OIL-BASED FOOD EXTRACTS FROM DISTILLERS DRIED GRAINS

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Joseph Michael Kallenbach

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Joseph Michael Kallenbach

The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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Dr. Clifford Hall III
Chair

Dr. Frank Manthey

Dr. Julie Garden-Robinson

Approved:

11/15/16
Date

Dr. Richard Horsely
Department Chair

ABSTRACT

The goal of this project was to examine benefits of developing a DDGS-based extract for human food consumption. The antioxidant activity of extracts derived from corn and distillers' dried grains with solubles (DDGS) was determined. Adding 1% DDGS extract to chips significantly decreased peroxide value and hexanal content compared to the control and potentially lengthened the oxidation induction period. Supercritical carbon dioxide was successful in producing extracts with similar phytochemical content, but adding 0.05% extract to crackers did not significantly reduce oxidation, although the DDGS extract may have lengthened the induction period. Tocopherols and lutein significantly decreased during oxidation and likely provided antioxidant benefits while phytosterol content did not significantly change.

ACKNOWLEDGEMENTS

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More people deserve to be acknowledged, thanked, and rewarded than is possible with the few words available in the space allotted. Suffice it to say that I am grateful to anyone who had even the slightest hand in getting me to where I am today. I will do my best to make it worth your efforts and spread the benefits as far and wide as possible.

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LIST OF ABBREVIATIONS

[C] .....Celsius

[ACS] .....American Chemical Society

[AOCS] .....American Oil Chemists' Society

[BHA] .....butylated hydroxyanisole

[BSTFA] .....N,O-bis(trimethylsilyl) trifluoroacetamide

[CDO] .....corn distillers' oil

[CDS] .....condensed distillers' solubles

[CO_2] .....carbon dioxide

[DDG] .....dried distillers' grains

[DDGS] .....distillers' dried grains with solubles

[DWG] .....distillers wet grains

[EISA] .....energy independence and security act

[F].....Fahrenheit

[FID] .....Flame ionization detector

[g].....gram

[GC] .....gas chromatography

[HPLC].....high performance liquid chromatography

[HSE] .....hydroxycinnamate steryl ester

[HS- SPME] .....headspace solid phase microextraction

[IPA] .....isopropyl-alcohol (isopropanol)

[kg].....kilogram

[KOH] .....potassium hydroxide

[m].....meter

[meq].....milliequivalent

[mg].....milligrams

[min].....minute

[mL] .....milliliters

[mm].....millimeter

[MMT] .....million metric tons

[MPa] .....mega pascal

[MTBE].....methyl-tert-butyl-ether

[N].....normal

[NCI].....Northern Crops Institute

[NDSU].....North Dakota State University

[nm].....nanometer

[NR] .....not reported

[OSI] .....oxidative stability index

[p-AnV].....p-anisidine value

[Pa].....Pascal

[PDA].....photodiode array detector

[Pet Ether].....petroleum ether

[ppm].....parts per million

[PV].....peroxide value

[QDA] .....quantitative descriptive analysis

[RCBD].....random complete block design

[RFS].....renewable fuel standards

[rpm] .....revolutions per minute

[SAS].....statistical analysis software

[SC-CO_2].....supercritical carbon dioxide

[SE] .....steryl ester

[SFX].....supercritical extraction

[TBA].....thiobarbituric acid

[T] .....tocopherol

[T3] .....tocotrienol

[TMCS].....trimethylchlorosilane

[µg].....microgram

[UHP].....ultra high purity

[µL] .....microliter

[UV/Vis] .....ultraviolet–visible spectroscopy

LIST OF SYMBOLS

[°] .....degrees

[%].....percent

[α].....alpha

[β].....beta

[δ].....delta

[γ].....gamma

[v/v].....volume per volume percent

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GENERAL INTRODUCTION

Production of ethanol from corn has increased more than 10 fold in the last decade (USDA-ERS 2016). Despite increased production, profit margins for ethanol have tightened. The net return per gallon of ethanol has been cut in half since 2005 (Hofstrand 2016). Ethanol byproducts are a substantial source of revenue in ethanol production (Liu & Rosentrater 2011). Distillers Dried Grains with Solubles (DDGS) are the principal coproduct of ethanol production. An average of 35 million metric tons (MMT) of DDGS are produced annually from ethanol production with a record 40 MMT produced in 2015; corn is the primary source for ethanol in the USA (USDA-ERS 2016; RFA 2016). DDGS account for over 20% of the revenue from ethanol production, with additional revenue from corn distiller's oil (CDO) (Hofstrand 2016; Irwin 2016). Ethanol production would not be profitable without revenue from coproducts. DDGS have the potential for further development to increase the economic viability of the ethanol industry (Liu & Rosentrater 2011).

DDGS are a nutritionally dense byproduct of corn ethanol production. DDGS are sold as a commodity, mostly for animal feed. Phytochemicals, including tocopherols, carotenoids and phytosterols, are naturally found in corn and DDGS. The antioxidant potential of these phytochemicals has been demonstrated for decades; however, a lack of research exists on DDGS oil for human consumption. DDGS-based oil extract could provide antioxidant benefits, including extending the shelf-life of food.

Antioxidants are important to extending shelf-life by preserving food quality. Oxidation produces negative sensory attributes that can make food undesirable. Synthetic antioxidants are effective at preventing oxidation at low levels of inclusion. Approved synthetic antioxidants,

such as BHA and TBHQ, are limited to addition of 0.02% (200 ppm) on a fat basis (Pokorny 2007).

Consumers are demanding more foods with natural ingredients and thus the food industry is striving to meet this demand. Natural antioxidants are extracted from plants for use as food additives. While natural antioxidants are effective at reducing oxidation, limited sources are available. Natural antioxidants generally require higher addition concentrations than synthetic antioxidants to be effective. Tocopherols and carotenoids are generally recognized as safe (GRAS) and regulated under good manufacturing practices (GMP) in the United States (Pokorny 2007). International standards limit carotenoids to addition of 100 ppm in snack foods. Tocopherol additive limits are not defined in snack foods, but are defined for many other food products from cheese to chocolate. Addition of tocopherols in fat spreads and cereal based desserts is limited to 500 ppm (Codex Alimentarius 2016). As natural antioxidants would meet the needs of both consumers and food producers, developing DDGS based antioxidants could be beneficial. In addition, with food waste emerging as a major societal concern, finding new ways to preserve food quality using ingredients from waste products could improve relations between the consumer and the food industry.

The goal of this project was to examine benefits of developing a DDGS-based extract for human food consumption. Natural extracts were tested in low moisture foods, such as chips and crackers, to identify a potential antioxidant benefit. Phytochemicals were quantified through high performance liquid chromatography (HPLC) and gas chromatography (GC). Primary and secondary lipid oxidation products were monitored. Sensory evaluation was conducted in crackers over 90 days to demonstrate oxidative stability and consumer acceptance. This study

showed the benefits of a DDGS extract at higher concentrations. Future work should aim to raise antioxidant concentration in the DDGS extract and optimize the incorporation level.

1. LITERATURE REVIEW

1.1. Ethanol Production

1.1.1. Overview of the Ethanol Industry

The modern ethanol industry emerged in the United States in the 1970's. The production of ethanol increased gradually until a surge in production in the early 2000's; government mandates promoted increases in the ethanol production (Liu and Rosentrater 2011). The Energy Policy Act of 2005 implemented Renewable Fuel Standard (RFS). Renewable energy standards were further strengthened in 2007 with the passage of the Energy Independence and Security Act (EISA), which required the United States to increase renewable fuel production to 36 billion gallons by 2022, from 9 billion gallons in 2008 (NDEC 2014). These policies have encouraged long-term investment in ethanol that has greatly impacted the Nation's energy sector (RFA 2013). The ethanol industry contributes $44 billion to the Unites States economy per year and accounts for about 10% of the oil produced in the United States annually (RFA 2013; EIC 2014; NDEC 2014).

Ethanol has many uses and is produced from a variety of raw goods. Cereal grains were the original source, as they were fermented to produce alcohol for human consumption. Alcohol distilleries now comprise a very small fraction of ethanol production, as biofuel manufacturing has grown to the primary sector. Biofuels can be produced from many plants including cereal grains, oilseeds, sugary crops, legumes and perennial grasses (Liu and Rosentrater 2011). Corn has become the dominant source of fuel ethanol production in the United States and corn based DDGS are nearly ubiquitous.

Dry-grind corn ethanol production is the dominant method for producing ethanol from corn. In dry milling, the whole kernel is milled, cooked, and fermented (Figure 1). The

fermentation process utilizes the starch fraction of corn. Starch is converted to sugar and fermented to produce ethanol. The remaining non-fermentable components are collectively known as whole stillage. Whole stillage is separated into a liquid and solid fractions. The liquid fraction becomes thin stillage, which is partially recycled and dried to become condensed distillers solubles (CDS). Recent advances in the ethanol industry have promoted the extraction of oil from CDS by centrifugation; the extracted oil can be used in animal feed or biodiesel production, adding value to the ethanol process (Ciftci and Temelli 2014; RFA 2014).

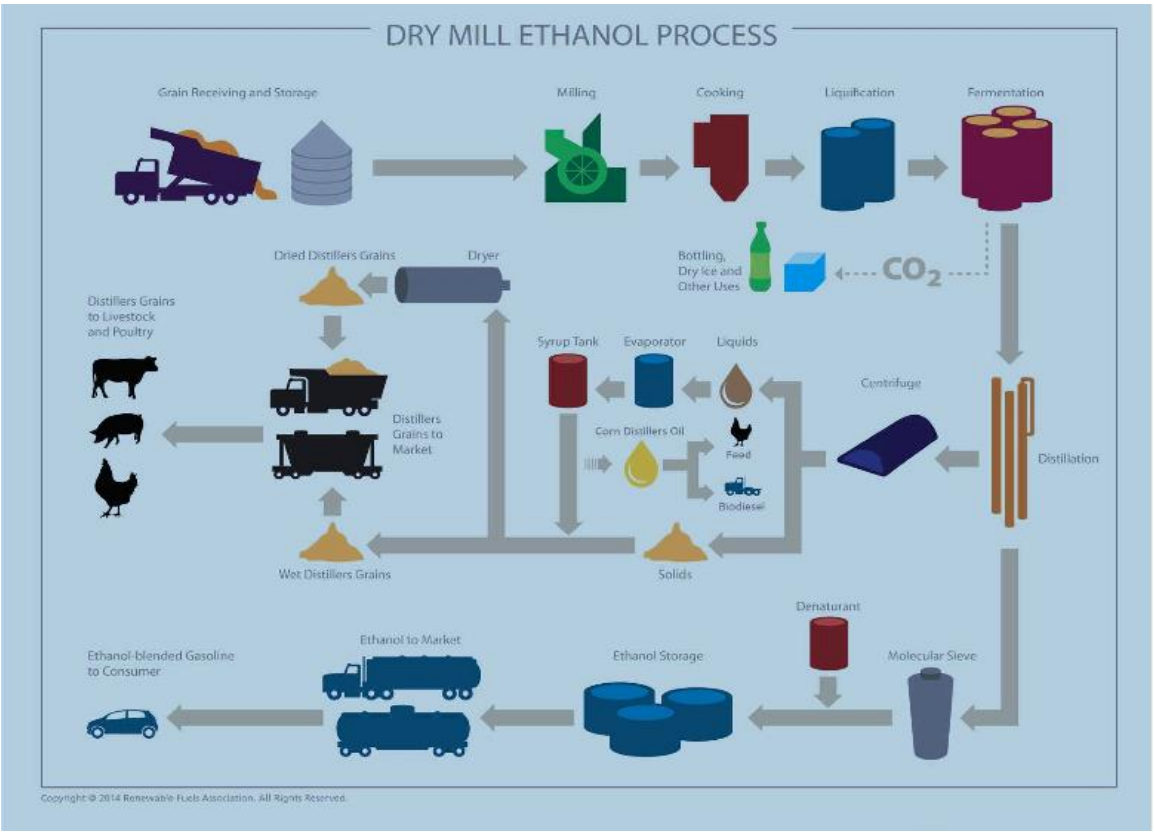


Figure 1. Dry grind ethanol process (RFA 2014).

The solid fraction of whole stillage, known as wet cake, can be sold as feed or dried. Wet cake has a short shelf life due to high moisture content and thus has few practical large-scale uses. Distillers wet grains (DWG) often spoils in less than a week. In addition, DWG has different flow characteristics than dried distillers' grains (DDG) and requires special handling

techniques, which make it less ideal for commercialization (Liu and Rosentrater 2011). Typically, wet cake is incrementally dried from approximately 65% moisture to 12% moisture, thus forming DDG. CDS often is recombined during the drying process to produce distillers dried grains with solubles (DDGS). Many consider DDGS a coproduct of ethanol production, as opposed to a byproduct, due to its importance to the ethanol industry (Liu and Rosentrater 2011). For example, DDGS accounted for over 20% of revenue from ethanol production in 2015, which is increased from around 12% of total revenue in 2005 (Hofstrand 2016).

1.1.2. Corn

1.1.2.1. Corn Composition

Corn is the most widely used crop for ethanol production. Corn, also known as maize, is a member of the grass family Gramineae (Kent and Evers 1994). Dent corn is the most prominent class grown in the United States and is preferred for ethanol production. Corn produces the largest kernel of the cereal grains. The corn kernel is composed of a tip cap, pericarp, endosperm and germ. The endosperm is 82% of the kernel, while the pericarp and germ consist of 6% and 12%, respectively (Liu and Rosentrater 2011). Starch can compose more than 80% of nutrients, but is often 60-80%, and entirely located in the endosperm. Fiber, protein and ash make up about 10%, 8% and 1% of the kernel, respectively (Shukla and Cheryan 2001). Lipids comprise only 3-6% of the whole kernel. However, lipids are concentrated in the germ, containing approximately 45-50% oil, which is 85% of the total kernel lipid content (CRA 2006; Majoni and Wang 2010). Most lipids are in the form of triacylglycerides. Linoleic acid is the predominant fatty acid in corn, followed by oleic, palmitic, stearic and linolenic with 49.8%, 33.4%, 14.0% 2.0% and 1.5%, respectively (Kent and Evers 1994). Phospholipids, glycolipids, and free fatty acids also are found in corn (Moreau et al 2001; Moreau and Hicks 2005). A

distinct feature of corn is the relatively high amounts of phytochemical compounds, including phytosterols, tocopherols and carotenoids, found in the lipid extracts of corn (Hall III and Zhao 2011).

1.1.3. Distillers Dried Grains with Solubles

1.1.3.1. Overview of DDGS

DDGS are the primary coproduct of ethanol production. Thirty-five million metric tons (MMT) of DDGS are produced annually from ethanol production with 40 MMT being produced in 2015. Approximately $5 billion worth of DDGS were produced from corn ethanol production in 2015, of which, one third is exported (RFA 2014; 2016; USDA-ERS 2016). DDGS account for more than 20% of revenue for ethanol production (Hofstrand 2016).

DDGS are currently sold almost exclusively as livestock feed. Distillers grains have been proven to be valuable in the diets of beef cattle, dairy cattle, poultry, sheep, swine and other animals (Babcock et al 2008). DDGS are desirable as an animal feed, in part, due to the high protein and energy density. Macro- and micro- nutrient contents within DDGS are often inconsistent, which may reduce the value as animal feed (Cromwell et al 1993; Belyea et al 2006). However, DDGS are still a popular source of nutrition at a controlled portion of animal diets (US Grain Council 2012). Utilization of distillers' grains as animal feed can reduce feed costs to farmers (Masa'deh et al 2011; 2012). The price of DDGS is highly dependent on corn and, to a lesser extent, soybean prices (Hoffman and Baker 2011).

Removing oil from DDGS has been used as an additional source of revenue for ethanol producers; therefore, reduced fat DDGS have been researched as feed for various animals. High-fat diets reduce milk production in dairy cows; research shows that low-fat DDGS increased milk production compared to other feed sources (Babcock and other 2008). Lowering fat content in

DDGS did not seem to negatively affect egg production when incorporated into hen diets (Purdum et al 2014). In addition, fat content of DDGS has little effect on swine metabolism (US Grain Council 2012). This research suggests that reduced lipid DDGS can be an advantageous source of animal feed.

1.1.3.2. DDGS Composition

The ethanol production process significantly affects the composition of DDGS. Nutrient composition often differs between ethanol facilities. Variability can be attributed to the processing conditions, but may also be an effect of the methods used for composition analysis (Belyea et al 2004; Spiehs et al 2002). In addition, corn genotype and growing location affects DDGS nutritional content. Compared to corn, the percent of protein, fat and fiber is increased by approximately three-fold in DDGS, mostly due to the removal of starch. Leguizamon et al (2009) found approximately 4 times the lipid content in DDGS compared to corn. DDGS contain approximately 5% starch, 30% protein, and 5% ash (Liu 2011).

Approximately 90% of ethanol is produced by dry grind production (RFA 2014). Dry grinding is often preferred over wet milling due to lower capital investment (Liu and Rosentrater 2011). Dry mills use the entire kernel whereas wet mills concentrate the starch component by separating various components of the corn kernel. Dry milling lends itself to producing distillers' grains with higher phytochemical content compared to wet milling. Phytochemical concentrations are dependent on location. Tocopherols are more concentrated in the germ whereas tocotrienols are more evenly distributed between germ and fiber. Tocols are likely degraded somewhat due to heat used during ethanol production, but total tocol concentration in DDGS is comparable to corn kernel oil (Moreau et al 2011). Carotenoids are highly concentrated

in endosperm, but nearly void in the germ (Moreau et al 2000). This explains why DDGS oil has up to 33 times more carotenoids than corn germ oil (Moreau et al, 2007).

Reduced-oil DDGS have become more popular due to increased efficiency in the ethanol industry. Various processes have since been developed for removing oil from ethanol byproducts (Cantrell and Winsness 2009). Over 85% of ethanol plants have added the capacity to extract oil, known as Corn Distillers Oil (CDO), from ethanol stillage (RFA 2014). As a result, the nutritional composition of the majority of DDGS has changed over the last decade. Lipid content of DDGS, prior to CDO extraction, varied between 9.7-12.7% of dry matter (Cromwell et al 1993; Spiehs et al 2002; Belyea et al 2004; Winkler et al 2007; Robinson et al 2008; Kim et al 2008; Liu 2009; Stein et al 2009; Leguizamon et al 2009; Masa'deh et al 2011; Moreau et al 2011). CDO extraction can remove between 30-70% of oil from stillage; most DDGS now contain 7-9 percent oil. DDGS sold as feed can be as low as 5% fat (Shurson et al 2012). An evaluation of multiple ethanol plants from Illinois, Indiana, Iowa, Michigan, Minnesota, Nebraska, North Dakota and South Dakota showed that fat content in DDGS varied between 6-13% during 2011 and 2012 (Anonymous 2012). Recent macronutrient data from DDGS using the dry grinding ethanol process (with CDS oil extraction) consist of approximately 89.4% dry matter, 28.3% protein, 7.3% fat and 6.9% fiber (RFA 2014). With this amount of fat, oxidation could be a problem.

1.2. Oxidation

1.2.1. Overview of Oxidation

Oxidative stability is important to nutritional and sensory attributes of food (Kamal-Eldin 2006). Lipid oxidation encompasses many complex interactions between lipids, oxygen and other various components. A consequence of lipid oxidation is the decomposition of fatty acids

into volatiles compounds; volatiles affect sensory characteristics and often promote further oxidation. Lipid oxidation reduces food quality. Oxidative rancidity is a general term for the production of unwanted aromas and flavors due to lipid oxidation (McClements and Decker 2008).

Oxygen is important to food oxidation because it is a powerful oxidant. Oxidants remove electrons from other reactants. The oxidant power of oxygen can change depending on its outer electron state. Ground state oxygen is referred to as triplet oxygen. Singlet oxygen appears in a higher energy state; the difference is the position of electrons in the outer orbital. Singlet oxygen has electrons in both orbitals whereas the ~~the~~ triplet oxygen contains electrons within a single orbital. The singlet state is less stable and thus more highly reactive.

The oxygen state is important as it affects both the products of oxidation and circumstances under which oxidation will occur. Singlet oxygen is formed by photo-excitation. Storage conditions can inhibit photooxidation thus preventing the formation of singlet oxygen species. Autoxidation by triplet oxygen is more dependent on other factors. Oxidation can be catalyzed by temperature, enzymes, and metals (St. Angelo 1992). Understanding the mechanism of oxidation is important for choosing the correct method of quantifying oxidation (Decker et al 2010).

The basic steps in oxidation include initiation, propagation and termination. Lipid radical first form during initiation. Initiation can occur by abstraction of a hydrogen atom or homolytic cleavage of hydroperoxides. Propagation is the formation of new radicals from existing radicals. Proliferation of free radicals is an integral part of lipid oxidation. Termination is the interaction of free radicals to form non-radical molecules (Frankel 1998l; Pokorny et al 2001).

Lipid oxidation products are affected by the location of hydrogen extraction. Initiation often occurs when a hydrogen is removed from an unsaturated lipid, creating an alkyl radical (Figure 2). The allylic carbon (the carbon adjacent to a carbon-carbon double bond) has low bond dissociation energy; therefore, hydrogen abstraction occurs preferably from allylic carbons in autoxidation. Increasing unsaturation increases the likelihood of initiation. Bis-allylic carbons (i.e. carbon located directly between 2 sets of double bonds) are often found in polyunsaturated fatty acids. A bis-allylic carbon has a high potential for hydrogen abstraction due to surrounding instability. For example, linoleic acid (18:2) is 10-40 times more susceptible to autoxidation compared to oleic acid (18:1) and the induction times for each fatty acid at 25°C are 19 and 82, hours respectively (McClements and Decker 2008; Belitz et al 2009). Hydrogen abstraction takes place at the bis-allylic carbon, which is followed by a rearrangement of double bonds to stabilize the free radical (Figure 3).

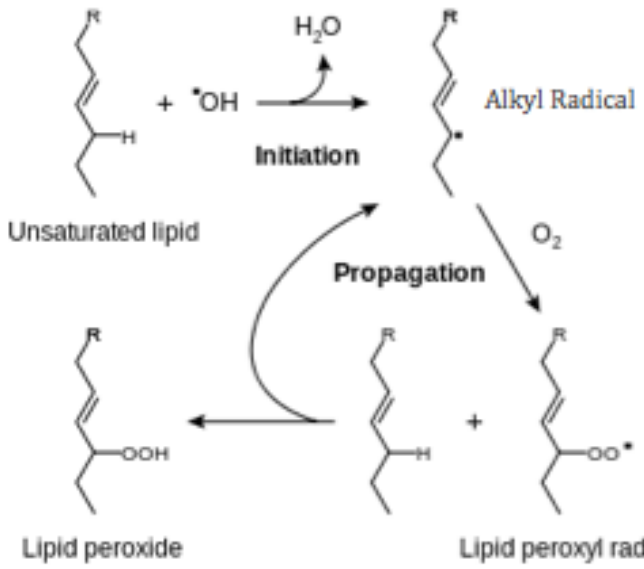


Figure 2. Initiation and propagation of unsaturated lipids (Vickers et al 2001).

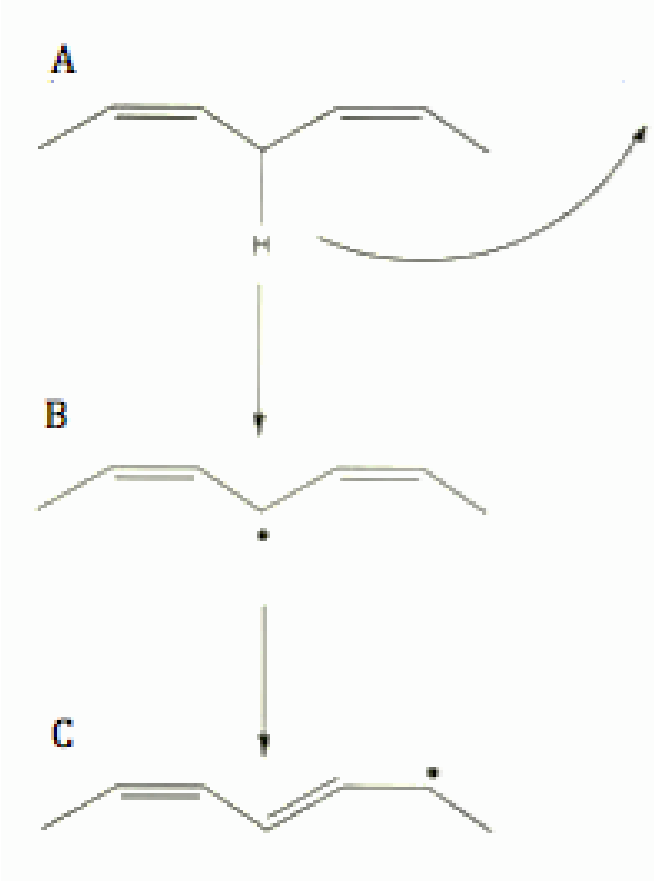


Figure 3. Hydrogen abstraction in lipid oxidation (McClements and Decker 2008).
Location of hydrogen abstraction within polyunsaturated lipids (A), creation of a carbon centered radical (B) and shift in double bond placement to stabilize the radical (C).

Hydrogen abstraction can occur at different locations for autoxidation and photooxidation, as a result of the oxygen species involved. Flavors and aromas generated are dependent on the type of oxidation and the specific lipid being oxidized. Oleic acid (18:1) can result in four different alkyl radicals in autoxidation, which in turn can each produce a variety of volatile compounds (Pokorny et al 2001).

