < .0001$) and a relatively higher average value for bound phenolics (12.97 mmol TE/g).

The increase of free radical scavenging activities of DDGS may be caused by the release of specific phenolic acids which altered the composition and content of phenolic compounds. Herein, correlation analysis of phenolic compounds and the DPPH+ and ABTS•+ free radical scavenging activities was conducted and summarized in Table 6. Overall, the contents of p-coumaric acids (r=0.639 for free phenolics and r=0.810 for bound phenolics), and ferulic acid (r=0.785 for free phenolics and r=0.775 for bound phenolics) were highly correlated to both DPPH+ and ABTS•+ radical scavenging activities, which was in agreement with the study of Zavala-López et al. (2018). As shown in Table 6, ferulic acid was the major phenolic compound representing about 70% of the total and it was mainly found in bound forms. Researchers found both ferulic acid and p-coumaric acid had a strong antioxidant capacity because of their phenolic nucleus and unsaturated side chain which could easily form a resonance stabilized phenoxy radical (Kiliç & Yeşiloğlu, 2013; M. Srinivasan et al., 2007). Srinivasan et al. (2007) explained that any reactive free radical colliding with ferulic acid easily abstracts a hydrogen atom to form a phenoxy radical, and the extended conjugation in the unsaturated side chain provides additional stabilization of the phenoxy radical. Besides, vanillic acid and sinapic acid were highly correlated to both DPPH+ and ABTS•+ radical scavenging capacities of free phenolic compounds with all r values higher than 0.69, while 4-hydroxybenzoic acid and syringic acid highly contributed to those antioxidant activities of bound phenolics with all r value higher than 0.75. There is limited research on the comparison of the antioxidant capacity of different types of phenolic compounds, but some research reported that the antioxidant potential of phenolics generally depends on their chemical structure and substitution pattern of hydroxyl groups on the side chain (Acosta-Estrada et al., 2019; J. Chen et al., 2020; Lopez-Martinez et al., 2009; Méndez-Lagunas et al., 2020; Rice-Evans et al., 1996; M. Srinivasan et al., 2007; Wojdylo et al.,

2007). In sum, DDGS showed higher antioxidant potential in terms of the DPPH+ and ABTS•+ radical scavenging activities. The higher scavenging capacity is not only affected by the increase of phenolic content but also by the phenolic composition. More detailed studies should be performed in the further on the comparison on the antioxidant capacity of individual phenolic compounds, and the correlation between individual phenolic compounds and antioxidant activities.

3.4 Conclusions

The fermentation using four yeast strains improved the TPC in corn DDGS, and the corn variety was also a key factor. Yeast fermentation enhanced the TPC by destroying the linkages between phenolic compounds and cell walls or proteins to ease the release of phenolic compounds during extraction, and their effects were highly dependent on their strains. Antioxidant activities of phenolic fractions were also improved after fermentation. This work confirmed that fermentation can improve the yield and antioxidant activity of phenolic compounds. But further study is needed to fully explain the interaction between yeast cells and the molecules in corn kernel during fermentation.

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Table 3.1. Effect of corn variety and yeast strain on the total phenolic content[1]

Corn variety	Total phenolic content of free phenolic compounds (μg/g)					
	Yeast strains					
	Control[2]	DADY	GR-2	HG-1	USW-6	Average[3]
Blue corn	852.45±59.03[A]	2362.35±108.34	2397.60±139.65	2515.35±126.08	2266.35±141.40	2078.82a
Red corn	620.20±25.75[B]	1694.10±103.05	2081.10±164.72	1941.60±103.94	1364.85±100.71	1540.37b
White corn	452.45±29.77[C]	1316.10±37.23	1563.60±49.26	1394.10±68.10	1120.35±83.94	1169.32c
Yellow corn	447.70±28.32[C]	1223.10±73.03	1271.10±23.37	1595.85±55.66	1493.10±193.17	1206.17c
Average[4]	593.20c	1648.91b	1828.35a	1861.72a	1561.16b	
Corn variety	Total phenolic content of bound phenolic compounds (μg/g)					
	Yeast strains					
	Control	DADY	GR-2	HG-1	USW-6	Average
Blue corn	1988.78±118.34[A]	3548.03±90.03	3720.15±107.94	3614.4±202.73	3544.65±76.98	3283.20a
Red corn	1939.28±141.56[A]	3426.53±205.87	3759.53±250.99	3707.78±189.44	3109.28±182.88	3188.48ab
White corn	2030.40±89.13[A]	3474.90±171.81	3341.03±124.46	3640.28±139.88	3008.03±173.68	3098.93b
Yellow corn	1923.53±150.48[A]	3624.53±103.69	3373.65±88.72	3470.40±92.84	3623.40±86.52	3203.10ab
Average	1979.49c	3518.49a	3548.59a	3608.21a	3321.34b	

[1]All data are presented as mean ± standard deviation based on four replicates.

[2]For the Control column, different uppercase letters indicate significant difference across corn varieties at $p < 0.05$.

[3]For the Average column, different lowercase letters indicate significant difference across yeast strains at $p < 0.05$.

[4]For the Average row, different lowercase letters indicate significant difference across corn varieties at $p < 0.05$.

Table 3.2 Analysis of variance for the effects of maize variety, yeast strain, and their interaction on the final ethanol concentration and ethanol yield

Source of Variation	Free phenolic compounds								
	TPC			DPPH			ABTS		
	df	*F-value*	*P-value*	*df*	*F-value*	*P-value*	*df*	*F-value*	*P-value*
Corn variety	3	366.90	<.0001	3	2045.43	<.0001	3	2511.84	<.0001
Yeast strains	4	449.45	<.0001	4	746.71	<.0001	4	794.00	<.0001
Interaction	12	16.43	<.0001	12	39.62	<.0001	12	56.80	<.0001
Source of Variation	Bound phenolic compounds								
	TPC			DPPH			ABTS		
	df	*F-value*	*P-value*	*df*	*F-value*	*P-value*	*df*	*F-value*	*P-value*
Corn variety	3	5.24	0.0028	3	66.83	<.0001	3	2.01	0.1228
Yeast strains	4	351.85	<.0001	4	208.13	<.0001	4	351.85	<.0001
Interaction	12	6.21	<.0001	12	8.62	<.0001	12	6.21	0.0008

Table 3.3 Phenolic content of six phenolic acids

Corn varieties	Yeasts	Phenolic content (μg/g)											
		4-hydrobenzoic acid		**Vanillic acid**		**Syringic acid**		***p*-Coumaric acid**		**Ferulic acid**		**Sinapic acid**	
		Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound
Red	Control	2.26±0.10j	nd	2.86±0.00l	17.50±0.34b	18.5 ±2.25l	7.43±0.05g	10.08±0.80hi	167.37±6.26f	6.40±0.55j	2074.80±49.60g	7.93±0.42l	2.11±0.11i
	DADY	116.10±3.10ef	22±0.39b	26.04±0.04hi	11.79±0.43cd	202.08±2.44i	33.63±1.25ab	14.29±1.22fg	347.41±8.03ab	48.36±2.61c	3920.98±44.09bcd	39.28±0.39g	68.54±3.71b
	GR-2	99.97±0.76fg	21.15±0.73b	45.88±0.65e	12.13±0.81c	187.04±1.65ij	28.51±0.22cd	7.35±0.49ij	318.12±21.06bc	65.99±0.18a	4052.67±66.22b	8.98±0.16l	59.70±0.24c
	HG-1	259.35±8.12a	29.33±0.33a	43.87±0.07e	12.86±0.36c	574.15±35.68a	33.98±1.77ab	15.01±0.20f	358.81±6.11a	54.2±1.14b	3958.72±15.94bcd	16.61±1.05k	15.75±1.09efg
	USW-6	88.45±3.57gh	20.77±0.15bc	32.23±0.82fg	10.42±0.27de	346.74±6.15efg	28.29±0.31cd	6.42±0.54j	271.68±0.32d	37.20±2.07de	3533.74±0.63f	51.25±0.64de	11.44±1.51gh
White	Control	3.27±0.14j	nd	2.34±0.02l	4.53±0.14jkl	17.52±1.39l	6.12±0.02g	3.36±0.05h	118.96±3.97g	16.04±0.00j	2018.08±75.90g	16.04±0.01k	2.27±0.24i
	DADY	115.37±1.76ef	9.43±0.21ij	21.39±0.36j	5.64±0.27ij	336.04±6.21fg	26.59±3.43de	34.50±0.20e	251.15±9.49de	31.89±0.38ef	3936.08±18.11bcd	31.89±0.07h	21.44±0.46de
	GR-2	97.45±0.69gh	7.79±0.12j	26.98±0.51hi	2.11±0.09m	158.12±5.68ig	21.55±0.29f	30.75±0.20fg	242.09±338de	20.22±1.04fgh	3823.94±61.32bcde	20.22±0.77hi	15.65±0.39efgh
	HG-1	199.18±3.78c	15.74±0.06ef	26.43±0.20hi	3.89±0.25kl	256.70±10.04h	26.63±0.38de	29.84±0.28gh	264.74±5.70d	29.38±1.66gh	4016.01±41.00bc	29.38±0.10jk	14.99±0.59efgh
	USW-6	101.52±0.86fg	10.16±1.10i	23.34±0.66ij	5.16±0.18ijk	599.20±8.33a	23.34±0.00ef	16.59±0.07k	221.18±3.26e	25.97±0.69i	3714.23±27.51def	25.97±0.65i	63.59±3.21bc
Blue	Control	26.96±1.69i	nd	13.87±0.46k	29.38±1.01a	161.53±4.16ij	6.89±0.34g	9.89±0.29hi	123.85±1.43g	5.28±0.01j	1715.22±102.84h	48.32±0.22ef	25.86±0.46d
	DADY	149.23±2.59d	18.94±0.23cd	123.12±5.05c	5.46±0.38ij	390.62±22.47bcd	32.66±2.51abc	76.57±2.41a	225.93±0.07e	63.17±1.76a	3744.11±86.24def	105.49±1.48c	85.11±3.60a
	GR-2	128.02±4.35e	12.15±0.02h	133.05±1.59b	4.91±0.11ijkl	298.66±4.36gh	29.49±0.16	28.15±0.21c	239.86±2.71de	40.35±0.23d	3912.08±1.45bcd	128.12±3.30a	19.34±0.55def
	HG-1	227.01±1.91b	21.84±1.04be	152.15±0.12a	3.52±0.20lm	409.82±18.96bc	27.52±0.09de	39.2±0.32b	228.11±1.97e	50.02±0.20bc	3758.36±11.91cdef	115.62±0.32b	14.23±0.87fgh
	USW-6	101.46±0.96fg	16.96±0.54e	112.13±0.94d	4.47±0.18jkl	424.91±7.14b	31.25±0.85bcd	25.20±0.08d	240.59±11.05de	31.85±0.19fgh	3606.75±208.24ef	111.61±3.25b	63.59±3.21b
Yellow	Control	3.56±0.07j	nd	1.91±0.10l	7.73±0.24gh	21.21±1.45l	8.31±0.43g	2.35±0.05h	146.48±6.63fg	2.16±0.00j	1847.71±71.34gh	25.09±0.64ij	8.52±0.44hi
	DADY	103.74±2.77fg	14.12±0.52fg	19.7±0.53j	9.33±0.22ef	362.72±22.80cde	36.93±1.18a	13.93±0.29fg	349.09±7.52ab	34.24±073efg	3927.45±27.66bcd	54.36±0.44d	85.54±2.99a
	GR-2	80.15±3.88h	12.15±0.06h	29.97±0.71gh	8.02±0.34fg	142.56±4.16j	29.84±0.18bcd	15.39±0.17ef	306.73±7.12c	29.32±0.80h	3754.20±15.46cdef	44.08±0.71fg	16.72±0.63efg
	HG-1	257.59±15.92a	12.79±0.50gh	36.05±1.81f	6.23±0.20hi	312.07±12.75fg	33.88±0.83ab	13.41±0.55fg	317.11±16.33bc	36.97±1.90de	5023.42±28.74a	43.05±2.29fg	14.51±0.17ab
	USW-6	117.06±3.27ef	17.28±0.79de	34.05±0.5fg	3.72±0.13kl	348.52±11.56efg	33.10±1.50abc	9.05±0.15ij	339.48±1.77ab	33.85±0.95efgh	3896.19±3.02bcd	46.12±1.15ef	9.79±0.44gh

Table 3.4 Effect of corn variety and yeast strains on DPPH scavenging activity

Corn variety	DPPH of free phenolic compounds (mmol TE/g)					
	Yeast strains					
	Control[1]	DADY	GR-2	HG-1	USW-6	Average[3]
Blue corn	2.03±0.11[A2]	3.71±0.03	3.76±0.04	3.75±0.05	3.79±0.09	3.41a
Red corn	1.51±0.11[B]	2.96±0.22	3.49±0.15	3.02±0.11	2.51±0.09	2.70b
White corn	0.92±0.04[C]	1.83±0.11	2.10±0.10	1.86±0.10	1.66±0.09	1.67c
Yellow corn	0.98±0.05[C]	1.66±0.08	1.81±0.14	1.97±0.08	1.96±0.06	1.68c
Average[4]	1.36d	2.54c	2.79a	2.65b	2.48c	
Corn variety	DPPH of bound phenolic compounds (mmol TE/g)					
	Yeast strains					
	Control	DADY	GR-2	HG-1	USW-6	Average
Blue corn	3.16±0.10[AB]	4.28±0.06	4.27±0.17	4.18±0.11	4.17±0.29	3.92b
Red corn	3.30±0.27[A]	4.87±0.10	4.97±0.04	4.59±0.15	4.27±0.08	4.40a
White corn	2.98±0.10[BC]	4.12±0.25	4.23±0.10	4.21±0.05	3.80±0.27	3.87bc
Yellow corn	2.79±0.16[C]	4.30±0.08	3.92±0.30	3.76±0.25	4.20±0.09	3.79c
Average	3.06d	4.39a	4.26ab	4.19bc	4.11c	

[1]For the Control column, different uppercase letters indicate significant difference across corn varieties at $p < 0.05$.

