EFFECTS OF CORN QUALITY AND STORAGE ON DRY GRIND ETHANOL PRODUCTION

BY

DIVYA RAMCHANDRAN

DISSERTATION

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Doctoral Committee:

Professor Vijay Singh
Associate Professor Kent D. Rausch
Professor Emeritus M. E. Tumbleson
Professor Stephen P. Moose
Research Chemist Mila Hojilla-Evangelista, NCAUR, ARS, USDA

Abstract

Corn dry grind industry is the major contributor of ethanol production in the US. Ethanol plants incur economic losses due to seasonal variations in ethanol yields. Ethanol yields typically are low during the first month after harvest, increase for the next six to seven months and decrease again three to four months before next harvest. There is little published information on factors causing variation in dry grind ethanol concentrations. One possible cause associated with ethanol yield variability is incoming grain quality. The main objectives of this study were to quantify ethanol yield variation over time at a dry grind facility, evaluate relationships among corn quality attributes and ethanol yields and determine physiologic changes in corn protein quality during storage and its effects on ethanol yields.

Corn from a Midwestern ethanol plant (commodity corn) and an identity preserved corn hybrid from a seed company (control corn stored at 4° C) were used to study the effects of incoming corn on ethanol concentrations. Ethanol concentrations were determined every two weeks for one year using conventional dry grind procedure. Variations in ethanol concentrations were significant and variability patterns for commodity and control corn followed the same trend. Highest ethanol concentrations were seen in the month of January. Variation with control corn suggested that storage time is a significant factor affecting ethanol concentrations. Effects of different enzyme treatments on mean ethanol concentration over a year were evaluated. Two liquefaction enzymes (optimum pH – 5.8 and 5.1, respectively), two saccharification enzymes (optimum pH – 5.0) and one protease were used in five enzyme treatments (I, II, III, IV and V). Final ethanol concentration with enzyme treatment V was (17.5 ± 0.486)% v/v. This was 0.6% higher than enzyme treatment I resulting in an additional ethanol production of 600,000

gallons/year in a 100 million gallon/year ethanol plant. Using effective enzymes increases overall dry grind ethanol production and ethanol plant profitability.

Commodity corn samples were analyzed for physical quality parameters (test weight, kernel weight, true density, percent stress cracks and moisture content) and composition (starch, protein, oil and soluble sugars contents). There were variations in corn quality parameters and ethanol concentrations. Correlation coefficients were significant but low ($-0.50 < r < 0.50$) between starch content and final ethanol concentrations (72 hr) and total soluble sugar content and ethanol concentrations at 72 and 48 hr. Ethanol concentrations (at 24, 48 and 72 hr) were predicted as a function of a combination of grain quality factors using multiple regression methods; however, the R^2 values obtained were low. Ethanol concentration variations were not related to physical and chemical composition quality factors.

Physiologic changes in corn protein quality (soluble protein contents, initial free amino nitrogen (FAN) content and susceptibility to enzyme hydrolysis) during storage at refrigerated and ambient conditions were investigated. Albumin, prolamin, glutelin contents and initial FAN contents of corn slurry varied with storage time; however, there were no effects of storage temperatures (ranging from -7 to 23°C) on soluble protein and FAN contents. Albumin content decreased; whereas, prolamin content increased from wk 8 to 40. Susceptibility to enzyme hydrolysis was affected during storage; highest rate of protein hydrolysis was observed during wk 20. Variation in ethanol yields for corn stored at ambient and refrigerated conditions followed similar trends. Final ethanol yields (72 hr) had no correlations with protein quality attributes; however, ethanol yields at 24 hr correlated with glutelin ($r = -0.76$) and prolamin ($r = +0.74$) content.

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Chapter 1

Introduction

Depletion of fossil fuels, reducing the dependence on foreign oil and mitigating greenhouse gas emissions have been the main motivations for driving the fuel ethanol industry in the US. Annual US ethanol production was 14.7 billion gallons in 2015 (RFA 2016). The major feedstock for ethanol production in the US is corn grain. Annual production of corn was 13.6 billion bushels of corn in 2015 of which 4.1 billion bushels were used for fuel ethanol production (NCGA 2016). The 2007 Energy and Independence Act (EISA) mandated Renewable Fuel Standard (RFS) with a target of 36 billion gallons of ethanol production by 2022; however, ethanol production from corn grain was limited to 15 billion gallons. Concerns on grain based ethanol production, such as reduced production of other grains, increased cost of food markets and energy usage led to the cap on corn ethanol production implying the remaining amount must be met by advanced biofuels, including cellulosic ethanol (Yacobucci and Capehart 2009). However, corn grain based ethanol continues to aid in achieving the renewable fuel target.

In 2008, the corn dry grind industry accounted for 86% of ethanol production in the US (Mueller 2010). In the dry grind process, whole corn is ground and corn starch is hydrolyzed to glucose using enzymes; glucose is fermented further to ethanol by yeast. The rest of the kernel components are recovered in the coproduct, distillers dried grains with solubles (DDGS) which is used primarily as animal food. Profitability of this industry largely depends on consistent ethanol and DDGS production. Ethanol yield variations have been reported by dry grind processors. Ethanol yields typically are low during the first month after harvest, increase for the next six to seven mo and decrease again three to four mo before next harvest (Singh 2012). This study was

conducted at a dry grind ethanol plant for a period of 7 years *(unpublished data)*. The source of this variation is not known; it is possible that the variation in ethanol yields is due to the variation in incoming grain quality. In a 100 million gallon ethanol plant, an average 3% loss of ethanol yield due to grain quality is equivalent to 3 million gallons of ethanol per year (Singh 2012). The US Federal Grain Inspection Service (FGIS) has established grading criteria for corn based on four quality attributes: test weight, broken corn and foreign material (BCFM), heat damage and total damage. However, several other factors govern the quality of grain such as true density, percent stress cracks, moisture content and chemical composition.

In the conventional dry grind process, starch is hydrolyzed into shorter chain oligosaccharides during liquefaction by the action of alpha amylase at high temperatures (85 to 105°C) for 90 to 120 min. Liquefied mash is treated with glucoamylase converting oligosaccharides into mono, di and trisaccharides, such as glucose, fructose, maltose and maltotriose, which are consumed by yeast to produce ethanol (Wang et al. 2005). In addition to amylases, using proteases in modified dry grind processes have resulted in higher ethanol yields (Vidal et al. 2009; Vidal et al. 2011). Use of proteases resulted in more efficient starch separation from proteins in the wet milling process (Johnston and Singh 2001). Addition of proteases helps in hydrolysis of the protein matrix surrounding the starch granules thereby increasing accessibility of amylases to starch granules. Proteolysis of the protein matrix also results in production of free amino nitrogen (FAN) which is utilized by yeast during fermentation (Vidal et al. 2011). It is possible that certain enzyme treatments consisting of specific amylases and proteases can aid in reducing the overall variability of ethanol yields or increase the mean ethanol yield, thereby reducing economic losses of the dry grind industry.

The observed variability in grain quality is attributable to three main factors: genetics, growing environment and postharvest practices (Singh et al. 2012). While the effects of hybrid variability, planting locations and postharvest drying of corn on dry grind ethanol yields have been studied (Singh et al. 2005; Haefele et al. 2004), there are few studies available on the effects of postharvest storage on ethanol yields. Effects of different drying conditions on ethanol yields were reported by Medić et al. (2011) and Murthy et al. (2012); high temperature drying (above 85°C) had a detrimental effect on ethanol yields. In the dry grind process, not all starch is hydrolyzed to sugars. The left over starch is known as unconverted or residual starch and is recovered in the DDGS (Sharma et al. 2010). Higher residual starch in DDGS can correspond to lower ethanol production. Plumier et al. (2015) reported the effects of storage time and temperature on unreacted starch contents from the dry grind process and found that storage time had an effect on unreacted starch contents. In 2011, unreacted starch contents decreased during the first 10 wk after harvest and thereafter, had an increasing trend from wk 10 to 48 (Plumier et al. 2015). Physiologic changes in corn due to long term storage may have an influence on dry grind processing and impact ethanol yields. Availability of starch granules to be converted to ethanol is dependent on the protein matrix surrounding the starch granules. Corn proteins are categorized into four main groups- albumins, globulins, prolamins (primarily zeins) and glutelins, depending on their solubility in different solvents. Changes in different protein classes during storage have been reported in rice (Chrastil 1990; Chrastil and Zarins 1992) and to a lesser extent in corn (McDonough et al. 2004); however, it is not known if these changes correspond with ethanol yield variation. The main goal of this dissertation was to understand seasonal variations in dry grind ethanol yields over time. Specific objectives of this study were to:

1) Quantify ethanol concentration variation at a dry grind facility for a year and determine effects of using enzyme treatments on variations in ethanol and mean ethanol concentrations.
2) Evaluate relationships between incoming corn quality parameters and ethanol concentrations.
3) Investigate effects of long term storage of corn on dry grind ethanol concentrations, examine physiologic changes in corn protein matrix during storage and determine relationships among protein quality parameters and ethanol concentrations.

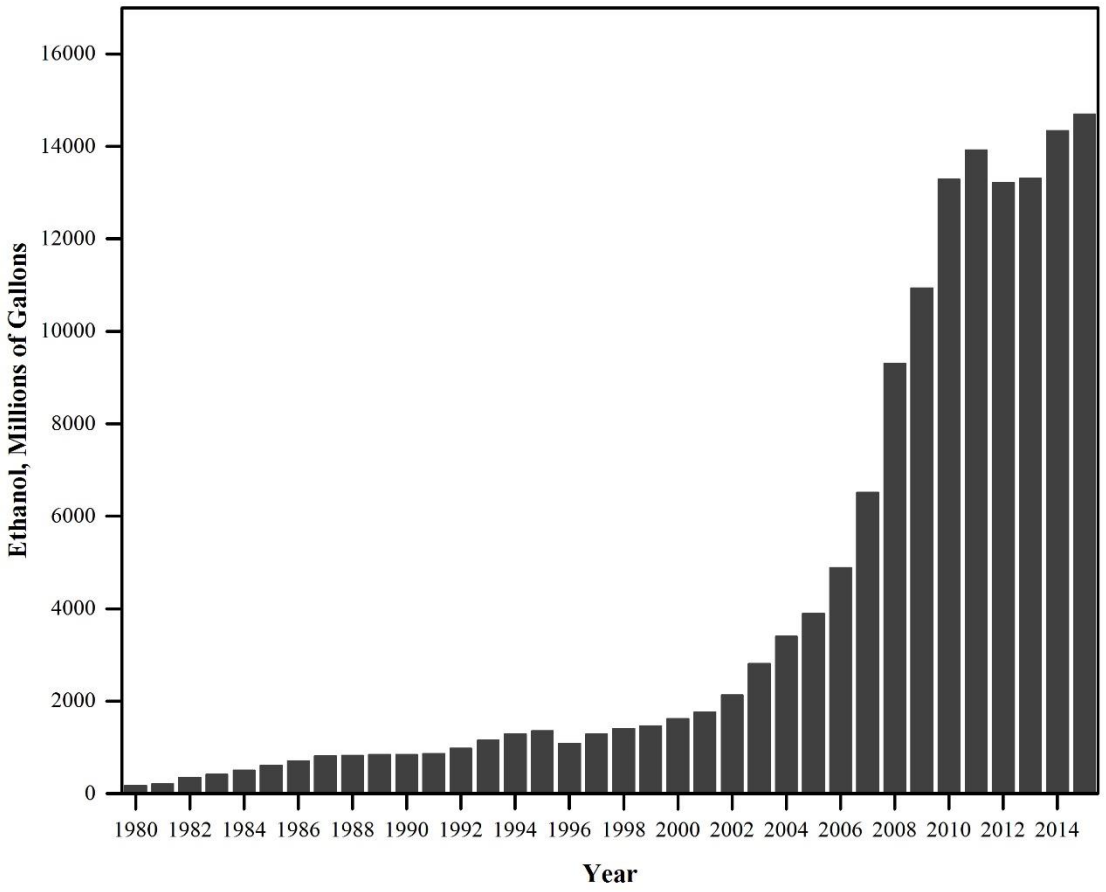
Chapter 2

Literature Review

2.1. Fuel Ethanol Market in the US

The use of ethanol as a transportation fuel dates back to 1908 when Henry Ford built the "Model T" vehicle with an ethanol powered engine. His vision was to "*build a vehicle affordable to the working family and powered by a fuel that would boost the rural economy*" (Rosillo-Calle and Walter 2006). The ethanol industry in the US has expanded from 175 million gallons in the 1980's to 14.7 billion gallons in 2015 (Fig. 2.1) (RFA 2016). The Clean Air Amendment Act passed in 1990, which required use of oxygenated fuel and reformulated gasoline to reduce carbon monoxide emissions, spurred ethanol industry growth (Dien et al. 2002). Methyl tertiary butyl ether (MTBE) was used as a gasoline oxygenate but was identified as a major ground water contaminant (Dien et al. 2002). MTBE use was banned in several states of the US in 2002 which led to using ethanol as gasoline additive. The Renewable Fuel Standard (RFS) was passed in 2005 by the Energy Policy Act which further promoted the ethanol industry.

Currently, corn grain is the main feedstock (>95%) for ethanol production in the US; whereas, the rest of the production comes from other grains such as wheat, barley and milo, as well as cheese whey and beverage residue (Soloman et al. 2007). The two main biological conversion processes practiced in the US for the conversion of corn starch to ethanol are the dry grind and wet milling processes.



Source: Adapted from RFA 2016

Fig. 2.1. Historic ethanol production in the US.

2.2. Dry Grind Process

With the conventional dry grind process, 1 bu corn (56 lb or 25.4 kg) produces 2.8 gal (10.6 L) ethanol, 17 lb (7.2 kg) DDGS and 17 lb (7.2 kg) carbon dioxide (CO_2). A schematic of the dry grind process is presented in Fig. 2.2. Whole corn is ground using a hammer mill to reduce the particle size to ≤ 4 mm and make starch more accessible to enzyme hydrolysis. Ground corn is mixed with water (fresh water and recycled water from thin stillage) to make a slurry of 20 to 40% solids (Dale and Tyner 2006). Slurry is passed through a jet cooker (>100°C

for 5 to 8 min) where one third of the alpha amylase dose is added. Alpha amylase is an endo enzyme that hydrolyzes α-1,4 bonds of starch and helps in reducing slurry viscosity and breaking down starch granules to dextrins. High temperature cooking exceeds the gelatinization temperature of starch which increases the speed and efficiency of starch hydrolysis. Additional thermostable alpha amylase is added to continue the liquefaction process (≤ 85°C, pH 5 to 6) for 60 to 90 min. The resulting product, called mash, undergoes the saccharification process where glucoamylase (an exo enzyme that hydrolyzes α-1,6 and α-1,4 linkages of starch) is added to hydrolyze dextrins to glucose. The saccharification step usually is combined with the fermentation process and is called simultaneous saccharification and fermentation (SSF). Yeast is added simultaneously with glucoamylase and sugars produced from hydrolysis of dextrins are metabolized to produce ethanol and CO_2. The SSF process is the adopted practice in dry grind industries as it prevents high glucose concentrations and reduces osmotic stress on yeast. The SSF process is more energy efficient and lowers the chance of microbial contamination (Bothast and Schlicher 2005).This process usually takes place at 32°C and pH 4.5 to 5.0 for 48 to 72 hr. Beer produced after fermentation is distilled using distillation and stripping columns and passed through molecular sieves to remove residual water. Resulting product is neat ethanol (200 proof) which is denatured with gasoline to produce fuel grade ethanol. Whole stillage from distillation columns is centrifuged to separate wet grains (solid fraction) and thin stillage (liquid fraction). A part of thin stillage (30 to 50%) known as backset is recycled back to the slurry. Remaining thin stillage is concentrated and dried with wet grains to produce distillers dried grains with solubles (DDGS) (Bothast and Schlicher 2005).

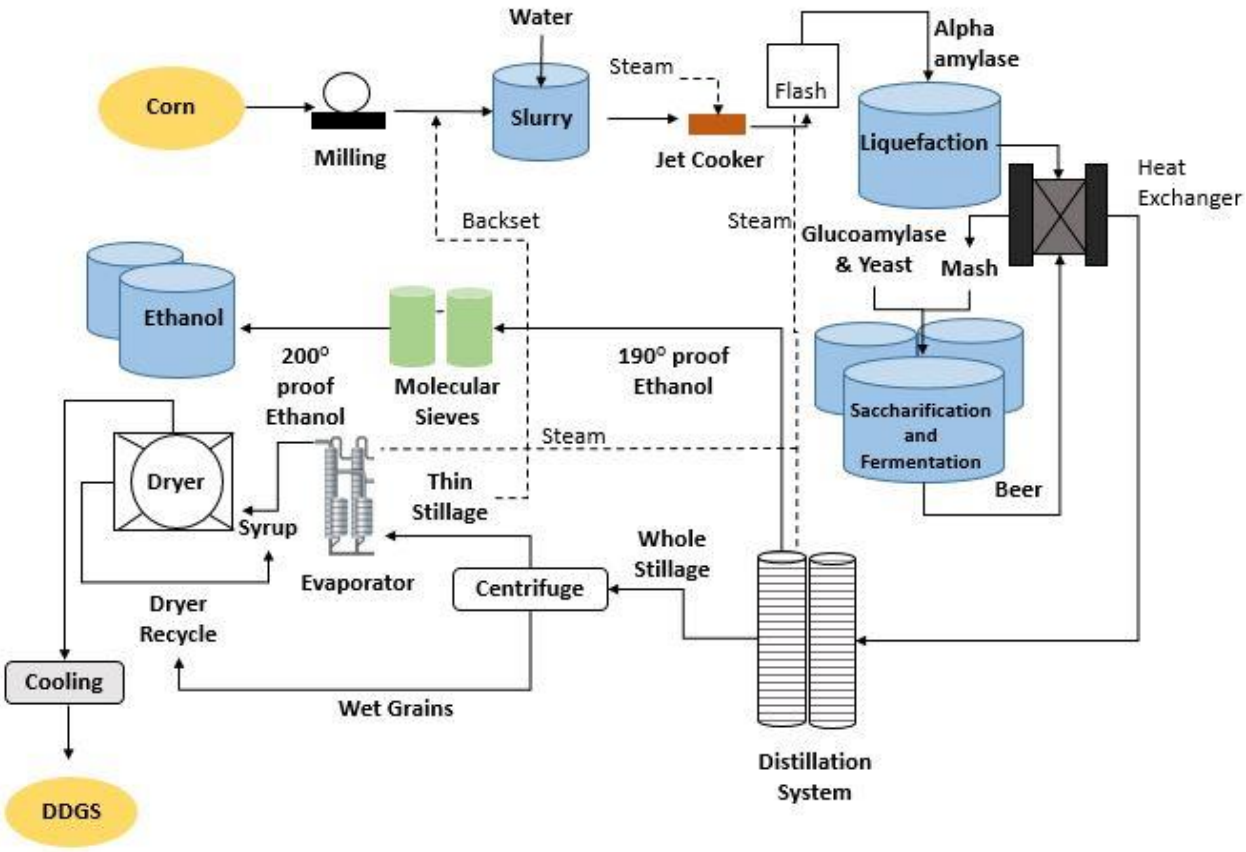


Fig. 2.2. Schematic of the dry grind process.

Being the main products of the dry grind process, sustainability of dry grind plants is dependent on the marketing of ethanol and DDGS. Consistent ethanol and coproduct yields are essential for economic viability of dry grind plants. One of the primary sources of variation is the quality of corn coming to the dry grind plant. Three main factors affecting corn quality are: genetics, growing environment and postharvest practices.

2.3. Extent of variability on product yields due to genetics, environment and postharvest practices

Corn hybrid variability and planting location contributes to the variability in ethanol yields (Singh and Graber 2005). Eighteen corn hybrids grown at eight locations were processed

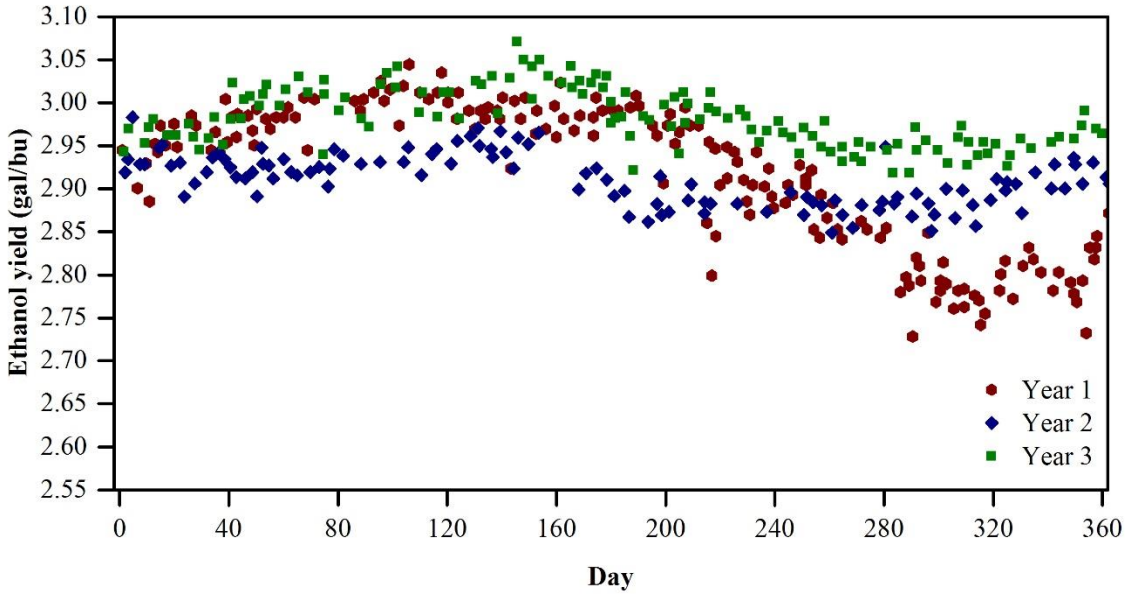
using a laboratory dry grind procedure to study effects of hybrid variability and planting locations on final ethanol yields. Ethanol concentrations varied from 11.2 to 13.8% v/v (with 25% solids content) representing a variability of 22.7% among hybrids across growth locations. Hybrid variation in ethanol concentration (12.9 to 14.6% v/v, with 24.5% solids content) has been reported with enzymatic milling dry grind process (Sharma et al. 2006). There are studies in the literature on effects of corn and other crop hybrids on ethanol yields; however, it is not clear how genetics affect ethanol yields (Singh et al. 2012). Different hybrids were evaluated for the selection of factors responsible for high wet milled starch yields; starch extractability was lower for certain hybrids than others (Zehr et al. 1996). Similarly, different corn hybrids were evaluated to study the effects of starch contents on dry grind fermentation efficiency (Dien et al. 2002). Highest starch producing hybrid did not produce highest fermentation efficiency. Nine sorghum cultivars were evaluated for ethanol fermentation efficiency and higher fermentation efficiency was obtained with a waxy sorghum cultivar (Wu et al. 2008).