Propagation occurs when oxygen reacts with the alkyl radical to form a peroxyl radical. The peroxyl radical extracts a hydrogen from another unsaturated lipid, initiating additional radical formation, and forming a hydroperoxide (Figure 4) (McClements and Decker 2008). The interaction of two radicals to destroy both radicals is known as termination. Termination rarely occurs at low radical concentrations (Pokorny et al 2001)

Hydroperoxide breakdown results in lower molecular weight volatiles associated with rancidity. Alkoxyl radicals are highly energetic intermediate products of hydroperoxide oxidation. The carbon bond adjacent to the alkoxyl radical is cleaved to form smaller compounds (McClements and Decker 2008). Secondary oxidation products are often aldehydes or ketones that produce flavors and aromas (Kamal-Eldin 2006).

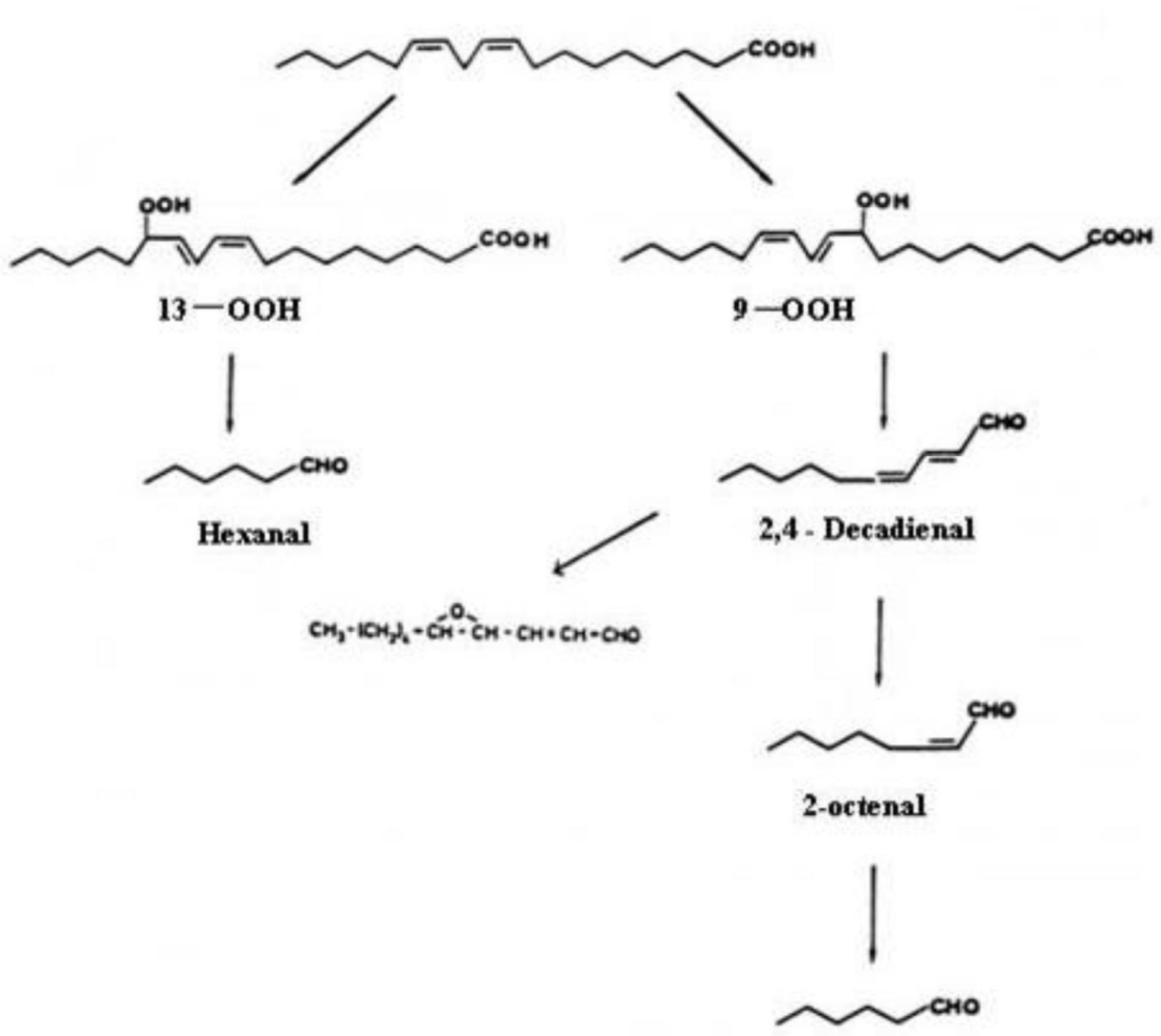


Figure 4. Formation of oxidation products from linoleic acid (Shi and Ho 1994).

1.2.2. Quantification

Primary oxidation products are an important assay for oxidation. Lipid hydroperoxides are primary oxidation products. The iodometric titration method is the standard for calculating

peroxide value. Hydroperoxides will oxidize iodide to iodine, which turns purple in the presence of starch. Titration with sodium thiosulfate returns iodine to iodide. Peroxide value is expressed as milliequivalents of oxygen per kilogram of oil (meq/kg) (McClements and Decker 2008). The titration method has many positive aspects, but can be time consuming and labor intensive, and requires relatively large amounts of sample. In addition, titration involves visually determination of an end point, which may increase human error and reduce test sensitivity. Iodometric titration is sensitive to approximately 0.5 meq/kg. Other methods have been developed for quantifying primary oxidation products, but the titration method is still generally favored (Shahidi and Zhong 2005).

Secondary oxidation products also must be quantified to produce an accurate indication of oxidation (Shahidi and Zhong 2005). Primary oxidation products increase during the initial phase of oxidation, but will eventually deteriorate as secondary oxidation products form (Figure 5).

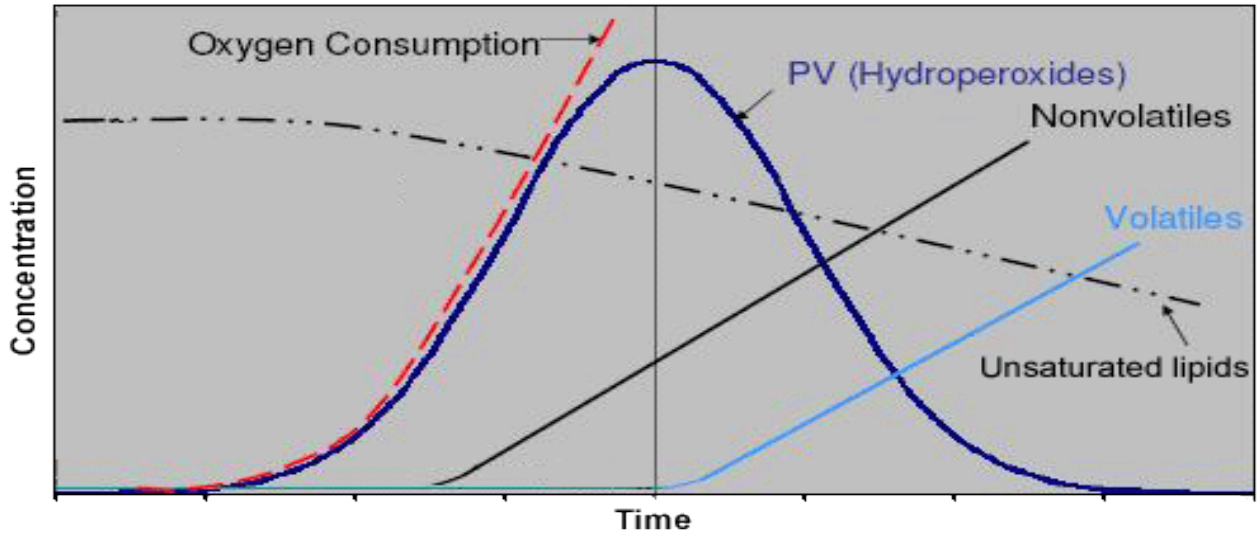


Figure 5. Concentration of primary and secondary oxidation products over time (Labuza and Dugan 1971).

Secondary oxidation products include a wide variety of compounds. Lipid content and oxidation conditions should determine the method for quantifying secondary products. Some

common methods for quantifying secondary oxidation products include the thiobarbituric acid (TBA) test, p-anisidine value (p-AnV), totox value, carbonyl content, oil stability index (OSI) and hydrocarbon assays (Shahidi and Zhong 2005).

The content of certain hydrocarbons are known to change with the degree of oxidation. Hexanal is the predominant secondary product of linoleic acid oxidation (Belitz et al 2009). Hexanal has been useful in measuring antioxidant efficiency and shows a relationship to sensory perceptions (Sanches-Silva et al 2004; Shahidi and Zhong 2005). The path for hexanal production from linoleic acid involves peroxide formation followed by decompostition (Figure 4). Other volatiles may have greater impact on rancid flavors and aromas, but high hexanal concentration can be better for analysis (Pokorny et al 2001).

Hexanal can be quantified by various gas chromatography methods. Static headspace methods are relatively easy to perform and reliably measures hexanal in foods such as potato chips (Azarbad and Jeleń 2014). Static heaspace is not as sensitive as some other methods, but these methods are good for measuring hexanal at high concentrations. The headspace solid-phase microextraction (HS-SPME) method has been used for GC determination of hexanal in potato crisps. In HS-SPME, a coated fused silica fiber sits above a sample where the volatiles are allowed to equilibrate in the headspace and onto the fiber. The volatiles are desorbed from the fiber when placed in the injection port of the GC and separated as volatiles move throught the column (Sanches-Silva et al 2004).

1.2.3. Antioxidants in Corn and DDGS

1.2.3.1. Overview of Mechanism of Antioxidant Action

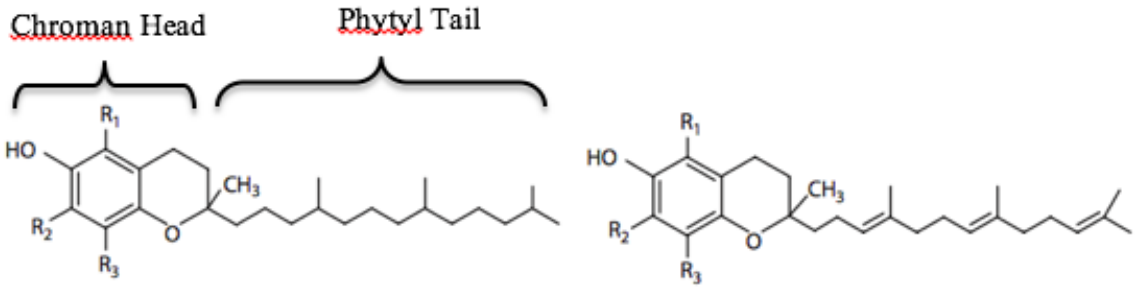
Antioxidants inhibit oxidation by interacting with free radicals. Antioxidants work by various methods; the main antioxidant categories include inhibitors and chain breaking

antioxidants. Singlet oxygen quenchers are preventative inhibitors by reducing the high-energy singlet oxygen to the triplet state, a less reactive oxygen species. Carotenoids and, to a lesser extent, tocopherols have single oxygen quenching potential (Pokorny et al 2001). Antioxidants may also inhibit free radicals or scavenge radical species (Hall III and Zhao 2011). Hydrogen donation is a chain-breaking mechanism where the antioxidant donates a hydrogen to terminate a radical; the antioxidant is generally stabilized by resonance within a phenolic structure. Antioxidants also can work synergistically by combining multiple oxidation prevention mechanisms (Pokorny et al 2001).

Natural antioxidants, such as tocopherols, are nearly ubiquitous in nature and play a vital role in maintaining life. Consumers often prefer natural antioxidants for perceived health benefits. Some natural antioxidants are more favorable than their synthetic counterparts. Synthetic tocopherols are racemic whereas natural tocopherols have right-handed chirality making natural tocopherols more effective as a dietary source of vitamin E (Pokorny et al 2001). Additionally, natural carotenoids are mostly found in the all-trans form, which has the greatest vitamin A activity (McClements and Decker 2008).

1.2.3.2. Tocols

Tocochromanols, also referred to as tocols, are a group of monophenols that can be divided into two groups: tocopherols and tocotrienols. Tocopherols consist of a chromal ring with a saturated phytyl side chain. The phytyl tail of tocotrienols is unsaturation at the 3, 7 and 11 carbons. Each group has four isomers (α-, β-, γ-, or δ-), which differ by the number and position of the methyl groups (Figure 6).



Tocopherols

	R1	R2	R3
α	CH_3	CH_3	CH_3
β	CH_3	H	CH_3
γ	H	CH_3	CH_3
δ	H	H	CH_3

Tocotrienols

	R1	R2	R3
α	CH_3	CH_3	CH_3
β	CH_3	H	CH_3
γ	H	CH_3	CH_3
δ	H	H	CH_3

Figure 6. Methyl group location in tocopherol and tocotrienol isomers (adapted from Hall III and Zhao 2011).

Corn contains approximately 30 ppm tocopherols, but can vary between 7 and 86 ppm depending on genotype (Kurilich and Juvik 1999). Tocopherols are approximately three-fold greater than tocotrienols in corn (Panfili et al 2003). The most prominent tocol is γ-tocopherol, usually constituting greater than 50% of total tocol content. The β-isomers of both tocopherol and tocotrienol are sparsely found in corn. While tocopherols are highly concentrated in the germ (90%), tocotrienols are more evenly distributed throughout the kernel (Messias et al 2015; Weber 1987). Milling can have a significant impact on tocol concentration considering the disparity between kernel components (Table 1).

Table 1. Tocol content from various corn fiber oil, refined corn oil, whole kernel oil and corn germ oil shown as the mean data of multiple sources.

	Total Tocols	α-T[a]	γ-T	δ-T	α-T3[b]	γ-T3	δ-T3
Oil	---------- μg/g ----------						
Corn Fiber[c]	578	0	79	36	15	378	70
Refined Corn Oil[d]	787	185	555	26	9	13	3
Whole Kernel[e]	2000	407	1051	93	156	269	25
Corn Germ[f]	2164	184	1758	107	26	97	28

[a] T = tocopherols
[b] T3 = tocotrienols [(c)] (Moreau and Hicks 2006)
[d] data adapted from (CRA 2006; Schwartz et al 2008)
[e] data adapted from (Moreau and Hicks 2006; Moreau et al 2010)
[f] data adapted from (Kamal-Eldin and Anderson 1997; Moreau and Hicks 2006; Moreau et al 2010; Tuberoso et al 2007; Winkler-Moser and Breyer 2011)

Lipids extracted from DDGS are of some interest as a source of valuable nutrients including tocopherols (Liu 2011). The non-starch nutrients in corn can become concentrated to 3-4 times higher levels in DDGS (Moreau et al 2011). However, tocols are sensitive to heat and may be destroyed in processing (CRA 2006; Čukelj et al 2010; Fardet et al 2008; Moreau et al 2010). Refined corn oil contains about 1/3 the total tocol concentration compared to corn germ oil, which is due to the oil refining process. Depending on the study, DDGS and corn can contain relatively similar amounts of tocols; however, Winkler-Moser et al (2009) found much higher levels of tocols in DDGS (Tables 2). DDGS oil has better oxidative stability than natural vegetable oils, possibly due to the high concentration of antioxidants (Winkler-Moser and Breyer 2011).

Table 2. Tocol content (μg/g) in DDGS oil from literature.

Solvent	Total Tocols	α-T[a]	γ-T	δ-T	α-T3[b]	γ-T3	δ-T3
	---------- μg/g ----------						
Hexane[d]	1820	190	950	51	16	460	15
Hexane[e]	1801	194	948	51	155	453	NR[c]
Hexane[f]	2866	296	761	48	471	1210	80
Hexane[g]	1803	178	875	250	100	250	19
Pet Ether[h]	1265	111	687	NR	123	116	228
$SCCO_2$[d]	1710	260	830	58	13	450	NR
$SCCO_2$[i]	1682	144	889	NR	163	112	374
Mean	1849	196	848	91	149	436	143
(% of total)		(10.6%)	(47.9%)	(3.5%)	(7.3%)	(21.2%)	(6.4%)

[a] T = tocopherols
[b] T3 = tocotrienols
[c] NR = not reported
[d] data adapted from (Winkler et al 2007)
[f] data adapted from (Winkler-Moser et al 2009)
[g] data adapted from (Winkler-Moser and Breyer 2011)
[h] data adapted from (Winkler et al 2007)
[i] data adapted from (Ciftci et al 2012)

Vitamin E is a collective group of lipid-soluble antioxidants, of which, tocopherols are the most prominent (Schwartz et al 2008). Tocols act as a chain breaking antioxidants by donating a hydrogen to a free peroxyl radical (Traber 2007). The hydroxyl group on the C6 position of the phenolic ring functioning to reduce free radicals by hydrogen donation (Figure 7). The resonance within the phenol group stabilizes the tocol, preventing the spread of radical species. Tocopherols can also act as antioxidants by trapping alkoxyl radicals (Hall III and Zhao 2011).

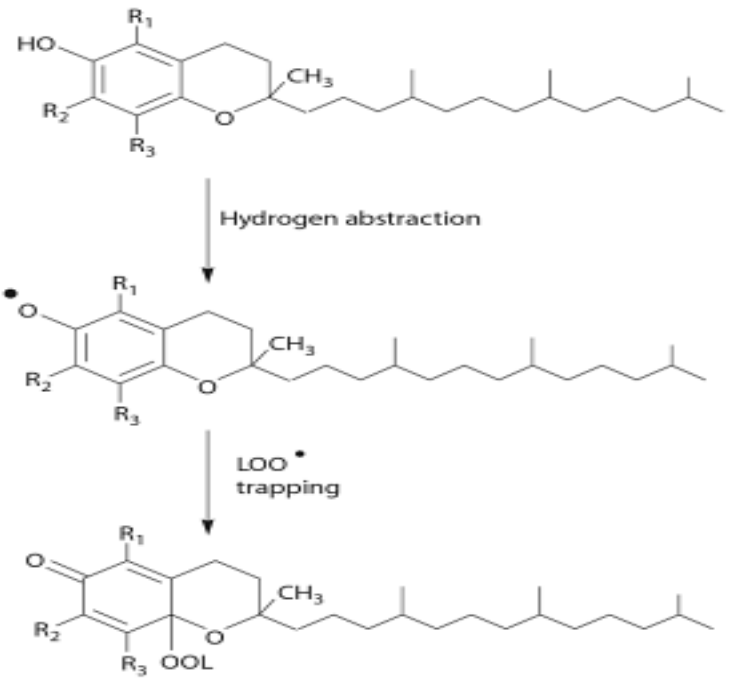


Figure 7. Proposed tocopherol radical scavenging mechanism (Hall III and Zhao 2011).

Tocopherols are known to have very high antioxidant properties (Winkler-Moser 2011). Burton et al (1985) considered tocopherols to be the main antioxidant in stabilizing vegetable oils. Tocopherols have been shown to extend the lag phase of oxidation in corn oil (Lindsay 2008). Vegetable oils are stabilized most effectively at an optimal level of supplementation (Kamal-Eldin and Appelqvist 1996). Natural levels of tocopherols stabilize corn oil effectively while excessively high amounts of tocopherol can facilitate oxidation. High concentrations promote prooxidant interactions within oil systems. For example, α-tocopherols are considered the most efficient peroxyl radical scavenger among tocopherols, but side reactions may promote propagation (Kamal-Eldin 2006; Yanishlieva et al 2002). Furthermore, α-tocopherols are better antioxidants at low concentration, while γ-tocopherols are more effective at higher concentrations (Fuster et al 1998; Yanishlieva et al 2002). While tocotrienols are not as prominent in nature and have not been studied as thoroughly, they have many benefits and potentially greater antioxidant properties in some circumstances (Sen et al 2007).

The vitamin E activity found in both tocopherols and tocotrienols may be linked to health benefits (Sen et al 2007). Human and animal studies suggest E-vitamins reduce oxidative stress (Martin et al 1996; Golestani et al 2006). Tocopherols and tocotrienols prevent the oxidation of polyunsaturated lipids in cell membranes by the same mechanism described above (Traber, 2007). Tocopherols may reduce risk of cancer and heart disease (Burton and Traber 1990; Burton 1994). Some research suggests that α-tocopherols are the primary tocochromanol isomer in preventing cellular oxidation within humans due to the greater affinity for transport proteins (Traber 2007; Traber and Atkinson 2007). Plasma concentrations of α-tocopherols are correlated with reduced rates of cause-specific mortality; however, vitamin E supplementation does not seem to correlate with reduced risk of mortality, possibly due to reduced affinity of artificial tocopherols by human tocopherol transport proteins (Wright et al 2006; Traber 2007).

1.2.3.3. Phytosterols

Phytosterols are plant steroids that can be subdivided into sterols and stanols. Phytosterol structures consists of three 6-carbon rings, a 5-carbon ring, and a variable carbon side chain. Stanols are saturated while sterols have an unsaturated ring structure. Hundreds of known phytosterol compounds exist due to variations in the carbon side chain and degree of saturation (Moreau et al 2002).

The aleurone layer of the corn caryopsis is likely the source of phytosterol production (Moreau et al 2000). In contrast to tocopherols, the germ contains the lowest percentage of free phytosterols in corn. The germ still contains the most total phytosterols due to higher lipid content, but the aleurone and fiber have the highest concentration (Figure 8). Commercial corn oil contains only germ oil; therefore, the loss of phytosterols in fiber and aleurone results in a substantial reduction of phytosterols in commercial corn oil (CRA 2006).

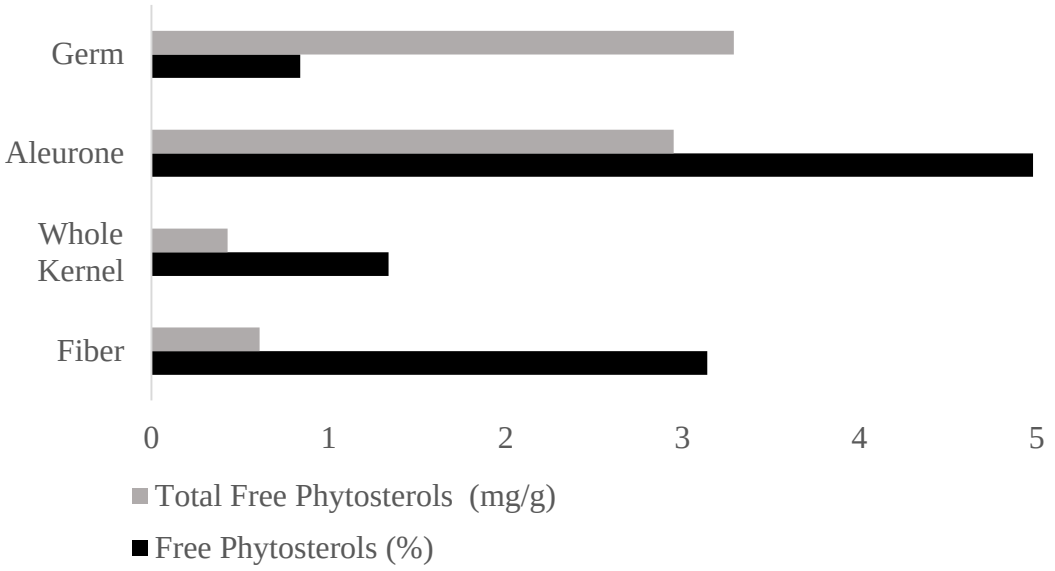


Figure 8. Percent free phytosterol in extracts from fiber, aleurone, germ and whole kernel compared to total concentration of free phytosterols in each fraction.
Data adapted from (Jiang and Wang 2005; Leguizamon et al 2009; Liu 2009; Moreau et al 2000; 2001; 2002; Stein et al 2009).

Sitosterol is predominant in corn oil, but stigmasterol, campesterol, sitostanol, cycloartenol campestanol and sigmastanol are also present (Figure 9) (Jiang and Wang 2005).

Distribution of common phytosterols is relatively similar throughout the kernel (Figure 10).

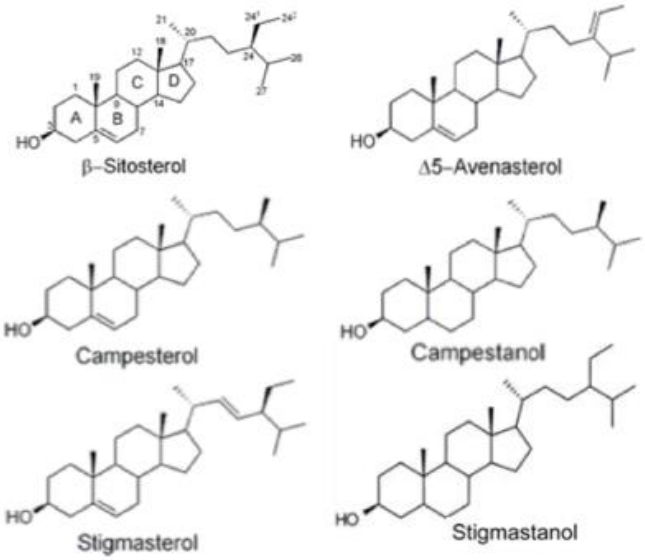


Figure 9. Structure of common phytosterols found in corn (Winkler-Moser 2011).