[2]Mean±standard deviation. All data were based on four replicates.

[3]For column means, different letters indicate significant difference across yeast types at $p < 0.05$.

[4]For row means, different letters indicate significant difference across corn varieties at $p < 0.05$.

Table 3.5 Effect of corn variety and yeast strains on ABTS scavenging activity

Corn variety	ABTS of free phenolic compounds (μmol Trolox equiv./g)					
	Yeast strains					
	Control[2]	DADY	GR-2	HG-1	USW-6	Average[3]
Blue corn	7.72±0.28[A1]	13.43±0.04	13.64±0.02	13.7±0.09	13.05±0.11	12.31a
Red corn	5.58±0.26[B]	9.22±0.09	11.21±0.71	9.78±0.26	7.28±0.30	8.61b
White corn	4.24±0.02[C]	6.66±0.02	7.27±0.06	6.73±0.21	6.51±0.09	6.3d
Yellow corn	4.3±0.02[C]	6.77±0.39	7.17±0.17	7.44±0.18	7.5±0.34	6.63c
Average[4]	5.46e	9.02c	9.82a	9.41b	8.58d	
Corn variety	ABTS of bound phenolic compounds (μmol Trolox equiv./g)					
	Yeast strains					
	Control	DADY	GR-2	HG-1	USW-6	Average
Blue corn	10.8±0.39[A]	13.09±0.13	13.29±0.10	13.01±0.18	13.17±0.18	12.35a
Red corn	10.86±0.31[A]	12.03±0.89	13.02±0.12	13.08±0.36	12.78±0.41	12.41a
White corn	11.79±1.02[A]	11.96±1.00	12.73±0.14	12.97±0.11	12.61±0.19	12.67a
Yellow corn	11.02±0.25[A]	13.03±0.23	12.81±0.24	12.67±0.27	12.93±0.12	12.49a
Average	11.12c	12.53b	12.97a	12.93ab	12.87ab	

[1]Mean±standard deviation. All data were based on four replicates.

[2]For the Control column, different uppercase letters indicate significant difference across corn varieties at $p < 0.05$.

[3]For column means, different letters indicate significant difference across yeast types at $p < 0.05$.

[4]For row means, different letters indicate significant difference across corn varieties at $p < 0.05$.

Table 3.6 Correlation coefficient analysis between phenolic compounds and antioxidants activities in samples

Phenolic compounds	Antioxidant activities of free and bound phenolic compounds			
	DPPH-free	DPPH-bound	ABTS-free	ABTS-bound
4-hydrobenzoic acid	0.482	0.872	0.477	0.752
Vanillic acid	0.861	-0.271	0.932	-0.603
Syringic acid	0.463	0.829	0.462	0.839
p-coumaric acid	0.639	0.810	0.720	0.658
Ferulic acid	0.785	0.775	0.729	0.815
Sinapic acid	0.691	0.465	0.787	0.331

Chapter 4 - Sequential extraction of phenolic and protein hydrolysate antioxidants from corn distillers' dried grains with solubles and evaluation of their antioxidant performance

Abstract

There is limited information about the sequential extraction of phenolic compounds following hydrolysis of proteins of corn DDGS. In this study, five generally recognized as safe (GRAS) organic solvents at 50% (v/v) were used to extract phenolic compounds from DDGS including acetone, ethanol, methanol, 1-proponal, and 2-propnal. The extracted phenolics were named Phenolic-A, Phenolic-E, Phenolic-M, Phenolic-1P, and Phenolic-2P. The residues were then hydrolyzed using 0.1 AU/g of Alcalase, and the prepared protein hydrolysates were named Hydrolysate-A, Hydrolysate-E, Hydrolysate-M, Hydrolysate-1P, and Hydrolysate-2P. Phenolic antioxidants indicated significantly higher TPC than hydrolysate antioxidants, and the highest was observed on Phenolic-A (67.54 mg GAE/g). The composition and each phenolic extract were determined using the UPLC-DAD-ESI-Q-TOF-MS/MS with six phenolic acid standards. And results indicated the extraction solvents would affect the phenolic acid amount and types. The hydrolysates antioxidants showed degree of hydrolysis in the range of 6.39-7.60%, and no significant difference was observed on the peptide content (501.58 -532.69 mg/g) as well as molecular weight distribution. Both phenolic and hydrolysate antioxidants had high antioxidant capacity against DPPH free radical scavenging and ferrous ion chelating. Considering the yield and antioxidant activities, 50% (v/v) of acetone and 50% (v/v) ethanol were the most efficient to produce antioxidants with promising antioxidant capacity. Phenolic-A, Phenolic-E, Hydrolysate-

A, and Hydrolysate-E were added into o/w emulsions at 1.0 and 2.5 mg/mL to evaluate their antioxidant performance. The selected phenolics and hydrolysate showed high efficiency in the prevention of lipid oxidation, and higher dosage showed relatively better prevention which was even better than 1 mg/mL rosemary extract. The results provide evidence that corn DDGS is a potential source of natural antioxidants, and sequential extraction would be a potential way to produce both corn phenolic and peptide antioxidants.

4.1 Introduction

Antioxidants are substances used in food products at low concentrations to retard or prevent lipid oxidation (Halliwell et al., 1995). Lipid oxidation could occur during food processing, handling, and storage. Food-grade antioxidants have been widely used in food industries to prevent the loss of food texture, aroma, taste, and nutrients caused by oxidation, especially for high-fat/oil foods or ingredients (Amaral et al., 2018; Saiga et al., 2003; Zhou et al., 2013). Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), ethoxyquin (EQ), and tert-butylhydroquinone (TBHQ) are commonly used in various food and feed products (Shahidi & Zhong, 2008; Shahidi, 2000). The use of synthetic antioxidants in foods can date back to the 1940's when BHA was found to retard oxidation and the effectiveness of several alkyl esters of gallic acid was also unraveled (Shahidi et al., 1994). However, with more and more research on those synthetic antioxidants being conducted, scientists found that ingestion of such antioxidants possibly increases health risks due to their toxicity and carcinogenicity (Gharavi et al., 2007). Hence, there is a growing interest in natural antioxidants with high efficiency and low cost.

In recent years, antioxidative phenolic compounds and bioactive protein hydrolysate or peptides have been considered as potential sources of natural and safe antioxidants. Fruits and vegetables are top antioxidants sources, and precious studies have reported that phenolic extracted from berries, cherries, kiwi, beans, ginger, and teas showed high antioxidant capacities (Dimitrios, 2006; S. Y. Wang & Lin, 2000; Yanishlieva-Maslarova & Heinonen, 2001.). Moreover, co-products generated during food processing are also a potential source of antioxidative phenolics like nuts co-products (skin, shell, and hull/pellet) (Chang et al., 2016),

date co-products (Martín-Sánchez et al., 2014), milled-rice co-products (Hih & Daigle, 2003), and wheat milling co-products (Sarfaraz et al., 2017). There are also various studies conducted to investigate the antioxidant properties of bioactive peptides from plant or animal sources or some co-products. The hydrolysates or peptides produced from rice bran (Revilla et al., 2009), corn gluten meal (Hu et al., 2022; Li et al., 2008), frog skin (Qian et al., 2008), and alga protein waste (Sheih et al., 2009) were reported high biopeptide activity due to their specific amino acid composition and sequences (Chen et al., 1998).

Corn is one of the most cultivated crops worldwide. It contains up to three times more phenolics compared to wheat, rice, and oats, and usually contains about 10-15% or proteins (Adom & Liu, 2002; Hu et al., 2020). The dramatic increase in bioethanol production in the USA has resulted in millions of tons of corn co-products, such as corn gluten meal (CGM), corn germ meal, and corn distiller's dried grain with solubles (DDGS). Corn DDGS is one of those co-products generated from corn dry-milling processing. Corn DDGS is a high nutrient by-product and contains about 27-35% of protein and 3-5% of phenolic (Hu et al., 2020; Y.-H. Wang et al., 2016). Some studies indicated phenolic compounds and hydrolysates or peptides extracted from either corn or corn co-products (most used CGM) showed high antioxidant capacity (Dimitrios, 2006; Li et al., 2008; Nuez Ortín & Yu, 2009), while there lack of study using sequential extraction to produce both phenolic and hydrolysate antioxidants from corn DDGS.

Hence, the objectives of this study were to prepare phenolic and hydrolysate antioxidants from corn DDGS using sequential extraction procedure, and to investigate the effects of different solvents on their antioxidant properties. The antioxidants activities of those prepared phenolic

and hydrolysate antioxidants were determined by different assays. The phenolic antioxidants were characterized by ultra-high performance liquid chromatography coupled with diode-array detection and electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-DAD-ESI-Q-TOF-MS/MS), and the peptide antioxidants were characterized by measuring the degree of hydrolysis, peptide content, as well as molecular weight distribution using SDS-PAGE. Promising antioxidants were further evaluated in oil-in-water (O/W) emulsions to assess their antioxidant performance.

4.2 Materials and methods

4.2.1 Chemicals and reagents

Corn distillers' dried grains with solubles (DDGS) that contained 28.7% crude protein were provided by the Grains Processing Corporations (Muscatine, IA, USA). Alcalase (protease from Bacillus licheniformis with activity of ≥ 2.4 U/g) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Vegetable oil was purchased from the local market. The rosemary extract was purchased from the BulkSupplements (Henderson, NV, USA). All other chemicals, solvents, and reagents used were of at least analytical grade and purchased from Sigma-Aldrich (St. Louis, Mo, USA) or Fisher Scientific (Fairlawn, NJ, USA).

4.2.2 Pretreatments of corn DDGS

The commercially sourced corn DDGS was milled using a Wiley mill with a 0.2-μm screen (Thomas Scientific LLC, NJ, USA). Fat was removed by mixing DDGS with hexane at 1:6 (w:v) and stirring for 30 min at room temperature. The mixture was then filtrated, and the defatted DDGS was dried in a fume hood for 24 h to evaporate all hexane residue. Defatting was

repeated three times. Before further extraction, defatted DDGS was stirred in boiled deionized water (D.I water) for 1 h which could help with the extraction and release of both phenolic and proteins in further extraction (Li et al., 2019; Wan et al., 2011; Wang et al., 2013). The hot water treated DDGS was then filtrated, and precipitant was lyophilized using a freeze dryer (Labconco corp., Kansas City, MO, USA). The lyophilized precipitant was collected as pretreated DDGS and was kept at -20 C until further treatments.

4.2.3 Extraction of phenolic compounds

Pretreated DDGS was first used for extraction of phenolic antioxidants. Five generally recognized as safe (GRAS) solvents were used for the extraction including acetone, ethanol, methanol, 1-proponal and 2-proponal. The optimum concentration of each solvent was used according to preliminary tests (Table S1). In brief, pretreated DDGS was mixed with each solvent (1:10, w/v) and stirred for 1 h at room temperature. The slurry was then centrifuged at 8000 *g* for 15 min. The supernatant was collected and dried using a rotary evaporator, and the dried phenolic compounds were stored at -20˚C until further analysis. The precipitant was freeze dried and stored at -20 ˚C until further treatments.

4.2.4 Preparation of protein hydrolysates

After phenolic extraction, the dried residue was then used for the production of protein hydrolysates. The hydrolysis was conducted according to our previous publication with some modifications (Hu et al., 2020). In brief, DDGS residue was mixed with DI water (10%, w/v) and the pH was adjusted to 8.0. After pH adjustment, Alcalase was added at 0.1 AU/g (protein basis) and then the mixture was incubated in a water bath shaker at 50˚C and 150 rpm. Reaction was

stopped after 4 h in boiling water to deactivate enzymes for 15 min. The mixture was cooled and then centrifuged at 8000 *g* for 15 min to collect the supernatant. All prepared hydrolysate antioxidants were then freeze dried and kept at -20˚C until further analysis.

4.2.5 Determination of antioxidant yield

The yield of antioxidant was calculated using the equation as follow:

$$\text{Yiled of antioxidant} = \frac{\text{Weight of antioxidant}}{\text{Weight of pretreated DDGS}} * 100\%$$

4.2.6 Determination of degree of hydrolysis (DH) and peptide content

The DH of hydrolysates was measured following the method of (Hu et al., 2022). Peptide content of the hydrolysates was measured using the pierce quantitative colorimetric peptide assay kit (ThermoFisher Scientific, Waltham, MA, USA), and results were expressed as mg peptide per gram of sample.

4.2.7 Identification of phenolic compounds by UPLC-DAD-ESI-QTOF-MS

To better understand the phenolic composition of all phenolic antioxidants, an ultra-high performance liquid chromatography coupled with diode-array detection and electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-DAD-ESI-Q-TOF-MS/MS, Waters Corporation, Milford, MA, USA) was used to qualify and quantify those phenolic compounds. The setting of UPLC and MS parameters was according to our previous publication (Tian et al., 2019). Six phenolic acid standards were used to for quantification including 4-hydrobenzoic acid, vanillic acid, syringic acid, p-coumaric acid, ferulic acid, and sinapic acid.

4.2.8 MW Determination of protein hydrolysates using SDS-PAGE

The molecular weight distribution of proteins was determined by SDS-PAGE according to the modified method of Hong et al. (2022). Protein hydrolysates for SDS-PAGE under non-reducing condition were prepared by mixing samples in a sample buffer (2% SDS in PBS, pH 6.80) and shaking for 2 h. The supernatant was collected. For non-reducing condition, 60 µL protein supernatant was mixed with 20 µL of 4x Laemmli buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and boiled for 5 min. After cooling down to room temperature, a 15 µL mixture is loaded into wells of a 16.5% Mini-Protein Tris-Tricine gel (Bio-Rad Laboratories Inc., Hercules, CA, USA). Proteins were separated at 200 V for around 1 h in Tris-Glycine-SDS running buffer at ambient temperature. The gel was stained with 30 mL of diluted Brilliant Blue R Concentrate dye (Sigma-Aldrich, St. Louis, MO, USA) for 8 min with gentle shaking. Then, it was washed in 30 mL destaining solution containing 10% (v/v) acetic acid and 30% (v/v) methanol until the background became clear.