Growing environment includes cultivation site, agronomic practices and weather conditions and has an effect on dry grind ethanol concentrations (Singh and Graeber 2005). Hybrids were ranked based on their ethanol production for all planting locations; corn hybrids giving high ethanol concentrations at one growth location did not have the same result in other growth locations. Interactions among genetics and planting locations have been reported (Reicks et al. 2009; Wu et al. 2008). Location affected chemical composition (starch, fat, protein, crude fiber and ash contents) and kernel hardness of sorghum cultivars and ethanol yields; however, no effects of locations on fermentation efficiency were found (Wu et al. 2008).

Effects of postharvest drying of corn on ethanol yields have been reported (Reicks et al. 2009). Corn harvest moisture contents (20 and 25%) and drying temperatures (38, 52 and 60°C)

resulted in interaction effects on ethanol yields. Ethanol yields for corn harvested at 25% moisture decreased as drying temperatures increased from 38 to 60°C. This effect was not seen for corn harvested at 20% moisture content. This typically was attributed to the longer heat exposure during drying for high moisture corn. Drying beyond 60°C reduced ethanol concentrations from corn regardless of their harvest moisture contents (Reicks et al. 2009).

After drying, corn is stored to ensure a continuous supply of grain for processors throughout the year (Singh et al. 1998). Storage time ranges from a few days to two years. There are studies on the effects of storage time on wet milling starch yields. Roushdi et al. (1979) reported a decrease in starch yield by 1.1, 1.9 and 2.5% as storage time increased to 3, 6, and 9 mo, respectively. Variation in extractable starch yields with stored corn was reported by Singh et al. (1998). Extractable starch yields across storage time were lower for corn stored at ambient conditions compared to cold room (4°C) for two of the three tested hybrids grown in 1994. However, starch yield for one of the same hybrids grown in another year (1996) did not vary across storage temperature. Starch yields of all the tested hybrids were not affected by storage time at a particular storage condition (4°C or ambient) (Singh et al. 1998). Ethanol production for incoming corn at a dry grind ethanol plant has been monitored for seven years (2003 to 2009) by a seed company (Elliot et al. 2010 and Singh et al. 2012). Data for three crop years from this study are presented in Fig 2.3. Ethanol yields were low during the first month after harvest of new corn. Ethanol yields were higher for the next 6 to 7 months and decreased thereafter**.** Mean ethanol yields in 2008 during first, second, third and fourth quarters were 2.96, 2.99, 2.92 and 2.80 gal/bu, respectively. Overall, range of ethanol yields was from 2.72 to 3.04 gal/bu representing a variation of 11.6% (Elliot et al. 2010). This has been the only reported study on estimating ethanol yield variation with time at a dry grind ethanol plant.



Source: Adapted from Elliot et al., 2010

Fig. 2.3. Variations in ethanol yields monitored at a dry grind plant.

2.4. New crop season phenomenon

'New crop season phenomenon' has been defined by Shelke et al. (1992a) as the uncontrolled variation in product quality due to the introduction of freshly harvested wheat. As a result, processors have to make adjustments in formulations and modify processes to rectify this problem and maintain product quality. This process is laborious and time consuming and often unsuccessful. Millers in the wheat industry incorporate freshly harvested wheat into the mill mix either by storing the new wheat for two to three months before blending it or by blending 5 to 15% of new wheat with the old wheat mix (Posner and Deyoe 1986). Blending freshly harvested wheat with the old wheat mix was observed to be beneficial to reduce the fluctuations of the mill. Storage time of 14 wk was the cut off time after which storage cost overrode the improvements in milling value by blending new wheat with old wheat mix (Posner and Deyoe 1986). The

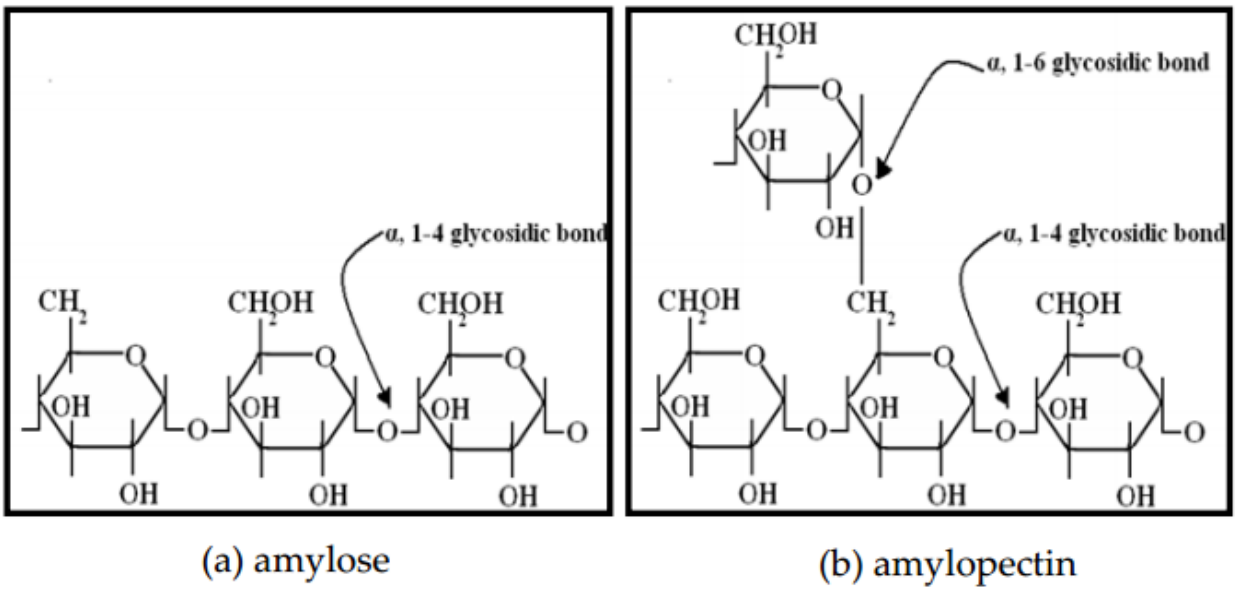
effects of whole wheat and wheat flour age (soft red winter wheat) on cake baking quality (batter specific gravity, cake volume and uniformity) were evaluated; cake characteristics improved with wheat and wheat flour age. Cake volume and symmetry increased; whereas, batter specific gravity decreased as a function of wheat and flour age (Shelke et al. 1992a). Similarly, in corn processing industries, wet millers reported difficulties in processing freshly harvested corn compared to two to three mo old corn such as increased foaming during steeping and other process adjustments to maintain milling quality (Singh et al. 1998).

2.5. Enzymatic hydrolysis of starch in the dry grind process

Starch consists of α-glucose units linked together either by α-1,4 glycosidic bonds or α-1,6 glycosidic bonds. The two types of glucose polymers present in starch are amylose and amylopectin. Amylose is a linear chain of glucose units (up to 6000 glucose units) with α-1,4 glycosidic bonds. Amylopectin is a branched polymer consisting of short chain α-1,4 linked linear chains of 10 to 60 glucose units and α-1,6 side chains with 15 to 45 glucose units (Fig. 2.4) (Fallal et al. 2012).

The main class of enzymes involved in starch hydrolysis are amylases which are classified into three major categories based on their mode of action: endoamylases, exoamylases and debranching enzymes. Alpha amylase belongs to the class of endoenzymes (also known as liquefying enzymes) and hydrolyzes α-1,4 glycosidic bonds in the inner regions of starch resulting in rapid decrease in viscosity of the starch solution. Resulting products of alpha amylase action are linear and branched oligosaccharides (Fallal et al. 2012). Exoamylases, also known as ‘saccharifying enzymes’, cleave α-1,4 glycosidic bonds from the nonreducing ends of starch chains by successive removal of glucose or maltose in a stepwise manner (Muralikrishna and Nirmala 2005). Glucoamylases and beta amylases belong to this category. Glucoamylase

(also known as amyloglucosidase or α-glucosidase) cleaves α-1,4 bonds of starch to produce glucose but also can cleave α-1,6 bonds at a slower rate. Beta amylase cleaves α-1,4 bonds of starch or glycogen to produce maltose. Debranching enzymes consists of pullulanases capable of cleaving α-1,6 linkages of starch. Pullulanases are classified into two categories: type I and type II. Type I pullulanases cleave only α-1,6 linkages and produces maltotriose and linear oligosaccharides; whereas, Type II pullulanases hydrolyze α-1,4 linkages in addition to α-1,6 linkages and produces glucose, maltose and maltotriose (Bertoldo and Antranikian 2002).



Source: Fallal et al., 2012

Fig. 2.4. Structure of amylose and amylopectin.

2.6. Grain quality and its effects on end product properties

Understanding the relationships of grain quality factors to its end use value is crucial as it will be helpful to predict the end product properties at the time of purchase. It also provides valuable information by identifying the important traits which can be used in the development of improved hybrid varieties that are easier to process and result in high value products in greater proportions (Fox et al. 1992).

Efforts have been made in establishing interrelationships among grain quality parameters and wet milled coproduct yields. Weller et al. (1988) studied the relationships between grain quality parameters such as test weight, kernel density, stress cracks percentage, ethanol soluble protein, gross composition, moisture content and starch recovery in the wet milling process. Starch recovery was calculated as the ratio of the total weight of starch recovered from the wet milling process to the total weight of starch present in the corn. Starch recovery was found to be a function of starch content, test weight and ethanol soluble protein ($R^2 = 0.60$). Fox et al. (1992) studied different characteristics of corn (physical properties and composition) to predict starch yield and protein content in recovered starch from the wet milling process. Physical properties such as 1000 grain weight, test weight, kernel density, kernel hardness, water absorption index and breakage susceptibility and proximate composition factors such as starch, protein, oil and grain moisture contents were analyzed. No single grain quality trait accounted for more than 40% variation in starch yields or more than 60% variation in protein content of recovered starch.

Considering multiple factors together, starch yields were best predicted by a model including corn protein content and test weight ($R^2 = 0.61$) (Fox et al. 1992). Similarly, protein content in recovered starch was best predicted as a function of grain protein and oil contents ($R^2 = 0.66$). Correlations among wet milling attributes of sorghum (starch, gluten and fiber

yields, starch and protein recoveries, protein content of starch and gluten fractions) and properties such as kernel weight, density, water absorption rate, test weight and proximate composition (starch, protein, oil contents) were studied by Buffo et al. (1998). Initial water absorption rate correlated positively with gluten yield (r = 0.43), starch recovery (r = 0.48) and protein recovery (r = 0.52) indicating faster absorption of steeping solution by the kernel causes efficient disruption of starch and protein matrix and releases starch and protein easily. Gluten yield had correlations with 1000 kernel weight (r = -0.40) and grain starch content (r = -0.51). Correlation coefficients among quality factors and wet milling attributes were found to be low. Relationships among multiple quality factors and wet milling attributes were not evaluated.

There are few studies in the literature relating the quality of incoming corn to dry grind coproducts (ethanol and DDGS). Relationships among compositions of corn (fat, protein and starch contents) and DDGS (fat, protein, fiber and starch contents) were studied by Belyea et al. (2004) and no correlations were found. Variations in composition of DDGS were quantified by Belyea et al. (2010) where they evaluated the effects of plants and sampling periods. Most nutrient concentrations were affected by plant × period interactions, suggesting fermentation batches were a more important source of variation compared to plants or periods alone. Relationships among grain quality factors and dry grind ethanol yields are not well documented. Effects of sorghum starch content on ethanol yields have been reported (Zhan et al. 2003 and Wu et al. 2007). Zhan et al. (2003) evaluated 16 hybrids grown at 2 locations and reported a positive correlation between starch content and ethanol yield; however, the correlation coefficient was low (r = 0.012). Wu et al. (2007) studied 70 sorghum genotypes and reported a strong positive correlation (r = 0.88) between starch content and ethanol yields. Awole et al. (2012) evaluated the relationship among wheat quality factors (starch and protein contents, grain weight, grain

density and grain size parameters) and ethanol yields. Correlation between starch concentration and ethanol yield was low ($R^2 = 0.16$), while protein content had a negative correlation with ethanol yield ($R^2 = 0.53$). Protein and 1,000 grain weight factors together accounted for ethanol yield variation with $R^2 = 0.67$. Highest variation ($R^2 = 0.82$) in ethanol yield was explained when variety of the grain and cultivation site were considered together with protein content and 1,000 grain weight. Without including site and variety of the grain, variation in ethanol yield was best explained by a model consisting of protein content, starch content, packing efficiency, grain length and perimeter ($R^2 = 0.73$, $P < 0.0001$). In a study conducted by Kindred et al. (2008), a combination of grain starch and protein contents explained 69.7% of variation in ethanol yields from winter wheat.

2.7. Starch-protein interactions and its effect on starch conversion

While it is the starch portion of corn grain that is converted to ethanol, fermentability of corn depends on several other factors such as size and shape of starch granule, amylose:amylopectin ratio and starch-protein interactions. The four main components of a corn kernel structure are endosperm, pericarp, germ and tip cap. Starch granules are concentrated mainly in the endosperm fraction of corn kernel and are held together by a protein matrix. The mature endosperm of a yellow dent corn consists of a soft central core, known as the soft or floury endosperm surrounded by a glassy appearing region known as the horny or hard endosperm (Watson 1987). Recovery of starch granules from soft/floury endosperm is easier than from hard/horny endosperm, primarily due to the type of protein matrix surrounding it. Soft endosperm primarily consists of large spherical starch granules surrounded by a thin protein matrix which ruptures during kernel drying resulting in air spaces and easy recovery of starch granules. Hard endosperm is surrounded by a thicker protein matrix, which shrinks during

drying, resulting in polygonal shaped starch granules. The amount and type of protein determines endosperm hardness and starch recovery (Dombrink-Kurtzman and Bietz 1993).

Protein solubility is considered to be an excellent indicator of protein functionality (Hung and Zayas 1992). Proteins in corn kernel were first classified by Osborne (1924) into four groups based on their solubilities: albumins dissolve in pure water, globulins are soluble in dilute salt solutions, prolamins dissolve in 70% ethanol solvent and glutelins are soluble in dilute acid or base. Albumins and globulins are biologically active proteins such as enzymes, membrane proteins, glycoproteins and nucleoproteins. Prolamins and glutelins are storage proteins; prolamins are arranged to form protein bodies and consist mostly of zeins; whereas, glutelins form the protein matrix and are believed to hold the starch granules together in the endosperm (Christianson 1969).

Factors affecting starch digestion (amount of starch hydrolyzed to glucose relative to initial starch present in the sample) by ruminal microbes were studied by McAllister et al. (1993). Ground corn and isolated corn starch samples were inoculated with ruminal microbes (isolated from ruminal fluid from a Holstein steer) for 24 hr. Starch digestion was found to be lower for ground corn compared to isolated corn starch samples. This implied that starch digestion was more influenced by the structural components within the corn endosperm rather than properties of starch granules alone. Pretreating the ground corn sample with protease resulted in higher starch digestion after 24 hr of incubation compared to the untreated sample further indicating the role of protein matrix on starch conversion (McAllister et al. 1993). Effects of protein distribution in corn endosperm of different corn types (waxy, high amylose and normal dent corn) on ruminal degradation of starch were studied by Philippeau et al. (1998). Ruminal starch degradation was studied in situ by supplying ground corn in nylon bags directly

into the rumen and taking out samples after different time intervals to analyze the starch content. There was no relationship between total protein content and ruminal starch degradability but accessibility of starch granules to ruminal microbes was related to protein distribution. Rapidly degrading starch fraction was linked positively to salt soluble proteins (globulins); whereas, slowly degradable fraction of starch was correlated positively with glutelin contents. Effect of high temperature cooking (heating slurry at 95°C for 10 min) on in vitro protein digestibility (protein digested by pepsin enzyme) of maize and sorghum were studied by Duodu et al. (2002). Protein digestibility was measured as the difference between total and residual nitrogen as a percentage of total nitrogen. Cooking reduced the protein digestibility of sorghum but not maize; treatment with α-amylase during cooking improved protein digestibility of both corn and sorghum indicating starch protein interactions (Duodu et al. 2002). Further analysis of the effects of cooking parameters (temperature and time) on maize and sorghum starch digestibility was conducted by Ezeogu et al. (2005). In this study, starch digestibility was determined by incubating the samples with alpha and glucoamylase enzymes and then measuring the amount of starch hydrolyzed relative to the initial amount of starch present. Polymerization of prolamin protein network during cooking, possibly due to disulfide bond crosslinking, reduced starch digestibility in sorghum and to a lesser extent, in maize (Ezeogu et al. 2005). Similar results were reported by Zhao et al. (2008), where they evaluated the effects of high temperature mashing on sorghum ethanol fermentation. Ethanol yields and conversion efficiency were correlated positively with protein solubility. During mashing, strong protein weblike microstructures were formed which had negative correlation with conversion efficiency due to the entrapment of starch granules inside the protein structures. Protein solubility was found to be low for cultivars with lowest conversion efficiency (Zhao et al. 2008).

Use of proteases in the dry grind process has elucidated further the role of starch protein interactions on starch conversion. Proteases hydrolyze protein matrix and increase the accessibility of starch granules to amylolytic enzymes (Vidal et al. 2011). Protease treatment during dry grind fermentation resulted in increased ethanol yields and rates of fermentation (Johnston and McAloon 2014). Addition of protease prior to liquefaction resulted in higher starch hydrolysis rates for both sorghum and corn (Pérez-Carillo and Serna Saldívar 2007).

2.8. Role of free amino nitrogen (FAN) in yeast fermentation

Free amino nitrogen consists of individual amino acids, short peptides (≤ tripeptides) and ammonium ions which are used as a nitrogen source for yeast growth during fermentation. *Saccharomyces cerevisiae* can assimilate only amino acids and small molecular weight peptides (typically ≤ tripeptides) but not proteins (Pérez-Carillo and Serna-Saldívar 2007). In brewing, amount of usable nitrogen must be in the range of 140 to 150 mg/L with normal gravity wort (12° Plato where 1°P = 1 g of sugar as sucrose per 100 g of wort at 20°C) for achieving adequate fermentation rates and complete utilization of fermentable sugars (O'Connor-Cox et al. 1991). Factors affecting yeast growth, rates of fermentation and final alcohol yields are determined not only by the amount of FAN content but also the source of nitrogen. Complete fermentation was possible for wheat mashes (16% solids content) containing only 58 mg/L FAN content in 8 days compared to brewing worts (where this level of FAN content is very low). This was indicative that there were qualitative differences in FAN of wheat mashes and worts (Thomas and Ingledew 1990). Shorter fermentation rates were observed with wheat mashes when amino acids such as casamino acids or glutamic acid were used as a nitrogen source. Glycine had an inhibitory effect on fermentation rates; rates were slower for mash supplemented with glycine compared to unsupplemented controls (Thomas and Ingledew 1990). It was noted that lysine when used alone

with wheat mash inhibited yeast growth but when supplemented with other amino acids (such as arginine, asparagine, glutamine, methionine and histidine) increased the fermentation rates (Thomas and Ingledew 1992).

Effectiveness of proteases can be assessed by measuring the breakdown of proteins into amino acids (FAN measurement) (Vidal et al. 2009). This method also can be applied to determine the nature of protein hydrolysis in cereal grains. FAN acts as a nitrogen source for yeast during fermentation. FAN content of the original sample can be crucial to determine its performance in ethanol fermentation. FAN content of the original sorghum sample had a positive correlation with ethanol fermentation efficiency at 30 hr ($R^2 = 0.90$) (Yan et al. 2011). Effects of germ derived FAN contents on corn endosperm fermentation was studied by Vidal et al. (2011); relationships between initial FAN contents of mash and ethanol yields followed a second order regression curve with R^2 value of 0.72.

The standard procedure using ninhydrin colorimetric assay was developed by European Brewery Convention (EBC) for determining the amount of total free α-amino nitrogen in brewing worts (Lie 1973). The principle of the procedure was as follows: diluted sample is mixed with ninhydrin reagent at pH 6.7 and the resulting color is measured at 570 nm. Ninhydrin, being an oxidizing agent, causes oxidative decarboxylation of α-amino acids resulting in CO_2, NH_3 and an aldehyde with one less carbon atom than the parent amino acid. A blue complex is formed by the reaction of the reduced ninhydrin, unreduced ninhydrin and ammonia (Lie 1973).