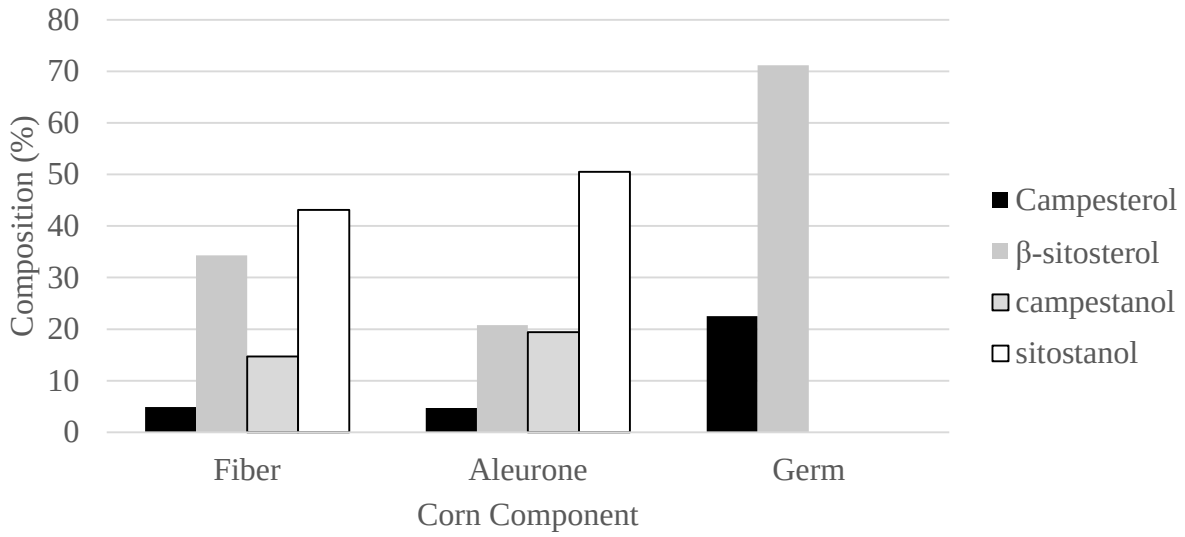


Figure 10. Distribution of prominent phytosterols in the fiber, aleurone and germ of corn (adapted from Jiang and Wang 2005; Moreau et al 2000; 2002).

Phytosterols can be found in free form or bound to fatty acids, phenolics and carbohydrates (Toivo et al 2001). The 3β-OH group on the "A ring" becomes an ester or glycosidic linkage in bound phytosterols (Moreau et al 2002). Sterol esters can be unique to cereals such as corn, including some hydroxycinnamate esters (Figure 11), also known as ferulates (Moreau et al 2002). In studies by Moreau et al (1999; 2001), approximately 54% and 12% of sterols from corn were bound to fatty esters and ferulates, respectively; only 33% kernel phytosterols were unbound. The germ contains about half of all free phytosterols in the kernel. Most esterified phytostanols are in the aleurone layer, which is removed in commercial corn oil extraction (Moreau et al 2000; Winkler-Moser et al 2012).

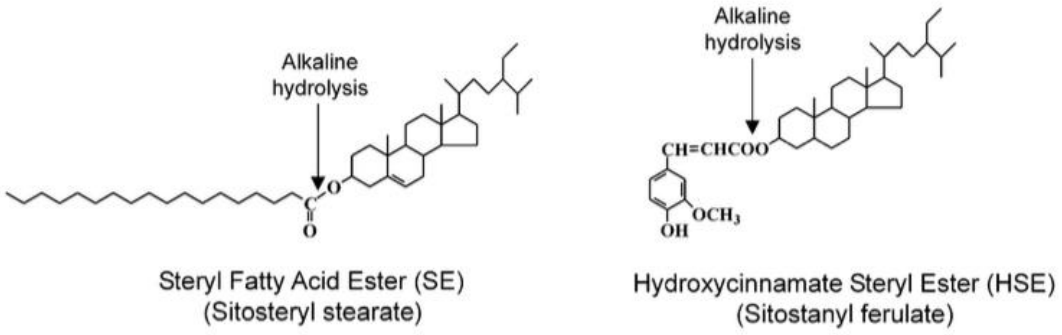


Figure 11. Example of phytosterol fatty esters and ferulates found in corn (adapted from Moreau et al 2002).

Phytosterol can be quantified by gas chromatography. Sample preparation generally involves releasing bound phytosterols to their free form. Alkaline hydrolysis with ethanolic KOH is sufficient for breaking phytosterol ester bonds (Figure 11). Glycosidic linkages require acid hydrolysis, but steryl glycosides are rare in corn (Moreau et al 1996; 2000; 2002). Phytosterol derivitization often is used for GC analysis to improve volatility and peak resolution. Trimethylsilyl ether derivitization is common (Moreau et al 2002; Winkler-Moser 2011).

A relatively wide range of phytosterol quantities have been reported in DDGS. This can likely be attributed to multiple reasons including extraction techniques and the variability in corn. Total phytosterol content appears to be about twice as high in DDGS oil compared corn oil (Leguizamon et al 2009). Sitosterol and campesterol are the most prominent sterols in both corn and DDGS oil (Table 3).

Table 3. Phytosterol content (mg/g) in DDGS oil from literature.

Solvent	Total Phytosterols	Campe-sterol	Stigma-sterol	β-sito-sterol	5-aventa-sterol	Campe-stanol	Sito-stanol
	---------- mg/g ----------						
Hexane[b]	16.2	2.5	0.8	8.1	0.7	1.1	2.7
Hexane[c]	11.5	NR[a]	NR	NR	NR	1.1	2.6
Hexane[d]	21.7	3.0	1.1	10.3	0.9	NR	NR
Hexane[e]	22.0	NR	NR	NR	NR	1.4	3.7
Pet Ether[f]	12.4	3.0	1.1	7.9	0.3	NR	NR
SCCO2[b]	15.8	2.5	0.1	7.9	0.1	NR	NR
SCCO2[g]	15.9	3.6	1.6	10.2	0.5	NR	NR
Mean (% of total)	16.5	2.9 (18.4%)[b]	1.0 (6.0%)	8.9 (54.9%)	0.6 (3.0%)	1.2 (6.6%)	3.0 (16.8%)

[a] NR = not reported
[b] data adapted from (Winkler et al 2007)
[c] data adapted from (Leguizamon et al 2009)
[d] data adapted from (Winkler-Moser and Breyer 2011)
[e] data adapted from (Moreau et al 2011)
[f] data adapted from (Ciftci et al 2012)

Phytosterols may contribute to antioxidant effects in some foods. The antioxidant activity of phytosterols appears to be linked to reduced polymerization in frying oil held at high temperatures. Polymerization is a common sign of oxidation in frying oils and thus steryl ferulates maintain oil freshness. The antioxidant benefits appear to be linked to some specific structural components only found in some phytosterols. According to Gordon and Magos (1983), "sterols with an ethylidene group in the side chain are most effective as antioxidants." Some phytosterols can interrupt autoxidation by reacting with lipid free radicals to form stable free radicals. Stigmasterol, β-sitosterol and cholesterol were ineffective antioxidants in a frying oil (180°C) application while Δ^5-avenasterol was effective at 0.1% of lipid content (Gordon and Magos 1983). Yoshida and Niki (2003) suggests that campesterol and β-sitosterol act as antioxidants while stigmasterol accelerates oxidation.

Another proposed mechanism for antioxidant activity from phytosterols is due to the attachment of phenolic acids. Phenolic acids are effective antioxidants. They likely act as antioxidants through hydrogen donation or radical scavenging. Winkler and Vaughn (2009) suggest steryl ferulates in DDGS oil contribute antioxidant activity. Similar steryl ferulates found in other cereals have been shown to be good antioxidants (Nyström et al 2005; Xu et al 2001). Phytosterols esterified to phenolic acids, such as hydroxycinnamate esters found in corn, are chain breaking antioxidants (Hall and Zhao 2011).

The health effects of phytosterols are considered more significant than antioxidant activity (Hall III and Zhao 2011). Cereal oils are one of the best sources of natural phytosterols. Sterols are known for their cholesterol-lowering potential by preventing the absorption of cholesterol in the intestines (Ostlund et al 2002). Dietary phytosterol consumption reduces serum low density lipoproteins, which are related to increased heart disease, without altering high density lipoproteins, which are associated with good heart health. Free sterols are the physiologically active form, but bound sterols are mostly hydrolyzed in the intestines. Stanol and stanyl esters have been shown to have strong enough cholesterol-lowering effects to warrant a health claim for food containing sufficient amounts. In addition to containing tocopherols and carotenoids, corn fiber oil is considered a heart healthy oil for its natural phytostanol content (Leguizamon et al 2009; Moreau et al 2002).

1.2.3.4. Carotenoids

Carotenoids are 40 carbon tetraterpenoids that include two groups: carotenes and xanthophylls. Xanthophylls contain oxygen while carotenes do not. Carotenoids with at least one terminating non-hydroxylated β-ionone ring can be converted into retinal, and thus, contain

Vitamin A activity; β-carotene and β-cryptoxanthin are provitamin-A carotenoids. Lutein and zeaxanthin are hydroxylated, which prevent conversion to retinal.

Many fruits and vegetables are good sources of carotenoids. Tomatoes and carrots are relatively well known sources due to their vibrant color; however, other lesser known sources include spinach and dried apricots. Carotenoids are fairly unstable and so processing conditions such as temperature and pH can be destructive (Rao and Rao 2007). Corn is a unique source of carotenoids in cereals grains (Pokorny et al 2001). Carotenes are generally associated with orange pigments and make up a very small percentage of carotenoids in corn; the majority of carotenoids in corn are xanthophylls, which appear yellow. The most prominent carotenoids in corn include lutein, zeaxanthin, β-cryptoxanthin and β-carotene (Figure 12).

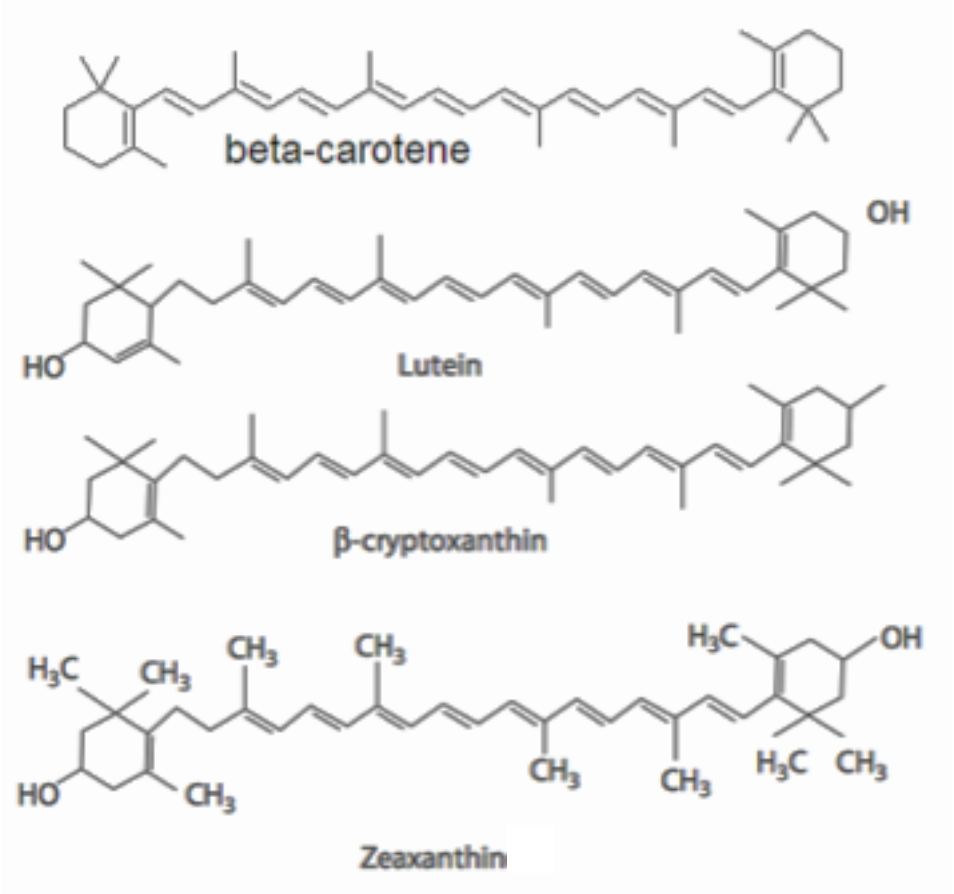


Figure 12. Structure of carotenoids found in corn (adapted from Ross 2007).

Carotenoid content is highly variable in corn. Average total carotenoid content is around 10 ppm (Kurilich and Juvik 1999; Panfili et al 2004). However, values depend on a variety of circumstances. Carotenoid content in corn can vary greatly by genotype (Kurilich and Juvik 1999; Weber 1987). Processing and extraction techniques also affect the carotenoid content (Moreau et al 2010). Ethanol extraction yielded a total carotenoid content of 324.5 μg/g in corn oil, compared to 60.7 μg/g from hexane extraction (Moreau et al 2007). In addition, carotenoids are not evenly distributed throughout the corn kernel. Unlike tocopherols and phytosterols, the endosperm contains a high concentration of carotenoids (Table 4) (Weber 1987; Moreau et al 2010). Conventional refining of corn oil removes the majority of carotenoids, making commercial corn oil a poor source (CRA 2006).

Table 4. Comparison of carotenoid concentration from oil extracted from the germ and whole kernel of corn.

	Total Carotenoids	β-Carotene	Lutein	Zeaxanthin	β-Cryptoxanthin
	---------- μg/g ----------				
Germ	2.3	0.0	1.4	0.9	0.0
Kernel	85.0	14.2	42.8	21.5	6.5

[a] data adapted from (Moreau et al 2010)

Carotenoid content is higher in DDGS compared to corn (Table 5). Winkler-Moser and Breyer (2011) found 75.02 μg total carotenoids per gram DDGS oil. The ratio of xanthophylls and carotenes was similar in both corn and DDGS, with xanthophylls being more prominent (Winkler and Vaughn 2009; Moreau et al 2010).

Table 5. Carotenoid content (μg/g) in DDGS oil from literature.

Solvent	Total Carotenoids	β-Carotene	Lutein	Zeaxanthin	β-Cryptoxanthin
	---------- μg/g ----------				
Hexane[a]	66.3	4.5	34.6	23.3	3.9
Hexane[b]	75.0	0.9	46.7	24.2	3.3
Pet Ether[c]	89.0	2.0	47.0	35.0	4.0
SC-CO_2[c]	107.0	3.0	57.0	42.0	5.0
Mean	84.3	2.6	46.3	31.1	4.1
(% of total)		(3.2%)	(55.1%)	(36.5%)	(4.9%)

[a] data adapted from (Winkler-Moser et al 2009)
[b] data adapted from (Winkler-Moser and Breyer 2011)
[c] data adapted from (Ciftci et al 2012)

Carotenoids are effective antioxidants by scavenging peroxyl radicals and singlet oxygen species. Carotenoids are among the best-known singlet oxygen scavengers. A series of conjugated double bonds make it possible for carotenoids to participate in physical quenching of reactive oxygen species. The conjugated structure dissipates energy from singlet oxygen. Carotenoids can return singlet oxygen to a stable triplet energy state without altering their structure (Akoh and Min 2002). Physical quenching allows carotenoids to participate in several antioxidant interactions. Carotenoids also can scavenge lipid peroxyl radicals; however, chemical quenching of radicals only accounts for 0.05% of carotenoid antioxidant activity (Stahl and Sies 2003). Radical species are stabilized by a variety of proposed mechanisms. Chemical quenching changes the carotenoid structure, resulting in color loss (Krinsky and Yeum 2003). Synergistic effects also have been seen with other phytochemicals such as tocopherols (Stahl and Sies 2003; Subagio and Morita 2001). Carotenoids can participate in prooxidant interaction. Carotenoids are highly reactive to molecular oxygen and can be unstable in certain environments. The prooxidant effect is increased at higher oxygen pressure and carotenoids concentrations (Stahl and Sies 2003).

Carotenoids are beneficial for human health. Carotenoids may prevent a variety of chronic diseases (Paiva and Russel 1999; Rao and Rao 2007). Their antioxidant properties protect cellular membranes from oxidative damage (Sies and Stahl, 1995). Some carotenoids have provitamin A activity because they are converted to retinol; vitamin A deficiency has many negative consequences (Akoh and Min 2002; Lindsay 2008). Non-provitamin A carotenoids also are linked to many health benefits. Strong evidence links lutein and zeaxanthin to eye health. Lutein and zeaxanthin are found in the macula lutea of the retina while other carotenoids are not. Stahl and Sies (2003) suggest that lutein and zeaxanthin are special because they "can be incorporated into membranes in higher amounts than other carotenoids". Dietary lutein and zeaxanthin are correlated to lower rates of macular degeneration, as well as reducing risk of some types of cancer (Ribaya-Mercado and Blumberg 2004). Antioxidant activity is the most likely mechanism for carotenoid health benefits but various alternative reasons have been suggested. While some epidemiological studies show benefits of increasing dietary intake, other studies suggest higher levels of carotenoids have been linked to negative health outcomes (Rao and Rao 2007). However, limited studies have been completed using 100% pure carotenoids. This is likely due to difficulty during extraction

1.3. Supercritical Fluid Extraction

The primary states of matter (solid, liquid and gas) are conditional on both temperature and pressure. Gases can be compressed or cooled to liquids, with the reverse being true when going from liquids to gases. When pressure and temperature are increased beyond a critical point, substances can exist in a state that exhibits properties inherent to both liquids and gases. This is considered a supercritical state (Figure 13) (Mukhopadhyay 2000).

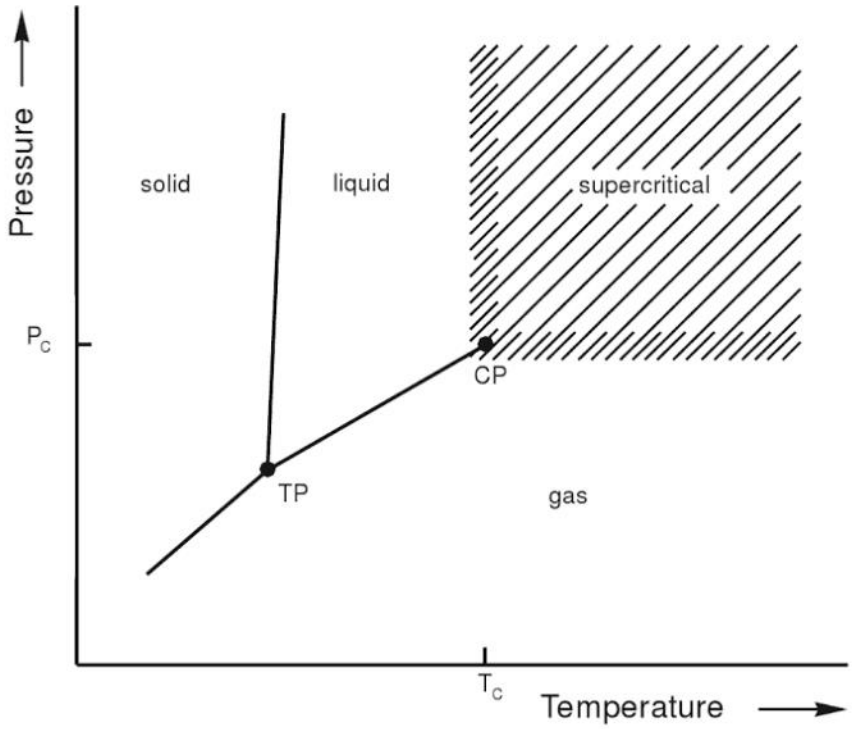


Figure 13. Pressure-temperature diagram (Mukhopadhyay 2000).

Carbon dioxide is a desirable solvent for use in supercritical extraction since it is inexpensive and readily available. Furthermore, carbon dioxide is "Generally Recognized as Safe" (GRAS) and leaves no residue in the extract. As a byproduct of ethanol production, CO_2 could potentially be recycled within an ethanol plant, reducing overall greenhouse effects (Mukhopadhyay 2000).

Supercritical carbon dioxide (SC-CO_2) has been used as a commercial solvent since the 1980's (King and Bott 1993). Supercritical carbon dioxide is considered a clean, safe, inexpensive and environmentally friendly solvent. Special solvent properties stem from the ability to flow as a liquid while penetrating matrices like a gas. Gaseous properties include low viscosity and high diffusivity, which promotes fast penetration into a matrix. SC-CO_2 also maintains the dissolving properties inherent to liquids. Furthermore, solute selectivity can be changed by adjusting pressure and temperature (Mukhopadhyay 2000).

Cosolvents can also increase extraction efficiency. Small concentrations of a cosolvent can increase extraction efficiency by altering polarity and solvent strength (Mukhopadhyay 2000). Cocero and Calvo (1996) found increasing ethanol concentration increased solubility of sunflower oil in SC-CO_2 extraction; the addition of ethanol may increase phospholipid extraction, but decrease free fatty acid content. Carotenoids are fairly insoluble in carbon dioxide, but addition of ethanol as a cosolvent considerably increases the solubility of both lutein and β-carotene (Jay et al 1991). Moreover, ethanol may open the structure of proteins found in corn to expose trapped carotenoids, allowing for greater extraction. The inclusion of 15% ethanol entrainer in SC-CO_2 extraction improved the removal of carotenoids from corn gluten (Sessa et al 2003). However, ethanol may decrease tocopherol separation; limiting to 5% cosolvent is recommended (Mukhopadhyay 2000).

Particle size is an additional factor in lipid extraction. Efficiency of phytochemical extraction in corn was increased tenfold by grinding (Moreau et al 1996). Corn is milled to less than 500 μm prior to ethanol extraction, but particles swell to create an average DDGS particle size of 700 μm with high variability (Liu 2008; 2009). Oil extraction is higher in corn and DDGS when particles are reduced to less than 700 μm (Liu 2010). This suggest that the hammer milling of corn prior to ethanol production is sufficient for oil extraction, but DDGS require further milling, possibly due to swelling during ethanol production. Although particle size was not specified, Ciftci et al (2012) reduced DDGS using a coffee grinder when determining optimal SC-CO_2 extraction parameters.

1.4. Conclusion

DDGS are a potential source of natural antioxidants. Tocopherols, phytosterols and carotenoids are more concentrated in DDGS than corn. Tocopherols and carotenoids are well

known antioxidants, in addition to having potential health benefits. Phytosterols benefit heart health and can convey antioxidant activity in some forms. The lipophilic properties of these phytochemicals allow them to be concentrated in lipid extracts. Supercritical fluid extraction can be used to create an antioxidant-rich extract from DDGS and corn. This extract can be tested in food for antioxidant activity.

2. PROBLEM STATEMENT

2.1. Summary of Literature Review

Distillers dried grains with solubles are a major byproduct of corn ethanol production. DDGS are primarily valued as animal feed due to high energy density and protein content; however, the DDGS has not been used as a source of phytochemicals. DDGS contain carotenoids, tocopherols and phytosterols. Utilizing the full nutritional potential from DDGS could provide additional revenue to the ethanol industry.

Phytochemical concentration can be highly variable in both corn and DDGS. The stated nutrient content is dependent on corn genotype, processing techniques and analytical methods (Liu 2011). Literature generally indicates that DDGS have a higher concentration of phytochemical than corn (Table 6). The dry grind ethanol process removes starch from corn, in effect, concentrating the non-starch components of corn. Phytochemicals may be degraded somewhat by heat used in ethanol production.

Table 6. Carotenoid, tocol and phytosterol content in hexane extracted corn and DDGS oil.

	Carotenoids	Tocols	Phytosterols
	---------- mg/g ----------		
DDGS oil[a]	0.075	2.9	21.7
Corn kernel oil[b]	0.061[c]	2.0	17.9

[a] Winkler-Moser et al 2011
[b] Moreau et al 2007; 2011

Lipid oxidation is a natural process that negatively affects food quality. Oxidation produces hydroxyl radicals by hydrogen abstraction from fatty acids. In the presence of oxygen, hydroperoxyl radicals are formed; lipid hydroperoxyl radicals can proliferate quickly and break down into smaller volatiles, such as aldehydes and ketones. Volatiles produce off flavors and aromas associated with rancidity.

Natural antioxidants are integral to controlling oxidation. Preventing oxidation is a major concern in the food industry. Tocopherols, carotenoids and phytosterols have all been shown to possess antioxidant activity in various ways. Tocopherols are monophenols with chain breaking potential by either donating a hydrogen or trapping a lipid radical. Carotenoids are highly efficient singlet oxygen quenchers due to the conjugated structure. Carotenoids also can scavenge lipid peroxyl radicals. Phytosterols are more known for dietary health benefits, but some steryl esters are considered good antioxidants.

Supercritical carbon dioxide extraction is a unique extraction method with potential benefits for producing a natural antioxidant extract. Supercritical fluids contain the flow characteristics and dissolving properties of a fluid while incorporating the low viscosity and high diffusivity of a gas. Solubility characteristics can be manipulated by adjustments in pressure and temperature. Supercritical CO_2 is a clean, safe, inexpensive and environmentally friendly solvent (Mukhopadhyay 2000).

Ciftci et al (2012) optimized corn DDGS extraction using SC-CO_2. The highest yield of total lipids, carotenoids, tocopherols and phytosterols were produced by SC-CO_2 at 49.6 MPa and 70°C. Both Ciftci et al (2012) and Winkler et al (2007) observed phytochemical extraction from DDGS by SC-CO_2 to be comparable to Soxhlet extraction using petroleum ether and hexane.

2.2. Objectives

Objective 1: Produce an antioxidant-rich extract from both DDGS and corn.

Objective 2: Determine the effect of extracts on oxidation in low moisture food and quantify potential antioxidants during oxidation of low moisture food.

2.3. Hypotheses

Objective 1: DDGS extract will have higher concentrations of tocopherols, phytosterols and carotenoids compared to a corn.