4.2.9 Determination of total phenolic content (TPC)

The TPC was determined using the Folin-Ciocalteu method following a previous method (Tian & Li, 2018). Sample was prepared at 5 mg/mL in either organic solvents (for phenolic antioxidants) or DI water (for hydrolysates antioxidants). The absorbance was measured at 765 nm using a VWR UV1600-PC spectrophotometer (Radnor, PA, USA). Gallic acid was used as a standard, and TPC of prepared antioxidants was expressed as mg gallic acid equivalent per gram of sample (mg GAE/g).

4.2.10 Determination of DPPH scavenging activity

The DPPH radical scavenging activity was measured following the method of Subbiah et al. (2020) with slight modification. DPPH radical solution was prepared by dissolving 4 mg of DPPH in 100 mL methanol. For this, 20 μL sample, 20 μL methanol, and 260 μL DPPH solution was added into a 96-well plate. The mixture was incubated for 30 min in the dark and then the absorbance was measured at 517 nm. Trolox was used as a standard, and the results were shown as mM Trolox equivalents (mM TE).

4.2.11 Determination of ferrous ion chelating capacity

The ability of sample to chelate Fe^{2+} ions was measured according to the method of Hu et al. (2022) but samples were prepared at 5 mg/mL.

4.2.12 Preparation of O/W emulsions

The prepared phenolic and hydrolysate antioxidants with promising antioxidant activities were selected and their antioxidant performance was evaluated in O/W emulsions. Antioxidants were added into the emulsions at 1.0 and 2.5 mg/mL, and the control emulsion was the one without any additives. Emulsions were prepared following a previous study with some modifications (Shen et al., 2019; Wang et al., 2020). Briefly, phenolic and protein hydrolysate antioxidants were stirred with 45 mL 0.1 M phosphate buffer (pH 7.0) for 2 h, and then stored overnight at 4ºC. Then, 5 mL vegetable oil and 0.45 mL Tween 20 were added into the buffer. The mixture was homogenized at 20,000 rpm for 2 min using a homogenizer (PowerGen 700, Fisher Scientific Inc., Ottawa ON, USA). The coarse emulsions were sonicated at 70% power for 2 min using a sonicator with a 1/8” tip (Branson Sonifier 250, Emerson Electric Co., St. Louis, MO, USA). The prepared emulsions were tightly sealed and stored at 37ºC for a total of 15 days

to determine their oxidation trend. The 1 mg/mL BHT and 1 mg/mL rosemary extract were used as a comparison.

4.2.13 Peroxide value (PV) determination

The peroxide value of emulsions was determined according to the method of Hu et al. (2020). Cumene hydroperoxide was used as a standard, and PV was quantified as mM hydroperoxide equivalent (mM hydroperoxide equiv.).

4.2.14 Thiobarbituric reactive substances (TBARS) value determination

The thiobarbituric reactive substances value was determined according to the method of Hu et al. (2020). Malonaldehyde was used as a standard, and TBARS value was expressed as mM MDA equivalent (mM MDA equiv.).

4.2.15 Statistical analysis

The results were carried out at least in triplicate and reported as mean ± standard deviation. Data were subjected to one-way analysis of variance (ANOVA) and Tukey's post-hoc test using SAS Studio software (SAS Institute, Cary, NC, USA). $P < 0.05$ was considered as significantly different.

4.3 Results and discussion

A series of preliminary tests had been conducted to decide which concentration was the optimum for extraction of phenolic compounds from the DDGS. Based on the amount of

phenolic produced and their antioxidant capacity (Table S1), the 50% (v/v) of each solvent was the most efficient and economic concentration to produce phenolic antioxidants from DDGS.

4.3.1 Characterization of phenolic antioxidants

The yield and TPC of phenolic antioxidants were shown in Table 4.1. The 50% (v/v) 1-proponal was the most efficient in the extraction, and the extracts (Phenolic-1P) indicated the highest yield with a value of 10.15%. The yield of other phenolic antioxidants ranged from 4.33% to 6.69% with no significant difference. However, the highest TPC was observed on the phenolics extracted using 50% (v/v) acetone (Phenolic-A) with a value of 67.54 mg GAE/g of extract, followed by the phenolic extracted using 50% (v/v) ethanol (Phenolic-E) with a value of 63.34 mg GAE/g of extract (Fig. 1A). Although Phenolic-1P has the highest yield, the TPC was the lowest which was 44.01 mg GAE/g of extract. For a better understanding of the phenolic composition of each phenolic antioxidant, quantification of extracted phenolic compounds was achieved by UPLC equipped with a DAD detector. Six external standards were used including 4-hydroxybenzoic acid, vanillic acid, syringic acid, p-coumaric acid, ferulic acid, and sinapic acid, and the results were shown in Table 4.2. Overall, Phenolic-A had the highest total content of the six phenolic acids which was as high as 548.58 μg/g of extract, followed by Phenolic-E and Phenolic-M (phenolic extracted using 50% (v/v) methanol) with values of 326.62 μg/g and 313.93 μg/g, respectively. The Phenolic-1P and Phenolic -2P (phenolic extracted using 50% (v/v) 2-proponal) had relatively lower total phenolic content with values of 215.57 μg/g and 248.92 μg/g, respectively. Comparing the content of each phenolic acid in different phenolic antioxidants, the sinapic acid was the major phenolic in both Phenolic-A and Phenolic-E (140.86 μg/g and 71.97 μg/g, respectively), followed by ferulic (114.21 and 64.54 μg/g, respectively).

While vanillic was the one with the lowest content of those two phenolic antioxidants. The Phenolic-1P and Phenolic-2P indicated similar distribution of each phenolic acid, and the highest amount was observed on the 4-hydroxybenzoic acid (62.48 and 66.56 μg/g, respectively), followed by the ferulic acid (51.00 and 45.30 μg/g, respectively). For the Phenolic-M, ferulic acid was the major phenolic compound with the highest content of 64.54 μg/g, and the content of vanillic acid was the lowest with a value of 39.12 μg/g.

Corn has a high phenolic content among major cereals. One previous study showed that corn had the highest total phenolic content (15.55 ±0.60 μmol GAE/g of grain) of the grains tested, which was up to three times higher compared to wheat, rice, and oats (Adom & Liu, 2002). The TPC and the content of each phenolic compound might be influenced by the polarity of the extracting solvent and the solubility of phenolic compounds in the extracting solvent (Babbar et al., 2014). Galanakis et al. (2013) reported that the solubility tendency can be explained by the stereochemistry of phenols (the polar and the non-polar fragment inside their molecules) and the intermolecular forces (mainly hydrogen bonds) occurred between them and the solvents.

The hydroxyl groups of phenolics can form hydrogen bonds with the electronegative oxygen of acetone, ethanol, or methanol. The hydroxyl groups of those alcohols can also develop hydrogen bonds with the oxygen atoms occurring inside the phenol molecules such as ferulic, vanillic, syringic, or synapic acid. This can explain why phenolics prefer to polar protic (e.g., methanol, ethanol, 1-proponal, and 2-proponal) instead of polar aprotic solvents (e.g., acetone) (Galanakis et al., 2013). Besides, methanol contains a smaller and more flexible aliphatic

fragment compared to ethanol and, thus surrounds easier phenols with substituted carbons inside their aromatic ring (Galanakis et al., 2013).

There were also other phenolic compounds detected by the ESI-QTOF-MS2, and it was obvious that extraction solvents had great effects on the phenolic composition (Table 4.3). The Phenolic-A contained all the detected compounds, and the 3,4-diacetoxybenzoic acid was only found in Phenolic-A. The 3,4-Dihydroxyphenylacetic acid (a type of hydroxyphenylacetic acid) and scopoletin (a type of hydroxycoumarin) were only detected in Phenolic-A and Phenolic-1P. Moreover, the luteolin 7,3'-disulphate (a type of flavone) was detected in the Phenolic-A and Phenolic-E. The differences may be caused by the compounds' structure and their solubility in different solvents.

4.3.2 Characterization of protein hydrolysates

The degree of hydrolysis and peptide content are listed in Table 4.4. The DH of protein hydrolysates was in the range of 6.39-7.60%. The highest DH was observed on protein hydrolysate prepared using DDGS residue extracted using 50% (v/v) acetone (Hydrolysate-A) and 50% (v/v) methanol (Hydrolysate-M) with DH of 7.36% and 7.60%, respectively. The peptide content of all samples was in the range of 501.58-532.69 mg/g of hydrolysate without significant difference. The MW distribution of protein hydrolysates was shown in Fig 4.3. There was no obvious difference of MW among all hydrolysates, and they all showed small MW which was below 14.2 kDa.

4.3.3 Antioxidant activities of phenolic and peptide antioxidants

The DPPH free radical scavenging activity (Fig. 1A) and Fe^{2+} chelating capacity (Fig. 1B) were used to evaluate the antioxidant activity of prepared phenolic and peptide antioxidants. Overall, the DPPH activity of the phenolic antioxidants was observed to be significantly higher than protein hydrolysate antioxidants, while hydrolysates indicated relatively higher metal chelating capacity than phenolic antioxidants. It may be caused by the different solubility of those two types of antioxidants in different solvents and different antioxidant mechanisms of those two assays. The DPPH assay used methanol to prepare reagents while all solvents for metal chelating were prepared using water. Prepared phenolic antioxidants had higher solubility in alcoholic solvents while hydrolysate antioxidants were water soluble.

Among all phenolic antioxidants, Phenolic-A has the highest DPPH scavenging activity (82.71 μmol TE/g extract), followed by Phenolic-E and Phenolic-M (76.17 μmol TE/g extract and 74.64 μmol TE/g extract, respectively) with no significant difference. The highest Fe^{2+} chelating capacity was observed on the Phenolic-E (53.93%), followed by Phenolic-2P (45.91%), and the lowest value was observed on the Phenolic-1P (14.31%). The study of Luthria et al. (2012) extracted phenolic acids from corn DDGS using ultrasonic assisted base hydrolysis and measured their antioxidant capacity as indicated by ferric reducing antioxidant power. They found that the antioxidant capacity of phenolic compounds from DDGS was 2.58-fold more than that of corn. Inglett et al. (2010) reported that high-shear, jet-cooking, and alkali treatment of corn DDG could enhance antioxidant activity of extracted phenolic. The DPPH value of their DDG extracts ranged from 2.65 to 8.18 μmol TE/g DDG, which was higher than that from wheat, rice, oat and millet extracted using 80% ethanol (4-7.5 μmol TE/g grain) (Stratil et al., 2007). Those studies provide evidence that DDGS was a rich source of phenolic antioxidants.

However, further study should be conducted to explain the effects of solvents on phenolic composition and their antioxidant activity.

The DPPH scavenging activity of hydrolysate antioxidants was in the range of 14.44-19.97 μmol TE/g, except for the Hydrolysate-2P. The Hydrolysate -2P showed the lowest DPPH activity with a value as low as 2.91 μmol TE/g, while it showed the highest Fe^{2+} chelating capacity which was up to 57.61%. The Hydrolysate -1P indicated the second highest activity in Fe2+ chelating (53.30%), followed by the Hydrolysate -E (49.20%) and Hydrolysate -M (48.32%). The different antioxidant activities between peptide antioxidants indicated the selection of organic solvent affected not only the phenolic but also the proteins and peptides that were extracted from DDGS. Although there was no difference in MW between peptide antioxidants, different solvents may alter the protein structures after phenolics extraction.

4.3.4 Antioxidant performance of selected antioxidants in O/W emulsions

The inhibition effect of phenolic and hydrolysate antioxidants in o/w emulsions was evaluated based on PV (Fig. 4) and TBARS value (Fig. 4.5). PV is an index used to quantify the amount of hydroperoxide in fat and oil (Gordon, 2001). Based on the antioxidant properties, Phenolic-A, Phenolic-E, Hydrolysate -A, and Hydrolysate -E were added into o/w emulsions at 1.0 and 2.5 mg/mL to evaluate their antioxidant performance. Overall, all selected antioxidants showed good prevention of hydro peroxidation of emulsions. The PV of the control (without antioxidants) was 0.17 mM hydroperoxide equiv. at day 0 and reached up to 3.41 mM hydroperoxide equiv. after 15 days of incubation at 37˚C (i.e., increased by 18.73 times). Efficient protection was observed for emulsion containing 1 mg/mL BHT, where the PV was

only 0.39 mM hydroperoxide equiv. at day 15, which only increased 1.60 times compared to day 0 (Fig. 4A). The prevention of rosemary extract was much weaker compared to BHT with PV value increased up to 12.04 times at day 15, and even higher than the selected antioxidants. A higher dosage of antioxidants showed better prevention of peroxidation, and 2.5 mg/mL of each antioxidant showed similar prevention capacity as the 1 mg/mL BHT. Emulsions with 1 mg/mL phenolic antioxidants showed a slow oxidation trend from day 0 to 8, but the PV value increased more rapidly after day 8. Moreover, Phenolic-E indicated a better oxidation inhibition effect than Phenolic-A at low dosage where the PV on day 15 was 0.84 and 1.37, respectively. For hydrolysate antioxidants, emulsions treated with those two hydrolysates showed similar oxidation stability when added at low dosage, while Hydrolysate-A was more efficient in protection when used at higher dosage.