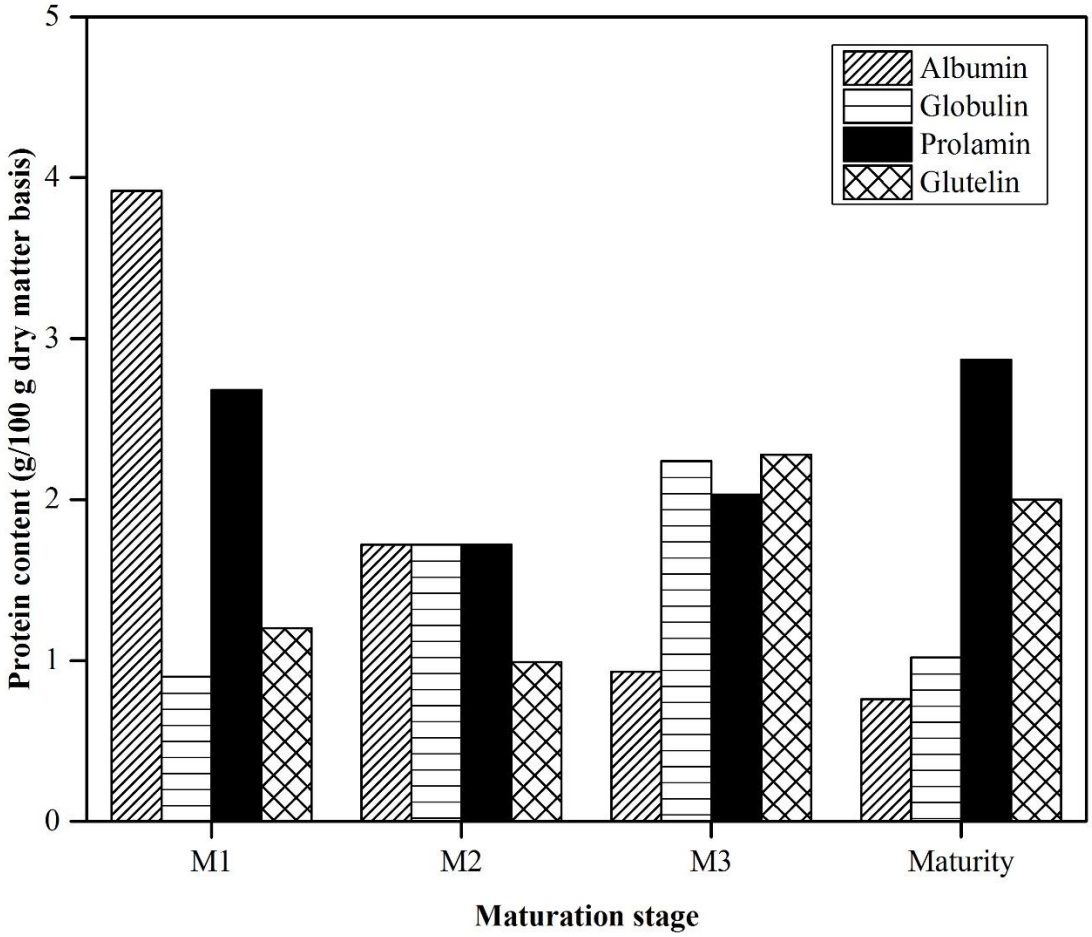
2.9. Changes in protein quality during maturation of corn

Changes in crude corn protein and soluble protein contents during maturation stages, namely, filling stage (M1, 74 days after seeding (DAS)), milk ripe stage (M2, 86 DAS), wax ripe

stage (M3, 98 DAS) and maturity stage (M4, 116 DAS) were studied by Xu et al. (2010). Crude protein content decreased from 13.1 to 10.2 g/100 g, db from M1 to M2 stages and then to 8.77 g/100 g, db during M4 stage. No changes in crude protein content were noticed between M2 and M3 stages. Albumin content decreased by 81.9% from 3.93 to 0.71 g/100 g db from M1 to M4 stage. Globulin content increased from stages M1 to M3 by 2.35 fold and then decreased by 55.2% during M4 stage (Fig. 2.5). Prolamin content decreased by 37.3% from stages M1 to M2 and then increased to 2.83 g/100 g, db during maturity (M4) stage. Glutelin content remained unchanged from M1 to M2 stages and then increased to 2.24 g/100 g at M3 stage and thereafter decreased to 1.98 g/100 g db during M4 (Xu et al. 2010; Fig. 2.5). Landry and Moreaux (1976) reported that albumins and globulins increased sharply during the first six weeks after pollination; and thereafter decreased during grain ripening stages (6 to 12 weeks after pollination). Glutelin content were low during the early stages of maturation and increased sharply during the later stages (6 to 12 weeks after pollination). Similar trend was observed with prolamin content which increased sharply after 2 weeks of pollination (Landry and Moreaux 1976).

In another study by Wall and Bietz (1987), changes in corn protein quality during maturation were compared for normal and *opaque-2* (*o2*) corn. Albumin and globulin contents in both corn genotypes declined with maturity; however, the rate of decline was greater in normal corn than *o2* corn. The total albumin and globulin content at 48 days after pollination (DAP) was higher in *o2* corn than in normal corn. Zien content increased rapidly in normal corn and was about 60% of total protein at 48 DAP; whereas, in *o2* corn, rate of increase of zein protein was slower and total zien content was only 20% at 48 DAP. Glutelins were characterized as alcohol soluble glutelins (soluble in 70% ethanol + 0.5% sodium acetate + 2% 2-mercaptoethanol) and

alcohol insoluble glutelins (soluble in borate buffer containing 2% 2-mercaptoethanol). Alcohol insoluble glutelins increased considerably in *o2* corn compared to normal corn (Wall and Bietz 1987).



Adapted from Xu et al. 2010

Fig. 2.5. Changes in soluble protein contents at different stages of maturation in corn grain (M1 – 74 days after seeding [DAS]; M2 – 86 DAS; M3 – 98 DAS; M4 – Maturity, 116 DAS).

2.10. Biochemical changes in cereal grains during storage

Effects of storage (10, 25 and 45°C for 6 mo) on nutritional quality (pH, titratable acidity, total soluble sugars, amino acid contents, starch and protein digestibility) of cereal grains (wheat,

rice and corn) were evaluated (Rehman 2006). Storage at 25 and 45°C resulted in lower pH, protein and starch digestibility, lysine and thiamine contents and higher titratable acidity in all cereal grains. Total soluble sugars increased after 6 mo for grains stored at 10 and 25°C and decreased at 45°C storage (Rehman 2006). Starch physiologic properties are characterized by amylose/amylopectin ratios, starch hydrolysis rates, pasting and thermal properties. Structural changes in rough rice such as a decrease in extractable starch yield, amylose:amylopectin ratios and shortened amylopectin average chain lengths were reported during storage at 38°C for 9 mo (Patindol et al. 2005). Setiawan et al. (2010) studied the effects of storage (27°C for 6 mo) for sun dried (35°C) and artificially dried (80°C) corn kernels on starch properties. Soluble sugar content of sun dried corn increased from 2.5 to 4.4% after 6 mo of storage; whereas, it did not vary for artificially dried corn kernel. Changes in soluble sugars for sun dried corn during storage were attributed to starch hydrolysis by endogenous amylases. Rate of starch hydrolysis decreased for sun and artificially dried corn samples poststorage. Based upon imaging analysis, there were increased number of damaged starch granules for both corn samples after storage. Lower molecular weight of starch and shortened amylopectin chain lengths poststorage were indicative of endogenous starch hydrolysis during storage (Setiawan et al. 2010). Changes in rice and corn starch pasting properties were observed during storage (Patindol et al. 2005; Park et al. 2012; Paraginski et al. 2014). Pasting temperatures increased; whereas, breakdown viscosity decreased for milled rice stored at 29°C for 6 mo and rough rice stored at 38°C for 9 mo, respectively (Park et al. 2012; Patindol et al. 2005).These changes were not reflected in the isolated starch samples which were stored at same conditions. Inconsistent trends between rice flour and starch samples implicated the role of nonstarch constituents present in grain along with starch during rice aging (Patindol et al. 2005). Similar results were observed by Paraginski et al. (2014) where corn starch

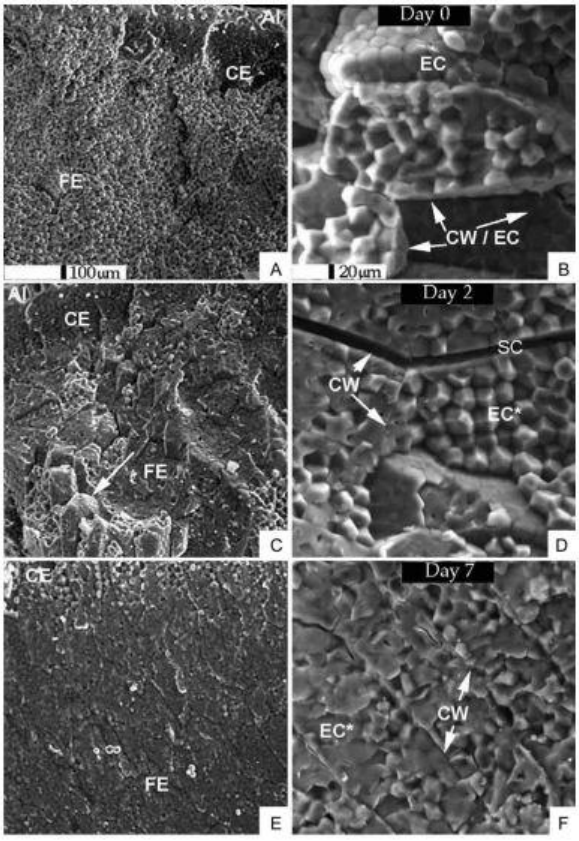
pasting temperatures increased and breakdown viscosity decreased after 12 mo of storage. These changes were more pronounced for corn samples stored at 35°C; however, they were reflected in corn stored at 5°C to a lesser extent. Protein solubility in water was analyzed for these corn samples as a function of storage time and temperature. There were 23.7, 6.8, 30.0 and 47.0% reductions in protein solubility for corn samples stored at 5, 15, 25 and 35°C, respectively. Changes in protein solubility were attributed as a factor responsible for changes in starch pasting properties (Paraginski et al. 2014).

2.11. Effect of postharvest practices on cereal protein properties and starch-protein interactions

There are physiologic changes in cereal grains during postharvest drying and storage which affect processing attributes and functionality of the final product. Postharvest drying of corn at temperatures above 82°C resulted in poor protein-starch separation in wet milling (Wall et al. 1975). High temperature drying of corn kernels resulted in excessive stress cracks and resulted in reduced separation of germ and pericarp and reduced the yield of prime grits in dry milling process (Wall et al. 1975). Effects of postharvest drying (temperatures from 54 to 130°C) on corn protein solubility was evaluated by Malumba et al. (2008). Globulin and zein (prolamin) contents decreased as drying temperatures increased; whereas, glutelin protein contents increased up to 110°C before decreasing slightly at 130°C. Total unextracted protein content increased as drying temperatures increased (Malumba et al. 2008).

Effects of an accelerated aging procedure on maize and sorghum properties were studied by McDonough et al. (2004). Accelerated aging methods have been used to mimic the long term storage effects encountered in industries without inducing microbiological growth. Maize and sorghum kernels were equilibrated to 12% moisture content at ambient conditions and stored in

sealed air tight jars for up to 15 days at 50°C. Protein solubility altered with storage time; albumin, globulin and prolamin contents decreased; whereas, the amount of glutelins increased during aging for whole sorghum and maize. As seen with the scanning electron micrographs (Fig. 2.6), after 2 days of accelerated aging, the floury endosperm region had more compacted appearance compared to 0 day old kernels. Starch, protein and cell walls of floury endosperm were as strongly associated as corneous endosperm after one week of storage (Fig. 2.6). The same phenomenon was observed with sorghum kernels. This further indicated there were stronger starch-protein associations as kernel aging progressed.



Reprinted from McDonough et al., 2004.

Fig. 2.6. Bisected maize kernels (using a razor blade) during accelerated aging (storage for 7 days at 50°C). Images taken using scanning electron microscope. (A,B – 0 day), (C,D – 2 days), (E,F – 7 days). CE (corneous endosperm), FE (floury endosperm), EC (endosperm cell), Al (aleurone), EC* (endosperm cell interior), CW (cell wall), SC (stress crack).

Changes in rice protein characteristics during long term storage (12 mo) have been reported by Chrastil (1990). Protein solubility in rice decreased post storage primarily due to the decreased solubility of oryzenin (glutelin) content. Average molecular weight of the oryzenins doubled during storage. Further analyses revealed this was due to changes in peptide unit composition and disulfide bridges (Chrastil and Zarins 1992). Lower molecular weight peptides decreased; whereas, higher molecular weight peptides increased and number of disulfide bridges increased during storage.

2.12. Nitrogen to protein conversion factor

In general, the conversion factor 6.25 is used to convert nitrogen content to protein in food materials. This originated several years ago when relatively few animal proteins were known such as serum albumin and serum globulin from blood and casein from milk and these contained 16% nitrogen content (100/16 = 6.25) (Jones 1931). Use of this conversion factor is dependent on two assumptions: all nitrogen in food material is protein nitrogen; all proteins contain 16% nitrogen. Jones (1931) analyzed the major protein fractions (zein and glutelins) in corn which was close to 16.1% and therefore recommended the commonly used 6.25 factor. They made a correction to the general conversion factor used for wheat (5.7) by considering the nitrogen contents in different fractions (embryo, bran and endosperm) of wheat kernel and recommended a factor of 5.83. This was further corrected by Tkachuk (1969) where the quantitative analysis of amino acid composition in wheat flour was done and the non-protein nitrogen content was taken into account in the calculations resulting in a conversion factor of 5.59. Mossé (1990) proposed a method for estimating the conversion factor by defining two conversion parameters: k_A and k_P. k_A was defined as the ratio of total weight of anhydrous amino acid residues to total nitrogen recovered from the 20 amino acids (including the amide N from

aspargine and glutamine). k_P was defined as the ratio of total weight of anhydrous amino acid residues to total nitrogen content. While k_A value tends to be overestimated as it does not take into account the nitrogen from non-protein constituents, k_P value is generally underestimated as it depends on the analytical recovery during amino acid analysis. It was recommended the average of k_A and k_P values to be used as the conversion factor as k_A and k_P values are higher and lower, respectively, than the true factor (Mossé 1990). However, k_A value is recommended for substances with minimal non-protein nitrogen content such as purified protein extracts from milk, cereals or soybean (Mariotti et al. 2008). Despite the information available on methods to analyze nitrogen to protein conversion factors, different conversion factors have been reported for corn protein content measurement: 5.65 (Mossé 1990), 5.61 (Sosulski and Imafidon 1990) and 5.68 (Sriperm et al. 2010). Sosulski and Imafidon (1990) reported the k_A value as the conversion factor; whereas, Sriperm et al. (2010) reported the average value of k_A and k_P.

Using different conversion factors would cause more confusion instead of improving accuracy (Lynch and Barbano 1999). Standard procedure for protein content estimation in cereal grains using combustion method has been defined by AOAC 2007. Conversion factor 6.25 has been reported for all cereal grains and oilseeds except for wheat with a conversion factor of 5.7 (Method 992.23, AOAC 2007).

Chapter 3

Seasonal variability in ethanol concentrations associated with incoming corn variability from a dry grind fermentation operation

3.1. Introduction

Dry grind processors report that ethanol yields are lower from freshly harvested corn compared to corn that has been stored for two to three mo (Singh et al. 2012). Plumier et al. (2015) evaluated effects of storage time and temperature on unreacted starch contents in DDGS following the dry grind process. In the dry grind process, not all starch is hydrolyzed to sugars. The left over starch is called unconverted or residual starch and is recovered in the DDGS (Sharma et al. 2010). Higher residual starch in DDGS can correspond to lower ethanol production. Unreacted starch contents decreased during the first 15 wk and then had an increasing trend during the course of the study (Plumier et al. 2015).

The main objective was to monitor variations in ethanol concentrations for corn received by a dry grind ethanol facility. Effects of two crop years on variation in ethanol concentrations were evaluated. Effects of enzyme treatments on ethanol variation and mean ethanol concentrations were investigated.

3.2. Materials and Methods

3.2.1. Substrates and Enzymes

Corn composite samples were obtained from a Midwestern ethanol plant for 2 yr (Oct 2012 to Sept 2014). Corn was purchased from a 50 mile radius around the plant. Samples were collected biweekly for the first year of the study (Oct 2012 to Sept 2013) and monthly from Oct 2013 to Sept 2014. A composite container was filled with samples collected on a daily basis directly from trucks before the corn was transferred to silos for storage; 5 kg composite samples were obtained. An identity preserved yellow dent corn hybrid obtained from a seed company was used as an experimental control during the first year of the study. Control corn was stored at 4°C for one yr. Subsamples (600 g) were used every 2 wk for analysis. Samples were cleaned using a 12/64" (4.8 mm) round hole sieve by removing the broken corn and foreign material (BCFM). Cleaned corn was ground using a 0.5 mm sieve in a laboratory hammer mill (1100W, model MHM4, Glen Mills, Clifton, NJ). Moisture content of ground corn was determined using a two stage standard oven method (Approved Method 44-19, AACC International 2000). Ground corn (100 g dry basis, db) was used for dry grind fermentation. Two replicates were used for each corn sample. Active dry yeast (Ethanol Red, Lesaffre Yeast Corp., Milwaukee, WI) was used for fermentation. A urea stock solution (50% w/v from 99.6% ACS grade) was prepared and used as the nitrogen source for yeast obtained from Fisher Scientific, Fair Lawn, NJ. Two liquefaction enzymes (AA-I and AA-II), two saccharification enzymes (GA-I and GA-II) and a protease (P) were provided by a commercial enzyme company. AA-II and GA-II were newer generation enzymes relative to AA-I and GA-I. Five enzyme treatments (I through V) were evaluated during the first yr of the study (Table 3.1). Enzyme treatment IV (AA-II and GA-II) was used for the

comparison of the effects of crop years on ethanol concentration variation. Enzyme treatments and dosages were selected based on manufacturer's recommendations.

Table 3.1

Enzyme Treatments and dosages[a] used in the dry grind process

	Enzyme Treatments				
	I	**II**	**III**	**IV**	**V**
Liquefaction Enzyme	AA-I (18.8 µL)	AA-I (18.8 µL)	AA-I (18.8 µL)	AA-II (25.7 µL)	AA-II (25.7 µL)
Saccharification Enzyme	GA-I (61.5 µL)	GA-II (56.3 µL)	GA-II (56.3 µL)	GA-II (56.3 µL)	GA-II (56.3 µL)
Protease	-	-	P (2.86 µL)	-	P (2.86 µL)

[a] Enzyme dosages in µL/100 g corn dry basis

3.2.2. Enzyme Activity Measurements

Enzyme protein contents were measured using Bradford's method (1976). Bovine serum albumin used as the protein standard and Bradford's reagent were purchased from Bio-Rad, Hercules, CA. Amylase activities were measured as an increase in reducing sugars and expressed as the amount of reducing sugars released/min by 1 mL enzyme (µmoles sugar/min mL). Alpha amylase and glucoamylase activities were determined using DNS assay using pregelatinized corn starch and maltodextrins as substrate, respectively. Standard sugars used for alpha amylase and glucoamylase assays were maltose and glucose, respectively (Fisher Scientific, Fair Lawn, NJ). Alpha amylase and glucoamylase assays were conducted at 85 and 32°C, respectively, for 5 min. Protease activity was determined at 32°C (Abe et al. 1977). Bovine hemoglobin was used as the substrate. Protease activity was expressed as the amount of tyrosine liberated by trichloroacetic acid soluble product/min by 1 mL enzyme at 280 nm. Amino acid tyrosine obtained from Fisher Scientific (Fair Lawn, NJ) was used as the standard. Each activity was determined in 20 mM

sodium phosphate buffer at the optimum pH of the enzyme. The optimum pHs of AA-I, AA-II, GA-I, GA-II and P enzymes were 5.8, 5.1, 5.0, 5.0 and 5.0, respectively.

3.2.3. Dry Grind Process

Ground corn (100 g, db) was mixed with water to obtain 30% slurry. Sulfuric acid (1*N*) was added to adjust the pH to 5.8 and 5.1 for AA-I and AA-II, respectively. Slurry was incubated at 85°C for 2 hr for liquefaction with AA-I (18.8 µL/100 g db) or AA-II (25.7 µL/100 g db). Liquefact samples (1 mL) were analyzed using HPLC. Before simultaneous saccharification and fermentation (SSF), pH of liquefied mash samples were adjusted to 5.0 using sulfuric acid (1*N*). Glucoamylase enzyme (GA-I: 61.5 µL/100 g db or GA-II: 56.3 µL/100 g db) and protease (2.86 µL/100 g db) were added as given in Table 3.1. Urea (0.4 mL) and yeast inoculum (2 mL) were added subsequently. Yeast inoculum was prepared by dispersing 1 g active dry yeast (ADY) in 5 mL water and incubating at 32°C and 30 min. SSF was carried out at 32°C and 120 rpm for 72 hr in a shaking incubator (Innova 42, Eppendorf, Enfield, CT). The fermentation process was monitored by withdrawing 1 mL samples at 72 hr and analyzed using HPLC.

3.2.4. HPLC Analyses

Each experiment was conducted with two replicates. Samples collected after liquefaction and SSF were centrifuged at 10,000 rpm (9,279 × *g*) (Model 5415 D, Brinkmann - Eppendorf, Hamburg, Germany) for 5 min; clear supernatant liquid was passed through 0.2 µm syringe filters (Nylon Acrodisc WAT200834, Pall Life Sciences, Ann Arbor, MI) into 150 µL vials. The resulting filtrates were analyzed by HPLC, using an ion exclusion column (Aminex HPX-87H, Bio-Rad, Hercules, CA) with the stationary phase maintained at 50°C. Glucose, ethanol, oligosaccharides and organic acids such as acetic and lactic acids were eluted out from the column using 5 mM sulfuric acid as a mobile phase with flow rate of 0.6 mL/min. Separated

components were detected by refractive index detector (model 2414, Waters Corporation, Milford, MA). Standard solutions for maltodextrins (DP4+) (0.44% w/v), maltotriose (DP3) (0.50% w/v), maltose (2.0% w/v), glucose (2% w/v), fructose (1% w/v), succinic acid (0.50% w/v), lactic acid (1.0% w/v), glycerol (2% w/v), acetic acid (0.5% v/v), methanol (1% v/v) and ethanol (20% v/v) were prepared and used to calibrate the column. Data were processed using HPLC software (Waters Corporation, Milford, MA).

3.2.5. Statistical Analyses

All statistical analyses used version SAS 9.4. Coefficient of variation of the dry grind procedure was 0.16%. This was computed by running 5 experiments (one experiment per week with three replicate samples per experiment) using samples from a single hybrid stored at 4°C. The trend in ethanol concentrations with time was determined by dividing the entire time series into three segments. The three segments were chosen based on the local maxima and minima points. A non-parametric regression method (PROC LOESS) with a smoothing parameter of 0.63 was applied to each dataset and values from the smooth curve were used to evaluate the local minima and maxima points using the first derivative test. Least squares regression lines (PROC AUTOREG) were fitted for the three regions after correcting for first order correlation using the maximum likelihood method. Slopes in each segment (rates of change of ethanol concentrations) were evaluated using t-test to check if they were different from 0.

Fisher's least significant difference (LSD) was used to determine differences in mean ethanol concentrations and sugar concentrations across a year due to different enzyme treatments. The level selected to show statistical significance in all cases was 5% ($P < 0.05$).

3.3. Results and Discussion

3.3.1. Temporal Trend of Ethanol Concentrations from Dry Grind Process

Ethanol concentrations varied with time (Fig. 3.1). Ethanol concentrations for commodity corn and control corn samples with enzyme treatment I ranged from 16.0 to 18.0% v/v and 16.5 to 17.9% v/v, respectively. Ethanol concentrations were lower immediately after harvest for commodity corn (16.6% v/v) and control corn (16.7% v/v).