Objective 2: Extracts from DDGS and corn will reduce oxidation in low moisture food with antioxidant benefits being related to the concentrations of carotenoids, tocopherols and phytosterols.

3. PAPER 1: ANTIOXIDANT ACTIVITY OF CORN AND DRY DISTILLERS' GRAINS IN CHIPS

3.1. Abstract

Antioxidant-rich extracts were produced by hexane extraction from corn and distillers dried grains with solubles (DDGS). Corn and DDGS extract contained 18.2 and 14.8 mg/g phytosterols, 719 and 929 μg/g tocopherols, and 20 and 74 μg/g xanthophylls, respectively. The phytochemical-rich extracts were incorporated in chip formulas at 1%, and evaluated for shelf stability. Peroxide value and hexanal were significantly lower in chips with DDGS extracts, but the extracts did not significantly increase phytochemical concentrations in the chips. DDGS extract appears to lengthen the oxidation induction period in chips. Tocopherols and lutein decreases significantly during oxidation, thus likely acting as an antioxidant while phytosterols did not change during oxidation.

3.2. Introduction

Limited research has been conducted regarding the use of DDGS in human food. Research has focused on the high fiber and protein content in distillers' grains. Dietary fiber promotes multiple health benefits including weight control, improved plasma lipid profile, glucose control and may help prevent Type 2 diabetes, colon cancer and obesity (AACC 2001; ADA 2002). Protein from distillers' grains may contribute to gluten replacement in foods for individuals with Celiac Disease (Liu and Rosentrater 2011). Researchers have tried incorporating distillers' grains in food for more than 30 years. Over 20 studies on nearly 50 food products were conducted during the 1980's; interest waned, due to negative sensory qualities and functional challenges (Rosentrater and Krishnan 2006). Wu et al (1987) successfully incorporated distillers' grains into spaghetti, but sensory characteristics were affected significantly by the addition of

distiller's grains. Researchers have continued to look for solutions to technical and sensory problems associated with distillers' grains.

More recent research has reexamined distillers' grains in food (Wu et al 1990; Liu et al 2011; Ciftci and Temelli 2014). Beverage distilleries provide the bulk of distillers' grains being tested in human food; DDGS from ethanol production have not been studied extensively in food applications (Liu and Rosentrater 2011). However, as DDGS have become more plentiful and remain inexpensive, more research has been incentivized. Breads and cookies have been popular vectors for incorporating DDGS in food. Other products include baked goods, pastas, extruded products and blended ingredients. While some studies claim successful incorporation of DDGS in food, utilization of DDGS in food does not appear to have become popular (Rosentrater and Krishnan 2006; Saunders et al 2013; Pourafshar et al 2014; 2015).

The lipid components of DDGS have not been as widely studied in human food. Winkler-Moser and Vaughn (2009) tested the DDGS oil extracts in stabilizing frying oil; the extract was a distillate of DDGS oil with a high concentration of steryl ferulates. The rate of polymerized triacylglycerols from oxidation in frying oils was reduced with the DDGS extract. In addition, the steryl ferulates appeared to protect added tocopherols, which also inhibit oxidation.

Phytochemicals of interest in DDGS include tocopherols, carotenoids and phytosterols, which are all lipophilic and thus extracted by non-polar solvents. The lipid extraction method is dependent on many factors including polarity, cost, and environmental impact. Multiple researchers have compared methods of from DDGS (Ciftci et al 2012; Winkler et al 2007). Hexane extraction is a popular solvent for lipid extraction due to its low polarity and moderate boiling point. Ethanol also has been explored as a solvent, but was not considered as effective (Singh and Cheryan 1998). However, ethanol extraction of DDGS produced far, i.e. about five

times more, carotenoids than hexane extraction, but the increased efficiency has not been found in other studies (Moreau et al 2007). While solvent extraction is considered the most efficient method for removing lipids, it poses many problems including capital investment, regulations, oil quality and safety. Solvent extraction of DDGS has not been adopted in ethanol plants in the United States. Centrifugation is commonly used for extracting CDO from DDGS for use in biofuels and animal food (Liu and Rosentrater 2011; Moreau et al 2010).

Oxidation in low-moisture foods is extremely complicated. *In vitro* studies can be used to measure the total antioxidant capacity; however, antioxidant capacity may not correlate well to oxidative stability in food due to the complexity of food systems. Oxidative stability is more predictive when tested on a case-by-case basis in food systems (Barden and Decker 2013).

Antioxidant activity in low-moisture food is difficult to predict for a variety of reasons (Barden and Decker 2013). Variables, such as polarity, concentration and side reaction volatility, can determine the effectiveness of antioxidants. Emulsions, generally seen in low-moisture food, require antioxidants be present at the interface to be effective. Low-moisture foods, such as chips and crackers, receive the best protection from hydrophobic antioxidants as they are most likely to partition at the oil-water interface (Barden et al 2015). Furthermore, many low-moisture foods are expected to maintain a relatively long shelf-life. Oxidation is affected by water activity. Oxidation is slowest in food with water activity between 0.2-0.5, which includes most low moisture foods (Labuza et al 1972). Extending the induction phase is critical to oxidative stability in low moisture food; however, antioxidants that are only effective after the induction phase may not provide any benefit to sensory qualities (Nanditha and Prabhasankar 2009). Lastly, the antioxidant should not themselves alter the sensory attributes of food.

Accelerated shelf-life studies are used to measure antioxidant effectiveness. Higher temperatures allow for faster analysis of oxidation and can be correlated to room temperature analysis. However, certain foods can produce anomalous results at temperatures above 50°C, possibly due to reactions such as non-enzymatic browning. Tocopherols have also been shown to be less effective at higher temperatures. Studies at, or below 40°C are common for accelerated oxidation studies (Labuza and Dugan 1971).

Corn and DDGS contain substantial amounts of lipid-soluble phytochemicals. The objective of this study was to test the antioxidant activity of a DDGS and corn extracts obtained from hexane extraction in chemically leavened pita chips. The amount of natural antioxidant added is regulated under GMP in the United States. The antioxidant-rich extracts should extend the shelf-life of chips by reducing oxidation.

3.3. Materials and Methods

3.3.1. Chemicals and Materials

3.3.1.1. Samples

Distillers' dried grains were obtained from three local ethanol facilities: Hankinson Renewable Energy (Hankinson, ND), Bushmills Ethanol (Atwater, MN), and Tharaldson Energy (Casselton, ND). Corn was obtained from 3 sources: O'Brien Seed Inc. (Mayville, ND), Greg LePlant (Fargo, ND) and Specialty Commodities (Fargo, ND). Corn and DDGS were stored at 40°C in closed 5 gallon buckets to prevent light exposure.

3.3.1.2. Chemicals

Hexane for oil extraction was purchased from Avantor Performance Materials (Central Valley, PA). Ethanol (95%) was purchased from NDSU chemical stockroom (Fargo, ND). HPLC-grade organic solvents, including hexane, isopropanol (IPA), methanol, methyl-tert-butyl-

ether (MTBE), dichloromethane, and chloroform, were purchased from VWR (Randor, PA). Laboratory grade granular potassium iodide and American Chemical Society (ACS) grade glacial acetic acid were also obtained from VWR. Sodium thiosulfate and potassium hydroxide (85%) were obtained from Thermo Fisher Scientific (formerly Alfa Aesar, Ward Hill, MA). Ultra-high purity (UHP) hydrogen (99%) was obtained from PraxAir Distribution, Inc. (Fargo, ND). Pyridine and N,O-bis(trimethylsilyl) trifluoroacetamide with trimethylchlorosilane (BSTFA + 1% TMCS) were purchased from Sigma-Aldrich (St. Louis, MO) in addition to standards for hexanal (98%), campesterol (65%), β-sitosterol (95%), 5α-cholestane (97%), lutein (95%) and zeaxanthin (95%). Tocopherol standards for α-, γ-, and δ-isomers (99% purity) were purchased from Supelco (Bellefont, PA).

3.3.2. Experimental Design

Shelf stability of chips over time was evaluated (Chapter 3.3.3.1). An extract was created by hexane extraction from corn and DDGS. Extracts were added to chips subjected to accelerated storage conditions and periodically sampled throughout the shelf life study (55 days). The experiment was organized as a Random Complete Block Design (RCBD) with 3 repetitions and 3 treatments. Treatments consisted of chips with DDGS extract, corn extract and a control group made without extract.

3.3.3. Methods

3.3.3.1. Preparation of extracts from corn and DDGS

Oil from corn and DDGS were extracted with hexane using a large soxhlet extraction unit overnight (≈12 hours). Prior to extraction, corn was milled via a Fitzmill hammer mill (Fitzpatrick Company Elmhurst, IL) at Northern Crops Institute (NCI). DDGS were milled using a lab scale Polymix Micro Hammer Mill (Kinematic, New York) with a 1.0 mm screen. All

particles passed through a 25 mesh (710 μm) sieve. Hexane was removed by rotary evaporator under vacuum at 60 MPa/40°C. Impurities were removed by separating the bottom organic layer after centrifuging oil for 20 minutes at 4000 rpm (2510 x g) and 0°C. The remaining oil was used as the antioxidant-rich extract. The final corn and DDGS extract was an equal mixture (by weight) of extract from the three corn and ethanol DDGS sources, respectively.

3.3.3.2. Chip Baking

To determine antioxidant potential, extracts were added to chemically leavened pita chips that were then subjected to accelerated shelf life storage. The chip formula was provided by NCI (Figure A1 and Table A3, Appendix). Chips were made using equipment in the NCI bake lab. Lipids in chips were contributed from White Spray Pastry Flour (ConAgra, enriched), and soy based shortening (Crisco, contains TBHQ and citric acid). All chips were made from the same ingredient batch of ingredients and all repetitions were completed within one week. Each batch of chips constituted an experimental unit. Extracts were added to dough at 1% total lipid weight. All ingredients were added together and dough was mixed for 6 minutes in a Hobart mixer at speed setting 2. Dough was hand rolled into 40 g balls and pressed for 1.2 seconds in a Dough-Pro tortilla maker at 180°C. Tortillas were cut into triangular chips. Chips were baked in a convection oven for approximately 2 minutes at 290°C. Batches of each treatment were made on three separate days for repetition.

3.3.3.3. Accelerated Shelf Life Study

Each batch of chips was placed in an open ziplock bag. The ziplock bag was placed in a brown paper bag to protect from photo-oxidation. The batches were stored in ovens at 40°C for the duration of the accelerated shelf life study. Approximately 60 g of chips were removed periodically and frozen (-13°C) until they could be analyzed.

3.3.3.4. Hexanal Analysis

Fatty acid profile showed linoleic acid (18:2) to be one of the major fatty acids in the chips; therefore, hexanal was assayed as an indicator for secondary oxidation products. Hexanal was measured by headspace using an Agilent 7820A Gas Chromatographer with a ZB-wax (60 m x 0.25 mm x 0.25 μm) column from Phenomenex (Torrance, CA). Samples were introduced by solid phase microextraction (SPME) according to a modified method for potato chips (Azarbad & Jeleń 2014). At least 1 g of chips were ground in a coffee grinder and approximately 0.2 g were added to a 4 mL vial with 1 mL water. The vials were capped with a Supelco PTFE/Silicone Septa, and vortexed for 60 seconds. The vial was partially submerged in 99°C water for 20 minutes. The tip of a Supelco SPME Fiber Assembly (Bellefonte, PA) was inserted through the septa so that the 100 μm polydimethylsiloxane coating was exposed in the headspace of the vial for at least 10 minutes as the vial was partially submerged in a 60°C water bath. The coating was retracted into the fiber assembly, transferred to the injection port of the GC and the filament exposed after inserting through the GC septa. At least 5 minutes were allowed for desorption of volatiles from the injection port (270°C). The helium carrier was 33.7 ml/min and initial oven temperature (40°C) was increased by 10°C/min to 180°C. Detector temperature was 290°C. The run was completed in 25.5 minutes and hexanal was quantified using a hexanal standard up to 500 ppm ($R^2 = 0.96$) in ground crackers. Hexanal eluted at 6.7 minutes (Figure A4).

3.3.3.5. Oil Sample Preparation

All remaining analysis methods were conducted on lipids extracted from chips. Extraction was done at room temperature using hexane. Approximately 50 g of chips were ground by a coffee grinder and evenly added to 50 mL centrifuge tubes. The centrifuge tubes

were filled to 45 mL with hexane and the lid was tightened. The centrifuge tubes were covered with aluminum foil to exclude light and revolved for 1 hour on a VWR platform shaker. The samples were then centrifuged for 20 minutes at 4000 rpm (2510 x g). The hexane was decanted into test tubes and dried under nitrogen.

3.3.3.6. Hydroperoxide Analysis

Peroxide value was measured as the primary oxidation product using titration with sodium thiosulfate AOCS Official Method Cd 8-53 (AOCS 1997) as modified by Crowe and White (2001). Approximately 0.5 g of oil were weighed into a 25 mL Erlenmeyer flask. Three mL of acetic acid:chloroform (3:2) were added to the flask. The reaction was initiated by pipetting 50 μL of saturated potassium iodide solution. After exactly 60 seconds, 3 mL of Millipure water was added; 0.5 mL of a 1% starch solution was added as an indicator followed by titration with 0.001-0.01 N sodium thiosulfate solution depending on expected peroxide value.

3.3.3.7. Tocopherol Analysis

Tocopherol and carotenoid contents were measured by HPLC using a Waters 2795 chromatography separation module with a Waters 2996 Photodiode Array Detector (PDA). The HPLC method for tocopherols was modified from Winkler et al (2007) by normal phase using a Luna-5μ-NH2-100A (250 x 4.6 mm) column from Phenomenex. The mobile phase consists of 98:2 v/v hexane/IPA with a continuous flow rate of 1.5 mL/min. Prior to analysis, oil samples were reconstituted in 100% hexane and vortexed for 60 seconds. Samples were not filtered. Tocopherols were analyzed at 295 nm (Figures A7 and A8). A standard curve was produced for α-, γ-, and δ- tocopherols for concentrations up to 10,000 ppm (all tocols $R^2 = 0.99$).

3.3.3.8. Carotenoid Analysis

The carotenoid separation method was modified from Gupta et al (2015); modifications include reverse phase separation using a YMC (Kyoto, Japan) Carotenoid-S-3 μm, 250 x 4.6 mm, column. The mobile phase consists of (A) methanol/water (98:2, v/v) and (B) MTBE. The gradient elutes 80:20 (% A/B) at 1.4 mL/min for the first 2 min, followed by linear gradient change to 60:40 (A:B) by 12 minutes. Starting at 12 min, the flow rate reduces to 1.0 mL/min and gradient of 0:100 (A:B) and finally returns to 1.4 mL/min and 80:20 (A:B) between 13-20 minutes. Standard curve was produced for lutein and zeaxanthin at concentrations up to 300 ppm (lutein and zeaxanthin $R^2 = 0.99$). Carotenoids absorbance was analyzed at 450 nm (Figures A5 and A6). Prior to analysis, oil samples were reconstituted in 25:75 v/v methanol/MTBE and vortexed for 60 seconds. Samples were not filtered due to preliminary studies showing a reduction of carotenoids from filtering.

3.3.3.9. Phytosterol Analysis

Phytosterol analysis occurred using a modified method described by Winkler et al (2007). Approximately 25 mg of oil were added to a test tube. The internal standard, 5α -cholestane, was added by reconstituting in chloroform and drying under nitrogen. Oil was saponified with 2 N ethanolic KOH for 1 hour at 60°C. Nonsaponifiable material was extracted twice with hexane and dried under nitrogen, then transferred to a 0.25 mL vial insert. Phytosterol derivatization occurred by adding 100 μL of both pyridine and BSTFA + 1% TMCS and heating at 60°C for 45 minutes. Samples were injected by auto sampler (1 μL) using a UHP hydrogen carrier with a 1:50 injector split.

Phytosterols were analyzed using an Agilent 7820A gas chromatograph with a flame ionization detector (FID). An Agilent J&W (Santa Clara, CA) DB-1701 (30 m x 0.32 mm x 1

μm) column was used for phytosterol separation based on methods from Winkler-Moser and Vaughn (2009). The column internal diameter and coating thickness were different from the referenced work due to column availability. The original elution parameters from referenced articles did not work with this column, so pressure was increased by increasing the flow rate to 1.5 mL/min, which resulted in good separation. Column temperature started at 250°C for 30 seconds and increased at 10 mL/min to 270°C, then held for 27 minutes, increased to 280°C at 10 mL/min and held for 3.5 minutes (Figure A9). Standards of campesterol and β-sitosterol were made by creating a serial dilution in chloroform up to 500 ppm (campesterol and β-sitosterol, R^2 = 0.99). Standards were added to 0.25 mL vial inserts and dried under nitrogen and derivitized as described above. Conversion factor from the nearest calculated phytosterol peak was used to quantify other phytosterols.

3.3.3.10. Statistical Analysis

Data was analyzed using Statistical Analysis Software (SAS). Data was arranged in an RCBD split plot in time design. Mean values for variables were compared using the Bonferroni correction. Differences were considered significant at 95% confidence.

3.4. Results

3.4.1. Extract Composition

Hexane was used to extract oil by soxhlet extraction to create an antioxidant-rich extract from corn and DDGS. Phytosterols, tocopherols and carotenoids were comparable to related research (Table 7). Extracts contained lower phytosterols and tocopherols than expected, but the DDGS extract contained more carotenoids than DDGS extracts found literature. High variability is known to be found in both corn and DDGS, so the differences are not unexpected (Kurilich and Juvik 1999).

Table 7. Phytochemical content of extracts from corn and DDGS compared to relevant literature.

Component	Corn				DDGS			
	Literature[a]		Extract		Literature[b]		Extract	
	(μg/g)							
Total Phytosterols	21100	(1200)	18200	(2900)	16200	(700)	14800	(400)
Campesterol	4200	(1900)	5700	(1100)	2000	NR	4500	(100)
β-sitosterol	9300	(400)	6600	(800)	7500	NR	5500	(100)
α-T	186	(53)	60	(11)	194	(5)	84	(1)
□-T	936	(14)	659	(123)	948	(17)	838	(55)
□-T3 (μg/g)	234	(35)	ND		453	(3)	ND	
□-T (μg/g)	119	(37)	ND		51	(2)	7	(12)
Lutein (μg/g)	10	(0)	10	(2)	35	(2)	43	(2)
Zeaxanthin (μg/g)	16	(1)	10	(10)	23	(0)	31	(2)

Results are the average of triplicate with standard deviation in parenthesis.
[a] data adapted from (Moreau et al 2007; 2011)
[b] data adapted from (Winkler-Moser and Vaughn 2009)

The total phytosterol content for corn (18.2 mg/g) and DDGS (14.8 mg/g) extracts were about 90% of comparable literature (Table 7). Six distinct peaks were identified in GC analysis of phytosterols. Campesterol and β-sitosterol peaks were identified by standards. Other peaks were present and identified by comparing to literature using similar methods. Phytosterol content was highest in β-sitosterol and campesterol, which accounted for about 37% and 30%, respectively. Estimates for stigmasterol, campestanol, sitostanol and avenasterol were approximately 10%, 9%, 9% and 4%, respectively, for both corn and DDGS extracts. Winkler-Moser and Vaughn (2009) suggest that campestanol and sitostanols are mainly bound as steryl ferulates, which would contribute 1.4 mg/g and 1.3 mg/g steryl ferulates, respectively; DDGS oil contains 4.0 mg/g steryl ferulates (Winkler-Moser and Vaughn 2009).

Total tocol content for corn (720 μg/g) and DDGS (930 μg/g) extracts were approximately half the levels found in literature. Tocotrienols were not identified, and account for much of the discrepancy compared to literature. However, tocopherols also were lower than

anticipated. The composition of tocopherols for both extracts was approximately 90% □-tocopherols, 9% α-tocopherols and less than 1% □-tocopherols. While ratios were similar, DDGS extract contained about 30% more tocopherols than corn extract and had far less variability between samples. Winkler-Moser and Vaughn (2009) utilized a dual UV and fluorescence detector, which would likely be more sensitive to smaller peaks than the UV detector alone, used in this study. The lack of fluorescence detections may explain why tocotrienols were not observed in most samples.

Xanthophyll content in the DDGS extract was more than 350% above that in the corn extract. Xanthophylls were higher in DDGS extract reported in literature levels reported in Winkler-Moser and Vaughn (2009), with 43 μg/g lutein and 31 μg/g zeaxanthin, while corn extract was lower reported in literature values, with 10 μg/g lutein and 10 μg/g zeaxanthin. There was extremely high variability in carotenoid content in corn extracts, which has been reported previously (Kurilich and Juvik 1999). Neither β-cryptoxanthin or β-carotene were identified in either extract.

3.4.2. Shelf-Life Study

3.4.2.1. Oxidation

Peroxide value and hexanal content are indicators of oxidation. Neither alone adequately represents oxidation because hydroperoxides decompose into secondary oxidation products, hexanal being one of many. The peroxide value was significantly lower in chips containing DDGS extract (15.9 meq/kg) than the corn extract (18.6 meq/kg) and control (19.3 meq/kg) treatments during the 55 days of storage (LSD = 2.3 meq) (Figure 14). Hexanal content also was significantly lower in chips containing DDGS extract (4.8 ppm) than corn extract (6.0 ppm) and control (6.0 ppm) treatments (LSD = 1.0 ppm) (Figure 15).

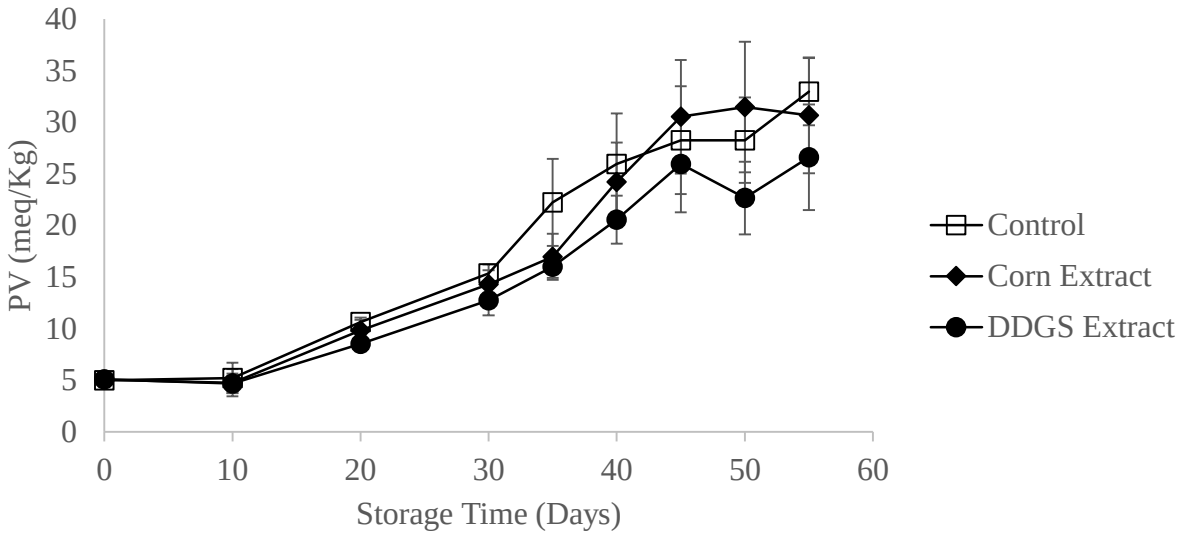


Figure 14. Peroxide value during accelerated storage of chips.
*Note: bars denote standard error.

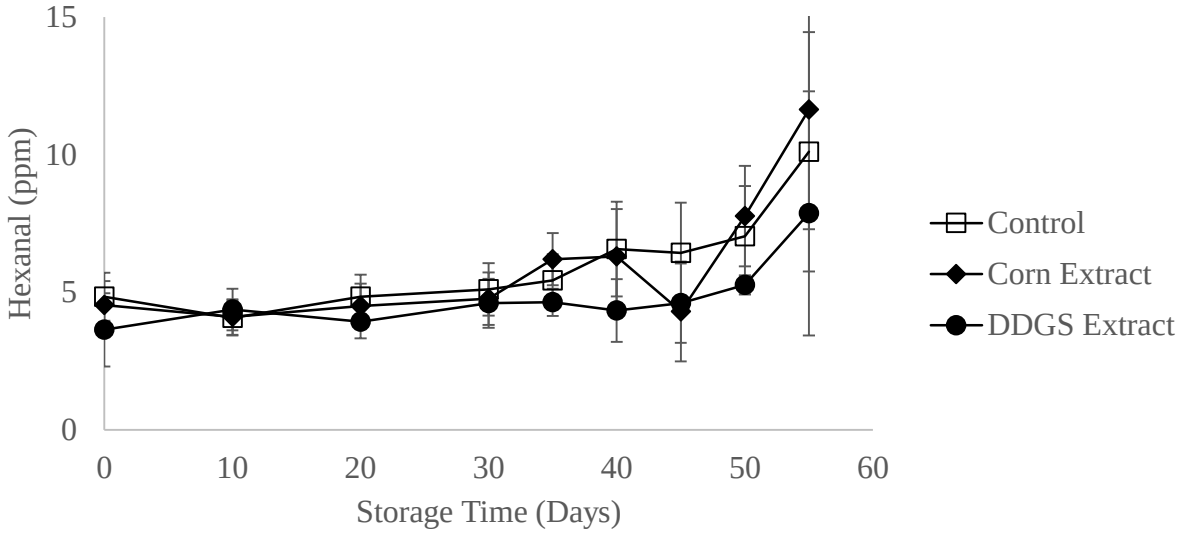


Figure 15. Hexanal content during accelerated storage of chips.
*Note: bars denote standard error.