The TBARS values of all the oil samples gradually increased during storage (Fig. 4.5). The TBARS value of the control emulsion was increased from 5.57 mM MDA euiqv. at day 0 to 94.63 mM MDA euiqv. at day 15, which was 17 times higher. The same as PV, 1 mg/mL BHT was the most efficient with the lowest increase of TBARS value after 15 days storage (increased by 0.36 times), and 1 mg/mL rosemary extract was also less efficient than BHT as well as some prepared phenolic and hydrolysate antioxidants. Higher oxidation prevention was observed for the emulsions with higher dosage of phenolic antioxidants (Fig. 4.5A), while no difference was observed on emulsions with different dosages of hydrolysates (Fig. 4.5B).

BHT is a synthesized antioxidant which is widely used in the food industry, especially for high fat/lipid products. It is a lipophilic organic compound and effective to prevent oil oxidation

in low dosage. However, most phenolic and hydrolysates from DDGS have low solubility in the oil system, which limits their oxidation protection performances. Moreover, the complex composition of various compounds in prepared phenolic and peptide antioxidants may be another factor contributing to the relatively lower efficiency in preventing oil oxidation.

4.4 Conclusions

This research was aimed at comparing effects of the type of solvents in phenolic acid content, composition, and antioxidant capacity of phenolic extracts, as well as their effects on the properties and antioxidant activities of protein hydrolysates that were produced using the residue after phenolics extraction. These observations indicate that there was a change in phenolic acid profiles and antioxidant properties of both phenolic and protein hydrolysates. Those two types of antioxidants indicated high antioxidant properties. Our study also provided evidence that those antioxidants from corn DDGS can enhance oxidation stability in o/w emulsion. Thus, DDGS is a rich source of phenolic and protein hydrolysate antioxidants. Further study could focus on the purification, identification, and modification of the specific antioxidant phenolic fraction and peptides in these hydrolysates, and *in vivo* evaluation should also be conducted in order to better characterize those antioxidants in a broader range of food, pet food, and animal feed products.

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Table 4.1 Yield of peptide and phenolic antioxidants

Antioxidants	Solvent/Source	Yield%	TPC (mg GAE/g)
Phenolic-A	50% Acetone	5.55 ± 0.16^{d}	67.54 ± 2.12^{a}
Phenolic-E	50% Ethanol	5.31 ± 0.05^{d}	63.34 ± 1.06^{b}
Phenolic-M	50% Methanol	4.33 ± 0.03^{d}	54.41 ± 1.81^{c}
Phenolic-1P	50% 1-Proponal	10.15 ± 0.19^{c}	44.01 ± 1.10d
Phenolic-2P	50% 2-Proponal	6.69 ± 0.20^{d}	55.34 ± 1.22^{c}
Hydrolysate-A	50% Acetone extracted residue	20.08 ± 1.43^{a}	25.21 ± 0.23^{f}
Hydrolysate-E	50% Ethanol extracted residue	16.57 ± 1.48^{b}	25.47 ± 1.30^{ef}
Hydrolysate-M	50% Methanol extracted residue	20.67 ± 1.05^{a}	36.74 ± 0.2^{ef}
Hydrolysate-1P	50% 1-Proponal extracted residue	16.83 ± 0.97^{b}	24.27 ± 1.27^{f}
Hydrolysate-2P	50% 2-Proponal extracted residue	16.15 ± 0.40^{b}	24.61 ± 0.5^{f}

Note: The statistical analysis was done on both phenolics and peptides together, and different letters indicated significant difference ($p < 0.05$).

Table 4.2 Quantification of phenolic acids extracted using different solvents

Extraction solvents	Phenolic content (ug/g)						
	4-Hybenzoic acid	Vanillic acid	Syringic acid	*p*-coumaric acid	Ferulic acid	Sinapic acid	Total
50% Acetone	85.40 ± 0.23^{Ac}	63.89 ± 0.04^{Af}	68.95 ± 0.64^{Ae}	75.27 ± 0.10^{Ad}	114.21 ± 1.19^{Ab}	140.86 ± 1.46^{Aa}	548.58 ± 0.54^{A}
50% Ethanol	53.59 ± 0.79^{Dc}	39.12 ± 1.08^{Cd}	48.39 ± 0.46^{Bc}	49.01 ± 2.52^{Bc}	64.54 ± 2.33^{Bb}	71.97 ± 0.87^{Ba}	326.62 ± 1.65^{B}
50% Methanol	60.52 ± 0.88^{Cb}	42.53 ± 0.84^{Be}	46.29 ± 1.06^{Bde}	47.77 ± 0.81^{Bcd}	65.06 ± 0.65^{Ba}	51.77 ± 1.89^{Cc}	313.93 ± 6.14^{B}
50% 1-proponal	$62.48\pm0.88B^{Ca}$	21.70 ± 0.96^{Dde}	18.74 ± 0.24^{De}	33.84 ± 0.27^{Cc}	51.00 ± 3.31^{Cb}	27.51 ± 0.87^{Ed}	215.27 ± 0.09^{D}
50% 2-Proponal	66.56 ± 2.12^{Ba}	23.49 ± 0.76^{De}	31.49 ± 1.50^{Cd}	37.69 ± 0.23^{Cc}	45.30 ± 2.41^{Cb}	44.39 ± 0.68^{Db}	248.92 ± 3.46^{C}

*The capitalized letter indicates significant differences of one phenolic acid among different solvent extracts (each column). The small letter indicates the difference of different phenolic acids in each solvent extract.

Table 4.3 Compounds identified by UPLC-DAD-ESI-QTOF-MS^2

Component name	Phenol type	Molecular Formula	Neutral mass (Da)	Observed neutral mass (Da)	[M - H]- (m/z)	MS/MS product ions	Samples	References
(2E)-3-(3,4-Dihydroxyphenyl)acrylic acid	Phenoilc acid	$C_9H_8O_4$	180.1574	180.1537	181.1564	135	A, E, M, 1P, 2P	Razgonova et al., 2022
3,4-Diacetoxybenzoic acid	Dihydroxybenzoic acid	$C_{10}H_{11}O_6$	238.1935	238.1254	237.0181	119	A	Razgonova et al., 2022
3,4-Dihydroxyphenylacetic acid	Hydroxyphenylacetic acid	$C_8H_8O_4$	168.0423	168.0410	167.0337	149, 123	A, 1P	Hong et al., 2021
Cyanidin 3-O-beta-D-Glucoside	Anthocyanidin	$C_{21}H_{21}O_{11}$	449.3848	449.3824	448.3802	285	A, E, M, 1P, 2P	Garg et al., 2016; Sharma et al., 2016
Daidzin	Isoflavonoid	$C_{15}H_{10}O_4$	416.1107	416.1102	415.1029	253	A, E, M, 1P, 2P	Rahman et al., 2018
Epicatechin	Flavonoid	$C_{15}H_{14}O_6$	290.0790	290.0771	289.0698	261, 173	A, E, M, 1P, 2P	Razgonova et al., 2022
Hydroxyferulic acid	Phenolic acid	$C_{10}H_{10}O_5$	210.1834	210.1856	219.1543	193,125	A, E, 1P, 2P	Aita et al., 2021
Isorhamnetin-3-O-glucoside	Flavonol	$C_{22}H_{22}O_{12}$	478.1111	478.1107	477.1034	271, 257, 243, 151	A, E, M, 1P	Chen et al., 2015
Luteolin 7,3′-disulphate	Flavone	$C_{15}H_{10}O_{12}S_2$	446.3627	446.3624	447.3613	287	A, E	Enerstvedt et al., 2016
Meta-Coumaric acid	Hydroxycinamic acid	$C_9H_8O_3$	164.0473	164.0461	163.0388	119	A, E, 1P	Hong et al., 2021
Myricetin-3-O-galactoside	Flavonol	$C_{21}H_{20}O_{13}$	480.0904	480.0889	479.0816	257, 262, 298, 355	A, E, 1P, 2P	Abeywickrama et al., 2016
Quercetin-3-O-malonylglucoside	Flavonol	$C_{24}H_{22}O_{15}$	550.0959	550.0934	549.0861	549, 505, 300, 271	A, E, M, 1P, 2P	Galvis Sánchez et al., 2003
Quercetin-3,7-di-O-beta-glucopyranoside	Flavonol	$C_{27}H_{30}O_{17}$	626.1483	626.1477	625.1404	465	A, E, M, 1P, 2P	Yin et al., 2019
Quercetin-3-arabinoglucoside	Flavonol	$C_{26}H_{28}O_{16}$	596.1377	596.1389	595.1316	300	A, E, 1P, 2P	Simonetti et al., 2022
Scopoletin	Hydroxycoumarin	$C_{10}H_8O_4$	192.0423	192.0412	191.0339	176	A, 1P	Barnaba et al., 2015
Vitexin	Flavone	$C_{21}H_{20}O_{10}$	432.1057	432.1049	431.0976	353, 341,311	A, E, M, 1P, 2P	Chatham et al., 2018

For samples, A, E, M, 1P, and 2P are phenolic antioxidants extracted using 50% acetone, 50% ethanol, 50% methanol, 50% 1-propona, and 50% 2-poponal, respectively.

Table 4.4 DH and peptide content of DDGS protein hydrolysates

Hydrolysate antioxidants	**DH**	**Peptide content (mg/g hydrolysates)**
Hydrolysate-A	7.36 ± 0.13^{a}	501.58 ± 23.39^{a}
Hydrolysate-E	6.77 ± 0.17^{b}	525.13 ± 7.90^{a}
Hydrolysate-M	7.60 ± 0.21^{a}	506.47 ± 14.00^{a}
Hydrolysate-1P	6.39 ± 0.08^{b}	524.02 ± 9.41^{a}
Hydrolysate-2P	6.41 ± 0.17^{b}	532.69 ± 18.05^{a}

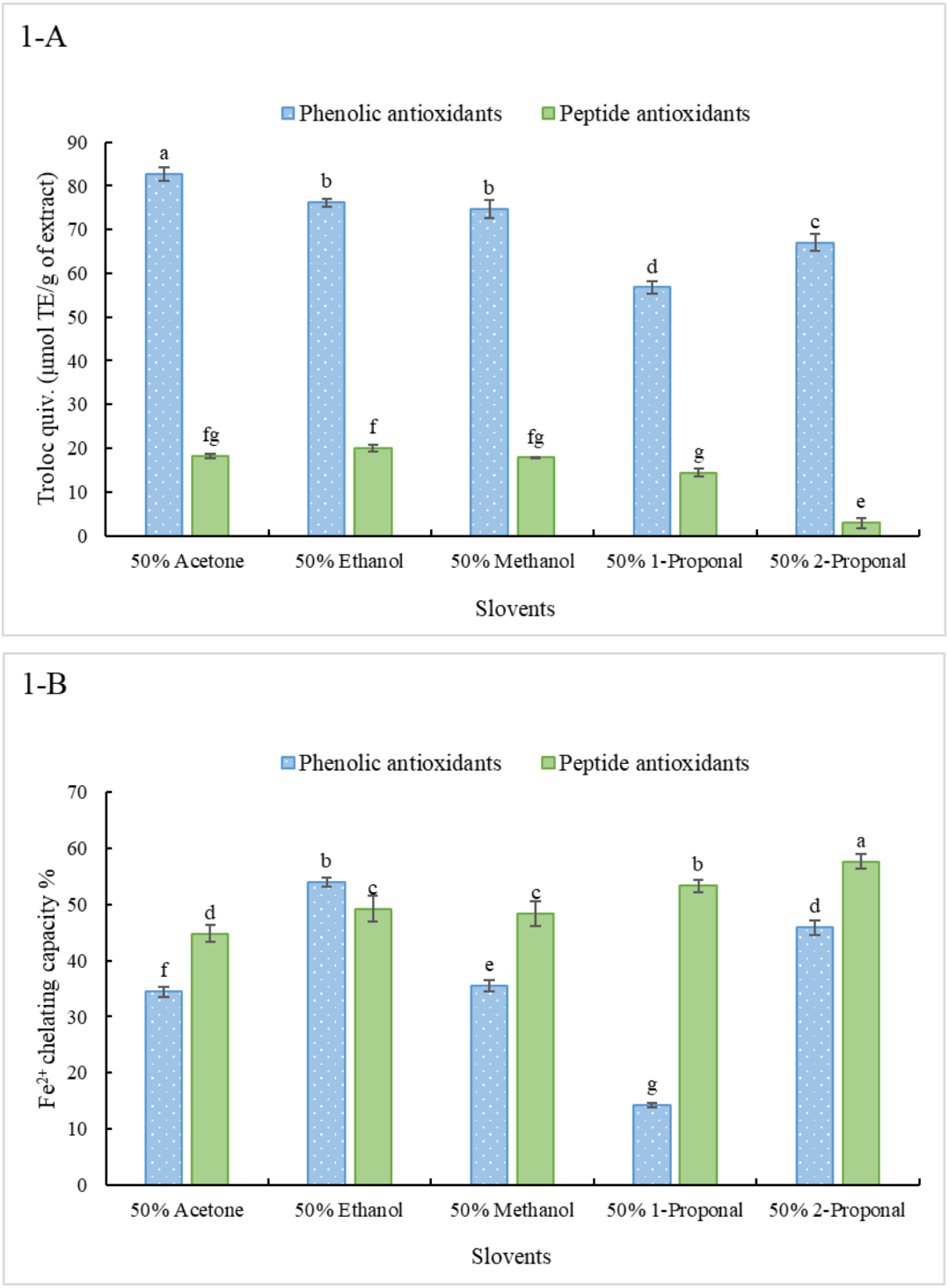

Figure 4.1 DPPH scavenging activity (1A) and Fe^{2+} chelating capacity (1B) of phenolic and peptide antioxidants.