For commodity corn samples, ethanol concentrations were between 16.4 and 17.1% v/v during the first quarter of the study (Oct to Dec, 2012) (Fig. 3.1A). Ethanol concentrations increased to 18.0 and 17.5% v/v during wk 12 and 14, respectively (Jan, 2012). Ethanol concentrations decreased during wk 16 and showed an increasing trend from wk 16 to 20 (Feb to Mar, 2012). During the third quarter of the study (end of Mar to Jun, 2012), ethanol concentrations ranged from 16.0 to 17.0% v/v. During the last quarter of study, ethanol concentrations increased from 16.5 to 17.3% v/v from wk 36 to 38 and thereafter, remained in the range of 17.3 to 17.4% v/v (Fig. 3.1A).

Ethanol concentrations followed the same pattern for control corn samples which were stored at refrigerated conditions (4°C) (Fig. 3.1B). Ethanol concentrations were 16.7 to 17.3% v/v during the first quarter after harvest (Oct to Dec, 2012) and increased to 17.9% v/v during wk 12 and 14 (Jan, 2012). It decreased to 16.5% v/v afterwards, and followed the same pattern as commodity corn samples. In the last quarter of study, ethanol concentrations increased to 17.7% v/v (wk 38) and remained consistent afterwards (wk 38 to 44).

Rates of change of ethanol concentrations were determined for three different time segments based on the local maxima and minima points during the first yr of study (Table 3.2; Fig. 3.1). The local maxima and minima points obtained from the smooth curve for commodity

corn dataset were wk 14 and 26, respectively (Fig. 3.1A). The local maxima and minima points for control corn dataset were wk 14 and 28, respectively (Fig. 3.1C). There was an increasing trend in ethanol concentrations from wk 0 to 14 for both commodity and control corn (Table 3.2; Figs. 3.1B and 3.1D). Slope in the second region (wk 14 to 26) for commodity corn was not significantly different from 0. For control corn, ethanol concentration had a decreasing trend (wk 14 to 28). Ethanol concentrations had an increasing trend in the last time segment (wk 26 to 44 and 28 to 44 for commodity and control corn, respectively). The resemblance of variability patterns for control and commodity corn samples showed that storage time had an effect on dry grind ethanol concentrations. Plumier et al. (2015) reported similar results where they looked at unreacted starch contents (USC) in DDGS from corn stored at different temperatures harvested in 2011 and 2012. For 2011 corn, USC decreased from 5.50% after harvest to a minimum of 1.50% in Jan, 2012 (after 10 wk storage). USC increased thereafter to 8.00%. For 2012 corn, a maximum value of USC (13.5%) after harvest (Oct, 2012) was reported; then USC decreased to 11% during wk 15 (Jan 2012) and increased from wk 15 to 23 (Feb to Mar, 2012). Rates of change in USC decreased from 0 to 15 wk and increased from 15 to 24 wk. The minima in USC was observed in the same month for both yr (January). Highest ethanol concentration in our study also was observed during the same time point (wk 12). Thus, variation of USC with storage time as reported by Plumier et al. (2015) was in agreement with this study.

Table 3.2

Rates of change of ethanol concentrations (% v/v per wk) for commodity and control corn (2012-13 year)

Segment	0	1	2
Commodity corn[a]	+0.06 *	-0.03	+0.05 *
Control corn[b]	+0.08 *	-0.06 *	+0.08 *

[a] Time segments for commodity corn: 0 = 0 to 14 wk; 1 = 14 to 26 wk; 2 = 26 to 44 wk.
[b] Time segments for control corn: 0 = 0 to 14 wk; 1 = 14 to 28 wk; 2 = 28 to 44 wk; On per segment basis, values followed by asterisk were significantly different from zero ($P < 0.05$). Comparison of slopes to a slope of 0 was calculated using t test using the following equation: $T = (b_1-0)/(S_{b1})$ where b_1 is the slopes of line and S_{b_1} is the standard error of slope.

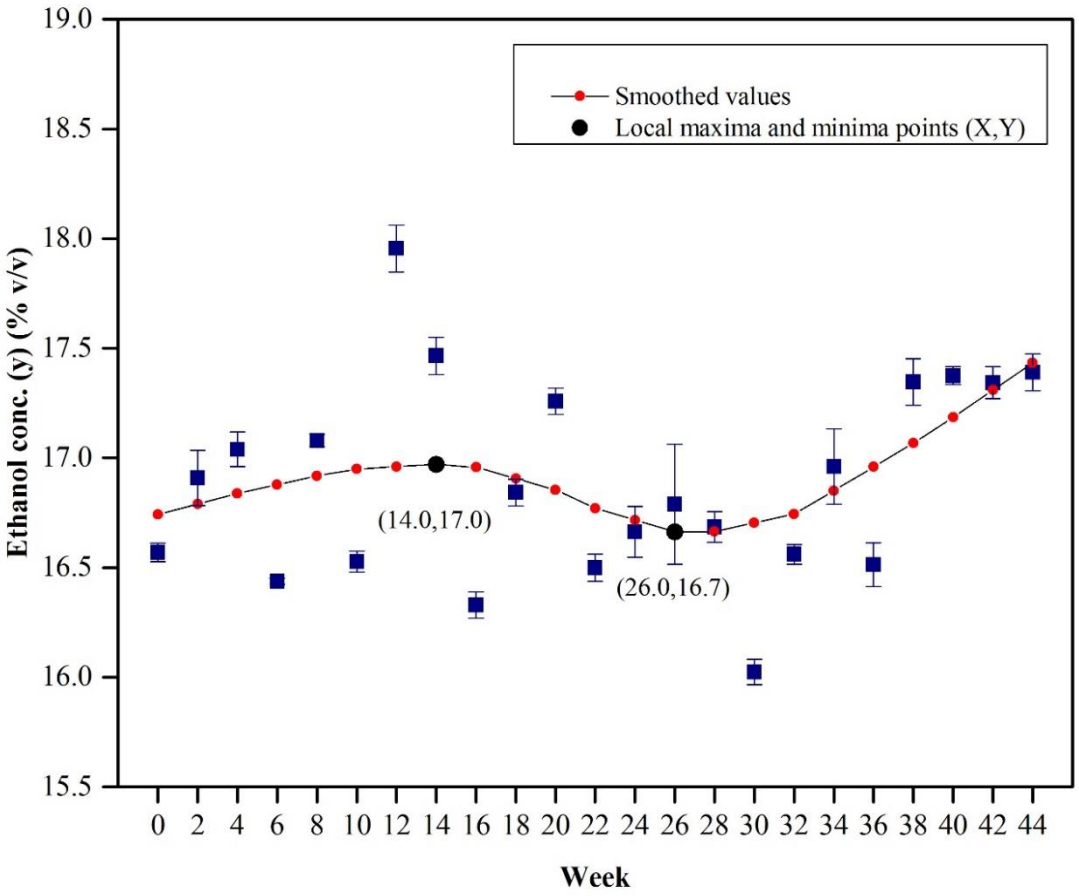


Fig. 3.1(A). Commodity corn (Smoothed values were obtained after smoothing the dataset).

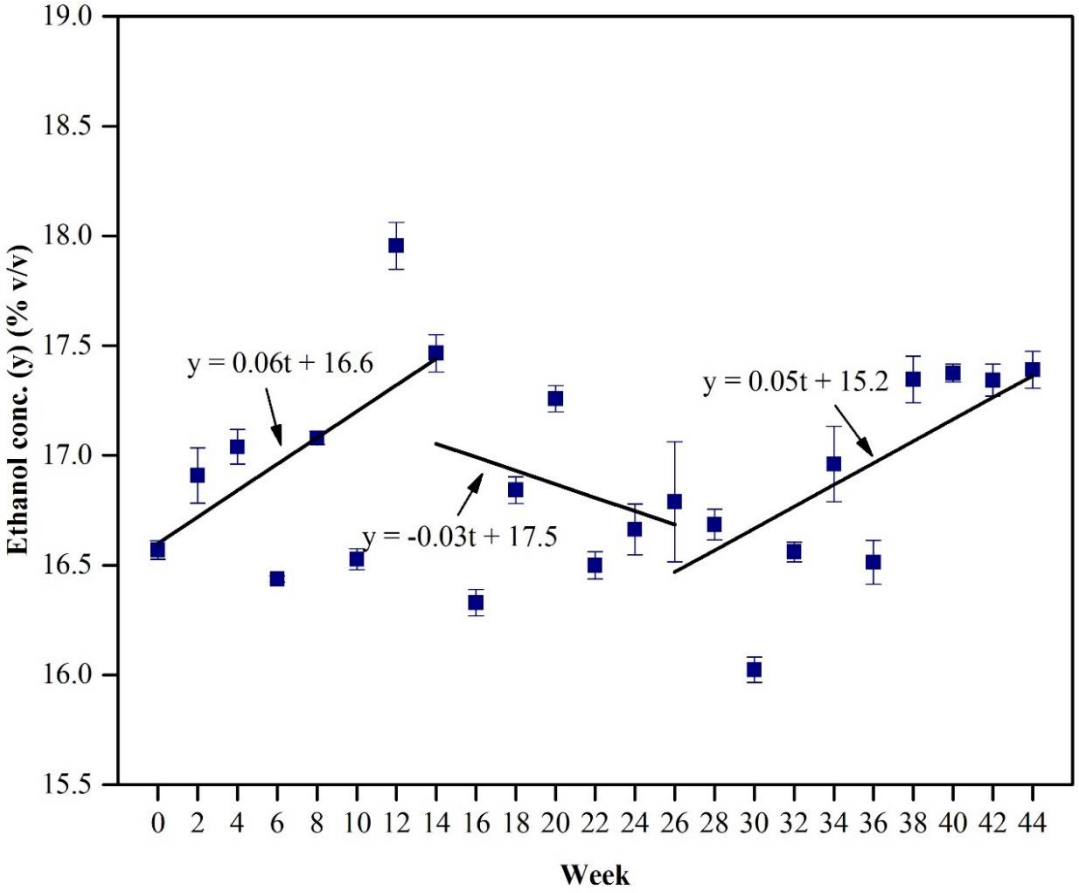


Fig. 3.1(B). Commodity corn (Least square regression lines fitted in the three chosen time segments). Slopes in time segments 0 (0 to 14 wks) and 3 (26 to 44 wks) were significantly ($P < 0.05$) different from 0.

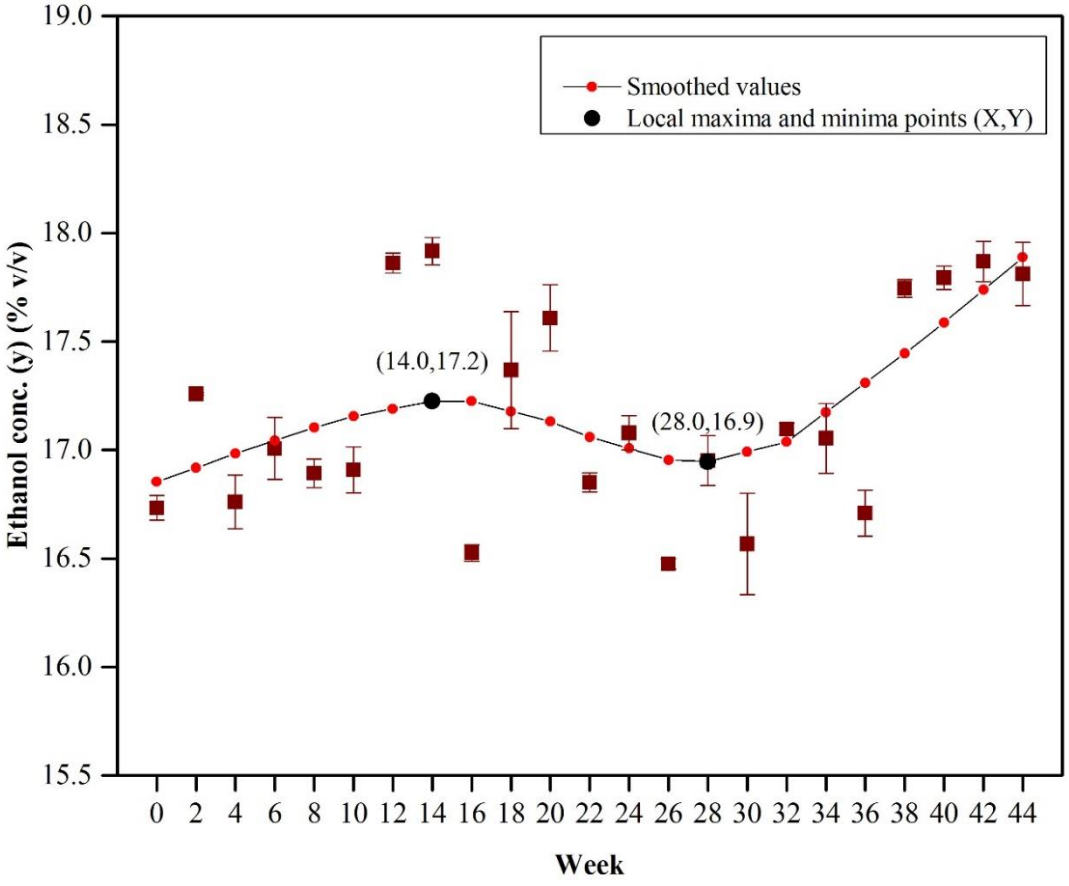


Fig. 3.1(C). Control corn (Smoothed values were obtained after smoothing the dataset).

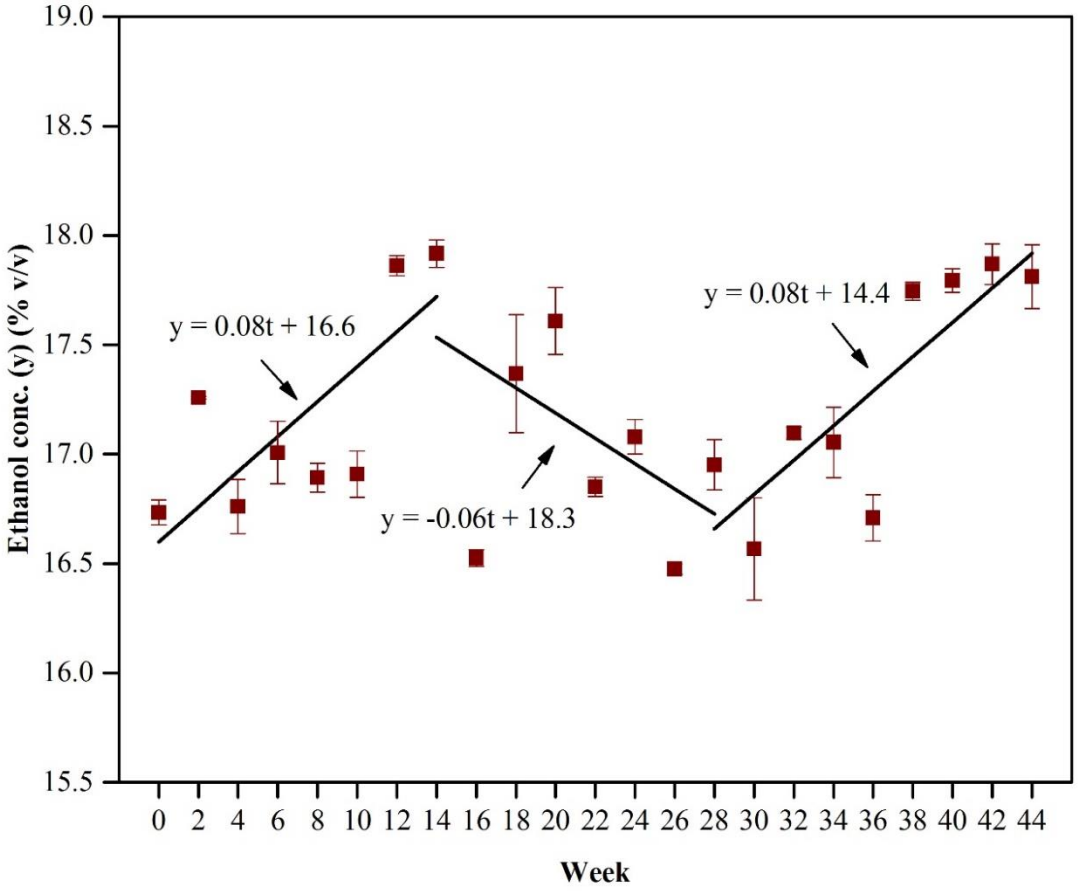


Fig. 3.1(D). Control corn (Least square regression lines fitted in the three chosen time segments). Slopes in all three time segments were significantly ($P < 0.05$) different from 0.

Fig. 3.1. Ethanol concentration variation with time using enzyme treatment I (2012-13). Two replicates were run for each corn sample. Error bars indicate standard deviations.

Ethanol production for corn coming in dry grind ethanol plants has been monitored in the past for 7 yr (2003 to 2009) by a seed company (Elliot et al. 2010 and Singh et al. 2012). Ethanol yields were low during the first month immediately after harvest; higher for the next 6 to 7 mo and decreased at year end. The highest ethanol production was observed in second quarter (from 120 to 150 days after harvest) for all 7 crop yr. Peak ethanol concentration in our study coincided

at the same time point; however, we noticed higher ethanol concentrations during the last quarter (wk 38 to 44). Based on the aforementioned studies (Plumier et al. 2015 and Elliot et al. 2010), the expected trend was that ethanol concentrations will decrease at year end. Plumier et al. (2015) observed an increasing trend in USC from wk 32 to 44 (last quarter) during the yr 2011; however, corn harvested in 2012 was only stored for 5 mo. Therefore, direct comparison of our results with Plumier et al. (2015) during the last quarter of 2012 was not possible. Average ethanol concentrations for commodity corn in our study during I, II, III and IV quarters were 16.8, 17.1, 16.6 and 17.4% v/v, respectively. Variation between the highest and lowest quarterly mean ethanol concentrations was 4.61% comparable to 6.80% reported by Elliot et al. (2010) during 2008 crop yr.

3.3.2. Effect of crop years on ethanol concentration variability for commodity corn

Ethanol concentrations in the 2013 crop year ranged from 17.1 to 18.6% v/v representing a variation of 8.65% (COV = 2.65%) which was comparable to the 2012 crop year when the range was 16.7 to 18.4% v/v with a variation of 10.2% (COV = 2.48%). Ethanol concentrations were in the range of 17.8 to 18.3%v/v during the first quarter of 2013 and were higher compared to 2012 year (17.0 to 17.4%v/v). The exact time points of highest (wk 24 and 12 for 2013 and 2012, respectively) and lowest (wk 36 and 30 for 2013 and 2012, respectively) ethanol concentrations did not coincide for the two crop years.

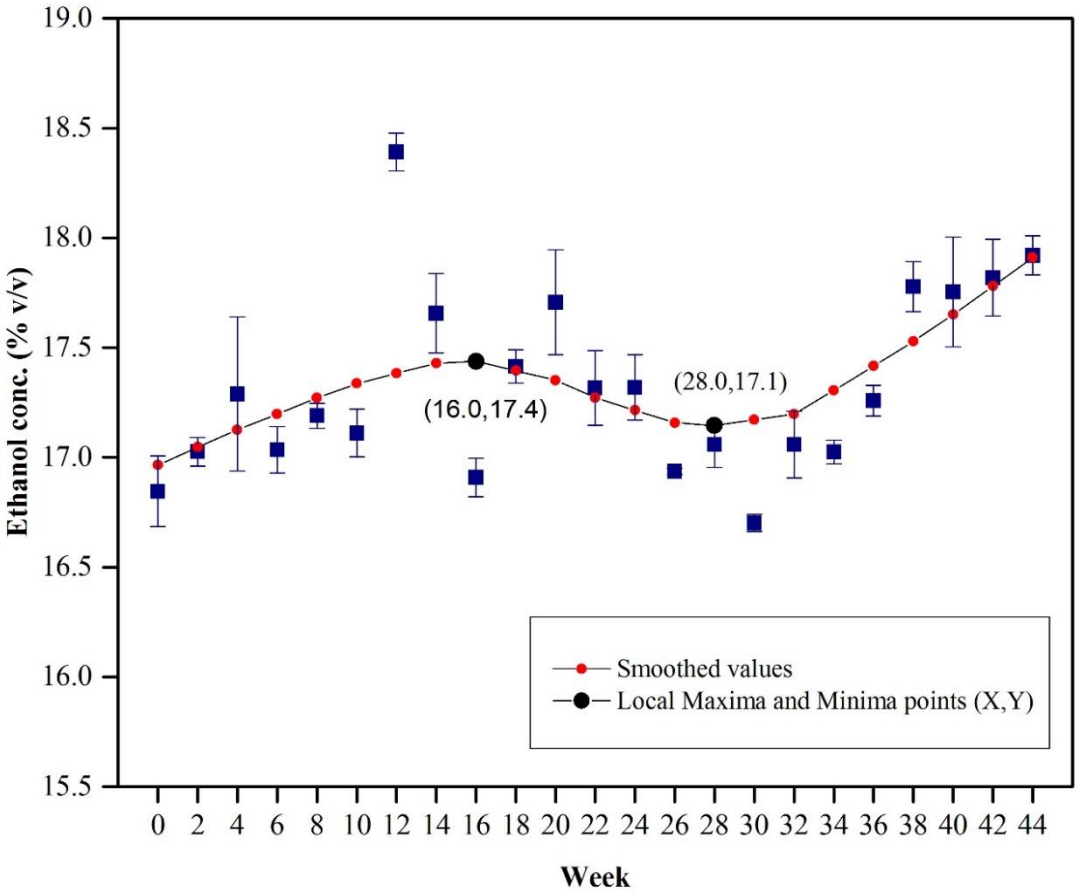
The local maxima and minima points for 2012 dataset (with enzyme treatment IV) were wk 16 and 28, respectively; whereas, the local minima and maxima points for 2013 dataset were wk 16 and 24, respectively (Figs. 3.2A and 3.2C). Ethanol concentration had an increasing trend with time (positive slope) from wk 0 to 16 (first region) and wk 28 to 44 (third region) in 2012 year (Table 3.3; Fig. 3.2B). In 2013, ethanol concentration had a decreasing trend from weeks 0

to 16, increasing trend from weeks 16 to 24 and decreasing trend from weeks 24 to 44 (Table 3.3; Fig. 3.2D).

Table 3.3
Rates of change of ethanol concentrations (% v/v per wk) for commodity corn with enzyme treatment IV for two crop years (2012-13 and 2013-14)

Segment	0	1	2
2012-13[a]	+0.04 *	-0.03	+0.07 *
2013-14[b]	-0.05 *	+0.16 *	-0.06 *

[a] Time segments for 2012-13: 0 = 0 to 16 wk; 1 = 16 to 28 wk; 2 = 28 to 44 wk.
[b] Time segments for 2013-14: 0 = 0 to 16 wk; 1 = 16 to 24 wk; 2 = 24 to 44 wk; On per segment basis, values followed by asterisk were significantly different from zero ($P < 0.05$). Comparison of slopes to a slope of 0 was calculated using t test using the following equation: $T = (b_1-0)/(S_{b1})$ where b_1 is the slopes of line and S_{b_1} is the standard error of slope.