Refined oil is considered rancid when peroxide value reaches 10 meq/kg (Gunstone 1996). The control group exceeded 10 meq/kg at 20 days of storage (10.6 meq/kg) while DDGS and corn extract stayed below this threshold at 20 days of storage (8.5 meq/kg and 9.8 meq/kg, respectively). However, the difference is not statistically significant. At no storage time was the mean statistically significant between treatments for peroxide value or hexanal content. An

effective antioxidant for low-moisture food should extend the oxidation induction period. It appears the induction period ends sometime between 10 and 20 days. The DDGS extract may in fact prolong the induction period by a few days. The exact number of days each treatment prolonged rancidity to the 10 meq/kg threshold can only be estimated because the 10 meq/kg level was not reached on days that the PV was measured. Instead, through extrapolating, the corn and DDGS extracts may prolong freshness by 1 and 5 days, respectively, at 40°C storage compared to the control (Figure 16). Additional shelf life studies can be conducted to analyze the benefits under non-accelerated conditions. A Q_{10} value is used for shelf-life at various temperatures. The Q_{10} value shows the relationship between storage when the temperature is raised by ten degrees. However, Q_{10} may be flawed as oxidation products are not produced linearly (Barden and Decker 2016). The complexity of food systems makes the translation from accelerated storage to natural conditions difficult, but methods are available to adequately analyze antioxidant activity (Frankel and Meyer 2000).

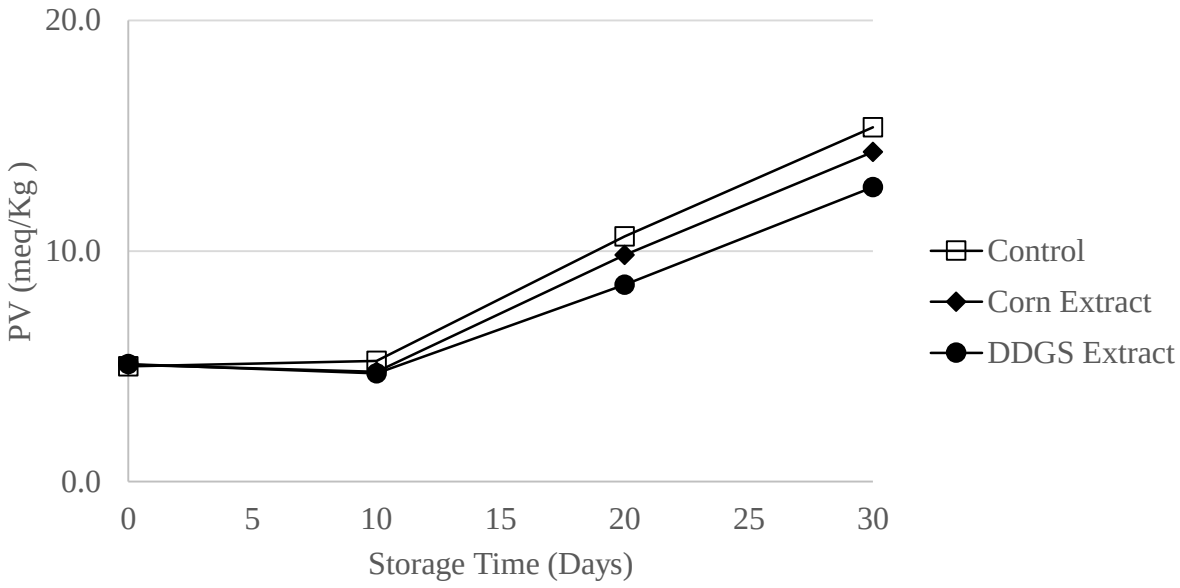


Figure 16. Accelerated storage of chips passing induction period.

3.4.2.2. Antioxidants

Tocopherols, phytosterols and lutein are found naturally in wheat flour and were detected in control and treatment groups. Conversely, zeaxanthin is not native to wheat flour; zeaxanthin content in 1% DDGS and corn extracts (0.3 μg/g and 0.1 μg/g, respectively) did not increase concentrations above the detection limit in chip oil. Treatments did not significantly increase tocopherol and lutein concentrations in chips. Tocopherol concentration in chips was highly variable compared to concentrations added from extracts. The mean tocopherol content in control chips at time zero was about 600 μg/g ± 57 μg/g. The tocol contribution from 1% corn and DDGS extracts were approximately 6 μg/g and 9 μg/g, respectively, well below the standard deviation. Likewise, lutein content from 1% corn extract (0.1 μg/g) and DDGS extract (0.4 μg/g) was similar to the 0.26 μg/g standard deviation for the control at time zero (0.127 μg/g). DDGS extract had a noticeable increase in lutein concentration, but it was not statistically significant. Phytosterols in chips were about 10 times more concentrated than tocopherols and 1,000 times more concentrated than lutein. Total phytosterol content was greater in corn (5.3 mg/g ± 0.7 mg) and DDGS (5.2 mg/g ± 0.5 mg) treatments than the control (4.9 mg/g ± 0.4 mg), but the increase was not statistically significant. The composition of phytosterols in chips is very similar to extracts, ordering from β-sitosterol > campesterol > stigmasterol ≈ campestanol ≈ sitostanol > avenasterol. Neither extract significantly increased phytosterol content of crackers.

Tocopherols and lutein likely contributed antioxidant activity in chips (Figure 17). Through the entire study, tocopherol content in chips ranged from 60-700 μg/g, which is within the range suggested to have antioxidant properties in literature (50-1,000 μg/g) (Deiana et al 2002; Winkler-Moser and Vaughn 2009). Autoxidation generally results in chemical alteration of an antioxidant, so depletion of a phytochemical may correlate to its antioxidant activity.

Tocopherols and carotenoids loss appear to have an induction period of about 20 days, where concentrations remain above 80%, followed by hastening decline. Both α-tocopherols and γ-tocopherols significantly decreased by 30 days of storage while γ-tocotrienols and δ-tocopherols did not significantly decrease until day 40 and 45, respectively. γ-Tocopherol had the highest rate of degradation in frying oils (Winkler et al 2012). γ-Tocopherol started at the highest concentration and declined to the lowest residuals by the end of the study; this may suggest that γ-tocopherol was the most active antioxidant, but may also indicate a lack of stability. Literature suggests antioxidant activity of tocopherols at 37°C proceeds from $\alpha > \beta > \gamma > \delta$, which follows closely to the degradation seen in chips (Nanditha and Prabhasankar 2009).

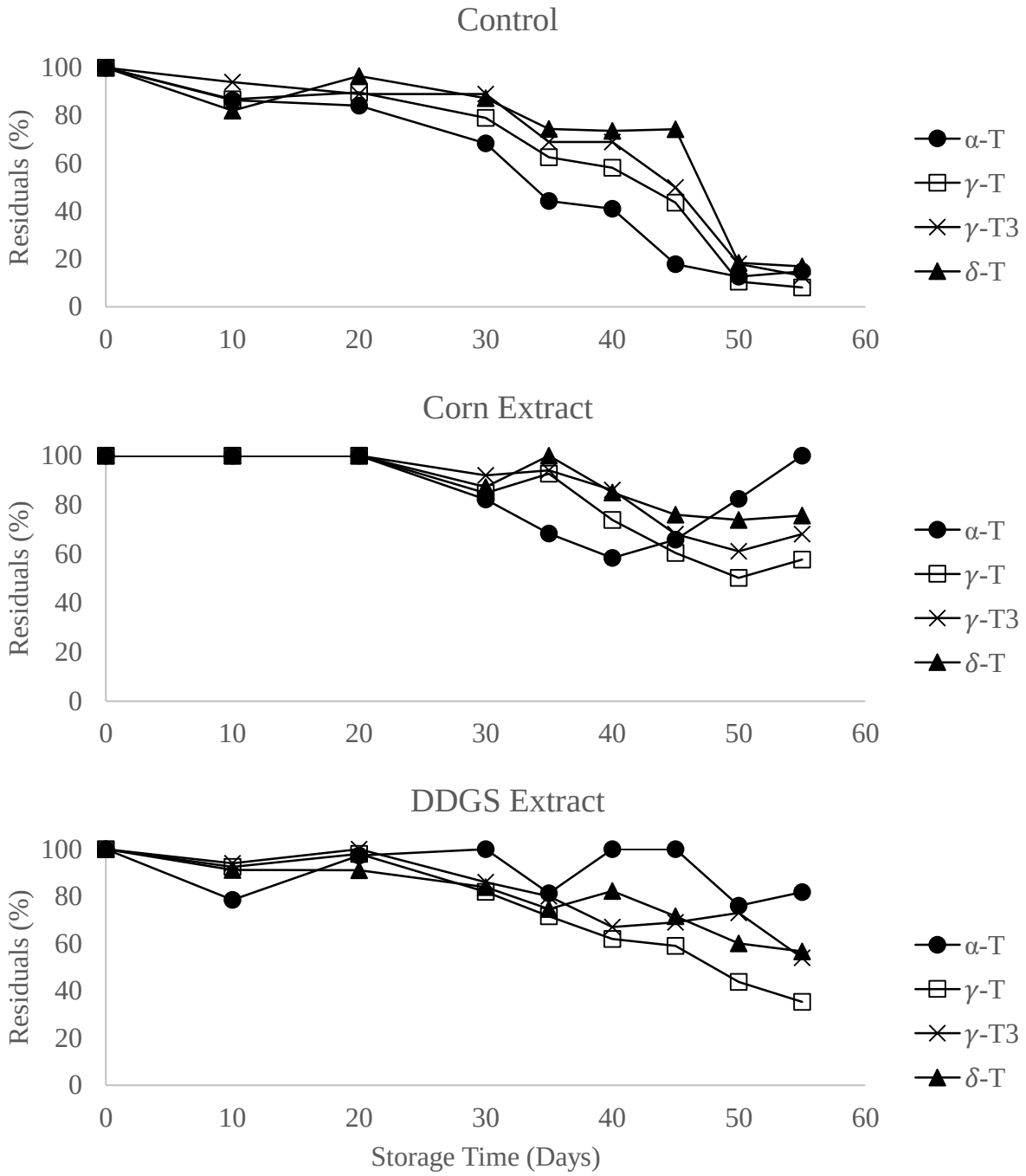


Figure 17. Tocopherol (T) and tocotrienol (T3) residuals in chips without (control) and with corn and DDGS extracts.

Lutein acted similar to tocopherols and likely produced some antioxidant benefits (Figure 18). Carotenoid content in chips was less than 1% the concentration of tocopherols. Low concentrations of carotenoids are very effective singlet oxygen quenchers, but have less impact

on autoxidation. Carotenoids and tocopherols have been shown to have synergetic properties, but synergistic effects were found at higher carotenoid concentrations than used in this study (Schroeder et al 2006). Similar to tocopherols, lutein significantly decreased by 30 days of storage and continued to decrease through the end of the study. The reduction in lutein appeared to coincide with the increase in peroxide value.

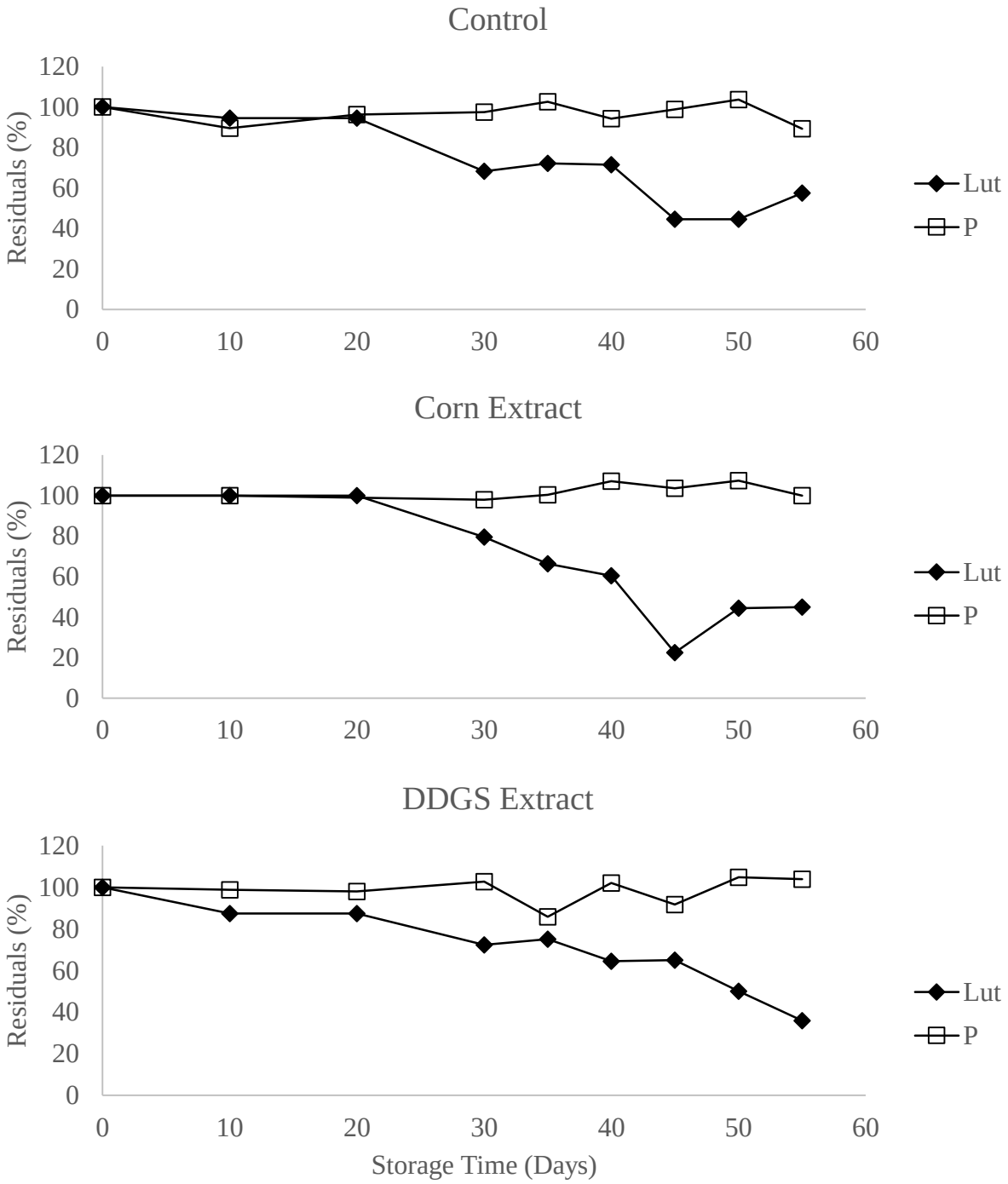


Figure 18. Lutein (Lut) and phytosterol (P) residuals in chips without (control) and with corn and DDGS extracts.

Phytosterols did not decrease throughout the study (Figure 18). Previous studies have shown phytosterols to be effective antioxidants at higher temperatures by preventing

polymerization in frying oil (Gordon and Magos 1983). Temperature may be a factor in phytosterol antioxidant activity. Furthermore, studies often focus on the activity of steryl ferulates rather than phytosterols alone (Winkler-Moser and Vaughn 2009; Wang et al 2002). Phytosterols required saponification before analysis, which cleaves the ester bond between the phytosterol and ferulate components; therefore, these methods would not measure ferulate content. Regardless, this study does not provide evidence for antioxidant activity from phytosterols in the chips.

3.5. Conclusion

Phytochemical-rich extracts can be made from corn and DDGS. The DDGS extract contained higher concentrations of tocopherols and carotenoids. Adding 1% DDGS extract significantly reduced oxidation products in chips while corn extract did not. Tocopherols and lutein appear to contribute antioxidant activity. Results suggest DDGS is a potential source of natural antioxidants in low-moisture food.

The actual contribution to antioxidant activity of tocopherols, carotenoids and phytosterols cannot be determined given that the compounds were not tested in isolation. Similar to the research by Winkler and Vaughn (2009), “the exact contribution of each component and possible synergisms would be impossible to elucidate under these conditions”. Tocols and lutein appear to be providing antioxidant activity, while phytosterols were unchanged. A better understanding of the antioxidant contributions may be possible if each treatment had a significantly different concentration of phytochemicals; future research could test increasing amounts of extract and increase sampling during important oxidation events.

4. PAPER 2: ANTIOXIDANT ACTIVITY OF CORN AND DRY DISTILLERS' GRAINS IN CRACKERS

4.1. Abstract

Antioxidant-rich extracts were produced by SC-CO_2 extraction from corn and DDGS. Corn and DDGS extract contained 16.9 and 14.6 mg/g phytosterols, 682 and 1127 μg/g tocopherols, and 71 and 75 μg/g xanthophylls, respectively. The phytochemical-rich extracts were incorporated in cracker formulas at 0.05% and evaluated for shelf stability. Extracts did not significantly reduce oxidation products or increase phytochemical concentrations in crackers. Tocopherols and lutein decreased significantly during oxidation, thus likely acting as antioxidant activity while phytosterols did not change during oxidation.

4.2. Introduction

Supercritical carbon dioxide (SC-CO_2) extraction is a "green" alternative to lipid extraction using organic solvents. Wang et al (2007) found superior extraction capability from sorghum DDGS using SC-CO2 extraction compared to hexane extraction. SC-CO_2 has been explored for corn and DDGS (Ciftci and Temelli 2011; 2013). Ciftci et al (2012) optimized SC-CO_2 extraction conditions for DDGS. The optimal extraction parameters for total lipids, carotenoids, tocols and phytosterols were produced at 49.5 MPa and 70°C; the concentration of each phytochemical was greater using SC-CO2 than soxhlet extraction with petroleum ether (Table 8).

Antioxidant activity of tocopherols and carotenoids has been established. Studies have shown tocopherol antioxidant activity to be best at 500 μg/g (ppm). Addition of 500 ppm for natural antioxidants is common in many lipid studies (Seppanen et al 2010).

Table 8. Comparison of soxhlet extraction (petroleum ether) and SC-CO_2 extraction reported by Ciftci et al (2012).

	Lipids	Carotenoids	Tocols	Phytosterols
	%	---------- mg/g ----------		
Soxhlet	9.2	0.09	1.3	12.4
SC-CO_2	11.2	0.11	1.5	15.9

The primary concern of lipid oxidation is the deleterious effect on sensory perceptions. The extent of lipid oxidation can be measured through laboratory assays; however, consumers choose food based on taste, which may not correlate perfectly with laboratory testing (Tepper and Trail 1998). The complexity of food systems makes it nearly impossible to test all the variables associated with oxidation. However, sensory analysis is very important in evaluating the oxidative stability of food. In addition, the antioxidants should not themselves alter the sensory perceptions of food. Phytosterols, tocopherols and carotenoids are well suited for bakery products because they do not alter sensory attributes (Quilez et al 2003; 2006).

4.3. Materials and Methods

4.3.1. Chemicals and Materials

4.3.1.1. Samples

Distillers' dried grains were obtained from three local ethanol facilities: Hankinson Renewable Energy (Hankinson, ND), Bushmills Ethanol (Atwater, MN), and Tharaldson Energy (Casselton, ND). Corn was obtained from 3 sources: O'Brien Seed Inc. (Mayville, ND), Greg LePlant (Fargo, ND) and Specialty Commodities (Fargo, ND). Corn and DDGS were stored at 40°C in closed 5 gallon buckets to prevent light exposure.

4.3.1.2. Chemicals

HPLC-grade liquid CO_2 was purchased from Praxair Distribution, Inc. (Fargo, ND) with a dip tube. Hexane for oil extraction was purchased from Avantor Performance Materials

(Central Valley, PA). Ethanol (95%) was purchased from NDSU chemical stockroom (Fargo, ND). HPLC-grade organic solvents, including hexane, isopropanol (IPA), methanol, methyl-tert-butyl-ether (MTBE), dichloromethane, and chloroform, were purchased from VWR (Randor, PA). Laboratory grade granular potassium iodide and American Chemical Society (ACS) grade glacial acetic acid were also obtained from VWR. Sodium thiosulfate and potassium hydroxide (85%) were obtained from Thermo Fisher Scientific (formerly Alfa Aesar, Ward Hill, MA). Ultra-high purity (UHP) hydrogen (99%) was obtained from PraxAir Distribution, Inc. (Fargo, ND). Pyridine and N,O-bis(trimethylsilyl) trifluoroacetamide with trimethylchlorosilane (BSTFA + 1% TMCS) were purchased from Sigma-Aldrich (St. Louis, MO) in addition to standards for hexanal (98%), campesterol (65%), β-sitosterol (95%), 5α-cholestane (97%), lutein (95%) and zeaxanthin (95%). Tocopherol standards for α-, γ-, and δ-isomers (99% purity) were purchased from Supelco (Bellefont, PA).

4.3.2. Experimental Design

The shelf stability of crackers over time was evaluated (Chapter 4.3.3.1). An extract was created by SC-CO_2 extraction from corn and DDGS. Extracts were added to crackers and then subjected to accelerated storage conditions and periodically sampled throughout the shelf life study (90 days). The experiment was organized as a Random Complete Block Design (RCBD) with 3 repetitions and 3 treatments. Treatments consisted of crackers with DDGS extract, corn extract and a control group made without extract.

4.3.3. Procedure

4.3.3.1. Supercritical Carbon Dioxide extraction of corn and DDGS

Unlike experiment 1, milled corn and DDGS were mixed prior to extraction from the three corn and ethanol DDGS sources, respectively. Two extraction cartridges were packed with

5-6 g of corn or DDGS. SC-CO_2 extraction was conducted using an ISCO SFX-210 supercritical extraction unit (Lincoln, NE) with a 260D syringe pump. Extraction pressure and temperature were set at 49.5 MPa and 70°C according to optimal parameters specified by Ciftci et al (2012). The 260D syringe pump fit 260 mL liquid CO_2. Flow rate was manually maintained near 10 mL/min, which generally allowed 20-minute dynamic extractions; however, extraction time was not always consistent. Extraction time slowly shortened (from a peak of 30 minutes to as low as 10 minutes at a flow rate of 10 ml/min), likely due to reduced CO_2 density as the volume of liquid CO_2 in the source tank dropped. Extract was collected in test tubes. Multiple extractions were conducted to collect a sufficient quantity of extract. Extract was not further processed because no wax precipitate formed after cooling and centrifugation, which has been reported in literature (Rebolleda et al 2012).

4.3.3.2. Cracker baking

To determine antioxidant potential, extracts were added to crackers and then subjected to accelerated shelf life storage. The cracker formula was adapted from the U.S. Wheat Association using equipment in the NCI bake lab (Figures A2 and A3). White Spray Pastry Flour (ConAgra, enriched), soy based shortening (Crisco, contains TBHQ and citric acid) and soy lecithin contributed to cracker lipid content. Extracts were added to dough at 0.05% of lipid weight. Ingredients were mixed at 35°C by mixing for 2 minute on the 1st speed and 9 minutes on the 2nd speed using a Hobart mixer with a double spiral attachment. Dough was rested for 90 minutes prior to sheeting with a Rondo dough sheeter. Dough was sheeted to about 2 mm, cut into squares and stacked into 6 layers. Laminated dough was slowly sheeted to 1mm and cut into approximately 3/4” squares. Crackers were baked on a mesh band in a deck oven at 225°C for approximately 6 minutes.

4.3.3.3. Accelerated Shelf Life Study

Each batch of crackers was placed in an open ziplock bag. The ziplock bag was placed in a brown paper bag to protect from photo-oxidation. Crackers were held at 40°C protected from photo-oxidation. Samples were removed periodically and frozen until analysis. Sampling frequency was dependent on degree of oxidation based on lab results.

4.3.3.4. Hexanal Analysis

Fatty acid profile showed linoleic acid (18:2) to be one of the major fatty acids in the chips; therefore, hexanal was assayed as an indicator for secondary oxidation products. Hexanal was measured by headspace using an Agilent 7820A Gas Chromatographer with a ZB-wax (60 m x 0.25 mm x 0.25 μm) column from Phenomenex (Torrance, CA). Samples were introduced by solid phase microextraction (SPME) according to a modified method for potato chips (Azarbad & Jeleń 2014). At least 1 g of chips were ground in a coffee grinder and approximately 0.2 g were added to a 4 mL vial with 1 mL water. The vials were capped with a Supelco PTFE/Silicone Septa, and vortexed for 60 seconds. The vial was partially submerged in 99°C water for 20 minutes. The tip of a Supelco SPME Fiber Assembly (Bellefonte, PA) was inserted through the septa so that the 100 μm polydimethylsiloxane coating was exposed in the headspace of the vial for at least 10 minutes as the vial was partially submerged in a 60°C water bath. The coating was retracted into the fiber assembly, transferred to the injection port of the GC and the filament exposed after inserting through the GC septa. At least 5 minutes were allowed for desorption of volatiles from the injection port (270°C). The helium carrier was 33.7 ml/min and initial oven temperature (40°C) was increased by 10°C/min to 180°C. Detector temperature was 290°C. The run was completed in 25.5 minutes and hexanal was quantified using a hexanal

standard up to 500 ppm (R^2 = 0.96) in ground crackers. Hexanal eluted at 6.7 minutes (Figure A4).

4.3.3.5. Oil Sample Preparation

Oil extraction for laboratory analysis was altered from the previous experiment to improve efficiency. Approximately 50 g of crackers were ground by mortar and pestle. Ground crackers were added to a 250 mL Erlenmeyer flask; hexane was added to approximately 150 mL, covered by tin foil and stirred for 1 hour. The mixture was centrifuged for 20 minutes at 4000 rpm (2510 g), then poured through Whatman number 1 filter paper. Hexane was removed by rotary evaporator at 40°C. Oil samples were frozen until they could be analyzed.