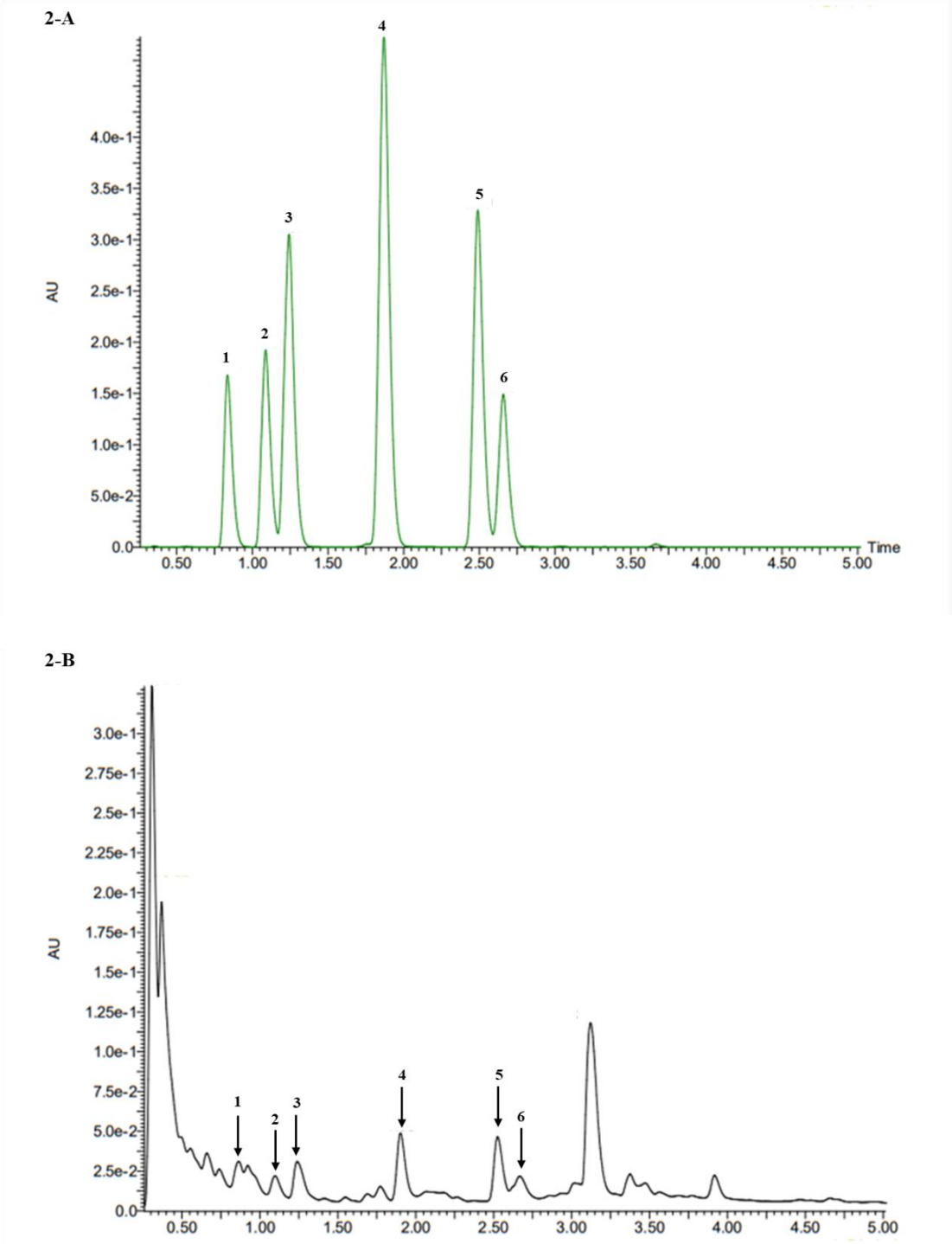

Figure 4.2 UPLC chromatograms of the phenolic acid standard (2A), and phenolic antioxidants extracted using 50% methanol (2B). The six phenolic acid standards were identified as: 4-hydrobenzoic aicd (1), vaninnic acid (2), syringic acid (3), *p*-coumaric acid (4), ferulic acid (5), and sinapic acid (6).

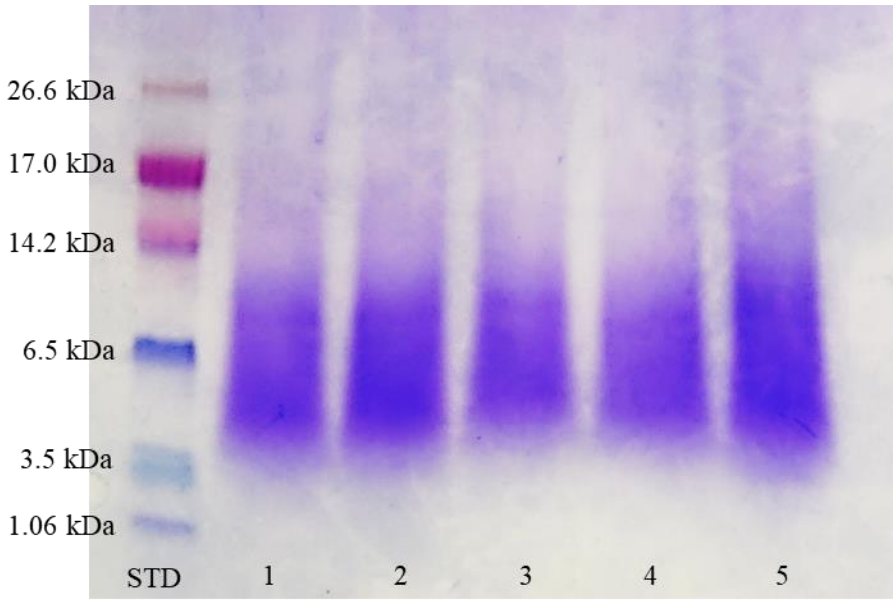

Figure 4.3 Molecular weight distribution of corn peptides that determined using SDS-PAGE. STD: standard. 1. Peptides from 50% acetone extracted DDGS residue; 2. Peptides from 50% ethanol extracted DDGS residue; 3. Peptides from 50% methanol extracted DDGS residue; 4. Peptides from 50% 1-proponal extracted DDGS residue; 5. Peptides from 50% 2-proponal extracted DDGS residue.

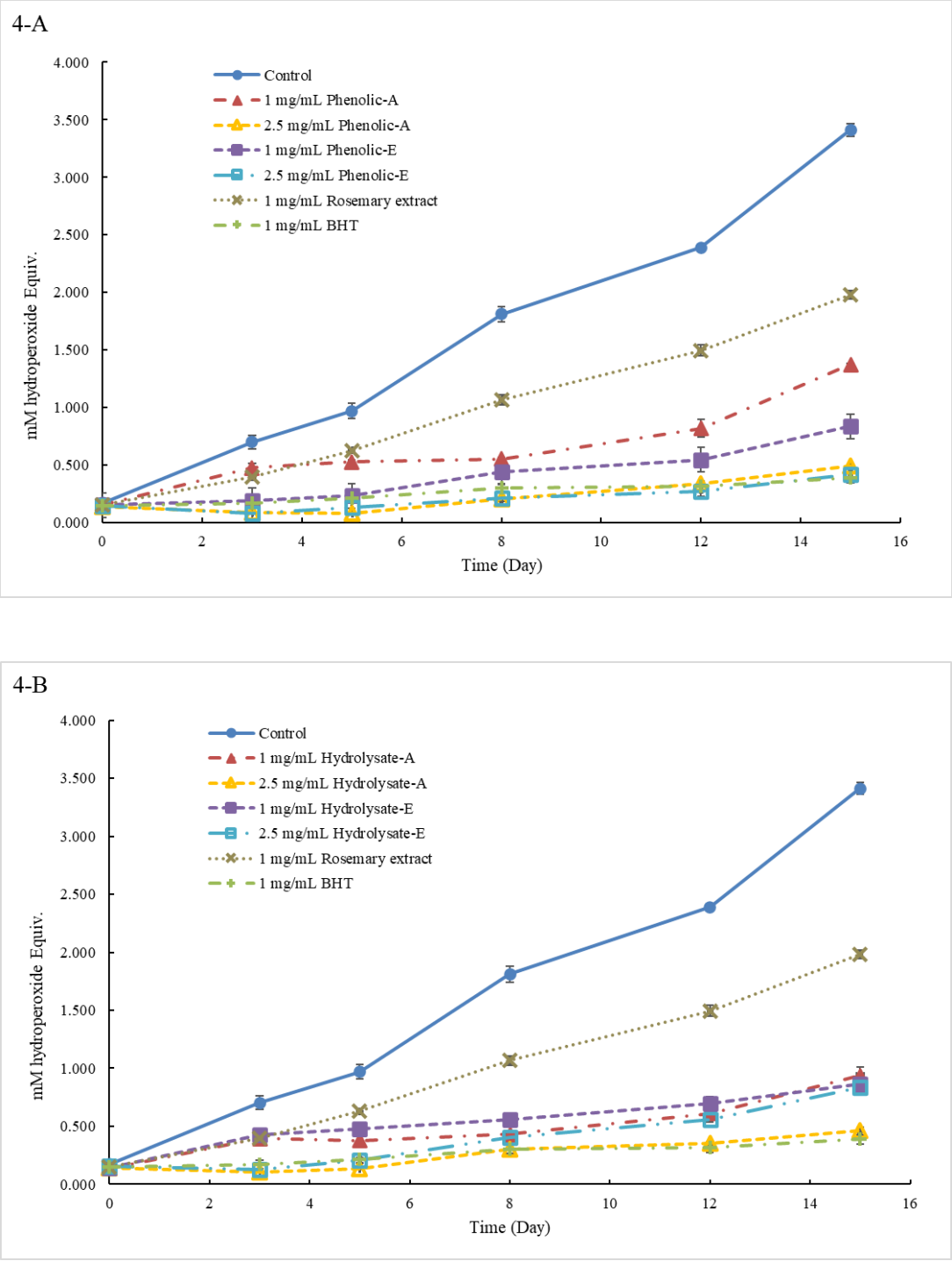

Figure 4.4 PV of o/w emulsion with phenolic antioxidants (4A) or peptide antioxidants (4B) at 1 and 2.5 mg/mL.

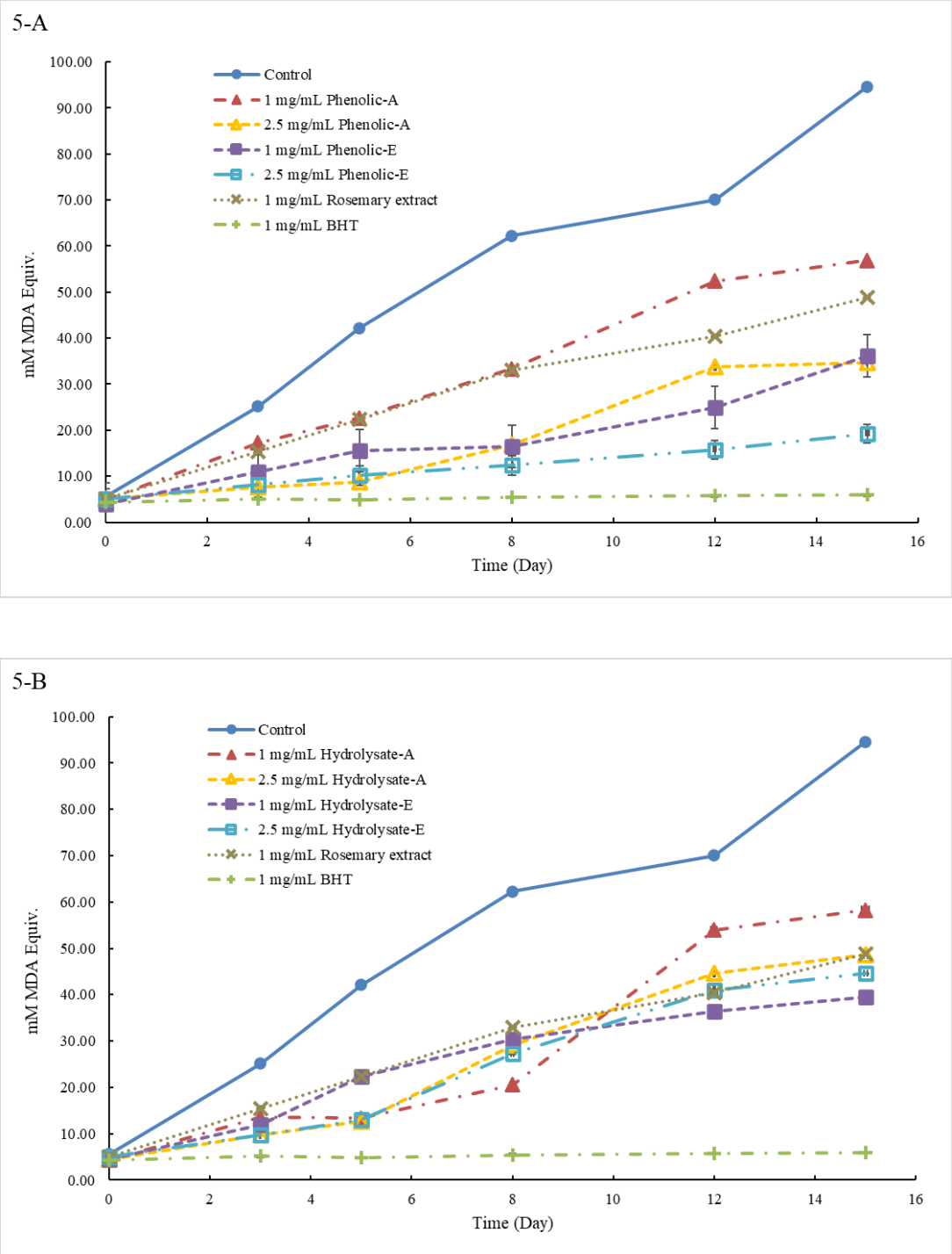

Figure 4.5 TBARS value of o/w emulsions with phenolic antioxidants (5A) and peptide antioxidants (5B) at 1 and 2.5 mg/mL.