3.2(A) 2012-13 year (Smoothed values were obtained after smoothing the dataset).

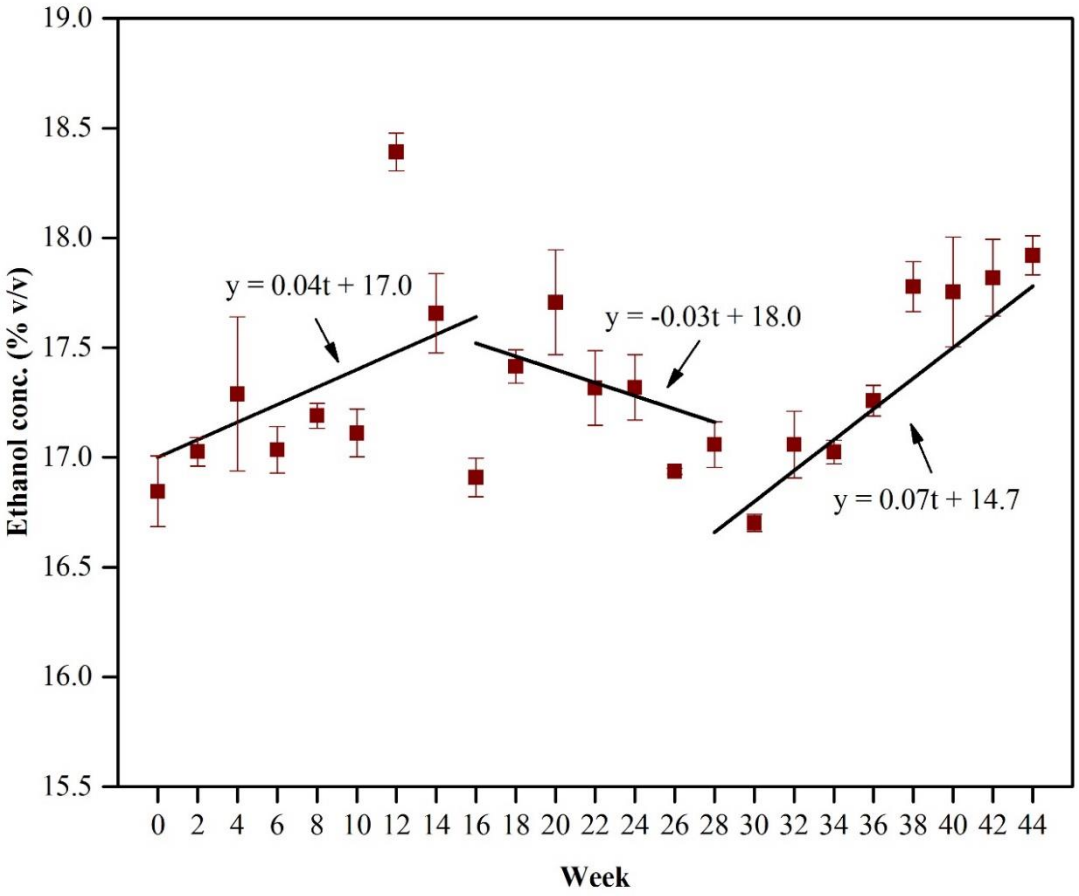
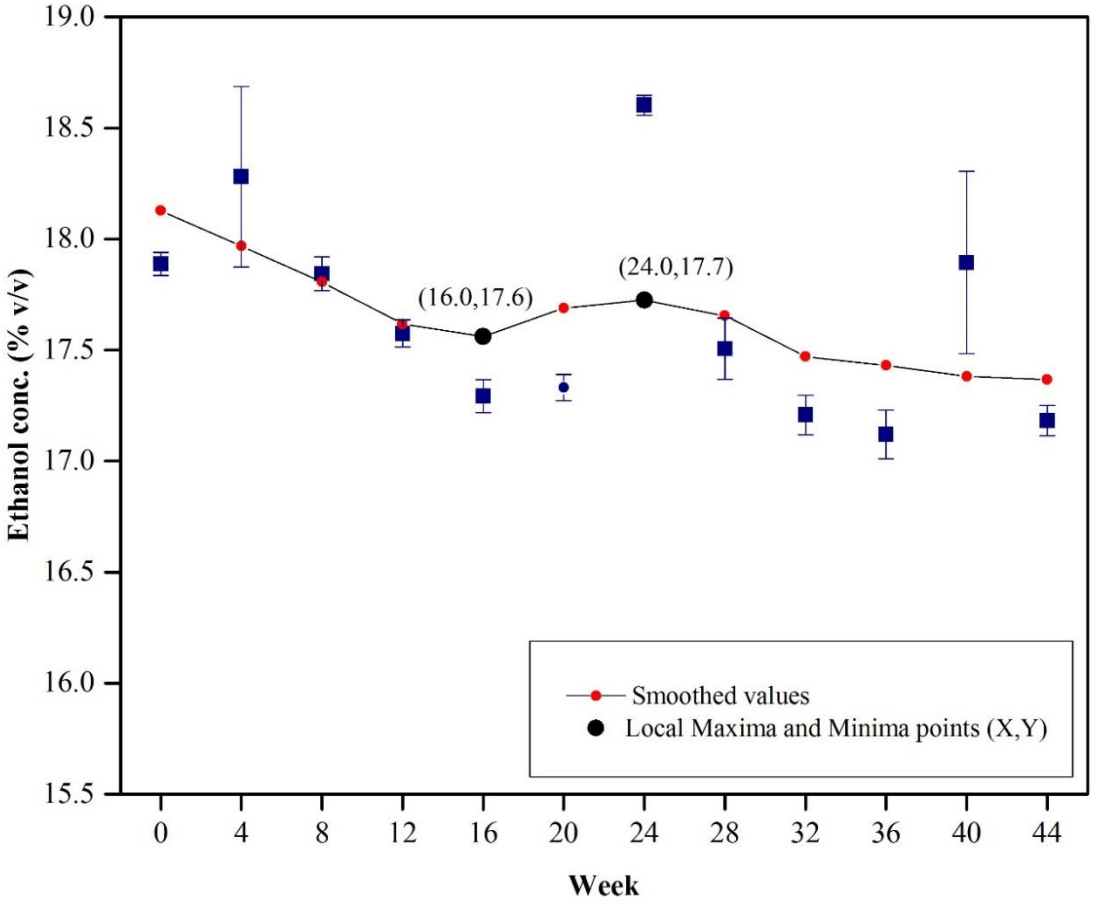


Fig. 3.2(B). 2012-13 year (Least square regression lines fitted in the three chosen time segments). Slopes in time segments 0 (0 to 16 wks) and 3 (28 to 44 wks) were significantly ($P < 0.05$) different from 0.



3.2(C) 2013-14 year (Smoothed values were obtained after smoothing the dataset).

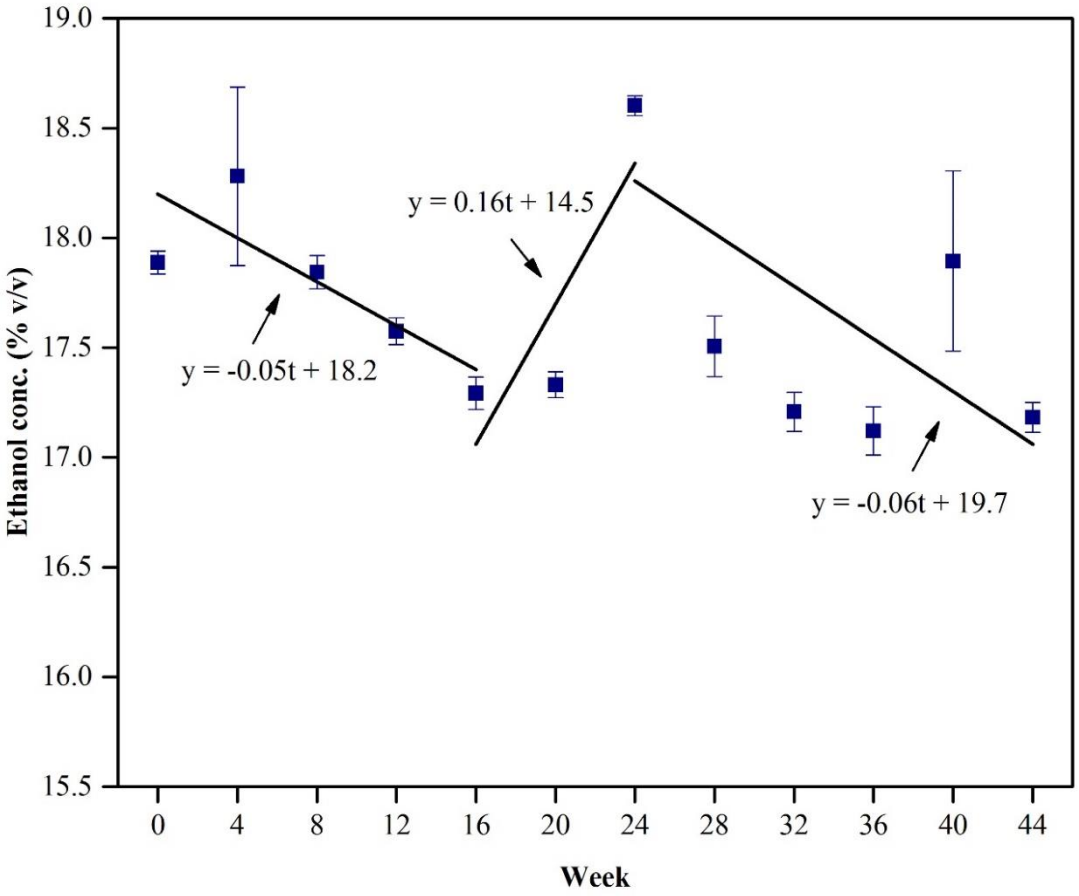


Fig. 3.2(D). 2013-14 year (Least square regression lines fitted in the three chosen time segments). Slopes in all three time segments were significantly ($P < 0.05$) different from 0.

Fig 3.2. Ethanol concentrations for commodity corn using enzyme treatment IV (2012-13 and 2013-14). Two replicates were run for each corn sample. Error bars indicate standard deviations.

3.3.3. Effects of enzyme treatments on ethanol concentration variation for commodity corn

Range of ethanol concentrations across time with enzyme treatments I, II, III, IV and V were 16.0 to 18.0, 16.3 to 18.2, 16.6 to 18.3, 16.7 to 18.4 and 16.7 to 18.8% v/v, respectively. (Fig. 3.3). Percent variability based on the lower and upper limits and COV of ethanol

concentrations across time points for enzyme treatments I, II, III, IV and V were 12.5 (COV = 2.85), 11.7 (2.29), 10.2 (2.51), 10.2 (2.48) and 12.6 (2.78)%, respectively. Variation in ethanol concentrations at different time intervals across enzyme treatments ranged from 16.0 to 18.8% v/v. Overall variation in ethanol concentration among different enzyme treatments across different time points was 17.1%. This was comparable to the ethanol variation previously reported. Haefele et al. (2004) reported 15.0% variability in ethanol concentration due to environment and planting location. Singh et al. (2005) reported 22.7% variation in ethanol concentration due to hybrid and planting location.

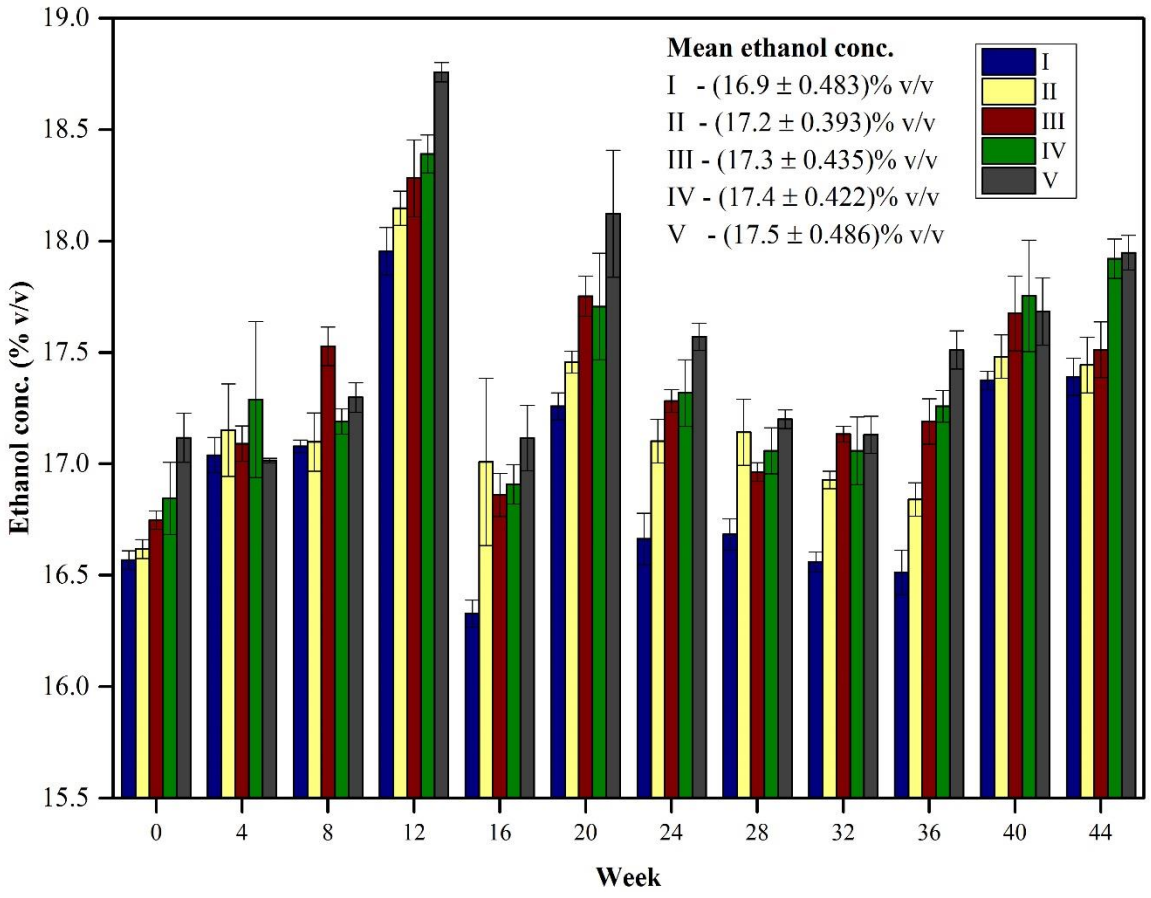


Fig. 3.3. Ethanol concentration variations for commodity corn with different enzyme treatments (2012-13 year).

3.3.4. Effects of enzyme treatments on mean ethanol concentrations for commodity corn

AA-II and GA-II were advanced liquefaction and saccharification enzymes owing to their higher protein contents and activities (Table 3.4). Alpha amylase activity of advanced liquefaction enzyme (AA-II) was twice as high as AA-I. AA-II also had higher side activities (glucoamylase and protease activities) than AA-I. No protease side activities were detected for AA-I, GA-I and GA-II enzymes. GA-II had 1.17 and 4.65 times higher glucoamylase and alpha amylase activities, respectively than GA-I (Table 3.4).

Table 3.4
Activity measurements of enzymes

Enzymes	Alpha amylase units [a]	Glucoamylase units [b]	Protease units [c]	Total Protein [d]
AA-I	3090	1721	ND [e]	451
AA-II	6420	7960	4.00	3010
GA-I	994	7638	ND [e]	5674
GA-II	4626	8951	ND [e]	8107
P	ND [e]	ND [e]	4.00	508

[a] Alpha amylase units - µmoles of maltose released per min per mL enzyme
[b] Gluco amylase units - µmoles of glucose released per min per mL enzyme
[c] Protease units - µmoles of tyrosine residues released per min per mL enzyme
[d] Total protein expressed in µg of protein
[e] Not detected

Enzyme treatment I (AA-I, GA-I) resulted in the lowest mean ethanol concentration (16.9 ± 0.483)% v/v (Table 3.5). Enzyme treatment II (AA-I, GA-II) with advanced glucoamylase enzyme and treatment III (AA-I, GA-II, P) with additional protease enzyme had mean ethanol concentrations of (17.2 ± 0.393) and (17.3% ± 0.435)% v/v, which were 0.3 and 0.4% higher than treatment I, respectively. Treatment IV (AA-II, GA-II) resulted in mean ethanol concentration of (17.4 ± 0.422)% v/v. Treatment V (AA-II, GA-II, P) gave higher mean ethanol concentration of (17.5 ± 0.486)% v/v compared to treatments I, II, III and IV. Mean ethanol

concentration with treatment V was higher by 0.6, 0.3, 0.2 and 0.1% compared to treatments I, II, III and IV, respectively (Table 3.5).

Table 3.5
Economics at a 100 MGY ethanol plant based upon mean ethanol concentrations

Enzyme treatment	Mean ethanol concentration[a] (% v/v)	Gain in ethanol concentration (% v/v)	Additional Income (million dollars)
I	16.9e	Baseline	Baseline
II	17.2d	0.3	0.71
III	17.3c	0.4	0.95
IV	17.4b	0.5	1.18
V	17.5a	0.6	1.42

[a] Mean ethanol concentrations are mean values of ethanol concentrations across entire year. Mean ethanol concentrations followed by the same letter in a column were not different ($P < 0.05$). LSD value was 0.057.

Protease and advanced liquefaction enzymes in treatments III, IV and V (with higher activities) resulted in higher mean ethanol concentrations. Mean ethanol concentrations for treatments IV and V with advanced liquefaction enzyme were 0.2% v/v higher compared to treatments II and III. Sugar yields after liquefaction with AA-II resulted in lower DP4+ concentrations and higher glucose, maltose and maltotriose concentrations compared to AA-I (Table 3.6). Mean sugar concentrations across time with AA- II were 8.55 (glucose), 3.53 (maltose), 2.26 (maltotriose) and 9.31 (DP4+) %w/v compared to 1.03 (glucose), 1.30 (maltose), 1.24 (maltotriose) and 21.3 (DP4+) % w/v with AA-I. The combined action of higher alpha amylase activity and protease activity resulted in the production of shorter chain oligosaccharides during liquefaction. Similar results were reported by Vidal et al. (2011) where they reported that addition of protease during liquefaction increased the overall concentrations of simple sugars (maltose, maltotriose and glucose) and decreased DP4+ concentrations. The reason being the

breakdown of protein matrix due to protease action and more available starch to be broken down to shorter chain sugars. Similarly, mean ethanol concentrations for treatments III and V with protease were 0.1% v/v higher than treatments II and IV, respectively. Protease addition at the beginning of SSF process increased fermentation rates and final ethanol yields for dry grind fermentations (Johnston et al. 2014). Protease treated samples had a higher concentration of amino acids compared to untreated control samples (Johnston et al. 2014). Similar results were seen by Vidal et al. (2011) where they looked at the effect of protease addition for corn endosperm fermentation. Utilization of these amino acids by yeast during fermentation resulted in faster fermentation rates and higher ethanol yields for protease treated samples. This is the most likely explanation for the observed higher ethanol concentrations for enzyme treatments III and V compared to treatments II and IV, respectively, in our study.

Table 3.6

Comparisons of sugar concentrations after liquefaction with AA-I and AA-II

	DP4+ (% w/v)		Maltotriose (% w/v)		Maltose (% w/v)		Glucose (% w/v)	
Week	**AA-I**	**AA-II**	**AA-I**	**AA-II**	**AA-I**	**AA-II**	**AA-I**	**AA-II**
0	26.9	8.89	0.62	1.91	0.63	2.64	0.53	8.22
2	23.1	6.62	1.40	2.12	1.53	3.82	1.34	9.64
4	25.2	6.82	1.74	2.01	1.52	3.59	1.24	9.62
6	24.9	7.89	1.52	2.39	1.51	3.12	1.10	7.76
8	19.3	9.59	1.32	2.23	1.52	2.87	1.06	6.81
10	19.0	15.5	0.75	1.60	0.94	1.67	1.21	4.22
12	24.1	17.0	1.26	1.88	1.23	1.96	0.94	4.09
14	16.5	15.5	0.76	2.42	0.99	2.94	0.99	5.69
16	30.5	14.9	1.74	3.62	2.20	5.13	1.47	9.93
18	34.5	11.5	2.03	3.20	1.83	5.28	1.37	12.4
20	21.5	7.98	1.27	2.53	1.36	3.55	1.10	8.88
22	20.2	7.76	1.22	2.07	1.19	4.10	0.95	11.4
24	19.9	8.82	1.54	2.48	1.70	3.91	1.06	10.2
26	18.8	7.23	1.21	2.39	1.24	3.58	0.89	8.89
28	17.1	6.17	0.98	2.13	1.10	3.99	0.82	8.90
30	17.8	8.79	0.95	2.32	0.99	3.00	1.00	7.94
32	19.8	7.11	1.15	2.50	1.11	4.08	0.88	9.65
34	18.1	9.02	0.98	2.05	1.09	2.48	1.22	6.79
36	19.7	9.79	1.22	2.30	1.24	2.94	0.94	7.61
38	19.1	6.97	1.09	2.40	1.11	4.24	0.92	9.86
40	19.4	5.40	1.53	0.67	1.57	5.77	1.04	12.3
42	18.7	9.13	0.95	2.22	0.97	2.81	0.81	7.28
44	18.1	7.90	1.06	2.33	1.12	3.46	0.88	8.55
46	18.4	7.02	1.38	2.39	1.42	3.74	0.94	8.66
Mean[a]	21.3*a*	9.31*b*	1.24*b*	2.26*a*	1.30*b*	3.53*a*	1.03*b*	8.55*a*
LSD	2.25		0.26		0.43		0.87	

[a] Mean concentrations followed by the same letter were not different ($P < 0.05$).

3.3.5. Economics of a 100 MGY ethanol plant based on mean ethanol concentrations

Considering treatment I to be the baseline, treatments II, III, IV and V resulted in average increases of ethanol concentration by 0.3, 0.4, 0.5 and 0.6% v/v, respectively (Table 3.5). In a 100 million gallon ethanol plant, this results in an additional production of 0.3, 0.4, 0.5 and 0.6

million gal/yr for enzyme treatments II, III, IV and V, respectively. Based on the average ethanol price (2.37 $/gal) during 2013 year (EIA 2015), this translates to an additional income of 0.71, 0.95, 1.18 and 1.42 million $/yr for treatments II, III, IV and V, respectively.