4.3.3.6. Hydroperoxide Analysis

Peroxide value was measured as the primary oxidation product using titration with sodium thiosulfate AOCS Official Method Cd 8-53 (AOCS 1997) as modified by Crowe and White (2001). Approximately 0.5 g of oil were weighed into a 25 mL Erlenmeyer flask. Three mL of acetic acid:chloroform (3:2) were added to the flask. The reaction was initiated by pipetting 50 μL of saturated potassium iodide solution. After exactly 60 seconds, 3 mL of Millipure water was added; 0.5 mL of a 1% starch solution was added as an indicator followed by titration with 0.001-0.01 N sodium thiosulfate solution depending on expected peroxide value.

4.3.3.7. Tocopherol Analysis

Tocopherol and carotenoid contents were measured by HPLC using a Waters 2795 chromatography separation module with a Waters 2996 Photodiode Array Detector (PDA). The HPLC method for tocopherols was modified from Winkler et al (2007) by normal phase using a Luna-5μ-NH2-100A (250 x 4.6 mm) column from Phenomenex. The mobile phase consists of

98:2 v/v hexane/IPA with a continuous flow rate of 1.5 mL/min. Prior to analysis, oil samples were reconstituted in 100% hexane and vortexed for 60 seconds. Samples were not filtered. Tocopherols were analyzed at 295 nm (Figures A7 and A8). A standard curve was produced for α-, γ-, and δ- tocopherols for concentrations up to 10,000 ppm (all tocols $R^2 = 0.99$).

4.3.3.8. Carotenoid Analysis

The carotenoid separation method was modified from Gupta et al (2015); modifications include reverse phase separation using a YMC (Kyoto, Japan) Carotenoid-S-3 μm, 250 x 4.6 mm, column. The mobile phase consists of (A) methanol/water (98:2, v/v) and (B) MTBE. The gradient elutes 80:20 (% A/B) at 1.4 mL/min for the first 2 min, followed by linear gradient change to 60:40 (A:B) by 12 minutes. Starting at 12 min, the flow rate reduces to 1.0 mL/min and gradient of 0:100 (A:B) and finally returns to 1.4 mL/min and 80:20 (A:B) between 13-20 minutes. Standard curve was produced for lutein and zeaxanthin at concentrations up to 300 ppm (lutein and zeaxanthin $R^2 = 0.99$). Carotenoids absorbance was analyzed at 450 nm (Figures A5 and A6). Prior to analysis, oil samples were reconstituted in 25:75 v/v methanol/MTBE and vortexed for 60 seconds. Samples were not filtered due to preliminary studies showing a reduction of carotenoids from filtering.

4.3.3.9. Phytosterol Analysis

Phytosterol analysis occurred using a modified method described by Winkler et al (2007). Approximately 25 mg of oil were added to a test tube. The internal standard, 5α -cholestane, was added by reconstituting in chloroform and drying under nitrogen. Oil was saponified with 2 N ethanolic KOH for 1 hour at 60°C. Nonsaponifiable material was extracted twice with hexane and dried under nitrogen, then transferred to a 0.25 mL vial insert. Phytosterol derivatization occurred by adding 100 μL of both pyridine and BSTFA + 1% TMCS and heating at 60°C for 45

minutes. Samples were injected by auto sampler (1 μL) using a UHP hydrogen carrier with a 1:50 injector split.

Phytosterols were analyzed using an Agilent 7820A gas chromatograph with a flame ionization detector (FID). An Agilent J&W (Santa Clara, CA) DB-1701 (30 m x 0.32 mm x 1 μm) column was used for phytosterol separation based on methods from Winkler-Moser and Vaughn (2009). The column internal diameter and coating thickness were different from the referenced work due to column availability. The original elution parameters from referenced articles did not work with this column, so pressure was increased by increasing the flow rate to 1.5 mL/min, which resulted in good separation. Column temperature started at 250°C for 30 seconds and increased at 10 mL/min to 270°C, then held for 27 minutes, increased to 280°C at 10 mL/min and held for 3.5 minutes (Figure A9). Standards of campesterol and β-sitosterol were made by creating a serial dilution in chloroform up to 500 ppm (campesterol and β-sitosterol, R^2 = 0.99). Standards were added to 0.25 mL vial inserts and dried under nitrogen and derivitized as described above. Conversion factor from the nearest calculated phytosterol peak was used to quantify other phytosterols.

4.4. Results

4.4.1. Extract Composition

Supercritical carbon dioxide extraction was used to create an antioxidant-rich extract from corn and DDGS. Compared to hexane extracts in the previous study, SC-CO_2 extracts contained more tocols and carotenoid, but less phytosterols for both corn and DDGS extracts. Phytosterols, tocopherols and carotenoids in DDGS were comparable to related research on SC-CO_2 extraction using the same parameters (Table 9). Extracts contained lower phytosterols and tocopherols than expected, but the DDGS extract contained more carotenoids than DDGS

extracts reported in literature. The standard deviation was far smaller in supercritical extraction compared to the previous study (chapter 3.4.1) using hexane extraction. Extracts were mixed and homogenized after extraction, whereas ground corn and DDGS were mixed together before SC-CO_2 extraction in this experiment. This suggests extraction method and sample handling may impact results; however, comparing the two extraction methods does highlight the large variation in phytochemical content in corn sources and, to a lesser extent, DDGS.

Table 9. Phytochemical content of SC-CO_2 extracts from corn and DDGS compared to DDGS extract in literature using optimal extraction parameters.

Component	Corn		DDGS		DDGS Literature[a]	
	---------- (μg/g) ----------					
Total Phytosterols	16900	(600)	14600	(1000)	15900	(900)
Campesterol	5200	(200)	4400	(200)	3600	(700)
B-sitosterol	6200	(300)	5500	(500)	10100	(2200)
α-T	61	(65)	254	(24)	144	(20)
□-T	565	(3)	609	(47)	889	(82)
□-T3	55	(17)	136	(0)	112	(12)
□-T	0	(0)	128	(29)	374	(36)
Lutein	41	(2)	41	(6)	57	(8)
Zeaxanthin	30	(23)	33	(5)	42	(6)

Results are the average of triplicate with standard deviation in parenthesis.
[a] adapted from (Ciftci et al 2012)

Extracts contained an estimated 16.9 and 14.6 mg/g phytosterols for corn and DDGs extracts, respectively. The total content for corn and DDGS extracts were lower than previous hexane extracts, but the composition of phytosterols was nearly identical.

Total tocol content for corn extract (682 μg/g) was slightly less compared to hexane extracts, but higher in the DDGS extract (1127 μg/g) by about 200 μg/g. Tocotrienols were identified, which accounts for a large portion of the greater tocol content in SC-CO_2 extracts, but α-tocopherols and □-tocopherols also were higher while □-T decreased. The □-tocopherols and □-tocopherols were only 68 and 34%, respectively, of the SC-CO_2 extract that was reported

previously (Ciftci et al 2012). The composition of tocols in DDGS extract consisted of 54, 23, 12, and 11% while corn extract contained 83, 9, 8, and 0% of □-tocopherols, α-tocopherols, □-tocotrienols and □-tocopherols, respectively.

Both extracts had similar carotenoid concentration, which also is comparable to hexane extracts. Lutein concentrations were highest among carotenoids, accounting for 41 and 43 μg/g in corn and DDGS extracts, respectively. Xanthophylls made up 33 and 30 μg/g of the corn and DDGS extracts, respectively.

4.4.2. Shelf-Life Study

4.4.2.1. Oxidation

Peroxide value and hexanal content were not significantly affected by either treatment. Compared to chip oxidation in paper 1, crackers oxidized more slowly; sampling intervals were lengthened to ensure adequate supply throughout the accelerated study. Peroxide value significantly increased by 70 days of storage at 40°C while hexanal content did not change significantly. Hexanal has been established as a good indicator of oxidation. However, peroxide values exceeded the rancidity threshold of 10 μg/g by 40 days of accelerated storage. The DDGS extract treatment contained lower peroxide values at days 20 and 40 (7.0 and 14.5 meq/kg, respectively) compared to the control (8.3 and 16.2 meq/kg, respectively) and corn extract treatments (8.4 and 19.4 meq/kg, respectively). Based on extrapolating between samples, peroxide values cross the 10 meq/kg threshold at 23, 24 and 28 days for corn extract, control and DDGS extract, respectively (Figure 19). DDGS extract provided approximately 4 days of extended freshness at 40°C.

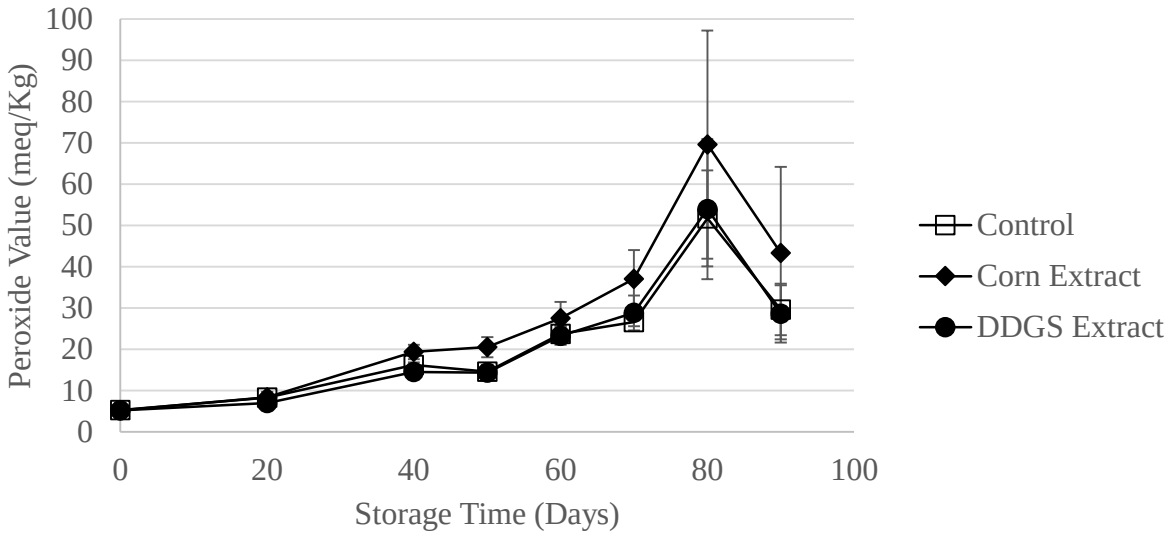


Figure 19. Peroxide value during accelerated storage of crackers
*Note: bars denote standard error.

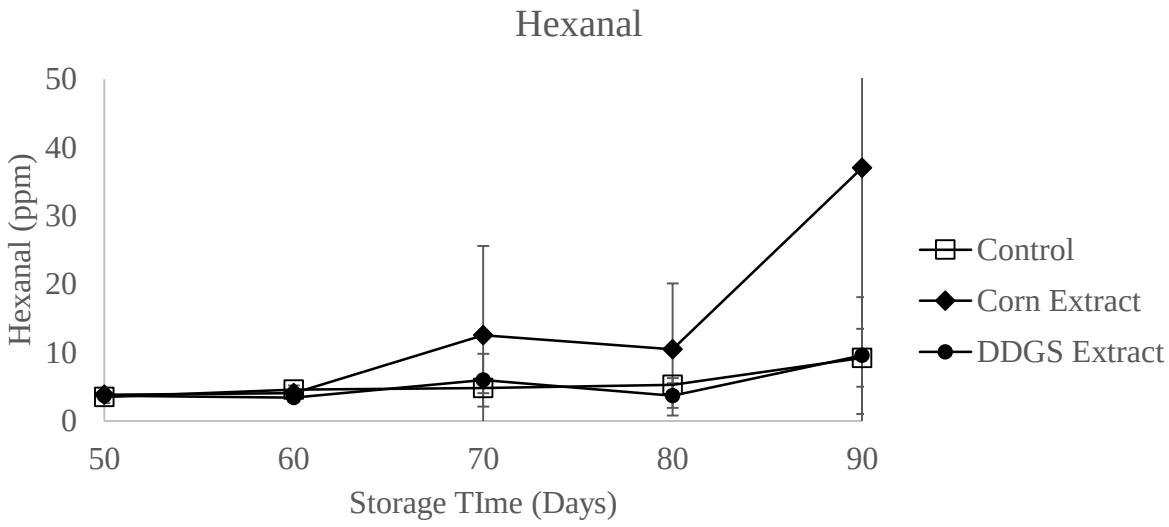


Figure 20. Hexanal during accelerated storage of crackers.
*Note: bars denote standard error.

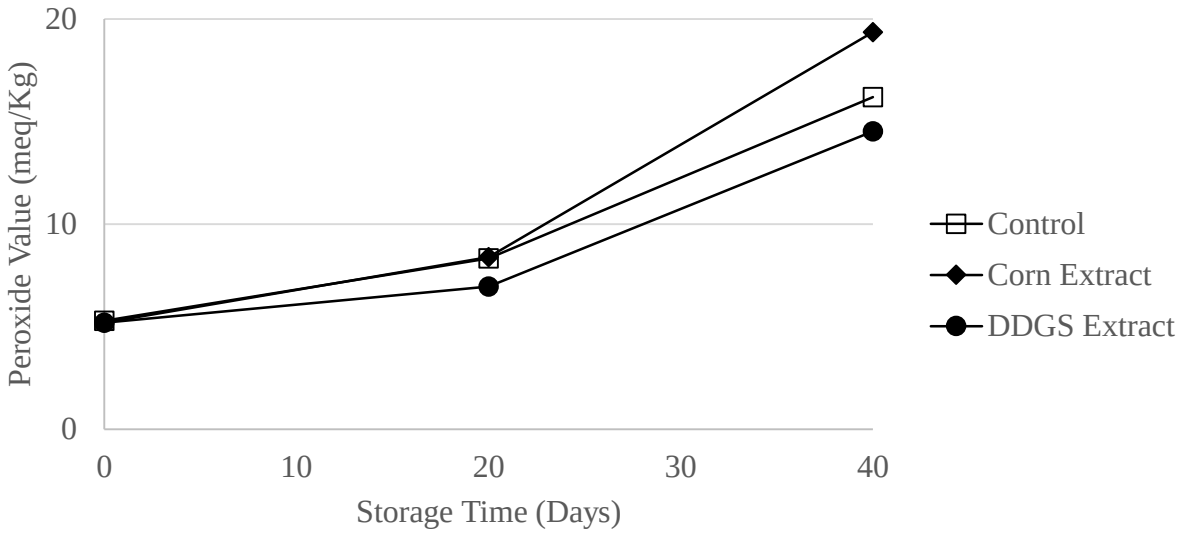


Figure 21. Accelerated storage of crackers passing induction phase (10 meq/kg).
*Note: bars denote standard error.

Oxidation was affected by the repetition (i.e. batches of crackers); crackers from the third repetition contained significantly higher oxidation products. The variation between repetitions becomes apparent only after 70 days of storage. The variability between repetitions may be responsible for the lack of significant results. Even the control was different in the third repetition, so the extracts are not likely responsible. A few hypotheses can be proposed for the significant difference between repetitions. Phytochemicals concentration was higher in third repetition samples. Some antioxidants have been shown to have prooxidant activity at high concentrations. Carotenoids can be unstable at high concentrations or oxygen pressure (i.e. higher partial pressure of oxygen in the surrounding atmosphere); lutein has prooxidant activity at 5 μg/g in purified corn oil (Subagio and Morita 2001). Tocopherols, especially α-tocopherols, can promote oxidation by participating in side reactions (Kamal-Eldin 2006). The reason for higher phytochemical content in the third repetition is unknown. Other prooxidants may also be present. Transition metal ions can induce prooxidative effect in phenolic antioxidants (Hall III and Zhao 2011). Tap water is a potential source of metal ions in the cracker recipe. Lastly, since

variability in the third repetition was only high later in storage, accelerated storage conditions may have altered the oxidation rate. Temperature or oxygen concentration could have fluctuated within the storage ovens. The third repetition may be a poor indicator of the treatment effects; however, removing the third treatment greatly decreases the power of the statistical model, making it much more difficult to find statistical significance. When removing repetition three, DDGS extract contains significantly less hexanal, but peroxide value was not significantly different between treatments.

4.4.2.2. Antioxidants

DDGS extract had significantly higher γ-tocotrienol and δ-tocopherol concentrations but other phytochemicals were not significantly different. Phytochemical content of corn extract was not significantly higher compared to other extracts

The mean tocopherol content in control crackers at time zero was 588 μg/g ± 102 μg. Extracts were added at 500 μg/g (0.05%) to convey commercial usage of natural extracts. Corn and DDGS extracts contributed 0.3 μg and 0.6 μg tocols per gram lipid, respectively, orders of magnitude below the standard deviation. The significantly higher γ-tocotrienol and δ-tocopherol contents in the DDGS treatment compared to the control were unexpected. Concentration of γ-tocotrienol in crackers at time zero was higher by 15 μg/g (control = 68 μg/g ± 11 μg/; DDGS treatment = 83 μg/g ± 6 μg/g) in crackers with DDGS extract that was expected to increase γ-tocotrienol concentration by only 0.07 μg/g. Similarly, concentration of δ-tocopherol in crackers at time zero was also higher by 15 μg/g (control = 128 μg/g ± 18 μg/g; DDGS treatment = 136 μg/g ± 15 μg/g) by adding the DDGS extract, which was expected to increase δ-tocopherol concentration by 0.06 μg/g. Considering none of the other phytochemicals were increased by the added extracts, this increase may be from analytical or sampling error, rather than an actual

benefit of adding 0.05% DDGS extract. However, interactions between tocopherols may be responsible for these results. α-Tocopherols could play a protective role for γ- and δ-tocols, similar to the interaction seen between ascorbic acid and tocols. Ascorbic acid has a well-established ability to protect α-tocopherol from degradation by regenerating tocopherol radicals back to the parent tocopherol (Frankel 1998; Pokorny et al 2001). Slow disappearance of γ-tocotrienols and δ-tocopherols may be explained by regeneration and recycling by α-tocopherols.

Corn and DDGS extracts at 500 μg/g were expected to contribute about 0.02 μg/g of both lutein and zeaxanthin to crackers. Zeaxanthin was not detected in crackers. Lutein content was not significantly increased in crackers with corn or DDGS extract (1.5 μg/g ± 0.1 μg/g and 1.6μg/g ± 0.2μg/g, respectively) compared to the control crackers (1.5 μg/g ± 0.1 μg/g) at time zero.

Phytosterols are prevalent in wheat flour and concentration in crackers was much greater than tocopherols and carotenoids. The composition of phytosterols was almost identical, ordering from β-sitosterol > campesterol > stigmasterol ≈ campestanol ≈ sitostanol > avenasterol. The extract addition did not have a significant effect on phytosterol concentration in crackers.

Tocopherols and lutein appear to provide antioxidant activity. Residual tocols had a lag period followed by a linear decline, which was similar to Winkler and Moreau (2009). All tocols significantly decreased within the first 20 days of storage while lutein took slightly longer, significantly decreasing by 40 days of storage. Lutein, α-tocopherol and γ-tocopherol decreased at a fairly consistent rate throughout storage, i.e. significant changes occurred every 20-40 days. In contrast, γ-tocotrienol and δ-tocopherol showed little change after the initial drop in concentration. Similar to the conclusion from paper 1, γ-tocotrienol and δ-tocopherol are likely

not as active in antioxidant reactions compared to lutein, α-tocopherol and γ-tocopherol. Phytosterols do not decrease through storage, and even appear to increase slightly, although not significantly. The increase is phytosterol concentration is likely due to sampling effect. Regardless, phytosterols are unlikely to be providing antioxidant benefits unless steryl ferulates are producing side reactions that cannot be observed in this study. Even though tocopherols and lutein are likely antioxidants, the added extract does not significantly improve stability of crackers. The treatment by time interaction was not significant for any phytochemical at any time.

While natural antioxidants, such as tocopherols, are generally added to food at concentrations at or below 500 μg/g, natural extracts are often added at higher concentrations (Bhale et al 2007; Seppanen et al 2010). Since the extract antioxidant potential is related to specific phytochemicals, the extract addition level may not have been sufficient.

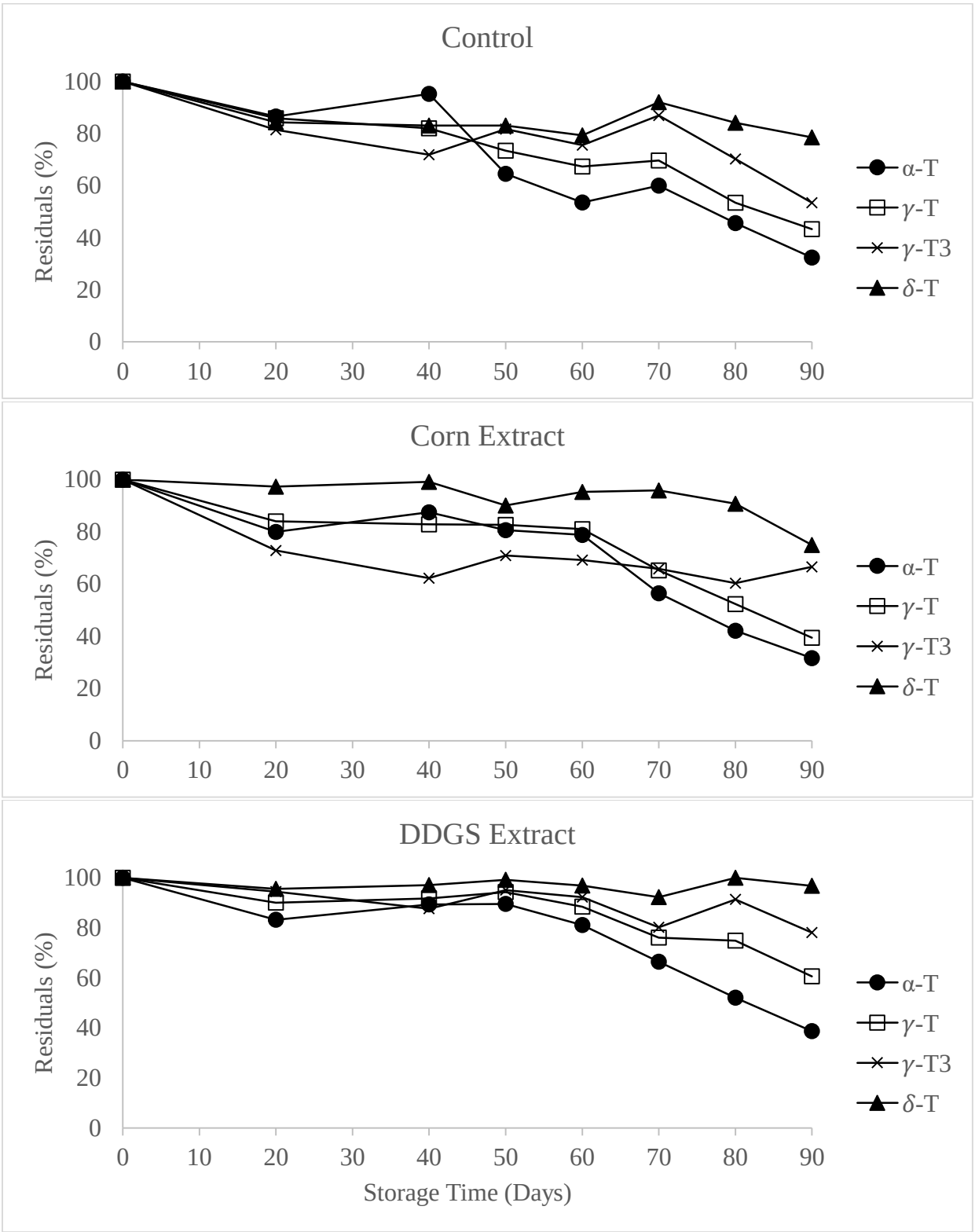


Figure 22. Tocopherol (T) and tocotrienol (T3) residuals in crackers without (control) and with corn and DDGS extracts.