Supplemental Materials

Table 4S1. Optimization of solvent concentration for phenolic extraction

Solvent	Concentration	TPC (mg/g)	DPPH % (at 5 mg/mL)
Acetone	25%	33.38 ± 1.80^{b}	27.28 ± 1.70^{ab}
	50%	39.11 ± 2.96^{ab}	29.12 ± 2.04^{a}
	75%	41.93 ± 2.83^{a}	32.72 ± 1.24^{a}
	100%	27.20 ± 1.54^{c}	21.28 ± 0.68^{b}
Ethanol	25%	29.84 ± 2.19^{a}	21.92 ± 0.23^{a}
	50%	35.29 ± 1.41^{a}	25.60 ± 1.13^{a}
	75%	36.11 ± 1.80^{a}	25.36 ± 1.24^{a}
	100%	19.20 ± 0.77^{b}	16.88 ± 1.02^{b}
Methanol	25%	28.56 ± 0.64^{b}	20.56 ± 1.47^{a}
	50%	33.65 ± 1.16^{a}	24.80 ± 1.13^{a}
	75%	36.20 ± 1.41^{a}	27.44 ± 3.96^{a}
	100%	25.84 ± 1.16^{b}	22.00 ± 1.70^{a}
1-Propanol	25%	35.29 ± 1.93^{a}	29.84 ± 0.79^{a}
	50%	35.20 ± 0.26^{a}	26.40 ± 2.49^{a}
	75%	34.56 ± 1.16^{a}	26.16 ± 2.15^{a}
	100%	17.47 ± 0.13^{b}	16.08 ± 0.57^{b}
2-Proponal	25%	32.84 ± 0.77^{a}	25.44 ± 0.23^{b}
	50%	35.65 ± 2.19^{a}	28.40 ± 0.79^{a}
	75%	37.02 ± 2.57^{a}	25.84 ± 0.57^{ab}
	100%	5.84 ± 0.39^{b}	11.44 ± 0.79^{c}

Chapter 5. Preparation and characterization of corn protein concentrate isolated from physical, enzyme, and wet milling treated corn flour and DDGS

Abstract

There is limited information about the comprehensive study on combination of physical, enzyme, and wet milling treatments on protein extraction from corn flour and corn distiller's dried grains with solubles (DDGS) as well as the treatments on protein properties. This study was conducted with a sequential extraction procedure including wet milling, sonication, homogenization, and enzyme hydrolysis (α-amylase and cellulase) to separate proteins from corn flour and DDGS. The extracted proteins have a high protein content with high purity and showed similar MW distribution with a clear band around 50 kDa and 20-25 kDa but with different content of each secondary structure. Protein extracted from liquid-fermented DDGS (protein-LD) had the highest random coil content (52.29%) compared to other proteins, but there was no significant difference of the secondary structure between the two protein extracts from protein-MC and protein-MT. Isolated proteins from wet milling followed by starch table and physical and enzyme treatments (protein-MT), and proteins from wet milling followed by centrifuge physical and enzyme treatment (protein-MC) showed more rupture of proteins such as unfolding of protein molecules, the consequent exposure of hydrophobic groups, and regions buried inside. Therefore, the protein-MC and protein-MT isolated proteins have relatively higher content of random coil (40.84% and 37.53%), higher SDS binding capacity (41.45 and 39.58 μg/mg), and higher free SH groups (3.04 and 3.14 μmol/g) and SS bonding (14.71 and 15.41 μmol/g). Protein

extracted from corn flour (Protein-flour) showed the highest water holding capacity (WHC) (4.70 g/g), but Protein-MC had the second lowest WHC (3.17 g/g), and protein-MT had the lowest WHC (2.91 g/g). The proteins from protein-MC, protein-MT, and DDGS have a higher oil holding capacity (3.52, 3.36, and 3.55 g/g, respectively). However, no significant difference was observed on the in vitro digestion rate which ranged from 83.13 to 85.03 g/ 100g protein.

5.1 Introduction

Corn is a principal source of food and is considered as an important source of protein and calories for millions of people in the world, especially people in Latin America, Africa, and Asia (Ufaz & Galili, 2008). Protein content in corn kernels varies among varieties but is usually around 10-15% (R. Hu, Chen, et al., 2020). Compared to other cereal crops, corn is relatively low in protein content as well as two essential amino acids, lysine and trypsin (Bressani, 1991). However, there are still about 42 million tons of corn proteins are produced yearly globally, which contributes to approximately 15% of the annual food-crop protein production (J. S. Li & Vasal, 2016). Therefore, the need to improve corn protein quality and quantity has been recognized for a long time.

The wet milling process separates the corn kernel into its constituent chemical components which are suitable for food ingredients, feeding materials, and/or other value-added co-products (Rausch et al., 2019). The conventional wet milling process includes steeping, separation (degemination, grinding, and screening), and recovery of different fractions (Wronkowska, 2016). During stepping, water diffuses into the kernel, and the sulfur dioxide (SO2) helps cleave protein disulfide bonds and disrupt the protein cross-link, which enhances the separation of protein, starch, and other components during further milling process (Jackson & Shandera, 1995; Neumann, 1984). However, complete separation and extraction of each component is difficult due to their different solubilities. The separated protein from wet milling is usually considered as protein concentrate with typically about 45-55% of crude protein with a significant proportion of starch and fiber(Dowd, 2003). Hence, it is important to use other treatments to improve the protein yield and purity.

Chemical treatments (acids, reducing agents, etc.), physical treatments (sonication, high pressure, etc.), and enzyme treatments (α-amylase, cellulase, protease, etc.) have been used individually or combined on protein extraction (Cameron & Wang, 2006; Du, 1996; Espinosa-Ramírez & Serna-Saldívar, 2016; Hojilla-Evangelista, 2012; Ozturk et al., 2021). Physical treatments are more widely used in industry due to easy operation, more economical and environmentally friendly than other treatments. Sonication can disrupt cell wall components through the energy from the sound waves. It was used to enhance protein extraction from different sources, such as rice bran and wheat flour (Messman & Weiss, 1993; Tang et al., 2021). Homogenization enhances protein extraction based on shear stress distribution in the materials which can disrupt intercellular and surrounding ext Corn is a principal source of food and is considered as an important source of protein and carbohydrate for millions of people in the world, especially people in Latin America, Africa, and Asia (Ufaz & Galili, 2008). The protein content of corn kernel varies among varieties but is usually around 10-15% (R. Hu, Chen, et al., 2020). Compared to other cereal crops, corn is relatively low in protein content as well as two essential amino acids, lysine and trypsin (Bressani, 1991). However, there are still about 42 million tons of corn proteins are produced yearly globally, which contributes to approximately 15% of the annual food-crop protein production (J. S. Li & Vasal, 2016). With the increased global production of bioethanol, a huge amount of corn co-products is generated each year. The distillers dried grains with solubles (DDGS) is a major co-products. There is about one-third of the corn mass is converted into DDGS through the dry-milling process to produce ethanol which makes DDGS quickly become a global commodity (Luthria et al., 2012). Currently, the livestock feed ingredient market is the ethanol industry's only outlet for those corn-coproducts. The

livestock feed market is well established but needs to be augmented if ethanol-processing residues are to retain their high-value return, especially as the quantities of these residues generated increase over time. To retain their value, novel uses for these residues (e.g., as ingredients in human food production) should also be investigated. Precious studies had shown that DDGS had high protein content (more than 30%)(Luthria et al., 2012). Since there is a high demand for plant proteins worldwide, DDGS may be a good source of corn proteins.

The wet milling process separates the corn kernel into its constituent chemical components which are suitable for food ingredients, feeding materials, and/or other value-added co-products (Rausch et al., 2019). The conventional wet milling process includes steeping, separation (degemination, grinding, and screening), and recovery of different fractions (Wronkowska, 2016). During stepping, water diffuses into the kernel, and the sulfur dioxide (SO2) helps cleave protein disulfide bonds and disrupt the protein cross-link, which enhances the separation of protein, starch, and other components during further milling process (Jackson & Shandera, 1995; Neumann, 1984). However, complete separation and extraction of each component is difficult due to their different solubilities. The separated protein from wet milling is usually considered as protein concentrate with typically about 45-55% of crude protein with a significant proportion of starch and fiber(Dowd, 2003). Hence, it is important to use other treatments to improve the protein yield and purity.

Chemical treatments (e.g., acids and reducing agents), physical treatments (e.g., sonication and high pressure), and enzyme treatments (e.g., α-amylase, cellulase, and protease) have been used individually or combined for protein extraction (Cameron & Wang, 2006; Du,

1996; Espinosa-Ramírez & Serna-Saldívar, 2016; Hojilla-Evangelista, 2012; Ozturk et al., 2021). The physical treatment has been more widely used in industry due to easy operation, more economical, and environmentally friendly than other treatments. Sonication can disrupt cell wall components through the energy from the sound waves. It was used to enhance protein extraction from different sources, such as rice bran and wheat flour (Messman & Weiss, 1993; Tang et al., 2021). Homogenization enhances protein extraction based on shear stress distribution in the materials which can disrupt intercellular and surrounding extracellular structures (Mesa et al., 2020). It has been reported that using homogenization increased about 30% of the protein extraction efficiency from wet-milled corn germ (Hojilla-Evangelista, 2012). To isolate and purify proteins, enzymes have also been used to remove other components. The α-amylase is usually used to remove starch residue in prepared proteins by hydrolyzing the α-1,4-linkage of starch. Treatment with α-amylase can enhance protein extraction due to the release of starch-bound proteins and the increase of solubility of unbound proteins (Tang et al., 2021). Fiber can also be removed by using enzymes. Cell walls are the main source of dietary fiber which comprise polysaccharides, structural proteins, and in some cases, lignin (Cui & Wang, 2009). Enzymes can degrade cell walls and induce the release of proteins during extraction (Fleurencel et al., 1995).

Most previous studies have reported extraction and purification of corn zein or glutenlin proteins from different raw materials, but the extraction of entire corn storage proteins has received little research for developing concentrates and isolates. A combination of different treatments maybe used to enhance corn protein extraction from corn flour and DDGS. The objectives of this study are to extract entire corn proteins from corn kernel and DDGS, and

compare the effects of sonication, homogenization, and enzyme with or without wet milling on protein extraction.

5.2 Materials and Methods

5.2.1 Materials

Corn kernel (Reid's yellow dent) was purchased from Hoss Tools (Norman Park, GA, USA). Cellulase (Cellic Ctec2, 1,000 BHU-2/g) was purchased from Novozyme (Novozymes North America Inc., Franklinton, NC, USA). The α-Amylase from Bacillus sp., α-Chymotrypsin from bovine pancreas (Type II, ≥40 units/mg protein), protease from Streptomyces griseus (Type XIV, ≥3.5 units/mg solids), trypsin from porcine pancreas (Type IX-S, 13,000-20,000 BAEE units/mg protein) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were at least analytical degree and purchased from either Sigma-Aldrich or Fisher Scientific (Fairlawn, NJ, USA).

5.2.2 Pretreatments of corn kernel and DDGS

The original materials including corn kernel, commercial DDGS, and dried DDGS generated from liquid fermentation (Weiss et al., 2023) were milled using an UYD Cyclone Sample Mill equipped with a 1.0-mm screen (UDY Corporation, Fort Collins, CO, USA) to produce corn flour and DDGS powder with a relatively uniform particle size.

Wet milling was used according to the method of a previous publication (D. Wang & Chung, 2002) to separate corn protein from the corn kernel. Part of the wet milled slurry was centrifuged at 5000 g for 10 min at 10 °C, and the top layer was collected as corn protein

fraction. Another part of wet milled slurry was used for starch tabling to separate starch and protein following the method of Eckhoff et al. (1996). The two crude protein fractions were both freeze-dried, ground into fine powder, and kept at -20 °C until further treatments.

Before protein extraction, the fat of all materials was removed by stirring materials with hexane (1:3, w: v) for 30 min at room temperature. The defatted materials were collected after centrifugation and were stored in a fume hood overnight to completely evaporate the hexane residual. Defatting was done in total 3 times. All defatted materials (dry-milled corn flour, two crude protein fractions, and two DDGS powder) were stored at -20 °C until further analysis.

5.2.3 Protein extraction

Before formal extraction, there were five different treatments used for defatted corn flour to help determined the most efficient treatments. Treatment #1 and #2 were enzymatic extraction referred to a previous method with some modifications (Paraman et al., 2006). Treatment #1 was using 5% (v/w, samples basis) α-amylase for 2 hr. Treatment #2 was using 5% (v/w, dry basis) α-amylase for 2 hrs and 5% (v/w, samples basis) cellulase for 1 hr, sequentially. Defatted corn flour was incubated with each enzyme and reacted under optimum conditions based on a precious study ((Paraman et al., 2006b). The precipitant was collected after reaction by centrifugation (8000 g, 20 ºC, 15 min) and then lyophilized. Treatment #3 was a combination of sonication with enzymatic extraction. D.I (deionized) water was added into defatted flour and then the mixture was sonicated in a sonicator (Branson 3800, Emerson Electric Co., St. Louis, MO, USA) at 150 W, 25 °C for 30 min. Then, the sonicated mixture was treated with 5% α-amylase and 5% cellulase following the same procedure as treatment #2. Treatment #4 was a combination of

homogenization with enzymatic extraction. D.I water was added into defatted flour and then the mixture was homogenized using a homogenizer (Polytron PT 2500 E, Kinematica AG, Switzerland) at 15,000 rpm for 2 min. The homogenized slurry was then treated with enzymes following the same procedure as treatment #2. For treatment #5, defatted corn flour was treated using sonication, homogenization, α-amylase, and cellulase, sequentially. The procedure and parameters for each treatment were the same as previous treatments mentioned above.

According to the protein recovery and protein content of extracted proteins, treatment #5 was considered the most efficient approach for protein extraction. It was then used for other defatted materials including the two crude protein fractions and the two DDGS samples.