3.4. Conclusions

Time variations for dry grind ethanol concentrations were significant for commodity and control corn during 2012-13. Ethanol concentrations were low during the first quarter of study, increased to a maximum in the month of January and then decreased. Trends in ethanol concentrations during 2013-14 were different from 2012-13 year. Nevertheless, variability in ethanol concentrations (10.2 and 8.65% in 2012 and 2013, respectively) were comparable in both years. Enzyme treatments did not reduce ethanol concentration variability; however, they had effects on mean ethanol concentrations. Enzyme treatment V (with advanced alpha amylase, glucoamylase and protease enzymes) resulted in the highest mean ethanol concentration over a year. Using effective enzyme treatments increased overall mean ethanol concentrations at a dry grind ethanol plant which can improve profitability, provided the cost of enzymes is covered by additional revenue.

Chapter 4

Maize proximate composition and physical properties correlations with dry grind ethanol concentrations

4.1. Introduction

Grain quality can be defined using a range of properties depending on grain type and end use. Corn grain quality can be divided into three main categories: physical, chemical and processing attributes (Khullar et al. 2009). Kernel size, shape, weight, test weight, true density, stress crack percentage, moisture content and endosperm hardness constitute the physical quality attributes. Chemical attributes are starch, protein, oil, fiber and sugar content. Processing attributes are starch extractability, starch fermentability, and wet and dry millability which are governed by the structure of corn kernel at the molecular level (eg. nature of starch granule and protein type and interaction between starch and protein matrix).

Corn hybrids with value added traits are developed by breeders for specific end use purposes desired by wet millers, dry millers and dry grind processors (Maier 2004). For corn dry milling industry, the yield of large flaking grits is the most crucial attribute. Paulsen and Hill (1985) reported that corn with low breakage susceptibility and high test weight increased the yield of large flaking grits. In the wet milling industry, corn with soft endosperm is desired as it has a shorter steeping time and the starch fraction is separated easily from the protein (Maier 2004). Existing studies on the effects of grain quality attributes on ethanol yields are limited and inconclusive. True density is used as a measure of endosperm hardness. Murthy et al. (2004) reported that corn with higher true density values resulted in higher ethanol yields. Khullar et al. (2011) found that corn with softer endosperm resulted in high ethanol concentrations. The

contradictory results were attributed to the different range of true densities of samples used in these studies. Wu et al. (2007) reported positive correlations between sorghum starch contents and ethanol yields (r = 0.88); whereas, a poor correlation was reported by Zhan et al. (2003) between sorghum starch content and ethanol yield. Zhan et al. (2003) reported a strong negative correlation between sorghum protein content and ethanol yield (r = -0.84).

Sources of variation for dry grind ethanol yields from corn is not well documented. One possible cause could be variability in the quality of corn arriving at ethanol plants. Our objective was to determine relationships between rapidly measurable corn quality parameters and dry grind ethanol concentrations. Corn quality was defined in terms of its physical properties (moisture content, test weight, kernel weight, stress cracks percentage and true density) and chemical composition (grain starch, protein and oil content and initial soluble sugar content).

4.2. Materials and Methods

4.2.1. Materials

Corn composite samples were obtained from a Midwestern ethanol plant for 2 years (Oct 2012 to Sept 2014). Samples were collected biweekly for the first year of the study (Oct 2012 to Sept 2013) and monthly from Oct 2013 to Sept 2014 (as detailed in section 3.2.1 of chapter 3). Enzymes used for the dry grind process were obtained from a commercial enzyme company. Details on enzyme activity measurements are mentioned in section 3.2.2 in Chapter 3. Enzymes used in this study were AA-II and GA- II with optimum pHs of 5.1 and 5.0, respectively. Dosages and activities of these enzymes are mentioned in Tables 3.1 and 3.4, respectively.

Ambient temperature and precipitation data were obtained from National Climate Data Center (NCDC 2015) to report the growing conditions for corn during 2012 and 2013. Temperature and amount of precipitation during the corn growing season are the two main

factors impacting corn quality (Martin et al. 1976). Temperature and precipitation data from April to September during the 2 years of the study are presented in Table 4.1. Mean daytime and nighttime temperatures for optimal corn growth are 21 to 27 and ≥ 13°C, respectively (Martin et al. 1976 and Belyea et al. 2004). Mean maximum daytime temperatures across both years ranged from 24.9 in May to 31.3°C in July. Within years, mean maximum daytime temperatures were greater than 21°C for all growing months (May to September). Mean minimum nighttime temperatures were greater than 13°C during the months of June, July and August. Minimum precipitation (20 cm) is required for optimal corn growth during the growing months (June, July and August) (Martin et al. 1976). Precipitation values were lower than 20 cm during the growing months in 2012 and 2013; however, drought conditions were more severe in 2012 due to the combined effect of higher average temperatures and lower rainfall. Irrespective of the low precipitation values that resulted in lower average yields of corn during these years, harvested corn quality was reported to be comparable to previous crop years in terms of the grading factors with high test weight, low damaged kernels and low broken corn and foreign material (US Grains Council 2012 and 2013).

Table 4.1

Temperature (°C) and precipitation (cm) data for corn growing months[a]

Month	Year		
	2012	**2013**	**Mean[b]**
April			
Max [c]	18.9	15.6	17.2
Min [d]	5.50	3.77	4.64
Precipitation [e]	3.07	7.24	5.16
May			
Max	26.5	23.4	25.0
Min	12.5	11.5	12.0
Precipitation	2.56	6.57	4.56
June			
Max	29.3	27.2	28.2
Min	15.5	16.2	15.8
Precipitation	1.91	5.36	3.64
July			
Max	34.3	28.3	31.3
Min	20.7	17.3	19.0
Precipitation	1.48	2.95	2.22
August			
Max	30.3	28.6	29.4
Min	15.8	16.5	16.2
Precipitation	3.55	1.50	2.52
September			
Max	24.8	27.1	26.0
Min	11.3	13.3	12.3
Precipitation	4.54	1.81	3.18

[a] *Data Source* NCDC, 2015
[b] Means for 2012 and 2013
[c] Mean maximum temperature for month
[d] Mean minimum temperature for month
[e] Mean precipitation for month

4.2.2. Methods

A subsample (600 g) of the composite corn sample was sent to Illinois Crop Improvement Association, Champaign, IL, for the measurement of composition (protein and oil contents), test weight, true density, stress cracks percentage and 100 kernel weight. All analyses were done in duplicates. Variations of physical quality parameters and corn composition were

quantified in terms of actual differences (highest – lowest values) and coefficient of variation (COV). Actual differences commonly are used to represent variation; however, it takes into account only the magnitudes of lowest and highest values; whereas, COV reflects magnitudes and distribution.

Physical properties of corn

Moisture content of whole corn (30 g subsample) was analyzed using a standard 103°C convection oven method (Method S352.2, ASABE Standards 2002). True kernel density was measured using a helium pycnometer (Model MVP-D160-E, Quantachrome, Boynton Beach, FL). Kernel test weight was determined according to the USDA Federal Grain Inspection service Method (USDA-GIPSA 1996). Hundred kernel grain weight was determined by weighing 100 randomly selected, unbroken kernels to within 0.01 g. For percent stress cracks, 100 unbroken kernels (with no external cracks) were selected and inspected visually using a light board and number of stress cracks were recorded. Percent stress cracks were reported as percentage of kernels having one or more cracks.

Chemical composition

Corn starch content was determined using an acid hydrolysis method (Vidal et al. 2009). The procedure was as follows: Ground corn samples (1g) in duplicates were weighed in 100 mL autoclavable bottles (GL45, VWR International, West Chester, PA). Samples were mixed with 50 mL dilute HCl (0.4*N*) and autoclaved at 126°C for 1 h (Napco Model 9000D, Thermo Fisher Scientific, Waltham, MA). After cooling, 1 mL aliquot samples were collected and centrifuged for 1,500 × *g* for 5 min (Model 5415 D, Brinkmann – Eppendorf, Hamburg, Germany). Supernatant samples were analyzed for glucose production using YSI analyzer (Model 7100 MBS, YSI Life Sciences, Yellow Springs, OH). Glucose recovery factor was determined by

running a known glucose solution to account for glucose lost during the acid reaction. Starch content was back calculated from the determined glucose concentration and was corrected using the glucose recovery factor. Moisture content of ground corn was determined using a standard two stage oven method (Approved Method 44-19, AACC International 2000). Protein and oil contents of corn (400 g whole corn sample) were analyzed using a near infrared reflectance (NIR) analyzer (Model Infratec 1229, Whole grain analyzer, FOSS, Eden Prairie, MN). Crude oil content was calibrated using petroleum ether extraction method (Method A-6). Crude protein content was calibrated using Kjeldahl method (Method A-18) according to Corn Refiners' Association (CRA 1980). Standard errors of predictions for protein and oil were 0.2 and 0.3%, respectively. For initial soluble sugars present in corn kernel, 1 g ground corn was mixed with 10 mL deionized (DI) water and mixed in a vortex mixer for 5 min. Samples were centrifuged at 1,600 × *g* (Model IEC CL30, Thermo Electron Corp., Asheville, NC) and supernatant was analyzed using HPLC. All analyses were in duplicate.

Dry Grind Process

Dry grind process of corn was carried out as mentioned in section 3.2.3 in chapter 3. Fermentation process was monitored by withdrawing 1 mL samples at 24, 48 and 72 hr, which were analyzed using HPLC. Details on HPLC analyses mentioned in section 3.2.4.

4.2.3. Statistical Analyses

Quality characteristics were analyzed for the effect of year using a simple block design (SAS version 9.3). When effects were significant, means were compared using least significant difference (LSD). Pearson's correlation coefficients were calculated using the correlation procedure, PROC CORR (SAS version 9.3) to determine relationships between corn quality characteristics and ethanol concentrations. Multiple correlations (R^2) and regression equations

were calculated using stepwise regression method in SAS regression procedure (PROC REG). The level selected to show statistical significance in all cases was 5% ($P < 0.05$).

4.3. Results and Discussion

4.3.1. Means and variations of corn quality parameters

There were significant ($P < 0.05$) effects of harvest year on mean protein and soluble sugar contents of corn (Table 4.2). Corn harvested in 2012 had higher mean soluble sugar contents (glucose, fructose and DP2 contents) and protein content compared with 2013 harvested corn (Table 4.2). Mean starch and fat contents of 2012 and 2013 harvested corn were similar. The US Grains Council reported starch content of 2012 harvested corn was lower than 2013 harvested corn. Differences in starch and protein content for 2012 and 2013 corn were attributable to the respective weather conditions in the grain filling period (July and August). Starch accumulation is promoted by moderate rainfall and cooler temperatures (Martin et al., 1976). Lack of rainfall and higher average temperatures in 2012 resulted in lower starch and higher protein content. Similarly, physical attributes of corn (test weight, true density, stress crack percentage and moisture content) were affected by year ($P < 0.05$) (Table 4.2).

There were variations in the physical quality parameters and chemical composition of corn within the two crop years (Table 4.2, Appendix A and B). Starch content varied from 66.6 to 74.4% (COV = 3.86%) and 67.0 to 74.8% (COV = 3.88%) for 2012 and 2013 harvested corn, respectively. Total soluble sugars in corn kernel consisted of glucose, fructose and DP2 sugars and ranged from 1.43 to 3.60% w/w (COV = 22.3%) for 2012 harvested corn and 1.21 to 2.60% w/w (COV = 23.8%) for 2013 harvested corn. Highest variation in corn composition for both harvest years was in soluble sugar contents of corn kernels (COV > 20%). Among the physical

quality parameters measured, the quality factor with highest variability was stress cracks percentage having a COV of 24.2 and 40.6% for 2012 and 2013, respectively (Table 4.2).

Final ethanol concentrations (72 hr) varied ranging from 16.7 to 18.4% v/v (actual difference of 1.7% v/v) in the first year of study (Oct, 2012 to Sept, 2013) and 17.1 to 18.6% v/v (actual difference of 1.5% v/v) in the second year of study (Oct, 2013 to Sept, 2012). This represents large impact on a dry grind facility as an average loss of 1.7% v/v in ethanol yield per year at a 100 million gallon ethanol plant would be a yield loss of 1.7 million gallons of ethanol per year.

Table 4.2

Variations and means of corn physical quality and chemical composition parameters

Parameter	Range (Difference[a])		COV (%)		Means[b]		
	2012-13	2013-14	2012-13	2013-14	2012-13	2013-14	LSD
Test weight (lb/bu)	58.1 - 60.5 (2.40)	57.0 - 58.8 (1.80)	1.00	0.86	59.4 a	57.9 b	0.38
True density (g/mL)	1.271 - 1.317 (0.05)	1.264 - 1.292 (0.03)	1.00	0.69	1.295 a	1.282 b	0.008
100 kernel weight (g)	32.5 - 36.7 (4.20)	31.9 - 34.8 (2.90)	3.82	2.70	34.8 a	33.9 a	0.86
Stress cracks (%)	23.0 - 60.0 (37.0)	16.0 - 53.0 (37.0)	24.2	40.6	45.0 a	31.0 b	8.20
Moisture content (%)	11.5 - 14.0 (2.50)	12.1 - 14.2 (2.10)	5.32	4.10	12.8 b	13.3 a	0.46
Starch (% db)	66.6 - 74.4 (7.80)	67.0 - 74.8 (7.80)	3.86	3.88	69.8 a	70.6 a	1.95
Protein (% db)	9.35 - 9.95 (0.60)	8.35 - 8.65 (0.30)	1.45	1.09	9.57 a	8.50 b	0.09
Oil (% db)	3.35 - 4.10 (0.75)	3.70 - 4.05 (0.35)	4.60	2.61	3.75 a	3.84 a	0.11
Glucose (%w/w)	0.47 - 1.20 (0.73)	0.41 - 1.08 (0.67)	20.7	30.0	0.82 a	0.61 b	0.12
Fructose (%w/w)	0.00 - 0.54 (0.54)	0.23 - 0.53 (0.30)	28.2	25.2	1.23 a	0.78 b	0.29
DP2 (%w/w)	0.36 - 2.03 (1.67)	0.50 - 1.32 (0.82)	37.5	29.4	0.40 a	0.32 b	0.07
Total soluble sugars (%w/w)	1.43 - 3.60 (2.17)	1.21 - 2.60 (1.39)	22.3	23.8	2.44 a	1.70 b	0.38

[a] Actual difference = highest – lowest

[b] Means within rows followed by different letters are different ($P < 0.05$).

4.3.2. Correlations between quality parameters and ethanol concentrations (pooled data from both years)

There were no correlations between any physical quality parameter and final ethanol concentrations (72 hr) (Table 4.3). The correlation coefficient between starch content and final ethanol concentration (72 hr) was significant but low ($r = 0.47$). No correlations existed between

final ethanol concentration and protein or oil content. Even though ethanol is produced from starch in the dry grind process, poor correlation between starch content and ethanol yields have been reported by several authors. Singh and Graeber (2005) studied the effects of hybrid variability and planting locations on ethanol concentrations and reported poor correlations between starch content ($r = 0.012$) and ethanol concentrations. Zhan et al. (2003) reported $R^2 = 0.12$ between starch content and ethanol yields for 16 sorghum hybrids.

The relationship between soluble sugars present in corn kernels and ethanol concentrations had not been studied. No correlations existed between final ethanol concentration (at 72 hr) and initial glucose or fructose or DP2 contents. Negative correlations were observed between the total soluble sugars and ethanol concentrations at 72 and 48 hr ($r = -0.38$ and $r = -0.37$, respectively) (Table 4.3). Higher amounts of sugars present at the beginning of fermentation can cause osmotic stress in yeast and result in lower ethanol concentrations; and this might be the reason for negative correlation between soluble sugars and ethanol concentrations. The effect of soluble sugars present in corn kernels on ethanol concentrations were low.

Table 4.3

Correlations of quality parameters and ethanol concentrations (Pooled data from 2012 and 2013)

	Ethanol concentration (%v/v) (24 hr)	Ethanol concentration (%v/v) (48 hr)	Ethanol concentration (%v/v) (72 hr)
Moisture content (%)	-0.25	-0.05	-0.06
Test weight (lb/bu)	0.31	-0.29	-0.32
True density (g/mL)	0.12	-0.03	-0.22
100 Kernel weight (g)	0.30	-0.02	-0.11
Stress cracks (%)	0.12	0.17	0.04
Initial glucose (%w/w)	0.13	-0.29	-0.29
Initial fructose (%w/w)	0.12	-0.18	-0.10
Initial DP2 (%w/w)	0.07	-0.30	-0.29
Total Soluble sugars (%w/w)	0.03	-0.37*	-0.38*
Starch content (% db)	0.02	0.42*	0.47**
Protein content (% db)	0.19	-0.19	-0.25
Oil content (% db)	0.12	-0.07	0.05

* and **indicate significance at $P < 0.05$ and 0.01, respectively.

Multiple linear regression combining different grain quality factors was evaluated to account for additional variation to predict ethanol concentrations at 24, 48 and 72 hr of fermentation (Table 4.4). Final ethanol concentration (72 hr) was best predicted as a function of starch content and true density with an R^2 value of 0.30. Ethanol concentration at 48 hr was predicted as a function of starch content and total soluble sugars with an R^2 value of 0.24. Second order models also were evaluated to predict ethanol concentrations as a function of grain

quality attributes; however, no improvements in R^2 were achieved. Low R^2 values were indicative that ethanol concentrations cannot be predicted as functions of these attributes.

Table 4.4

Prediction of ethanol concentrations using a combination of corn quality parameters

Model	R^2 value
Ethanol concentration $_{24\ hr}$ = 2.62 + 0.18 (test weight)	0.10
Ethanol concentration $_{48\ hr}$ = 13.2 + 0.07 (starch content) – 0.23 (total soluble sugars)	0.24*
Ethanol concentration $_{72\ hr}$ = 23.7 + 0.08 (starch content) – 9.55 (true density)	0.30*

* Significant at $P < 0.05$

There is little information available on the relationships between corn grain quality parameters and ethanol yields. Factors considered in this study were corn quality attributes which can be measured rapidly at the time of purchase. However, poor correlations between these factors and ethanol concentrations were indicative that fermentation performance cannot be predicted by these factors. Other physiological factors, such as nature of starch granules, amylose:amylopectin ratios and accessibility of starch granules for enzymatic attack governed by structural features of starch-protein matrix, may be more crucial in determining the ethanol concentrations.

4.4. Conclusions

Dry grind fermentations were conducted to evaluate the effects of maize proximate composition and physical properties on ethanol concentrations, which varied throughout the duration of study (16.7 to 18.4% v/v). Final ethanol concentrations had significant but low correlations with corn starch content (r = 0.47) and total soluble sugar content (r = -0.38). There

were no correlations between physical properties of maize and ethanol concentrations. Low R^2 values were obtained after considering multiple quality factors to predict ethanol concentrations.

Chapter 5

Physiologic changes in corn protein properties during postharvest storage

5.1. Introduction

There are physiologic changes in grains during storage that may cause variation in processing characteristics, end product quality and overall yields. Depending on intended applications, these changes may be favorable to some processors and unfavorable to others. In previous chapters, we noticed that variations in ethanol concentrations were similar for commodity and control corn (Chapter 3) and physical corn quality parameters and crude composition did not account for the ethanol variability (Chapter 4). It was indicative that ethanol yield variation was caused by intrinsic changes in corn during storage. Starch conversion to ethanol is governed by several factors, one of which is the quality of protein matrix (Chapter 2, section 2.7) and changes in protein quality during storage might have an effect on ethanol yield variability (Zhao et al. 2008). Protein quality is determined by the type of protein fractions (i.e., albumin, globulin, prolamin and glutelin). While albumin and globulin fractions are biologically active proteins located in the germ, prolamin and glutelin fractions are storage proteins found in the endosperm. Free amino nitrogen (FAN) content consisting of individual amino acids and short peptides is another protein quality parameter that has been known to affect fermentation rates and ethanol yields (Vidal et al. 2011).

Our objectives were to evaluate the effects of storage on dry grind ethanol concentrations and protein physiologic properties and determine relationships among protein properties and

ethanol concentrations. Protein quality in the corn kernel was characterized based on soluble protein contents, initial FAN content and its susceptibility to enzymatic hydrolysis.

5.2. Materials and Methods

5.2.1. Substrates and enzymes

Yellow dent corn (P1018YHR) was harvested and provided by Dupont Pioneer, Johnston, IA, in Oct, 2013. Samples were stored in airtight 5 gal plastic buckets within a cold room maintained at 4°C or under a shed without walls, exposed to ambient environmental conditions for a period of 12 mo. Ambient temperature data were obtained from National Climate Data Center (NCDC 2015) to report the outdoor storage temperature conditions in Urbana, IL, during the study period. Corn (1000 g) was mixed before it was sampled every 4 wk. Samples were cleaned using a 12/64" (4.8 mm) round hole sieve by removing broken corn and foreign material (BCFM). Moisture content of whole corn (30 g) was analyzed using a standard 103°C convection oven method (Method S352.2, ASABE Standards 2002). Cleaned corn was ground using a 0.5 mm sieve in a laboratory hammer mill (1100W, model MHM4, Glen Mills, Clifton, NJ). Moisture content of ground corn was determined using a two stage standard oven method (Approved Method 44-19, AACC International 2000).

Active dry yeast (Ethanol Red, Lesaffre Yeast Corp., Milwaukee, WI) was used for fermentation. A urea stock solution (50% w/v from 99.6% ACS grade obtained from Fisher Scientific, Fair Lawn, NJ) was prepared and used as the nitrogen source for yeast. All other chemicals were obtained from Fisher Scientific (Fair Lawn, NJ), unless stated otherwise. Enzymes used in the dry grind process were AA-II and GA- II with optimum pHs of 5.1 and 5.0, respectively. Activities and dosages of AA-II and GA-II are mentioned in sections 3.2.1 and 3.2.4, respectively. A protease enzyme (optimum pH 4.8 and activity 9.42 µmol/min mL)

provided by a commercial enzyme company was used for determining protein hydrolysis rate. Enzyme activity was measured as in section 3.2.2 (Chapter 3). All analyses were in duplicate.

5.2.2. Composition Analyses

Crude protein and oil contents of corn samples were analyzed using near infrared spectroscopy (NIR) at the Illinois Crop Improvement Association Laboratory, Champaign, IL. The NIR instrument (Model Infratec 1229, Whole grain analyzer, FOSS, Eden Prairie, MN) was calibrated using wet chemistry standard methods of the Corn Refiners' Association (CRA 1980). Oil and protein calibrations were based on methods A-6 and A-18, respectively (CRA 1980). Standard errors of predictions for oil and protein were 0.25 and 0.27%, respectively. Starch content was determined using the acid hydrolysis method as explained in Chapter 4 (Vidal et al. 2009).