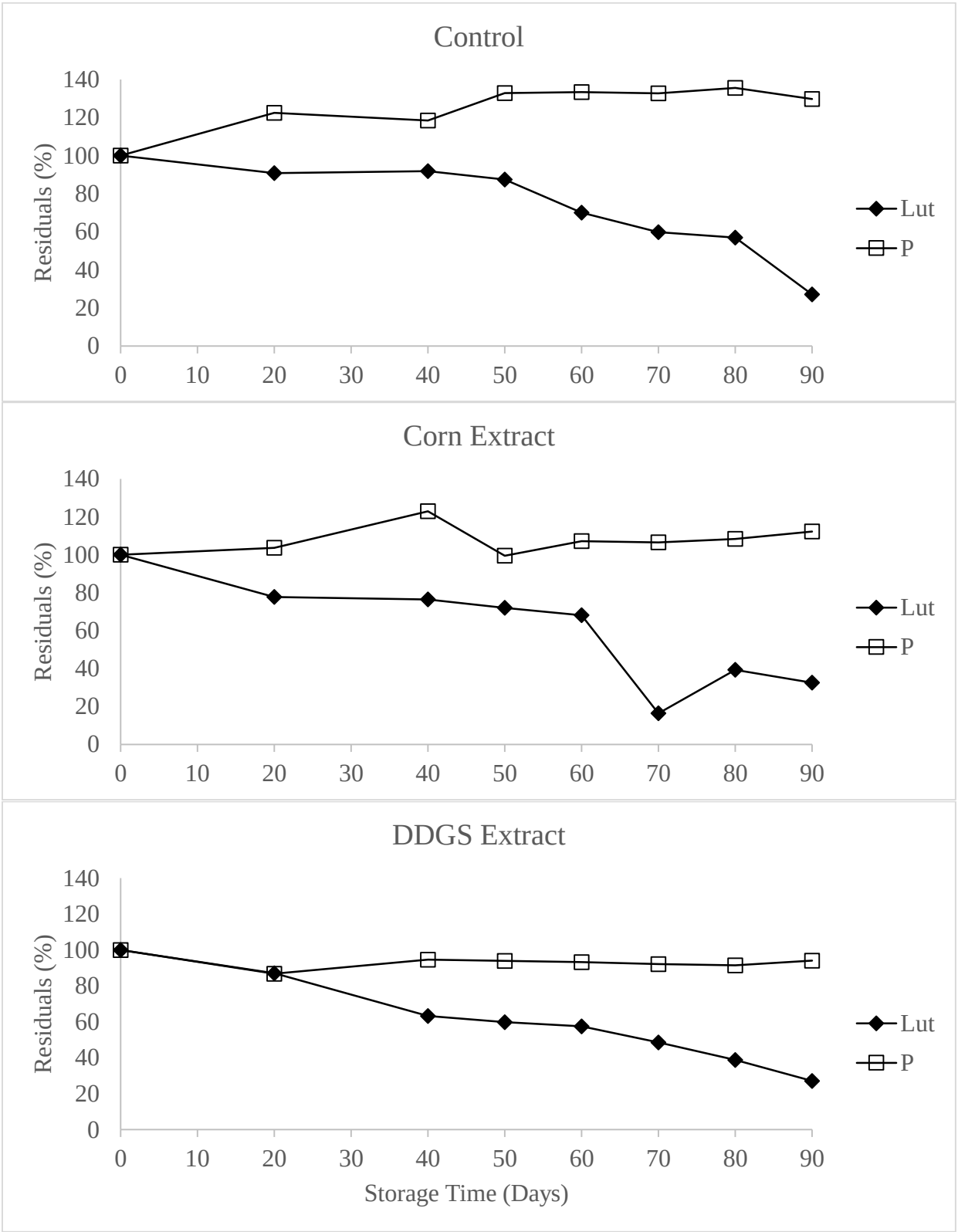


Figure 23. Lutein (Lut) and phytosterol (P) residuals in crackers without (control) and with corn and DDGS extracts.

4.5. Conclusion

Supercritical carbon dioxide extraction created very similar extracts as hexane extraction, but neither extract successfully reduced oxidation at 0.05%. Tocopherols and lutein appeared to contribute antioxidant benefits, but the concentration added by corn and DDGS extracts were too low to affect oxidation.

5. OVERALL CONCLUSION

DDGS are a plentiful coproduct of ethanol production with a high concentration of phytochemicals. Natural antioxidant extracts can be created by traditional solvent extraction with hexane, but SC-CO_2 extraction can create a very similar extract while being safer and more environmentally friendly.

Adding 1% DDGS extract reduced oxidation products in chips. DDGS extracts have the potential for extending shelf-life of low-moisture food. The corn extract contained less tocopherols and carotenoids, but more phytosterols and did not reduce oxidation. Tocopherols and carotenoids significantly reduced oxidation while phytosterols did not. This suggests that the antioxidant activity is largely related to tocopherols and carotenoids from the extract.

Adding extracts from SC-CO_2 at 0.05% to crackers did not affect oxidation. Tocopherols and carotenoids did not significantly increase once the extracts were added to crackers at 0.05%. However, the shortening (Crisco) in crackers contained antioxidants (TBHQ and citiric acid), which may have reduced the impact of the antioxidant extracts. Reducing the initial antioxidant content in crackers could have shown benefits from the antioxidant extract, but the conditions of this study suggest that the extracts' phytochemical concentration was too low to reduce oxidation in crackers at 0.05%.

6. FUTURE WORK

DDGS extract showed promise as an effective antioxidant at 1% addition while corn extract did not; however, 0.05% of either extract was too low. The oxidative stability of crackers appears to be related to tocopherol and lutein concentration.

Future research to increasing antioxidants should be considered. First, the extracts could be added at various concentrations above 0.05%. However, consideration to identifying an optimal addition level should be considered. Furthermore, the antioxidant activity could be tested on a reduced phytochemical baseline level. Changing the flour or shortening used in the baked product may increase the effect of an added antioxidant, or research to eliminate the antioxidant contribution of the commercial lipid could be evaluated. Another approach could be to increase phytochemical concentration in the extract. Non-phytochemicals can be removed by various processes to increase antioxidant concentration. For instance, free fatty acids, which are relatively high in DDGS oil (6.8%), might be removed by distillation (Winkler-Moser and Vaughn 2009). Saponification also could be explored. Steryl ferulates also could be analyzed for potential antioxidant properties.

Sensory analysis can be conducted in coordination with laboratory analysis. Quantitative descriptive analysis uses a trained panel to identify sensory attributes. This type of sensory analysis can be used to determine human perceptions of oxidation in food and sensory attributes relate to laboratory data.

REFERENCES

American Association of Cereal Chemists [AACC]. 2001. The definition of dietary fiber. Cereal Foods World. 46:112-29.

American Dietetic Association [ADA]. 2002. Position of the American Dietetic Association: Health implications of dietary fiber. Journal of the American Dietetics Association 102(7): 993-1000.

American Oil Chemists' Society [AOCS. 1997. Official Methods and Recommended Practices of the American Oil Chemists' Society, 4th Ed. Edited by D. Firestone. Method Cd 8-53.

Akoh, C. C., and Min, D. B. 2002. Food Lipids: Chemistry, Nutrition, and Biotechnology, Second Edition. CRC Press: New York

Anonymous. 2012. Distillers grains by-products in livestock and poultry feed: nutrient profiles. St. Paul, MN: University of Minnesota. Available at http://www.ddgs.umn.edu. Accessed on Nov. 10, 2016.

Babcock B. A., Hayes D. J., and Lawrence J.D. 2008. Using distiller's grains in the U.S. and International livestock and poultry industries. Midwest Agribusiness Trade Research and Information Center [MATRIC]. Ames, IA: USDA Award No. 2005-34285-15570.

Barden, L. and Decker, E. A. 2013. Lipid oxidation in low-moisture food: a review. Critical Reviews in Food Science. 56:2467-2482

Barden, L., Barouh, N., Villeneuve, P. and Decker, E. 2015. Impact of hydrophobicity on antioxidant efficacy in low-moisture food. Journal of Agricultural and Food Chemistry 63:5821-5827.

Belitz, H. D., Grosch, I., and Schieberle, P. 2009. Food Chemistry. 4th Ed. Springer: Berlin.

Belyea, R., Eckhoff, S., Wallig, M. and Tumbleson, M. 1998. Variability in the nutritional quality of distillers solubles. Bioresource Technology. 66:207-212.

Belyea R. L., Rausch K. D., and Tumbleson M. E. 2004. Composition of corn and distillers' dried grains with solubles from dry grind ethanol processing. Bioresource Technology. 94:293-298.

Belyea, R. L., Clevenger, T. E., Singh, V., Tumbleson, M. E., and Rausch, K. D. 2006. Element concentrations of dry-grind corn-processing streams. Applied Biochemistry and Biotechnology. 134:113-128.

Bhale, S. D., Xu, Z., Prinyawiwatkul, W., King, J. M., and Godber, J. S. 2007. Oregano and rosemary extracts inhibit oxidation of long-chain n-3 fatty acids in menhaden oil. Journal of Food Science. 72:C504-508

Burton, G.W., 1994. Vitamin E: molecular and biological function. Proceedings of the Nutrition Society. 53:251–262.

Burton, G. W., Doba, T., Gabe, E. J., Hughes, L., Lee, F. L., Prasad, L., and Ingold, K. U. 1985. Autoxidation of biological molecules. 4. Maximizing the antioxidant activity of phenols. Journal of the American Chemical Society. 107:7053–7065.

Burton, G. W., and Traber, M. G., 1990. Vitamin E: antioxidant activity, biokinetics, and bioavailability. Annual Review of Nutrition 10:357–382.

Cheng, M. H., Riess, S. M., and Rosentrater, K. A. 2014. Fractionation of distillers dried grains with solubles (DDGS) by combination of sieving and aspiration. Agricultural and Biosystestems Engineering Conference Proceedings and Presentations. Paper 379. http://lib.dr.iastate.edu/abe_eng_conf/379.

Ciftci, O. N., and Temelli, F. 2011. Continuous production of fatty acid methyl esters from corn oil in a supercritical carbon dioxide bioreactor. The Journal of Supercritical Fluids 58:79-87.

Ciftci, O. N., and Temelli, F. 2013. Continuous biocatalytic conversion of the oil of corn distiller's dried grains with solubles to fatty acid methyl esters in supercritical carbon dioxide. Biomass and Bioenergy 54:140-146.

Ciftci, O. N., and Temelli, F. 2014. Continuous bioconversion of the lipids of corn, wheat, and triticale distiller's dried grains with solubles to biodiesel in supercritical carbon dioxide and characterization of the products. Bioenergy Research 7:702-710.

Ciftci, O. N., Calderon, J., and Temelli, F. 2012. Supercritical carbon dioxide extraction of corn distiller's dried grains with solubles: experiments and mathematical modeling. Journal of Agricultural and Food Chemistry 60:12482-12490.

Cocero, M. J., and Calvo, L. 1996. Supercritical fluid extraction of sunflower seed oil with CO2-ethanol mixtures. Journal of the American Oil Chemists' Society 73:1573-1578.

Codex Alimentarius. 2016. General standard for food additives. Codex Stan 192-1995. Available from: http://www.fao.org. Accessed Nov 12, 2016.

Corn Refiners Association [CRA]. 2006. Corn oil. 5th Ed. Corn Refiners Association: Washington D.C.

Cromwell G. L., Herkelman K. L., and Stahly T. S. 1993. Physical, chemical, and nutritional characteristics of distillers dried grains with solubles for chicks and pigs. Journal of Animal Science. 71:679-86.

Crowe, D., and White, P.J. 2001. Adaptation of the AOCS official method for measuring hydroperoxides from small-scale oil samples. Journal of the American Oil Chemists' Society. 78:1267-1269.

Čukelj, N., Novotny, D., and Ćurić, D. 2010. Antioxidant properties of whole grain cereals. Hrvatski Časopis za Prehrambenu Tehnologiju, Biotehnologiju i Nutricionizam. 5:18-23.

Energy Information Administration [EIC]. 2014. Petroleum and other liquids. Washington, D.C.: U.S. Energy Information Administration. Available from http://www.eia.gov. Accessed Nov 10, 2016.

Decker, E. A., Elias, R. J., and McClements, D. J. 2010. Oxidation in foods and beverages and antioxidant applications: management in different industry sectors. Elsevier. Philadelphia.

Deiana, M., Rosa, A., Cao, C. F., Pirisi, F. M., Bandino, G., and Dessi, M. A. 2002. Novel approach to study oxidative stability of extra virgin olive oils: importance of alpha-tocopherol concentration. Journal of Agricultural and Food Chemistry. 50:4324-4326.

Fardet, A., Rock, E., and Rémésy, C. 2008. Is the in vitro antioxidant potential of whole-grain cereals and cereal products well reflected in vivo? Journal of Cereal Science. 48:258-276.

Frankel, E. N. 1998. Lipid oxidation. Oily Press. Dundee.

Frankel, E. N., and Meyer, A. S. 2000. The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. Journal of the Science of Food and Agriculture. 80:1925-1941.

Fuster, M. D., Lampi, A., Hopia, A., Kamal-Eldin, A. 1998. Effects of α-and γ-tocopherols on the autoxidation of purified sunflower triacylglycerols. Lipids. 33(7):715-722.

Golestani, A., Rastegar, R., Shariftabrizi, A., Khaghani, S., Payabvash, S. M., Salmasi, A. H., Dehpour, A. R., and Pasalar, P. 2006. Paradoxical dose-and time-dependent regulation of superoxide dismutase and antioxidant capacity by vitamin E in rat. Clinica Chimica Acta 365:153-159.

Gordon, M. H., and Magos, P. 1983. The effect of sterols on the oxidation of edible oils. Food Chemistry 10:141-147.

Gupta, P., Sreelakshmi, Y., and Sharma, R. 2015. A rapid and sensitive method for determination of carotenoids in plant tissues by high performance liquid chromatography. Plant Methods. 11:5.

Gunstone, F. D. 1996. Fatty Acid and Lipid Chemistry, 1st Ed. Black Academic & Professional: Glasgow, UK.

Hall III, C.A., and Zhao, B. 2011. Phytochemicals in cereals, pseudocereals and pulses. Pages 21-82 in: Tokuşoğlu, Ö., and Hall III, C. A., Fruit and Cereal Bioactives: Sources, Chemistry, and Applications. CRC Press: Boca Raton.

Hoffman L.A., and Baker A. 2011. Estimating the substitution of distillers' grains from corn and soybean meal in the US feed complex. FDS-11-01. US Department of Agriculture, Economic Research Service. Oct 2011.

Hofstrand, D. 2016. Tracking ethanol profitability. Agricultural Marketing and Resource Center: Ames, IA.

Irwin, S. 2016. The Profitability of ethanol production in 2015. Department of Agricultural and Consumer Economics: University of Illinois at Urbana.

Jay, A. J., Steytler, D. C., and Knights, M. 1991. Spectrophotometric studies of food colors in near-critical carbon dioxide. The Journal of Supercritical Fluids. 4:131-141.

Jiang, Y., and Wang, T. 2005. Phytosterols in cereal by-products. Journal of the American Oil Chemists' Society. 82:439-444.

Kamal-Eldin A. 2006. Effect of fatty acids and tocopherols on the oxidative stability of vegetable oils. European Journal of Lipid Science and Technology. 58:1051-61.

Kamal-Eldin, A., and Appelqvist, L.Å. 1996. The chemistry and antioxidant properties of tocopherols and tocotrienols. Lipids 31:671-701.

Kamal-Eldin, A., and Andersson, R. 1997. A multivariate study of the correlation between tocopherol content and fatty acid composition in vegetable oils. Journal of the American Oil Chemists' Society. 74:375-380.

Kent, N. L., and Evers, A. D. 1994. Technology of Cereals: An Introduction for Students of Food Science and Agriculture. Pergamon: New York.

Kim, Y., Mosier, N.S., Hendirckson, R., Ezeji T., Blaschek, H., Dien, B., Cotta, M., Dale, B., and Ladisch, M.R. 2008. Composition of corn dry-grind ethanol by-products: DDGS, wet cake, and thin stillage. Bioresource Technology. 99:5165-76.

King, M. B., and Bott, T. R. 1993. Extraction of natural products using near-critical solvents. Springer Science & Business Media: London.

Kirk, R. S., and Sawyer, R. 1991. Pearson's Composition and Analysis of Food, 9th Ed. Addison Wesley Longman Ltd: England.

Krinsky, N. I., and Yeum, K.J. 2003. Carotenoid–radical interactions. Biochemical and Biophysical Research Communications 305:754-760.

Kurilich, A. C., and Juvik, J. A. 1999. Quantification of carotenoid and tocopherol antioxidants in Zea mays. Journal of Agricultural and Food Chemistry. 47:1948-1955.

Labuza, T. P., and Dugan Jr. L. R. 1971. Kinetics of lipid oxidation in foods. Critical Reviews in Food Science & Nutrition. 2:355-405.

Labuza, T. P., McNally, L., Gallagher, D., Hawkes, J., and Hurtado, F. 1972. Stability of intermediate moisture foods. 1. Lipid oxidation. Journal of Food Science. 37:154-159.

Lawless, H. T., and Heymann, H. 2010. Sensory evaluation of food: principles and practices. Springer Science & Business Media.

Leguizamon, C., Weller, C. L., Schlegel, V. L., and Carr, T. P. 2009. Plant sterol and policosanol characterization of hexane extracts from grain sorghum, corn and their DDGS. Journal of American Oil Chemists Society. 86:707-716.

Lindsay, R. C. 2008. Food Additives. Pages 689-749 in: Damodaran, S., Parkin, K. L., and Fennema, O. R. 2008. Fennema's Food Chemistry. CRC press: New York.

Liu, K. 2008. Particle size distribution of distillers dried grains with solubles (DDGS) and relationships to compositional and color properties. Bioresource Technology. 99:8421-8428.

Liu, K. 2009. Effects of particle size distribution, compositional and color properties of ground corn on quality of distillers dried grains with solubles (DDGS). Bioresource Technology. 100:4433-4440.

Liu, K. 2010. Selected factors affecting crude oil analysis of distillers dried grains with solubles (DDGS) as compared with milled corn. Cereal Chemistry. 87:243-249.

Liu, K. S. 2011. Chemical Composition of distillers grains, a review. Journal of Agricultural and Food Chemistry. 59:1508-1526.

Liu, K. S., and Rosentrater, K. A. 2011. Distillers Grains: Production, Properties, and Utilization. Taylor & Francis: Boca Raton.

Liu, S. X., Singh, M., and Inglett, G. 2011. Effect of incorporation of distillers' dried grain with solubles (DDGS) on quality of cornbread. LWT - Food Science and Technology. 44:713-718.

Majoni, S., and Wang, T. 2010. Characterization of oil precipitate and oil extracted from condensed corn distillers solubles. Journal of the American Oil Chemists Society. 87:205-213.

Martin, A., Wu, D., Baur, W., Meydani, S. N., Blumberg, J. B., and Meydani, M. 1996. Effect of vitamin E on human aortic endothelial cell responses to oxidative injury. Free Radical Biology and Medicine. 21:505-511.

Masa'deh, M. K., Purdum, S. E., and Handord, K. J. 2011. Dried distillers grains with solubles in laying hen diets. Poultry Science. 90:1960-1966.

Masa'deh, M. K., Purdum, S. E., and Handord, K. J. 2012. Distillers dried grains with solubles in pullet diets. Journal of Applied Poultry Research. 21:531-539.

McClements, D. J., and Decker, E. A. 2008. Lipids. Pages 155-216 in: Damodaran, S., Parkin, K. L., and Fennema, O. R. 2008. Fennema's Food Chemistry. CRC press: New York.

Messias, R. S., Galli, V., Silva, S. D. D. A., Schirmer, M. A., and Rombaldi, C. V. 2015. Micronutrient and functional compounds biofortification of maize grains. Food Science and Nutrition. 55:123-139.

Moeller, S., Parekh, N., Tinker, L., Ritenbaugh, C., Blodi, B., Wallace, R., and Mares, J. 2006. Associations between intermediate age-related macular degeneration and lutein and zeaxanthin in the carotenoids in age-related eye disease study (CAREDS): Ancillary study of the Women's Health Initiative. Journal of the American Medical Association in Ophthalmology. 124:1151–1162.

Moreau, R. A., and Hicks, K. B. 2005. The composition of corn oil obtained by the alcohol extraction of ground corn. Journal of the American Oil Chemists' Society. 82:809-815.

Moreau, R. A., and Hicks, K. B. 2006. Reinvestigation of the effect of heat pretreatment of corn fiber and corn germ on the levels of extractable tocopherols and tocotrienols. Journal of Agriculture and Food Chemistry. 54:8093-8102.

Moreau, R. A., Powell, M. J., and Hicks, K. B. 1996. Extraction and quantitative analysis of oil from commercial corn fiber. Journal of Agriculture and Food Chemistry. 44:2149-2154.

Moreau, R. A., Singh, V., Eckhoff, S. R., Powell, M. J., Hicks, K. B., and Norton, R. A. 1999. Comparison of yield and composition of oil extracted from corn fiber and corn bran. Cereal Chemistry. 76:449-451.

Moreau, R. A., Singh, V., Nunez, A., and Hicks, K. B. 2000. Phytosterols in the aleurone layer of corn kernels. Biochemical Society Transactions. 28:803-806.

Moreau, R. A., Singh, V., and Hicks, K. B. 2001. Comparison of oil and phytosterol levels in germplasm accessions of corn, teosinte, and Job's tears. Journal of Agricultural and Food Chemistry 49:3793-3795.

Moreau, R. A., Whitaker, B. D., and Hicks, K. B. 2002. Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. Progress in Lipid Research. 41:457-500.

Moreau, R. A., Johnston, D., and Hicks, K. 2007. A comparison of the levels of lutein and zeaxanthin in corn germ oil, corn fiber oil and corn kernel oil. Journal of the American Oil Chemists' Society. 84:1039-1044.

Moreau, R. A., Hicks, K. B., Johnston, D. B. and Laun, N. P. 2010. The composition of crude corn oil recovered after fermentation via centrifugation from a commercial dry grind ethanol process. Journal of the American Oil Chemists Society. 87:895-902.

Moreau, R. A., Liu, K., Winkler-Moser, J. K., and Singh, V. 2011. Changes in lipid composition during dry grind ethanol processing of corn. Journal of the American Oil Chemists' Society. 88:435-442.

Mukhopadhyay, M. 2000. Natural extracts using supercritical carbon dioxide. CRC press: Boca Raton.

Nanditha, B., and Prabhasankar, P. 2009. Antioxidants in bakery products: a review. Critical Reviews in Food Science and Nutrition 49:1-27.

North Dakota Ethanol Council [NDEC]. 2014. National ethanol industry. Ethanol Council: Bismarck. Available from: http://www.ndethanol.org. Accessed Nov 10, 2016.

Nyström, L., Marjukka, M., Lampi, A., and Piironen, V. 2005. Antioxidant activity of steryl ferulate extracts from rye and wheat bran. Journal of Agricultural and Food Chemistry. 53:2503-2510.

Ostlund, R. E., Racette, S. B., Okeke, A., and Stenson, W. F. 2002. Phytosterols that are naturally present in commercial corn oil significantly reduce cholesterol absorption in humans. American Journal of Clinical Nutrition. 75(6):1000-1004.

Paiva, S. A. R., and Russel, R. M. 1999. B-carotene and other carotenoids as antioxidants. Journal of the American College of Nutrition. 18:426-433.

Panfili, G., Fratianni, A., and Irano, M. 2003. Normal phase high-performance liquid chromatography method for the determination of tocopherols and tocotrienols in cereals. Journal of Agricultural and Food Chemistry. 51:3940-3944.

Panfili, G., Fratianni, A., and Irano, M. 2004. Improved normal-phase high-performance liquid chromatography procedure for the determination of carotenoids in cereals. Journal of Agricultural and Food Chemistry. 52:6373-6377.

Pokorny, J. 2007. Antioxidants in food preservation. Pages 259-286 in: Rahmen, M. S., Handbook of Food Preservation, 2nd Ed. CRC Press: Boca Raton.

Pokorny, J., Yanishlieva, N., and Gordon, M. 2001. Antioxidants in Food: Practical Applications. CRC: New York.

Pourafshar, S., Rosentrater, K. A., and Krishnan P. G. 2014. Use of alternative flours as partial replacement of barbari bread formulation (traditional Iranian bread). Journal of Food Science and Technology. 52:5691-5699.

Pourafshar, S., Rosentrater, K. A., and Krishnan P. G. 2015. Changes in chemical and physical properties of Latin American wheat flour based tortillas substituted with different levels of distillers dried grains with solubles (DDGS). Journal of Food Science and Technology. 52:5243-5249.

Purdum, S., Hanford, K., and Kreifels, B. 2014. Short-term effects of lower oil dried distillers grains with solubles in laying hen rations. Poultry Science. 93:1-4.

Quílez, J., Rafecas, M., Brufau, G., García-Lorda, P., Megías, I., Bullo, M., Ruiz, J. A., and Salas-Salvado, J. 2003. Bakery products enriched with phytosterol esters, α-tocopherol and β-carotene decrease plasma LDL-cholesterol and maintain plasma β-carotene concentrations in normocholesterolemic men and women. The Journal of Nutrition. 133:3103-3109.

Quílez, J., Ruiz, J. A., Brufau, G., and Rafecas, M. 2006. Bakery products enriched with phytosterols, α-tocopherol and β-carotene. Sensory evaluation and chemical comparison with market products. Food Chemistry. 94:399-405.

Rao, A. V., and Rao, L. G. 2007. Carotenoids and human health. Pharmacological Research. 55:207-216.

Rebolleda, S., Rubio, N., Beltrán, S., Teresa-Sanz, M., González-Sanjosé, M. L. 2012. Supercritical fluid extraction of corn germ oil: Study of the influence of process parameters on the extraction yield and oil quality. Journal of Supercritical Fluids. 72:270-277.

Renewable Fuels Association [RFA]. 2013. Falling walls and rising tides: 2014 ethanol industry outlook. RFA: Washington, DC.

Renewable Fuels Association [RFA]. 2014. Fueling a nation feeding the world: The role of the U.S. ethanol industry in food and feed production. RFA: Washington, D.C.

Renewable Fuels Association [RFA]. 2016. 2015 ethanol co-product exports and imports statistical summary. RFA: Washington, D.C.

Ribaya-Mercado, J. D., and Blumberg, J. B. 2004. Lutein and zeaxanthin and their potential roles in disease prevention. Journal of the American College of Nutrition. 23:567S–587S.

Robinson, P. H., Karges, K., and Gibson, M. L. 2008. Nutritional evaluation of four co-product feedstuffs from the motor fuel ethanol distillation industry in the Midwestern USA. Animal Feed Science and Technology. 146:345-352.

Rosentrater, K. A., and Krishnan, P. G. 2006. Incorporating distillers grains in food products. Cereal Foods World 51:52-60.