5.2.4 Protein recovery

Protein recovery was calculated using the equations as follow:

$$\text{Protein recovery}\% = \frac{\text{Weight}_2 * \text{Protein content}_2}{\text{Weight}_1 * \text{Protein content}_1} * 100\%$$

In which Weight1 and Protein content1 were the weight and protein content of original sample, and Weight2 and Protein content2 were the protein content of extracted protein.

5.2.5 Chemical analysis

Ash, and crude fat content of prepared proteins was determined using AACC method 44-15.02, 08-01.01, 30-10.01. Crude protein content was determined using a FP928 LECO nitrogen analyzer (LECO Corporation, Saint Joseph, MI, USA).

5.2.6 SDS-PAGE

The molecular weight distribution of proteins was determined by SDS-PAGE according to the modified method of Hong et al. (2022). Protein samples for SDS-PAGE under both reducing and non-reducing conditions were prepared by mixing samples in a sample buffer (2% SDS in PBS, pH 6.80) and shaking for 2 h. The supernatant was collected after centrifuge at 8000 rpm for 5 min. Under non-reducing conditions, 60 μL protein supernatant was mixed with 20 μL of 4x Laemmli buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and boiled for 5 min. After cooled to room temperature, 15 μL mixture was loaded into wells of a mini-protein TGX precast polyacrylamide gel (Bio-Rad Laboratories Inc., Hercules, CA, USA). Under reducing condition, all procedures were the same except that the 4x Laemmli buffer contained 10% (v/v) β-mercaptoethanol. Proteins were separated at 200 V for around 30 min in Tris-MOPS-SDS running buffer at ambient temperature. The gel was stained with 30 mL of diluted Brilliant Blue R Concentrate dye (Sigma-Aldrich, St. Louis, MO, USA) for 8 min with gentle shaking and washed in 30 mL destaining solution containing 10% (v/v) acetic acid and 30% (v/v) methanol until the background became clear.

5.2.7 Surface hydrophobicity

Surface hydrophobicity of prepared proteins was determined using a sodium dodecyl sulfate (SDS) binding method with slightly modifications (Kato et al., 1984; Tang et al., 2021). In brief, 10 mg sample was mixed with 40 mL SDS solution (0.1 mM) and shaken for 1 h. The mixture was then dialyzed against DI water for 48 h with a dialysis bag (3.5 kDa). After dialysis, 10 mL of sample solution was mixed with 25 mL chloroform and 5 mL methylene blue (24 mg/mL). The mixture was vortexed, followed by centrifugation at 3000 g and 20°C for 10 min. The absorbance of the bottom layer was read at 655 nm. Solution of SDS at different

concentrations was used as a standard, and surface hydrophobicity of proteins was expressed as μg SDS equiv./mg of sample.

5.2.8 Free SH group and SS bonds

The SH and SS groups in prepared samples were determined according to the method of Beveridge et al. (1974). Free SH and SS content were expressed as μM/g of sample.

5.2.9 FTIR

The secondary structure of extracted proteins was measured using FTIR via a PerkinElmer Spectrum 400 FT-IR/FT-NIR Spectrometer (PerkinElmer, Inc., Waltham, MA, USA) equipped with an ATR accessory. A total of 64 scans were run for each analysis at an interval of 4 cm−1 in the range of 400–4000 cm−1. FTIR spectra were analyzed using the OriginPro software (version 2016, OriginLab Corporation, Northampton, MA, USA). The quantitative estimation of protein secondary structure was based on a previous study by Kong & Yu (2007).

5.2.10 Water and oil holding capacities

The water and oil holding capacity of proteins were determined following the method of a previous publication (Espinosa-Ramírez & Serna-Saldívar, 2016). Holding capacity was expressed as grams of water or oil that was held by one gram of sample (g/g sample).

5.2.11 Digestibility

The digestibility of protein samples was determined following the method of Hsu et al. (1977) with some modifications. The sample solution (30 mL) was prepared at 6.25 mg/ml (protein basis) in D.I. water. The pH of the sample solution was adjusted to 8.0 using 0.1M NaOH and re-adjusted every 10 min for a total of 50 min to make it stable at pH 8.0. During pH adjustment, the sample solution was stirred at room temperature for the first 20 min and then stored in a 37 °C water bath for the last 30 min. Enzyme solution containing 1.6 mg/mL trypsin, 3.1 mg/mL chymotrypsin, and 1.3 mg/mL protease was prepared in D.I. water and stirred at room temperature until use. The pH of the enzyme solution was also adjusted to 8.0 and tracked for 50 min using the same procedure that was used for the sample solution. After 50 min, 3 mL enzyme solution was added to 30 mL sample solution, and pH was recorded as the starting pH before digestion. The mixture was then incubated in a 37 °C water bath for 10 min, and pH was measured again which was recorded as the pH after digestion. The digestion rate of proteins was calculated according to the method of Tinus et al. (2012). The digestion rate was expressed as grams of digested proteins in 100 grams of the original sample in 10 min (g/100g protein).

5.2.12 Statistical analysis

All experiments were done by at least triplicates except for the SDS-PAGE, and results were presented as mean ± standard deviation. Data were analyzed with SAS Studio software (SAS Institute, Cary, NC, USA). One-way analysis of variance (ANOVA) was performed with Tukey's post-hoc test to determine significant differences between the means ($p < 0.05$).

5.3 Results and Discussion

5.3.1 Chemical composition of corn protein extracts

Five treatments were used for protein extraction from corn flour. As shown in Table 5.1, protein yields (16.98-18.44%) were not significantly affected by different treatments. Corn flour treated with 5% (v/w) α-amylase had the lowest protein content and the lowest protein recovery. The protein contents of corn flour using the other four treatments were in the range of 52-55% without any significant difference. The highest protein recovery was 96.85% which was observed on the corn sequentially treated with sonication, homogenization, and enzymes (treatment #5). Based on the protein content and protein recovery, treatment #5 was selected as the most efficient treatment and was used to extract corn protein I further experiments.

Table 5.2 summarizes the protein content and recovery of proteins extracted from different materials using treatment #5. Overall, the protein content of extracted protein increased compared to the raw materials without any treatments. The protein extracted from corn flour (protein-flour) showed a increased in corn protein content from 9.87% to 54.81% with protein recovery as high as 96.84%. Protein prepared from corn flour treated with wet milling and starch tabling (protein-MT) had a higher yield (40.05%) than that (28.61%) from corn flour treated with wet milling and centrifugation (protein-MC). Protein-MC and protein-MT showed the highest protein content (70.67% and 72.99%, respectively) and protein recovery (96.56% and 95.33%, respectively) without significant difference. For the two DDGS, there was no significant difference in their protein content before extraction. After extraction, the protein content of the extract from the commercial DDGS (protein-CD) increased from 30.58% to 47.17%, and the protein content of the extract from the liquid fermented DDGS (protein-LD) increased from 33.16% to 44.96%. Their protein contents did not show a significant difference. However, the

yield of Protein-LD (71.52%) was significantly higher than that of protein-CD (49.98%) and it was the highest among all extracted proteins.

The crude fat, and ash content were shown in Table 2. Fat content was in the range of 2.65-3.90%. Protein-flour had the highest rude fat content (3.90%), followed by protein-CD (3.32%). Ash content in each protein extract was significantly different. Compared to other proteins, proteins extracted from DDGS had higher ash content due to higher fiber content. Protein-LD yielded the highest ash (2.63%), followed by protein-CD (2.15%). Protein-MC and protein-MT yielded much lower ash content (0.61% and 0.69%, respectively).

5.3.2 SDS-PAGE

SDS-PAGE electrophoretic patterns of the corn proteins extracted from various pretreated materials under reducing and non-reducing conditions showed slightly different MW distributions (lines 1-5) (Fig.1). High molecular bands were found when non-reducing conditions were applied. All proteins had two clear bands between 20-25 kDa, which are the zein and glutelin fractions according to previously reported research (Malumba et al., 2008; Paraman & Lamsal, 2011; X. Yu et al., 2022). There was another band around 50 kDa for all proteins except for protein-CD. The protein-CD was extracted from the commercial DDGS. The production process may alter the protein structure which caused the difference in the MW distribution. Several other faint bands were observed and distributed in different MW ranges. When reducing conditions were applied, fewer bands were found on the MW above 75 kDa. The same as the non-reducing conditions, a band which was around 50 kDa was observed for all proteins except for protein-CD. Corn protein could be classified into four types including albumins, globulins,

prolamins, and glutenins based on their solubility. Most of the corn proteins were recovered in the Prolamins (also called zein, ~50%) and glutenin fractions (~40%) (Larkins, 2019). The extracted proteins in this study were a mixture of all corn proteins. It was hard to show which band belongs to which type of protein. Previous research reported that both albumins and globulins in corn showed 15-20 distinct fractions with MW in the range of 16-400 kDa. Glutelins contain ~20 components from 11-127 kDa in a reduced condition. Prolamins (zeins) had much smaller MW which have two major groups with MW of 22 and 45 kDa, respectively (Lasztity, 1984). Paulis et al. (1975) also reported the MW distribution of corn protein subunits in SDS-PAGE. There were several bands of each type of corn protein and there were some overlaps in the same MW range.

5.3.3 Secondary structure

The content of each secondary structure of the extracted corn proteins was determined using FTIR spectroscopy. The secondary structure is differentiated by their different C=O starching frequency in the amide region (1700-1600 cm-1) due to their unique molecular geometry and hydrogen bonding pattern (Kong & Yu, 2007; Meng et al., 2021). The extracted proteins from different materials showed different content of each secondary structure (Table 2). Compared to other proteins, protein-LD had the highest random coil content of 52.29%. It may be due to the fermentation process that altered the protein structure. Also, the commercial DDGS was much denser than the liquid fermented DDGS. The treatments were more difficult to rapture the protein structure during extraction. There was no significant difference in the secondary structure between protein-MC and protein-MT. Protein-MC and protein-MT showed relatively higher content of random coil (40.84% and 37.53%) than other proteins. According to the study

of Liu et al. (2021), ultrasonic before wet milling could increase α-helix and β-turn while increasing β-sheet and random coil in separated corn proteins. Guo et al. (2023) reported that sonication of isolated protein from purple corn flour could induce the changes of α-helix to random coil. The higher content of β-sheet and random coil in protein-MC and protein-MT may be caused by the higher protein content, which makes the sonication and other treatments more effective to alter the secondary structure of proteins.

5.3.4 Surface hydrophobicity

The surface hydrophobicity is a critical structure characteristic that reflects the number of exposed hydrophobic groups located on the surface of protein molecules (Y. Chen et al., 2019). As shown in Table 3, protein-MC had a similar SDS binding capacity (41.45 μg/mg) as protein-MT (39.58 μg/mg), while the SDS binding of protein-MC was significantly higher than that of other proteins. Yu et al. (2022) used ultrasound to separate corn proteins from starch after wet-milling, and the surface hydrophobicity of separated glutelin and zein was determined using the ANS fluorescence probe method. According to their study, ultrasonication could increase the surface hydrophobicity of proteins due to the unfolding of protein molecules and the consequent exposure of hydrophobic groups and regions buried inside (Zhou et al., 2013). They also found that untreated zein had remarkably higher surface hydrophobicity than untreated glutelin (101.32 vs. 41.80), which could be caused by the higher proportions of non-polar amino acid residues in zein (Shukla & Cheryan, 2001).

5.3.5 Free SH and SS content

The protein rigid structure was greatly influenced by the content of SH and SS groups in proteins. According to previous studies, the increase of free SH content in proteins reflects the interruption of SS bonds inside the protein molecules (Greenstein, 1938). Hence, the detection of free SH groups in proteins can indicate the cysteine residue available for conjugation, reveal incomplete disulfide bond formation, and help us understand the stability, functionality, and structure of proteins (J. Liu et al., 2021). The free and total SH groups as well as SS contents of extracted corn proteins were listed in Table 3. Protein-MC and protein-MT had 3.04 and 3.14 μmol/g free SH, respectively, which were significantly higher than that of other proteins. The content of free SH groups in other proteins had no significant difference. The content of SS groups in protein-MT (15.41 μmol/g) was significantly higher than that in other proteins, except for protein-MC (14.71 μmol/g). The higher SH and SS groups in protein-MC and protein-MT may be due to the higher protein content, as well as the wet milling process. To release starch from the corn kernel, sulfur dioxide was used in traditional corn wet milling process which could cleave disulfide bonds within corn proteins and then lose the regular compact matric structure of the corn kernel (J. Liu et al., 2021).

5.3.6 Oil/Water binding

The extracted proteins from different materials indicated different oil and water binding capacities even though the same protein extraction treatment was used (Table 5). The capacity of binding water and oil is important properties of protein materials, which determines their applications. Protein-flour showed the highest water holding capacity (WHC) of 4.70 g/g followed by the proteins extracted from two DDGS samples (protein-CD and protein-LC). Protein-MC had the second lowest WHC of 3.17 g/g, and protein-MT had the lowest WHC of

about 2.91 g/g. The highest oil holding capacity (OHC) was observed for protein-MC, protein-MT, and protein-CD, including 3.52, 3.36, and 3.55 g/g, respectively. The OHC of protein-flour was in the similar range as protein-MT but significantly lower than that of protein-MC and protein-CD. The lowest OHC was observed for the protein-LD with a value of 2.72 g/g. According to the study of Espinosa-Ramírez & Serna-Saldívar (2016), the WHC of corn proteins was in the similar range as karfirin rich protein extracts from sorghum (1.99-2.96 g/g), while the OHC of corn proteins was relatively higher than that of kafirin extracts. And the WHC of corn proteins are higher than soluble wheat proteins isolates which was only around 1.59 g/g dry matter (Ahmedna et al., 1999). Phillips & Sternberg (1979) extracted corn protein concentrate (CPC) from corn gluten filter cake, which was a by-product of the saccharification of the starch in corn flour. They measured the functional properties of CPC and compared it with soy protein concentrate (SPC). In their study, CPC indicated 2.2 g/g of WHC at room temperature, and 1.2 g/g of OHC, which were similar to those of a commercial SPC. Another study extracted corn proteins from corn oil cake, and the WHC and OHC prepared corn oil cake protein isolate was 4.03 and 0.55 g/g, and they were closer to peanut and coconut protein isolates (Premkumar et al., 2022). Although those values are in different range due to different measuring approaches, but those results indicated the corn proteins may has similar WHC and OHC as some commercial proteins concentrate or isolates, which could be used in some food systems in the future.