5.2.3. Dry Grind Process

Ground corn (100 g, db) was used for dry grind fermentation. Two replicates were used for each corn sample. Corn dry grind process was carried out as mentioned in section 3.2.3 in Chapter 3. Fermentation process was monitored by withdrawing 1 mL samples at 24, 48 and 72 hr, which were analyzed using HPLC.

5.2.4. Extraction of soluble protein classes

Defatted sample preparation

Corn was sampled at wk 0, 8, 16, 24 and 40. Ground corn (20 g) was extracted with hexane (1 g/10 mL, sample/hexane ratio) at 22°C and 150 rpm for 2 hr in a shaking incubator (Innova 42, Eppendorf, Enfield, CT). Each sample was centrifuged at 3234 × *g* (Model 5804R, Eppendorf, Enfield, CT) for 15 min at 22°C and supernatant fraction discarded. Sample was air

dried for 3 hr and extracted again with hexane for 1 hr. The defatted sample was air dried overnight to remove residual hexane. Defatted sample was used for the following protein fractionation process.

Protein fractionation

Soluble proteins were extracted from the defatted sample following the method of Evangelista et al. (2006) (Fig 5.1). Defatted ground corn (5 g, db) was extracted sequentially with water (5°C), 0.5 M NaCl (5°C), 70% ethanol (22°C) and 0.1 M NaOH (22°C) to obtain albumin, globulin, prolamin and glutelin fractions, respectively, in a shaking incubator at 150 rpm (Innova 42, Eppendorf, Enfield, CT). Sample was centrifuged at 3234 × *g* (Model 5804R, Eppendorf, Enfield, CT). All supernatant fractions except the water soluble fraction were dialyzed against deionized water for 72 hr at 4°C using Spectra/Por 3 molecular porous membrane tubing (Spectrum Laboratories, Inc., Rancho Dominguez, CA). Dialyzed supernatant fractions, water soluble supernatant fraction and solids remaining after extraction were freeze dried (Labconco Freezone 6 L Console Freeze dry system, Labconco, Kansas City, MO) for 72 hr. All freeze dried samples were analyzed for protein content (Method 990.23, AOAC 2007) at Illinois Crop Improvement Association Laboratory, Champaign, IL. Soluble and unextracted protein contents were calculated and reported with respect to sample total crude protein content.

Coefficient of variation (COV) of the protein extraction procedure was calculated for all four soluble and unextracted protein contents. This was determined by running 3 independent extraction experiments within a week using a corn sample from a single hybrid stored at 4°C during the course of experiment. All supernatant fractions were prepared (dialyzed and freeze dried) within the next 6 days and analyzed for protein content. COVs of protein extraction

procedure for albumin, globulin, prolamin, glutelin and unextracted contents were 26.4, 14.7, 12.0, 5.70 and 15.1%, respectively.

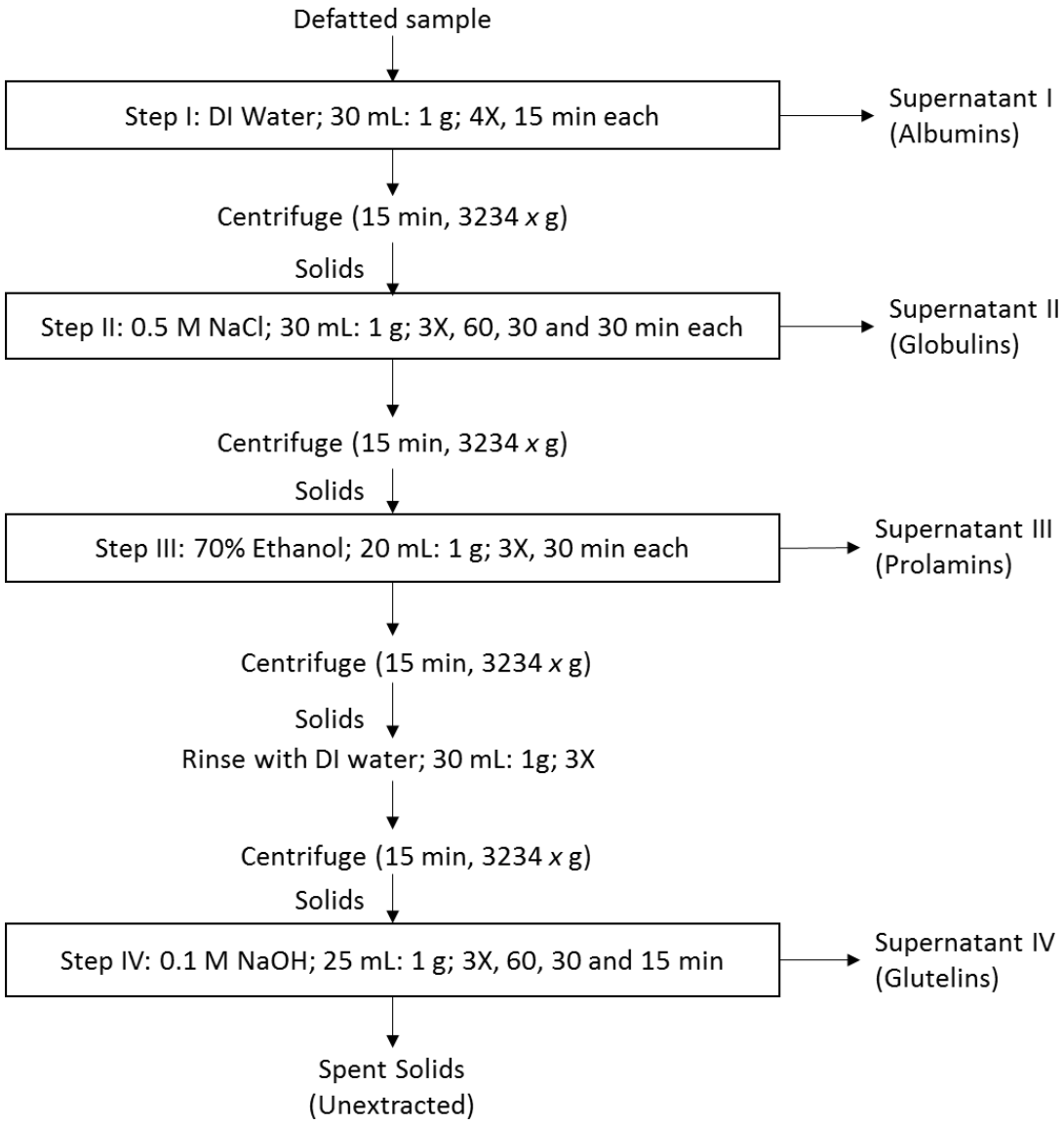


Fig 5.1. Schematic of sequential extraction of corn soluble proteins.

5.2.5. FAN protein hydrolysis

Proteolysis rate was determined using the procedure outlined by Vidal et al. (2009). Ground corn (50 g, db) was mixed with water to obtain 30% slurry. Slurry pH was set to 4.8 and

protease enzyme (0.25% w/w) was added. Aliquots of samples were taken at 0, 1, 2, 4 and 6 hr (incubation time) and frozen before analysis for FAN content. FAN was analyzed using the ninhydrin colorimetric method (Official Method 945.30L, AOAC 1980). COV of FAN analysis procedure was 3.23% which was measured by running 5 experiments within a week (one experiment per day with three replicates in each experiment) using a corn sample from a single hybrid stored at 4°C.

5.2.6. Statistical Analyses

All statistical analyses were conducted using SAS version 9.4 (SAS Institute Inc., Cary, NC) ($P < 0.05$). Fisher's least significant difference (LSD) was used to determine differences in ethanol concentrations, soluble and unextracted protein contents, composition analyses and FAN contents among values from different storage conditions. Pearson's correlation coefficients were calculated using the correlation procedure, PROC CORR, to determine relationships among protein quality characteristics and ethanol concentrations. FAN protein hydrolysis rates (slopes) were compared for the linear region of the FAN production profile (0 to 2 hr) using analysis of covariance (ANCOVA) with storage time as the classification factor. Slopes were tested to check if they were different from 0. Numerically highest and lowest slopes (that were different from 0) were chosen and pairwise comparisons of these chosen slopes were done against all other slopes. This was done separately for corn stored at ambient and refrigerated conditions. Effect of temperature conditions on protein hydrolysis rates were tested by comparing slopes for ambient and refrigerated conditions at different storage times.

5.3. Results and Discussion

5.3.1. Effects of storage conditions on dry grind ethanol concentrations and crude composition of corn

Ethanol concentrations varied with storage time. Ethanol concentrations for corn stored at ambient and refrigerated conditions varied from 17.1 to 19.0% v/v (COV = 2.85%) and 17.3 to 18.8% v/v (COV = 2.95%), respectively, which were higher than the experimental COV (0.16%) for dry grind procedure (mentioned in Chapter 3). Trends in ethanol concentrations with storage time were similar for corn stored at ambient and refrigerated conditions and to 2013 commodity corn (obtained from a Midwestern ethanol plant in 2013 as mentioned in Chapter 3, section 3.3.2). Local minima and maxima points were wk 16 and 24, respectively, for corn stored at ambient and refrigerated conditions (Appendix C, Figs. C.1 and C.2). For both storage conditions, ethanol concentrations had a decreasing trend from wk 0 to 16 and an increasing trend from wk 16 to 24 (Appendix C, Figs. C.1, C.2, C.3 and C.4). Slopes in the last time segment (wk 24 to 44) were not different from 0. Across storage time, average ethanol concentration of corn stored at ambient condition was higher than the corn stored at refrigerated condition (Table 5.1). However, overlaying the average temperature data for 2013 ambient conditions and refrigerated conditions with the ethanol concentrations, storage temperatures had significant but small effects on ethanol concentrations (Fig. 5.2). There was no correlation between storage temperature and ethanol concentrations ($r = -0.06$) but, storage time and ethanol concentrations had a negative correlation ($r = -0.49$). The reason for negative correlation between storage time and ethanol concentrations is not clear. Plumier et al. (2014) reported similar results that effects of storage temperatures used in their study (ranging from -3 to 20.7°C) on unreacted starch contents in DDGS were lower than storage time. They reported a positive correlation ($r =$

0.74) and negative correlation (r = -0.40) between unreacted starch contents and storage time in 2011 and 2012, respectively. More research is warranted to understand the effects of crop years (growing season) and its subsequent effects on ethanol concentration trends after harvest.

There were no changes in kernel crude starch or protein content for corn stored at refrigerated conditions (Appendix C, Table C.1). There were variations in starch and protein contents across storage time for corn stored at ambient conditions; however, starch variation was within the procedural COV from acid hydrolysis method (2.0%). Differences in overall protein content across different time points were < 1%.

Table 5.1. Ethanol concentrations from corn stored for one year at different conditions[3]

	Ethanol concentration (% v/v)		
Storage time (Week)	**Ref[1]**	**Amb[2]**	**Mean**
0	18.5 ± 0.098	18.5 ± 0.098	18.5 b
4	18.5 ± 0.091	18.5 ± 0.149	18.5 b
8	18.4 ± 0.014	18.5 ± 0.027	18.4 b
12	17.6 ± 0.051	18.1 ± 0.052	17.8 d
16	17.3 ± 0.052	17.5 ± 0.244	17.4 e
20	17.8 ± 0.038	17.8 ± 0.154	17.8 d
24	18.8 ± 0.118	19.0 ± 0.090	18.8 a
28	17.8 ± 0.061	17.8 ± 0.071	17.8 d
32	17.5 ± 0.092	18.0 ± 0.096	17.8 d
36	17.3 ± 0.030	17.1 ± 0.111	17.2 f
40	18.3 ± 0.128	18.0 ± 0.059	18.2 c
44	17.3 ± 0.113	17.7 ± 0.121	17.5 e
Mean	17.9 B	18.0 A	

[1] *Refrigerated;* [2]*Ambient.*

[3] Values are means of two replicates. *Means with the same lowercase letter within a column were not different. Means with the same uppercase letter within a row were not different.*

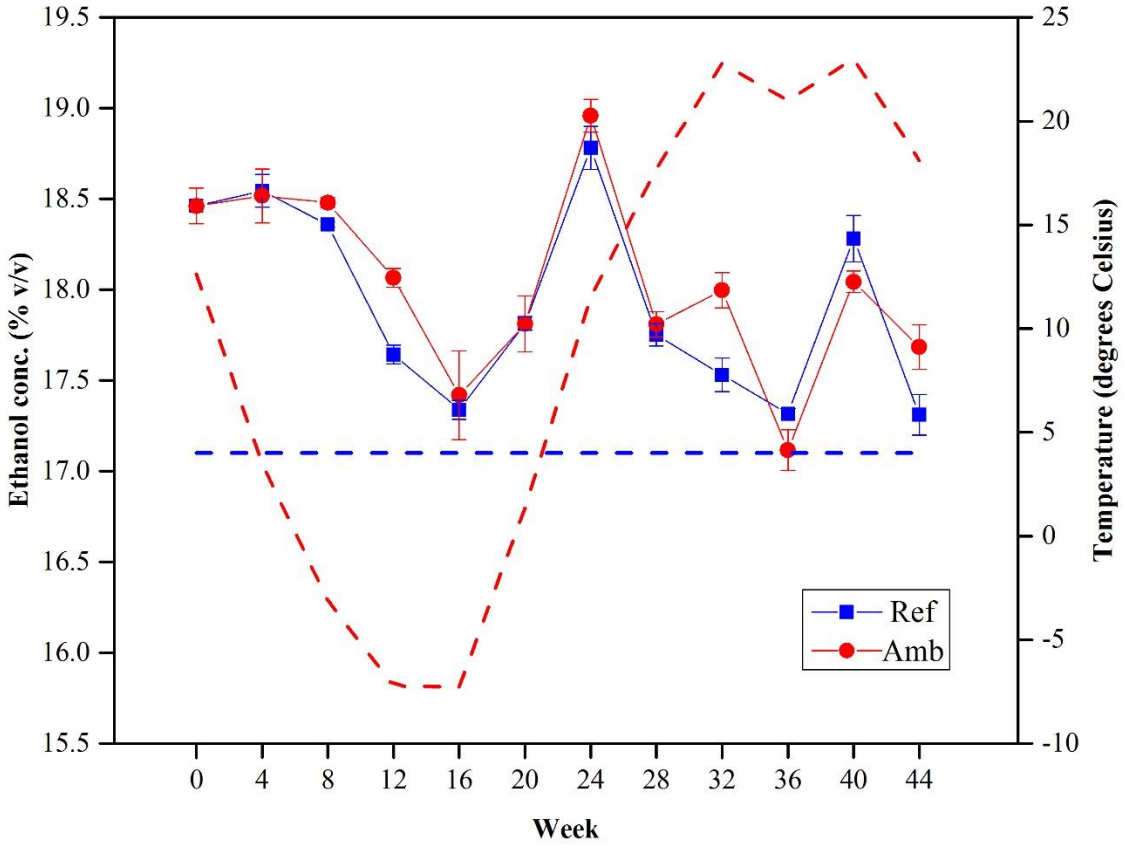


Fig. 5.2. Ethanol concentration variation with time for corn stored at refrigerated (Ref) and ambient (Amb) conditions (2013-14). *Red dashed lines denotes the average temperature conditions in Urbana, IL (obtained from NCDC 2015); blue dashed line denotes the constant refrigerated temperature (4°C).*

5.3.2. Changes in corn soluble protein contents during storage

The amounts of albumins, prolamins and glutelins varied significantly ($P < 0.05$) among various storage times for corn stored at ambient and refrigerated conditions (Figs. 5.3 and 5.4; Tables 5.2 and 5.3). COV across storage time for albumin, prolamin and glutelin contents for corn stored at ambient conditions were 37.0, 36.2 and 17.1, respectively, and were higher than their respective COV from the experimental procedure (Table 5.2).

All soluble and unextracted protein contents reported here are with respect to the crude protein content of the corn sample. Soluble protein contents for corn after harvest (wk 0) were 14.6% (albumin), 11.7% (globulin), 17.6% (prolamin) and 27.6% (glutelin). Boundy et al. (1967) reported albumin, globulin, prolamin and glutelin contents for dent corn as 10.9, 6.6, 22.6 and 26.6%, respectively. Landry and Moreaux (1980) have reported 15.6% albumin + globulin, 39.4% prolamin and 32.1% glutelin contents from whole corn. In our study, albumin and globulin contents were higher and prolamin content was lower compared to what has been reported in the literature. This is most likely due to the particular corn cultivar used in this study. Moueium et al. (1996) studied soluble protein classes in 10 corn cultivars and results were indicative that protein classes vary widely among corn hybrids. They reported a variation of 19.5 to 26.2% for albumin + globulin, 18.3 to 35.4% for prolamin (zeins) and 20.9 to 35.3% for glutelin contents.

For corn stored at ambient conditions, albumin content was 14.6% at wk 0 and increased to 32.1 and 32.6% during wk 8 and 16, respectively, and decreased to 18.1% after 44 wk of storage. Prolamin content increased from 17.6 to 40.0% from wk 0 to 24 and did not vary from wk 24 to 40 (40.0 to 36.0%). For glutelin content, no consistent pattern was found. Glutelin content was 27.6% during wk 0 and decreased to 18.9 and 19.1% during wk 8 and 24 and

increased to 22.2% which was lower than what was observed at the beginning of the study (wk 0). Similar results were obtained for corn stored at refrigerated conditions: COV across storage time for albumin, prolamin and glutelin contents conditions were 39.0, 33.3 and 16.9% (Table 5.3). Albumin content increased from 14.6 to 31.3 and 28.7% from wk 0 to 8 and 16, respectively, and then decreased to 16.0% during wk 40. Prolamin content increased from 17.6 to 42.4% from wk 0 to 24 and then decreased to 35.3% during wk 44 (Fig. 5.4). There was no variation in globulin content with storage time for corn stored at both refrigerated and ambient conditions. Unextracted protein content did not vary with storage time from wk 8 to 40 for corn stored at both ambient and refrigerated conditions (COV of data from different storage times < COV from procedure) (Tables 5.2 and 5.3). There were no effects of temperature on any of the protein contents; there were differences in mean albumin and globulin contents at wk 24 and 40, respectively, between refrigerated and ambient conditions but they were within the procedural COV (Appendix C, Tables C.2 and C.3).

There are few studies in the literature on the effects of storage on corn protein classes. Reduction in albumins was reported by Paraginski et al. (2014) for corn stored at different temperatures ranging from 5 to 35°C. Albumin content was reduced by 23.7, 6.80, 30.0 and 47.0% for corn stored at 5, 15, 25 and 35°C, respectively after 12 mo. McDonough et al. (2004) observed a reduction in corn albumin, globulin and prolamin contents and an increase in glutelin content with time when it was stored at 50°C. They reported that albumin and globulin contents decreased from 12 to 7 mg/g protein, prolamin content was reduced from 35 to 30 mg/g protein and glutelin content increased from 25 to 29 mg/g protein during 0 to 15 days of storage. Unextracted protein content increased from 5 to 12 mg/g protein during this storage duration. The differences in our results and those from McDonough et al. (2004) could be due to different

storage conditions. Temperature conditions used in our study were constant refrigerated conditions (4°C) and ambient conditions ranging from -7 to 23°C; whereas, McDonough et al. (2004) used high temperatures (50°C) and stored corn only for 15 days. McDonough et al. (2004) suggested that mechanisms similar to the grain maturation process might be responsible for changes in proteins during aging. Albumins and globulins decreased and are either degraded or transformed into storage proteins (glutelins) during grain maturation process. Chrastil and Zarins (1992) reported average molecular weight of oryzenin (glutelin) in rice doubled during storage, a mechanism observed during ripening of rice grains and proposed that biochemical ripening process is not completely stopped and continues during storage. Similar phenomenon was observed in our study when the albumin content decreased from wk 8 to 40 (2 to 10 mo) and prolamin content increased during this time.

Table 5.2. Amounts of protein classes (% of total protein) during storage for corn stored at ambient conditions[1]

TIME (WEEK)	ALBUMIN	GLOBULIN	PROLAMIN	GLUTELIN	UNEXTRACTED	TOTAL
0 (OCT)	14.6 ± 0.720 b	11.7 ± 5.00 a	17.6 ± 0.863 c	27.6 ± 1.30 a	NA	NA
8 (DEC)	32.1 ± 1.08 a	12.4 ± 2.29 a	26.1 ± 4.03 b	18.9 ± 0.493 c	9.41 ± 1.02 ab	98.9
16 (FEB)	32.6 ± 2.65 a	10.4 ± 0.434 a	19.0 ± 3.01 bc	25.7 ± 0.276 a	12.1 ± 1.44 a	99.7
24 (APR)	18.2 ± 0.182 b	12.5 ± 7.41 a	40.0 ± 3.15 a	19.1 ± 0.905 c	9.97 ± 0.571 ab	99.8
40 (AUG)	18.1 ± 4.11 b	13.6 ± 0.073 a	36.0 ± 1.74 a	22.2 ± 0.087 b	9.06 ± 0.723 b	99.0
LSD	7.08	12.4	8.34	2.10	2.76	
COV (%)	37.0	9.62	36.2	17.1	13.3	
PROCEDURAL COV (%)	26.4	14.7	12.0	5.70	15.1	

[1] *Values are reported with respect to the total crude protein content of the sample. All values are means of two replicates. Means with the same lowercase letter within a column were not different.*
NA = Not available (data point lost as weight of final residue was not measured).

Table 5.3. Amounts of protein classes (% of total protein) during storage for corn stored at refrigerated conditions[1]

TIME (WEEK)	ALBUMIN	GLOBULIN	PROLAMIN	GLUTELIN	UNEXTRACTED	TOTAL
0 (OCT)	14.6 ± 0.720 b	11.7 ± 5.00 a	17.6 ± 0.863 d	27.6 ± 1.30 a	NA	NA
8 (DEC)	31.3 ± 1.93 a	15.7 ± 4.15 a	24.7 ± 0.346 c	20.0 ± 0.137 bc	9.68 ± 3.84 a	101.4
16 (FEB)	28.7 ± 0.431 a	9.02 ± 0.050 a	25.8 ± 0.633 c	24.9 ± 0.222 ab	11.0 ± 0.075 a	99.5
24 (APR)	14.7 ± 0.225 b	12.7 ± 6.28 a	42.4 ± 1.58 a	18.1 ± 0.001 c	11.3 ± 0.394 a	99.2
40 (AUG)	16.1 ± 1.50 b	16.1 ± 0.009 a	35.3 ± 1.82 b	24.8 ± 3.86 ab	9.01 ± 1.10 a	101.2
LSD	3.61	11.7	8.34	2.10	5.57	
COV (%)	39.0	22.5	33.3	16.9	10.7	
PROCEDURAL COV (%)	26.4	14.7	12.0	5.70	15.1	

[1] *Values are reported with respect to the total crude protein content of the sample. All values are means of two replicates. Means with the same lowercase letter within a column were not different.*

NA = Not available (data point lost as weight of final residue was not measured).