Ross, C. A., and Harrison, E. H. 2007. Handbook of Vitamins. Taylor & Francis Group: Boca Raton.

Sanches-Silva, A., De Quirós, A. R.-B., López-Hernández, J., and Paseiro-Losada, P. 2004. Determination of hexanal as indicator of the lipidic oxidation state in potato crisps using gas chromatography and high-performance liquid chromatography. Journal of Chromatography A. 1046:75-81.

Saunders, J. A., Rosentrater, K. A., and Krishnan, P. G. 2013. Removal of color pigments from corn distillers dried grains with solubles (DDGS) to produce an upgraded food ingredient. Journal of Food Research. 2:1927-0895.

Schroeder, M. T., Becker, E. M., and Skibsted, L. H. 2006. Molecular mechanism of antioxidant synergism of tocotrienols and carotenoids in palm oil. Journal of Agricultural and Food Chemistry 54:3445-3453.

Schwartz, H., Ollilainen, V., Piironen, V., and Lampi, A.-M. 2008. Tocopherol, tocotrienol and plant sterol contents of vegetable oils and industrial fats. Journal of Food Composition and Analysis. 21:152-161.

Sen, C. K., Khanna, S., and Roy, S. 2007. Tocotrienols in health and disease: The other half of the natural vitamin E family. Molecular Aspects of Medicine. 28:692-728.

Seppanen, C. M., Song, Q., and Csallany, A. S. 2010. The antioxidant functions of tocopherol and tocotrienol homologues in oils, fats, and food systems. Journal of the American Oil Chemists' Society. 87:469-481.

Sessa, D. J., Eller, F. J., Palmquist, D. E., and Lawton, J. W. 2003. Improved methods for decolorizing corn zein. Industrial Crops and Products 18:55-65.

Shahidi, F., and Zhong, Y. 2005. Bailey's Industrial Oil and Fat Products, 6th Ed. John Wiley & Sons, Inc. Hoboken, NJ.

Shi, H., and Ho, C. T. 1994. The flavour of poultry meat. In: Shahidi, F. Flavor of Meat and Meat Products. Springer: Boston, MA, pp 52-70.

Shukla, R., and Cheryan, M. 2001. Zein: the industrial protein from corn. Industrial Crops and Products. 13(3):171-192.

Shurson, J., Tilstra, H., and Kerr, B.J. 2012. Impact of United States biofuels co-products on the feed industry. In: Makkar H.P.S., Biofuel Co-Products as Livestock Feed. Food and Agriculture Organization, Rome, pp. 35-60.

Sies H., and Stahl, W. 1995. Vitamin E and C, beta-carotene, and other carotenoids as antioxidants. American Journal of Clinical Nutrition. 62:1315S-1321S.

Singh, N., and Cheryan, M. 1998. Extraction of oil from corn distillers dried grains with solubles. Transactions of the ASAE. 41:1775-1777.

Spiehs, M. J., Whitney, M. H., and Shurson, G. C. 2002. Nutrient database for distillers' dried grains with solubles produced from new ethanol plants in Minnesota and South Dakota. Journal of Animal Science. 80:2639-2645.

Srinivasan, R., Moreau, R. A., Rausch, K. D., Tumbleson, M. E., and Singh, V. 2007. Phytosterol distribution in fractions obtained from processing of distillers dried grains with solubles using sieving and elutriation. Cereal Chemistry. 84:626-630.

St. Angelo, A. J. 1992. Lipid oxidation in food. ACS symposium series (USA). American Chemical Society. New York.

Stahl, W., and Sies, H. 2003. Antioxidant activity of carotenoids. Molecular aspects of medicine. 24:345-351.

Stein, H. H., and Shurson, G. C. 2009. Board-invited review: the use and application of distillers dried grains with solubles in swine diets. Journal of Animal Science. 87:1292-1303.

Stein, H. H., Connot, S. P., and Pedersen, C. 2009. Energy and nutrient digestibility in four sources of distillers dried grains with solubles produced from corn grown within a narrow geographical area and fed to growing pigs. Asian-Australian Journal of Animal Science. 22:1016-1025.

Subagio, A. and Morita, N. 2001. Instability of carotenoids is a reason for their promotion on lipid oxidation. Food Research International. 34:183-188.

Tepper, B. J., and Trail, A. C., 1998. Taste or health: A study on consumer acceptance of corn chips. Food Quality and Preference. 9:267-272.

Toivo, J., Phillips, K., Lampi, A.-M., and Piironen, V. 2001. Determination of sterols in foods: recovery of free, esterified, and glycosidic sterols. Journal of Food Composition and Analysis. 14:631-643.

Traber, M. G. 2007. Vitamin E regulatory mechanisms. Annual Review of Nutrition. 27:347-362.

Traber, M. G., and Atkinson, J. 2007. Vitamin E, antioxidant and nothing more. Free Radical Biology and Medicine. 43:4-15.

Tuberoso, C. I. G., Kowalczyk, A., Sarritzu, E., and Cabras, P. 2007. Determination of antioxidant compounds and antioxidant activity in commercial oilseeds for food use. Food Chemistry. 103:1494-1501.

United States Department of Agriculture Economic Research Service [USDA-ERS]. 2016. U.S. bioenergy statistics. Washington, D.C. U.S. Department of Agriculture Economic Research Service. Available at http://www.ers.usda.gov. Accessed on Nov. 1, 2016.

U. S. Grains Council. 2012. A guide to distiller's dried grains with solubles. Available at http://www.ethanolrfa.org. Accessed Nov. 10, 2016.

Vickers, T. after Young, I.S., and McEneny, J. 2001. Lipoprotein oxidation and atherosclerosis. Biochemical Society Transactions. 29(2)358-362.

Wang, T., Hicks, K. B., and Moreau, R. 2002. Antioxidant activity of phytosterols, oryzanol and other phytosterol conjugates. Journal of the American Oil Chemists' Society. 79:1201-1206.

Wang, L. J., Weller, C. L., Schlegel, V. L., Carr, T. P., and Cuppett, S. L. 2007. Comparison of supercritical CO2 and hexane extraction of lipids from sorghum distillers grains. European Journal of Lipid Science and Technology. 109:567-574.

Weber, E. J. 1987. Carotenoids and tocols of corn grain determined by HPLC. Journal of the American Chemists' Society. 64(8):1129-1134.

Winkler-Moser, J. K. 2011. Gas chromatographic analysis of plant sterols. Journal of the American Oil Chemists' Society. Available at http://lipidlibrary.aocs.org. Accessed Nov. 10, 2016.

Winkler-Moser, J. K., and Breyer, L. 2011. Composition and oxidative stability of crude oil extracts of corn germ and distillers grains. Industrial Crop Production. 33:572-578.

Winkler-Moser, J. K., and Vaughn, S. F. 2009. Antioxidant activity of phytochemicals from distillers dried grain oil. Journal of the American Oil Chemists Society. 86:1073-1082.

Winkler, J. K., Rennick, K. A., Eller, F. J., and Vaughn, S. F. 2007. Phytosterol and tocopherol components in extracts of corn distiller's dried grain. Journal of Agricultural Food Chemistry. 55:6482-6486.

Winkler-Moser, J. K., Rennick, K. A., Palmquist, D. A., Berhow, M. A., and Vaughn, S. F. 2012. Comparison of the impact of y-oryzanol and corn steryl ferulates on the polymerization of soybean oil during frying. Journal of the American Oil Chemists' Society. 89:243-252.

Wright, M. E., Lawson, K. A., Weinstein, S. J., Pietinen, P., Taylor, P. R., Virtamo, J., and Albanes, D. 2006. Higher baseline serum vitamin E concentrations are associated with lower total and cause-specific mortality in the alpha-Tocopherol, beta-carotene cancer prevention study. American Journal of Clinical Nutrition. 84:1200–1207.

Wu, Y. V., Youngs, V. L., Warner, K., and Bookwalter, G. N. 1987. Evaluation of spaghetti supplemented with corn distillers' dried grains. Cereal Chemistry. 64:434-436.

Wu, Y. V., Friedrich, J. P., and Warner, K. 1990. Evaluation of corn distillers dried grains defatted with supercritical carbon-dioxide. Cereal Chemistry. 67:585-588.

Xu, Z., Hau, N., and Godber, J. S. 2001. Antioxidant activity of tocopherols, tocotrienols, and γ-oryzanol components from rice bran against cholesterol oxidation accelerated by 2, 2'-azobis (2-methylpropionamidine) dihydrochloride. Journal of Agricultural and Food Chemistry. 49:2077-2081.

Yanishlieva, N. V., Kamal-Eldin, A., Marinova, E. M., and Toneva, A. G. 2002. Kinetics of antioxidant action of α- and γ-tocopherols in sunflower and soybean triacylglycerols. European Journal of Lipid Science and Technology. 104:262-270.

Yoshida, Y., and Niki, E. 2003. Antioxidant effects of phytosterol and its components. Journal of Nutrition Science and Vitaminology. 49:277-280.

APPENDIX

Table A1. References contributing to nutritional analysis of corn.

Macronutrients	**Tocopherols**
Jiang and Wang 2005	CRA 2006
Kent et al 1994	Kamal-Eldin and Anderson 1997
Leguizamon et al 2009	Kurilich and Juvik 1999
Liu 2009	Moreau and Hick 2006
Moreau et al 2000	Moreau et al 1996
Moreau et al 2001	Moreau et al 2001
Moreau et al 2003	Moreau et al 2010
Moreau et al 2006	Moreau et al 2011
Moreau 2011	Panfili et al 2003
Stein et al 2009	Schwartz et al 2008
	Tuberoso et al 2007
	Winkler-Moser and Breyer 2011
Phytosterols	**Carotenoids**
Jiang and Wang 2005	Kurilich and Juvik 1999
Leguizamon et al 2009	Moreau et al 2010
Liu 2009	Panfili et al 2004
Moreau et al 1996	Tuberoso 2007
Moreau et al 2000	Weber 1987
Moreau et al 2001	Winkler-Moser 2011
Moreau et al 2003	
Moreau 2011	
Stein et al 2009	

Table A2. References contributing to nutritional analysis of DDGS

Macronutrients	**Phytosterols**
Belyea et al 1998	Ciftci et al 2012
Belyea et al 2004	Leguizamon et al 2009
Cheng et al 2014	Majonji and Wang 2010
Ciftci and Temelli 2014	Moreau et al 2011
Ciftci et al 2012	Srinivasan et al 2007
Cromwell et al 1993	Winkler et al 2007
Kim et al 2008	Winkler-Moser et al 2010
Leguizamon et al 2009	Winkler-Moser and Breyer 2011
Liu 2009	**Tocopherols**
Masa'deh et al 2011	Ciftci et al 2012
Moreau et al 2011	Moreau et al 2010
Robinson et al 2009	Moreau et al 2011
Saunders et al 2013	Winkler-Moser et al 2007
Spiehs et al 2002	Winkler-Moser et al 2009
Srinivasan et al 2007	Winkler-Moser and Breyer 2011
Stein et al 2009	**Carotenoids**
Winkler et al 2007	Ciftci et al 2012
	Moreau et al 2010
	Winkler-Moser et al 2009
	Winkler-Moser and Breyer 2011

Table A3. Chip recipe.

	Control	Corn	DDGS
	----------%----------		
Pastry Flour	62	62	62
Shortening	4	4	4
Salt	1	1	1
Baking Powder	1	1	1
Water	32	32	32
Treatment extract	0	0.04[a]	0.04

[a] equal to 1% of lipid content

Basics of Wheat and Flour Quality Short Course - Tortilla formula

Tortilla	**Baker's %**	**Grams**
Pastry Flour	100	500
Shortening	6	30
Salt	1.5	7.5
Water	68	340
Baking Powder	1.28	6.4

Procedure:

1. Ingredients were mixed for 6-7 min.
2. Dough was placed in the proof cup for 10 min. resting.
3. Dough was cut (45 g. pieces) and molded.
4. Dough was baked for 1.2 sec in Dough-pro tortilla maker and sheeted (Scale: thick).
5. Sheeted tortilla was baked for 2 minutes at 550 °F.
6. Tortillas were tested for cooling and evaluated.

Figure A1. Chip recipe provided by NCI.

U.S. WHEAT ASSOCIATES

Good

NAME: Enzyme Cracker				04 - ENZ-2
TYPE OF PRODUCT: Cracker				
Program Number: Straight Dough Method				
FORMULA				
INGREDIENTS		%	Grams	REMARKS
Cookie Flour	✓	100.00	1000	Sift together and add it into the Hobart mixer with the McDuffy bowl & attachment
Icing Sugar	✓	8.00	80	
Milk Solids Non Fat	✓	1.50	15	
Salt	✓	1.00	10	
Mono Calcium Phosphate	✓	1.00	10	
Sodium Bicarbonate	✓	1.00	10	
Ammonia Bicarbonate	✓	1.00	10	Dissolve and set aside
Water		5.00	50	
Enzyme BK 5020	✓	0.02	0.2	Dissolve and add all the liquids into the mixing bowl.
Water		26.00	260	
Shortening	✓	12.00	120	Mix lecithin with shortening and add into the mixing bowl. 1st. Sp. - 2 minutes. Scrape. 2nd. Sp. - 9 minutes to achieve a dough temperature of 35°C Remove dough and allow it to stand for 90 minutes before further processing
Lecithin	✓	0.20	2	
GRAND TOTAL		156.72	1567.20	

*Sprinkle salt if needed before baking

*Spray with oil immediately after baking

Figure A2. Cracker recipe and baking instructions.

PROCESSING AND BAKING INSTRUCTIONS:

Pilot Line Section	Pilot Line Definition	ENZ -2 Enzyme Cracker	Actual
Reduction Roller 1	Gap (mm)	2.42	1.48
	Roller	8.39	5.17
	Conveyor	5.32	don't know
Reduction Roller 2	Gap (mm)	2.02	2.00
	Roller	8.20	4.39
	Conveyor	7.98	3.71
Laminator	Cross Conveyor	5.50	~~7.82~~ 3.0
	Retracting Speed	7.82	10.98
	Retracting Conveyor	15.38	7.82
	Number of layers	6.00	6
Reduction Roller 3	Gap (mm)	1.54	2.86
	Roller	3.32	2.63
	Conveyor	3.02	2.83
Reduction Roller 4	Gap (mm)	1.00	1.11
	Roller	4.39	3.71
	Conveyor	5.17	3.71
Cutting Section	Conveyor Speed	5.12	463
	Transfer Conveyor		20.81
	Rotary Cutter Speed	5.90	3.36
Panner	Panner Speed	15.00	14
Tunnel Oven	Zone 1 (°C)	235 220	227
	Zone 2 (°C)	251 225	227
	Zone 3 (°C)	251 190	196
	Bake Time (min)	5.99	6.01

Figure A3. Cracker processing instructions.

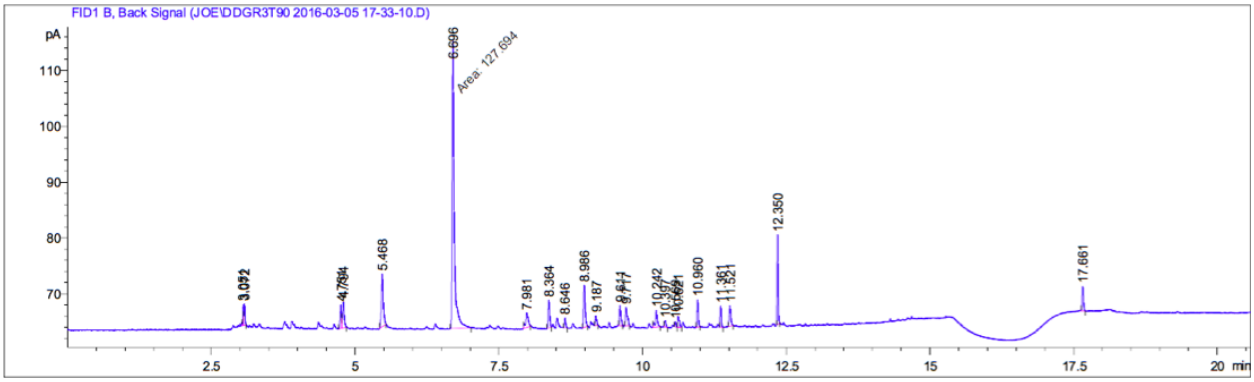


Figure A4. GC chromatogram of headspace analysis of rancid crackers. Hexanal peak is shown at 6.7 minutes.

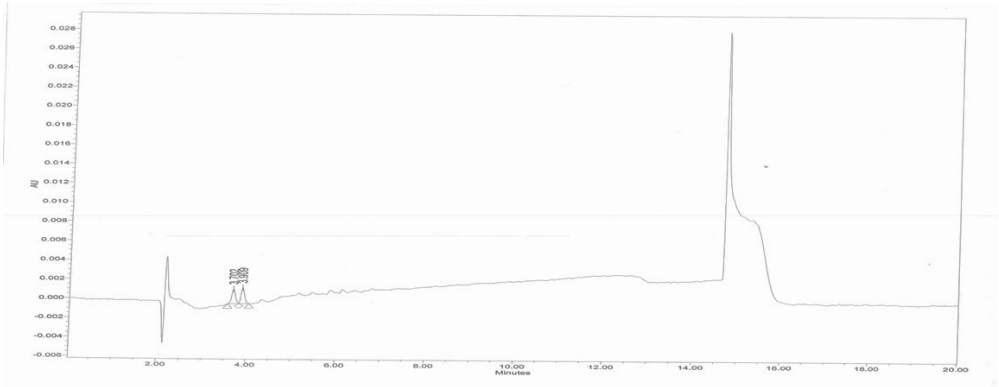


Figure A5. HPLC chromatogram of lutein (3.7 min) and zeaxanthin (3.9 min) standards.

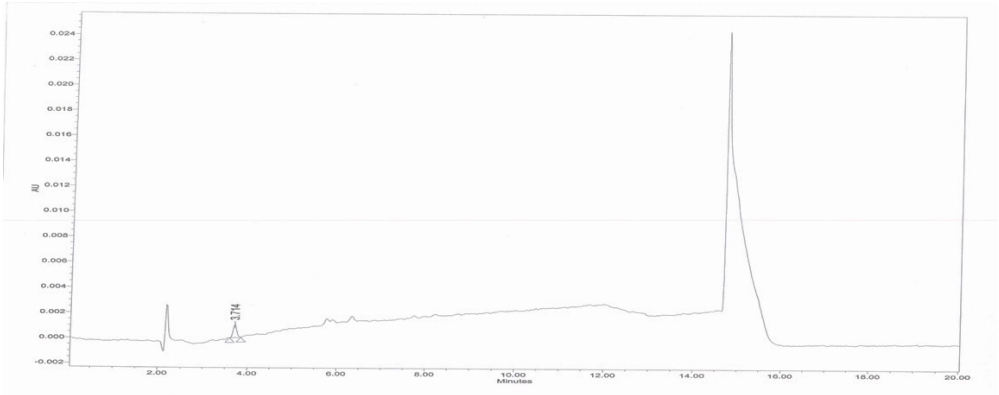


Figure A6. HPLC chromatogram showing carotenoid analysis of cracker oil. Lutein peak is observed at 3.7 minutes.

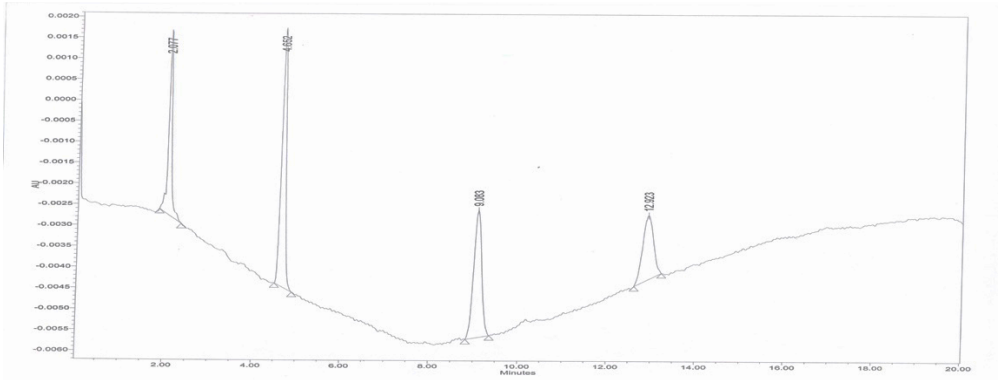


Figure A7. HPLC chromatogram of α-tocopherol (4.7 min), γ-tocopherol, (9.0 min) and δ-tocopherol (12.9 min) standards.

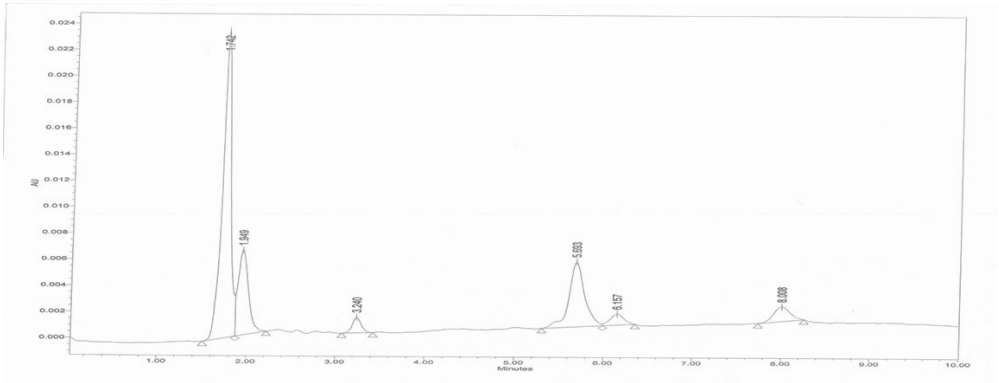


Figure A8. HPLC chromatogram of tocopherol analysis of cracker oil.
Tocopherols shifted from the retention times in standard analysis but tocopherols are identified through observing the fingerprint and analyzing spiked samples. The chromatogram shows α-tocopherols (3.2 min), γ-tocopherol, (5.7 min), γ-tocotrienols (6.2 min) and δ-tocopherol (8.0 min).

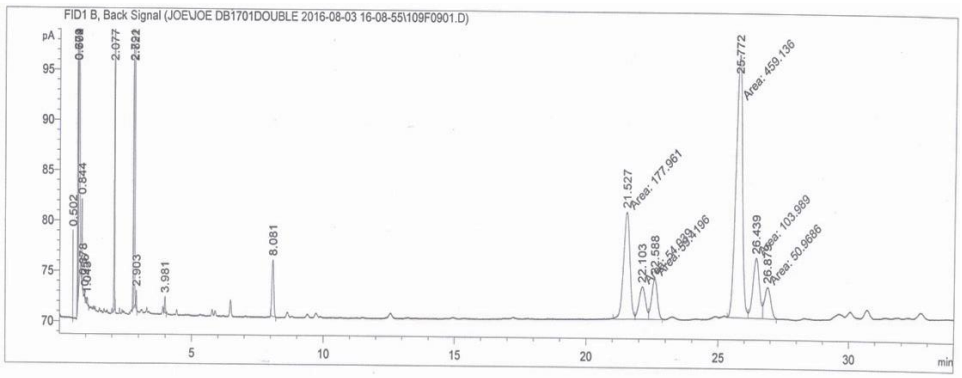


Figure A9. GC chromatogram of phytosterol analysis of cracker oil.
The chromatogram shows campesterol (21.5 min), campestanol, (22.1 min), stigmasterol (22.6 min), β-Sitosterol (25.7 min), sitostanol (26.4 min) and avenasterol (26.9 min).

Table A4. ANOVA of peroxide value in chips.

Source	DF	Sum of Squares	Mean Square	F-value	Pr > F
Batch	2	713	356	48.6	<0.0001
Treatment	2	180	90	12.3	0.0001
Batch*Treatment	2	180	90	9.4	0.0305
Time	8	7160	895	122.2	<0.0001
Batch*Time	8	7160	895	18.0	<0.0001
Treatment*Time	16	163	10	1.4	0.2074
Error	32	234	7		
Total	80	9282			

Table A5. ANOVA of hexanal content in chips.

Source	DF	Sum of Squares	Mean Square	F-value	Pr > F
Batch	2	51.8	25.9	27.1	<0.0001
Treatment	2	27.0	13.5	14.1	<0.0001
Batch*Treatment	2	27.0	13.5	7.4	0.0456
Time	8	227.6	28.5	29.7	<0.0001
Batch*Time	8	227.6	28.5	4.1	0.0083
Treatment*Time	16	29.2	1.8	1.9	0.0589
Error	32	30.6	1.0		
Total	80	485.7			

Table A6. ANOVA of peroxide value in crackers.

Source	DF	Sum of Squares	Mean Square	F-value	Pr > F
Batch	2	2715	1358	31.0	<0.0001
Treatment	2	758	379	8.7	0.0012
Batch*Treatment	2	758	379	2.0	0.2569
Time	7	18447	2635	60.1	<0.0001
Batch*Time	7	18447	2635	6.0	0.0023
Treatment*Time	14	544	39	0.9	0.5812
Error	28	1227	44		
Total	71	30670			

Table A7. ANOVA of hexanal content in crackers.

Source	DF	Sum of Squares	Mean Square	F-value	Pr > F
Batch	2	1111	555	4.7	0.0246
Treatment	2	673	337	2.9	0.0868
Batch*Treatment	2	673	337	1.1	0.4214
Time	4	1343	336	2.9	0.0585
Batch*Time	4	1343	336	1.4	0.3264
Treatment*Time	8	1031	129	1.1	0.4154
Error	16	1885	118		
Total	44	9253			