5.3.7 In vitro protein digestibility

The in vitro digestibility (IVPD) of the five protein extracts was also shown in Table 5. The IVPD of all proteins were in the range of 83.13-85.03 g/ 100g protein without any significant difference, which was close to the digestibility of proteins from general wheat flour

(85.7 g/100g protein) and from nonfat dry milk (82.5 g/100g protein). One previous study measured the in vivo digestibility of corn protein concentrates extracted from corn-milo distillers grains, corn-milo distillers grains, and yeast fermented feed grains using the same method (Hsu et al., 1977). The in vitro digestibility of those corn protein concentrates was in the range of 73.6-79.4 g/100g protein, which was lower than those protein extracts in this study. One reason may be due to the lower protein content in their samples due to different starting materials and extraction procedures.

5.4 Conclusion

Wet milling with either centrifuge or starch tabling following with sonication, homogenization, and two enzyme hydrolysis efficiently improve the protein extraction. The protein content was significantly increased from 9.87% (original corn flour) to about 70%, and the protein recovery was up to 95%. The same treatment could also improve protein extraction from corn DDGS but the source of DDGS (corn varieties, manufacturing processing, drying method, etc.) may affect the properties of final proteins. Comparing to the extract from corn flour, the MW of proteins extracted from wet-milling process were similar, but their secondary structure, free SH and SS groups as well as surface hydrophobicity were slightly different. It may be due to the different protein content in each extracted protein which affects the efficiency of physical and enzymatic treatments. Therefore, they also altered water and oil binding capacity. Compared to corn flour, extraction of protein from DDGS was much more difficult due to high fiber and oil content. However, extracted proteins from different sources showed the same in vitro digestibility. Overall, wet milling combined with sonication, homogenization, and enzyme treatments could improve corn protein extraction yield and purity.

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Table 5.1 Protein content and recovery of corn flour using different treatments.

Treatments		Protein content (%)	Protein recovery (%)
#1	5% α-amylase	44.53±1.64[b]	83.60±0.40[c]
#2	5% α-amylase+ 5% cellulsase	52.05±1.03[a]	92.63±1.22[b]
#3	Sonication + 5% α-amylase+ 5% cellulsase	53.29±0.42[a]	92.11±0.95[b]
#4	Homogenization + 5% α-amylase+ 5% cellulsase	53.19±0.30[a]	92.36±1.44[b]
#5	Sonication + homogenization + 5% α-amylase+ 5% cellulsase	54.81±0.23[a]	96.84±0.54[a]

Table 5.2 Composition of extracted corn proteins

Sample	Protein content before extraction (%, db)	Protein content after extraction (%, db)	Protein recovery (%)	Fat (%, db)	Ash (%, db)
Protein-flour[1]	9.87±0.11^{c}	54.81±0.23^{b}	96.84±0.54^{a}	3.90± 0.03^{a}	0.86±0.01^{c}
Protein-MC[2]	20.91±0.10^{b}	70.67±0.44^{a}	96.56±0.33^{a}	2.72±0.06^{c}	0.61±0.00^{e}
Protein-MT[3]	30.60±0.49^{a}	72.99±0.99^{a}	95.33±1.00^{a}	2.65±0.02^{c}	0.69±0.01^{d}
Protein-CD[4]	30.58±1.35^{a}	47.17±1.33^{c}	76.75±0.50^{b}	3.32±0.01^{b}	2.15±0.01^{b}
Protein-LD[5]	33.16±0.05^{a}	44.96±0.00^{c}	96.84±0.41^{a}	2.79±0.05^{c}	2.63±0.01^{a}

[1.] Protein-flour: protein extracted from dry-milled corn flour.

[2.] Protein-MC: proteins extracted from wet-milled corn kernel followed by centrifugation and other treatments.

[3.] Protein-MT: proteins extracted from wet-milled corn kernel followed by starch tabling and other treatments.

[4.] Protein-CD: protein extracted from commercial DDGS; [5.] protein-LD: proteins extracted from lab scale fermented DDGS.

Table 5.3 Contents of secondary structure analyzed by FTIR

Sample	α-Helix	β-sheet	β-Turn	Random
Protein-flour[1]	34.99±1.51[b]	42.60±0.53[a]	4.22±0.28[b]	18.21±1.76[c]
Protein-MC[2]	33.86±0.37[b]	12.77±1.44[c]	12.44±0.80[a]	40.84±2.02[ab]
Protein-MT[3]	33.75±0.96[b]	13.02±1.34[c]	12.71±0.91[a]	37.53±4.77[b]
Protein-CD[4]	41.66±0.88[a]	34.89±1.45[b]	3.30±0.11[b]	20.17±2.44[c]
Protein-LD[5]	30.93±1.58[b]	13.99±1.23[c]	2.78±0.08[b]	52.29±2.73[a]

[1.] Protein-flour: protein extracted from dry-milled corn flour.

[2.] Protein-MC: proteins extracted from wet-milled corn kernel followed by centrifugation and other treatments.

[3.] Protein-MT: proteins extracted from wet-milled corn kernel followed by starch tabling and other treatments.

[4.] Protein-CD: protein extracted from commercial DDGS; [5.] protein-LD: proteins extracted from lab scale fermented DDGS.

Table 5.4 Surface hydrophobicity and free SH and total SS groups

Sample	SDS binding (μg/mg)	Free SH (μmol/g)	SS (μmol/g)
Protein-flour[1]	38.92±1.02[b]	1.96±0.03[b]	14.01±0.53[bc]
Protein-MC[2]	41.45±1.12[a]	3.04±0.03[a]	14.71±0.37[ab]
Protein-MT[3]	39.58±0.83[ab]	3.14±0.11[a]	15.41±0.89[a]
Protein-CD[4]	39.43±0.35[b]	1.92±0.11[b]	14.36±0.31[bc]
Protein-LD[5]	37.81±1.02[b]	2.06±0.32[b]	13.55±0.23[c]

[1.] Protein-flour: protein extracted from dry-milled corn flour.

[2.] Protein-MC: proteins extracted from wet-milled corn kernel followed by centrifugation and other treatments.

[3.] Protein-MT: proteins extracted from wet-milled corn kernel followed by starch tabling and other treatments.

[4.] Protein-CD: protein extracted from commercial DDGS; [5.] protein-LD: proteins extracted from lab scale fermented DDGS.

Table 5.5 Oil/water binding capacity and *in vitro* digestibility

Sample	WHC (g/g sample)	OHC (g/g sample)	IVPD (g/100 g protein)
Protein-flour[1]	4.70±0.07[a]	3.18±0.07[b]	84.48±0.00[a]
Protein-MC[2]	3.17±0.00[c]	3.52±0.05[a]	84.94±0.38[a]
Protein-MT[3]	2.91±0.04[d]	3.36±0.00[ab]	85.03±1.02[a]
Protein-CD[4]	3.80±0.07[b]	3.55±0.06[a]	83.35±1.15[a]
Protein-LD[5]	3.75±0.05[b]	2.72±0.00[c]	83.13±0.64[a]

[1.] Protein-flour: protein extracted from dry-milled corn flour.

[2.] Protein-MC: proteins extracted from wet-milled corn kernel followed by centrifugation and other treatments.

[3.] Protein-MT: proteins extracted from wet-milled corn kernel followed by starch tabling and other treatments.

[4.] Protein-CD: protein extracted from commercial DDGS; [5.] protein-LD: proteins extracted from lab scale fermented DDGS.

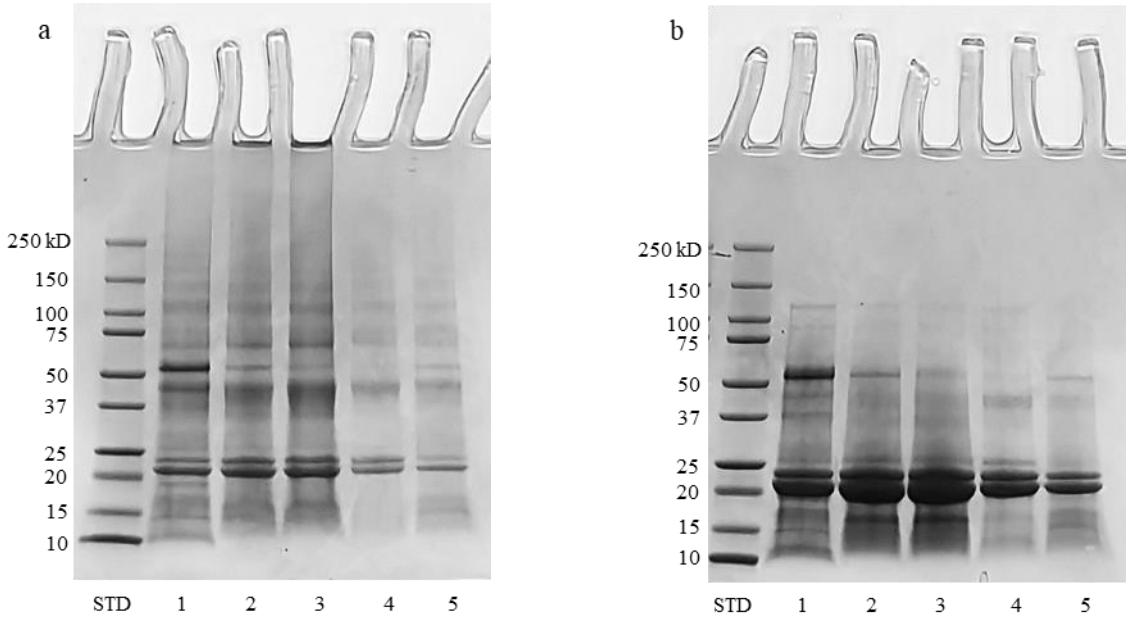

Figure 5.1 Molecular size profiles of corn proteins determined by SDS PAGE gel electrophoresis (1a. non-reducing; 1b. reducing). From left to right, the lines are standard (STD), protein-flour (1), protein-MC (2), protein-MT (3), protein-CD (4), protein-LD (5).

Chapter 6. Conclusions and Perspectives

Corn is an important cereal grain for food, feed, and biofuel industries. Besides providing energy to humans, corn proteins and phytochemicals exist in corn and corn co-products also indicated high antioxidant potential. The primary storage proteins in corn kernel are zein and glutelins, and the major group of phytochemicals are phenolic acids presenting in soluble-free, soluble-conjugated, and insoluble-bound forms. This study aimed to produce both protein and phenolic compounds with antioxidant properties from corn and corn co-products and evaluated their antioxidant capacity using different chemical assays and some model food systems.

Previous studies related to the topic were extensively reviewed in Chapter 1. Chapter 2 was a screening experiment which extracted and analyzed both free and bound phenolic compounds from seventeen strains of corn. Our results showed Although the specific profiles of phenolic compounds in these samples are undoubtedly related to the observed antioxidant activities, there appeared to be a general relationship between the enrichment of phenolic content and the relative level of antioxidant activity since only DPPH scavenging activity was highly correlated with the phenol content. The metal chelating capacity may be more related to some specific phenolic compounds in the corn kernel. Hence, when we determine the antioxidant activities, different assays should be conducted for better comparison among different samples. The colored corn indicated relatively higher total phenolic, flavonoid, and tannin content, but it highly depended on the corn type. Ferulic acid was among the groups of phenolic compounds that are prevalent in corn kernel and was strongly correlated to antioxidant activities.

Based on the study of chapter 2, chapter 3 selected four varieties of corn used for ethanol production. Four yeast strains were used, and results showed that the total phenolic content was increased two to three times after fermentation. Yeast fermentation could destroy the linkages between phenolic compounds and cell walls or proteins to enhance the release of phenolic compounds during extraction, and their effects were highly dependent on their strains. Antioxidant activities of phenolic fractions were also improved after fermentation. This work confirmed that fermentation can improve the yield and antioxidant activity of phenolic compounds. However, more study should be conducted for examples, the change of structure of corn inside granules before and after fermentation, which could help us understanding the how phenolic compounds was released during fermentation with different strain of yeast.

In the third experiment, our goal was to produce both phenolic and protein antioxidants from corn DDGS using a sequential extraction procedure. Our results showed that the extraction solvents for phenolic compounds would not only affect the final phenolic composition, but also affects the structure and antioxidant properties of corn hydrolysate which produced from the solvent extracted DDGS residue. It also provided evidence that those antioxidants from corn DDGS can enhance oxidation stability in o/w emulsion. Those phenolic and protein hydrolysate antioxidants may be used as neutral antioxidants in the food and feed industries in the future. Further study could focus on the purification, identification, and modification of the specific antioxidant phenolic fraction and peptides in these hydrolysates, and in vivo evaluation should also be conducted in order to better characterize those antioxidants in a broader range of food, pet food, and animal feed products.

The last experiments developed a new method for extraction of corn proteins from both corn kernel and DDGS. This method combined wet milling process with sonication, homogenization, and enzyme hydrolysis (cellulase and α-amylase). The protein content was significantly increased from 9.87% (original corn flour) to about 70% after treatment, and the protein recovery was up to 95%. The same treatment could also improve protein extraction from corn DDGS. Moreover, combined wet milling with physical and enzyme treatments could also improve water and oil holding capacity of corn proteins. This could be a potential production approach to achieve high purity corn concentrate with better functionality.