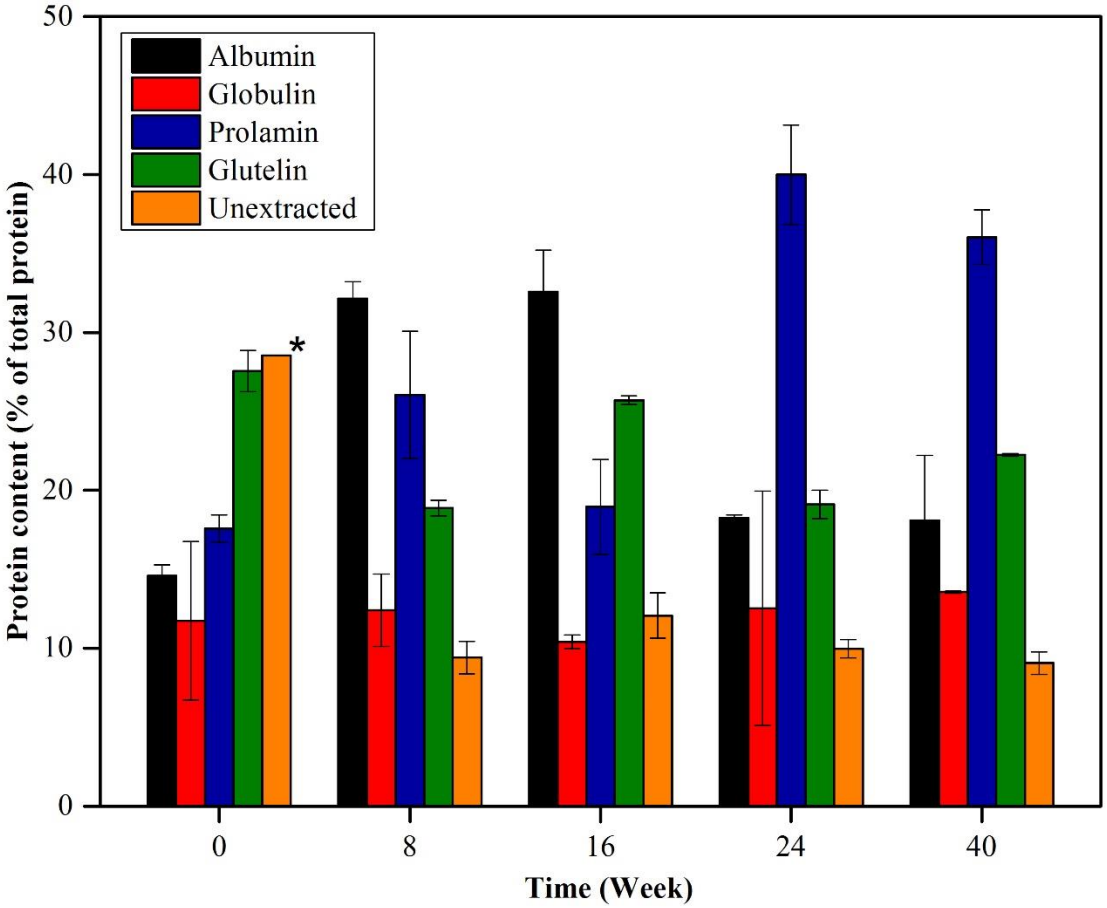


Fig. 5.3. Amounts of protein classes for corn stored at ambient conditions.
***** *Unextracted protein content for wk 0 was calculated as (100-sum of soluble protein contents).*

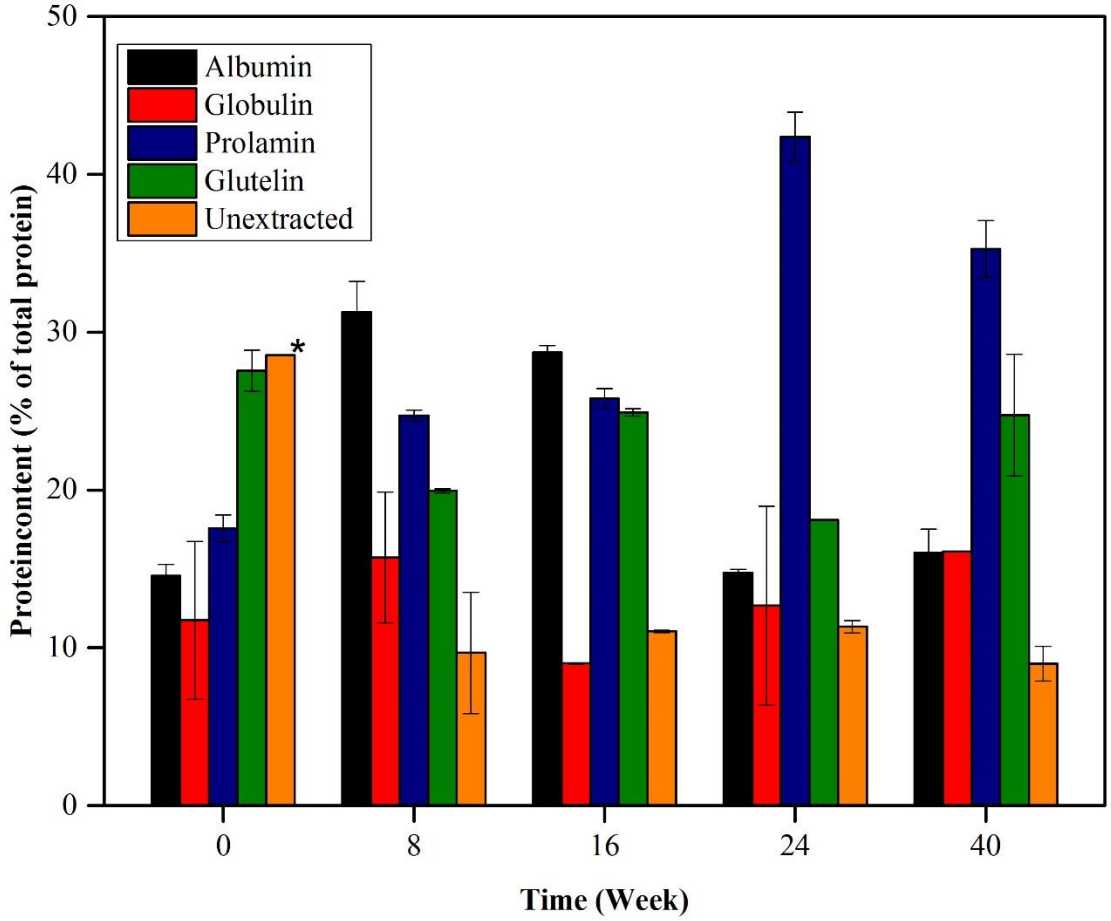


Fig. 5.4. Amounts of protein classes for corn stored at refrigerated conditions.
***** *Unextracted protein content for wk 0 was calculated as (100-sum of soluble protein contents).*

5.3.3. Changes in initial FAN content and FAN protein hydrolysis rates during storage

Initial FAN content of corn slurry (30% solids content) in the beginning of the study (0 wk) was 69 ± 12.0 mg/L (Fig 5.5; Table 5.4). This was within the reported range of FAN content in cereal grains. Vidal et al. (2009) reported initial FAN content of 80 mg/L for ground soaked corn (37% slurry). Wheat mashes with 16% solids content were reported to have 54 to 58 mg/L FAN content (Thomas and Ingledew 1990). There was notable variation in FAN content with storage time (Table 5.4). COV of initial FAN content across storage time for corn stored at

ambient and refrigerated conditions were 49.4 and 47.6%, respectively, (higher than the procedural COV of 3.23%). Highest FAN content was observed during wk 40 for corn stored at refrigerated (278 ± 15.7 mg/L) and ambient (301 ± 6.95 mg/L) conditions. Initial FAN content of corn had an overall increasing trend with storage time with a positive correlation coefficient of 0.60. There was no effect of storage temperature (ambient or refrigerated) on average FAN content across storage time (Table 5.4). Few studies have reported the effects of storage on FAN content of cereal grains. Onigbinde and Akinyele (1990) reported that there were no changes in FAN content of whole corn stored at ambient (18.0 to 35.6°C) conditions for 7 mo; however, in this study, the measurements were taken only before and after 7 mo of storage. Increased free amino acid content and proteolytic activity was reported for rice stored at ambient conditions for 12 mo (Dhaliwal et al. 1991).

Variation in initial FAN content with storage time was indicative of changes in protein structure during storage. Furthermore, changes in protein structure during storage were elucidated from variation in protein hydrolysis profiles with storage time. FAN production profile at selected storage time points is presented in Fig 5.6, while entire data are presented in appendix C (Tables C.6 and C.7). FAN production at the beginning of the study (0 wk) and after 0 and 6 hr of protease incubation were 69 ± 12 mg/L and 217.2 ± 5.93 mg/L. This was comparable to values reported by Vidal et al. (2009) where they noted FAN content for ground soaked corn (37% slurry) to be 80 and 312 mg/L after 0 and 6 hr of protease incubation. More than 50% of the FAN was produced in the first 2 hr of protease incubation and rates followed linear trends from 0 to 2 hr region ($R^2 = 0.99$).

For refrigerated conditions, protein hydrolysis rates were different from 0 for all wk except for wk 40 (11.8 mg/L.hr). Highest rate numerically was observed during wk 20 (129.2

mg/L.hr). This was compared to rates at all other storage times and was found to be higher than all of them (Fig 5.6 and Table 5.5). Lowest rate numerically was observed during wk 24 (25.1 mg/L.hr) which was similar to wk 0, 4, 8, 12, 28 and 32 but lower than wk 16, 20, 36 and 44. For ambient conditions, rates were different from 0 for all wk. Highest rate numerically was during wk 20 (127.7 mg/L.hr) and was higher than rates at all other storage times. Lowest rate numerically was during wk 24 which was similar to rates at 0, 4, 8,12, 28, 32 and 40 wk of storage but lower than 16, 20, 36 and 44 wk. Except for wk 40, rates at all storage time points were similar for ambient and refrigerated conditions (Fig 5.6 and Table 5.5).

Table 5.4. Initial FAN content (mg/L) during storage for corn stored at refrigerated conditions

	Storage temperature		
Storage Time (wk)	**Ref[1]**	**Amb[2]**	**Mean**
0	69.0 ± 12.0	69.0 ± 12.0	69.0 i
4	83.6 ± 0.746	82.4 ± 8.81	82.9 h
8	63.6 ± 0.895	77.2 ± 0.447	70.4 hi
12	103.6 ± 19.8	106.1 ± 7.65	104.8 fg
16	175.1 ± 13.2	159.0 ± 6.29	167.0 cd
20	235.3 ± 3.67	233.0 ± 10.1	234.2 b
24	96.8 ± 0.780	96.6 ± 4.29	96.7 g
28	110.3 ± 6.76	110.3 ± 1.58	110.3 f
32	131.2 ± 2.77	124.7 ± 0.161	127.9 e
36	167.6 ± 2.27	149.6 ± 6.57	158.6 d
40	277.7 ± 15.7	301.6 ± 6.95	289.6 a
44	174.1 ± 0.322	172.7 ± 0.276	173.4 c
Mean	140.6 A	140.2 A	

[1] *Refrigerated;* [2]*Ambient.*

[3] *Values are means of two replicates. Means with the same lowercase letter within a column were not different. Means with the same uppercase letter within a row were not different.*

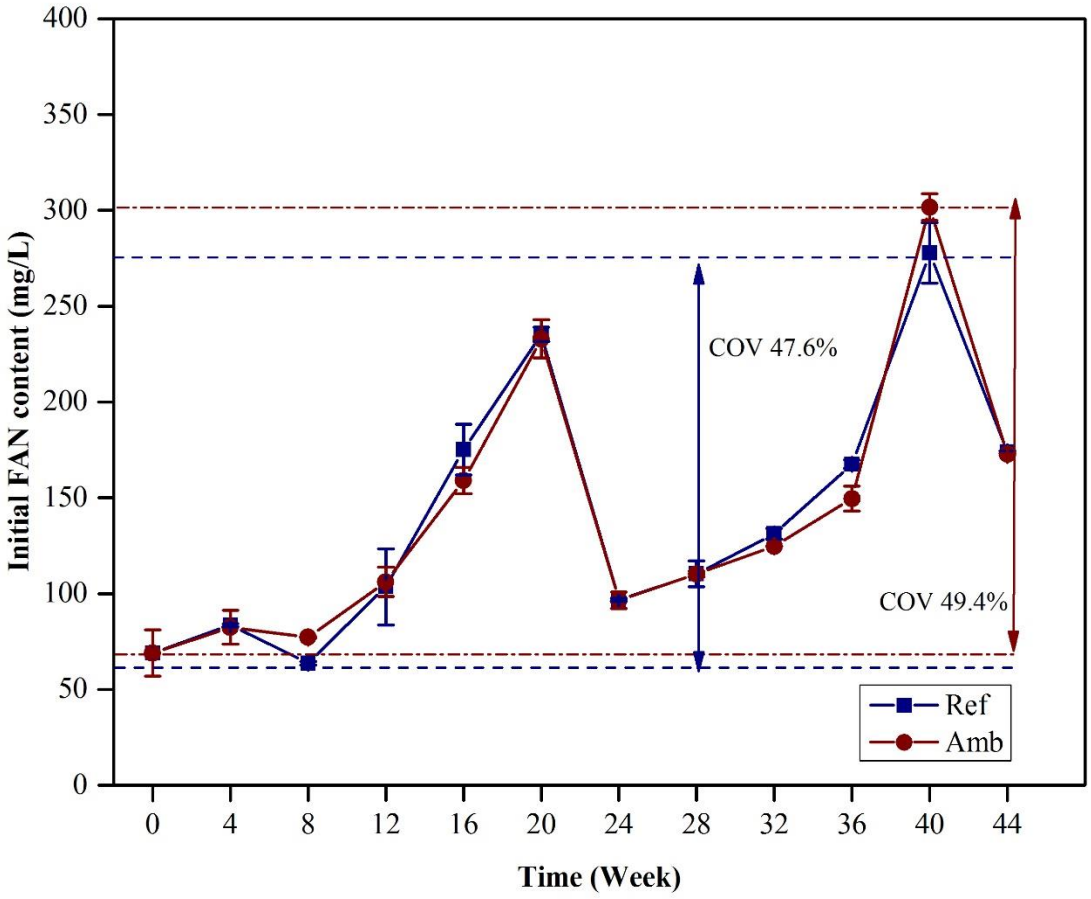


Fig. 5.5. Changes in initial FAN content during storage.

Table 5.5. Equations for linear portion of FAN production profile (t = 0 to 2 hr of protease incubation time).

	Ref[1]		Amb[2]	
Storage Time (wk)	**FAN (mg/L)**	**R^2**	**FAN (mg/L)**	**R^2**
0	38.5 t + 68.0	0.99	38.5 t + 68.0	0.99
4	59.1 t + 90.0	0.97	55.6 t + 82.8	0.99
8	40.8 t + 62.9	0.99	33.5 t + 77.8	0.99
12	55.5 t + 112.6	0.94	50.3 t + 104.6	0.99
16	72.4 t + 191.5	0.87	78.4 t +176.8	0.86
20[3]	**129.2** t + 252.6	0.95	**127.7** t +248.8	0.96
24[4]	**25.1** t +101.6	0.90	**26.3** t + 101.3	0.91
28	35.1 t +119.7	0.82	42.9 t + 117.2	0.93
32	42.4 t + 142.9	0.81	45.7 t + 137.1	0.82
36	62.2 t + 179.7	0.90	64.2 t + 166.1	0.84
40	11.8 t + 279.8	0.92	26.9 t + 294.9	0.84
44	91.1 t +194.4	0.87	95.5 t + 194.0	0.87

[1] *Refrigerated,* [2]*Ambient*

[3] *Numerically highest slope that was different from 0; this was higher than slopes at all other storage times within each storage condition (ref or amb).*

[4] *Numerically lowest slope that was different from 0; this was lower than slopes at wk 16, 20, 36 and 44 within each storage condition (ref or amb).*

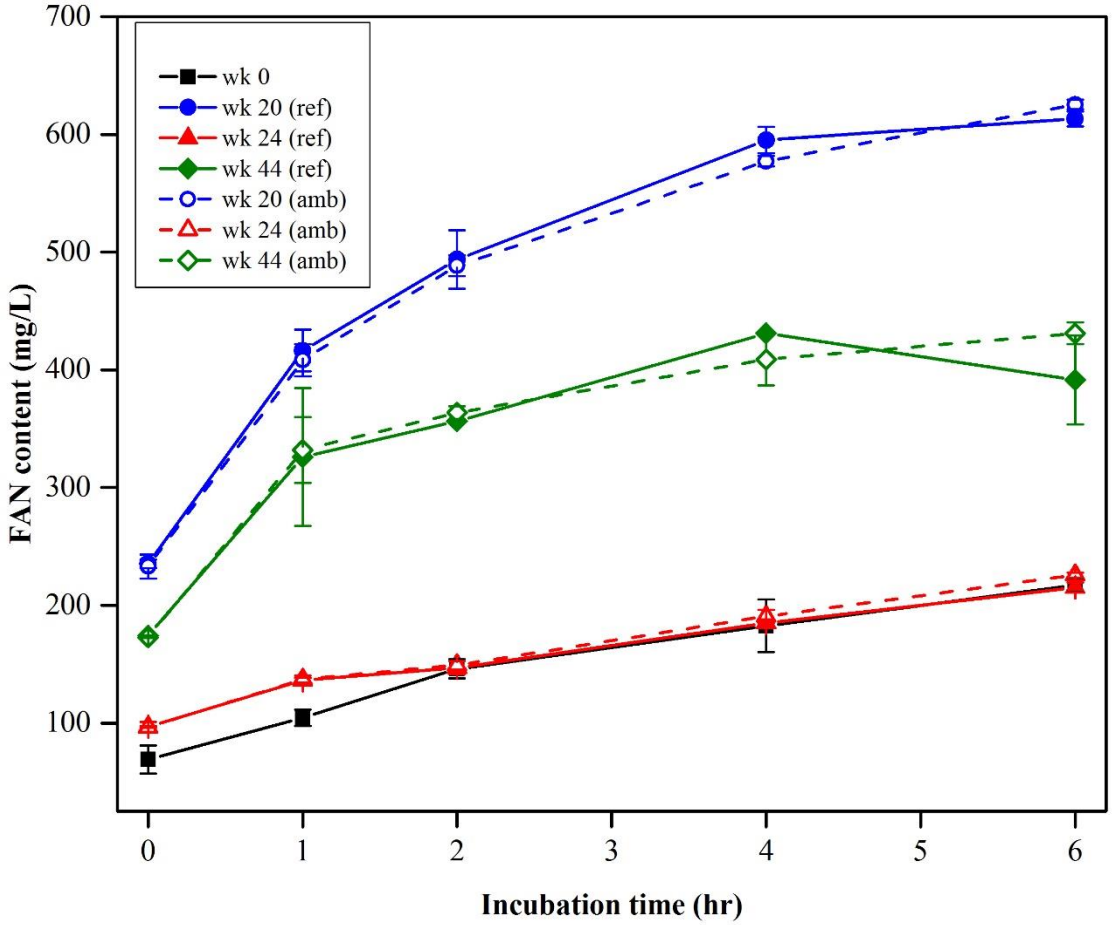


Fig. 5.6. FAN production profile for corn stored at different conditions.

5.3.4. Correlations among protein quality parameters and ethanol concentrations

There were no correlations among any of the protein quality parameters and final ethanol concentrations (72 hr) (Table 5.6). A negative correlation ($r = -0.76$; $R^2 = 0.58$) was found between glutelin content and ethanol concentration at 24 hr. As glutelins comprises the protein matrix surrounding the starch granules in the corn endosperm region, high glutelin content will correspond to lower accessibility of starch granules to enzymes to be converted to ethanol. Although the R^2 value is not high, these results are indicative that changes in glutelin content during storage might have a relationship with the variation in ethanol yields during storage. However, the correlation between prolamin content and ethanol concentration at 24 hr ($r = 0.74$; $R^2 = 0.55$) is not clear. Like glutelins, prolamins (zeins) are storage proteins and form protein bodies in the endosperm region; grains with hard endosperm have higher amount of prolamin content compared to soft grains (Chandrashekar and Mazhar 1999). It was expected that higher prolamin content also would limit the conversion of starch granules to ethanol. Dombrink-Kurtzman and Bietz (1993) reported that fractions of different types of zeins (α, β, γ and δ zeins) varied in the hard and soft endosperm portion of corn kernel. Amount of α and δ-zeins (19/22 and 10 kDa) in hard endosperm fraction were higher by an average of 3.3 times compared to soft endosperm fractions. In contrast, soft endosperm contained twice the amount of γ-zeins (27 kDa) compared to the hard endosperm fraction. Further characterization of prolamins can reveal the changes in different types of zeins during storage and may provide more insight into the relationship between the prolamin content and ethanol yield.

Investigators have shown that initial FAN content of the cereal grain has an effect on fermentation performance (Yan et al. 2011; Vidal et al. 2011). However, we did not find the initial FAN content of corn slurry to be a source of variation for ethanol concentrations during

storage. It is possible that changes in FAN content during storage were not associated with the right kind of peptides or amino acids that play a role during fermentation. For example, glycine, lysine and arginine are inhibitory to yeast growth and fermentation even though they are consumed readily by yeast, while, glutamic acid promotes yeast fermentation rates (Thomas and Ingledew 1990). Another related factor which was not taken into account was the amount of FAN produced during the dry grind process (the alpha amylase used in the dry grind process had protease activity). This along with the initial FAN content might have contributed to variation in ethanol yield.

Table 5.6. Correlations among protein quality parameters and ethanol concentrations (Pooled data from corn stored at ambient and refrigerated conditions)

Parameters	Ethanol concentration (% v/v) (24 hr)	Ethanol concentration (% v/v) (48 hr)	Ethanol concentration (% v/v) (72 hr)
Albumin (%)	-0.47	-0.42	-0.53
Globulin (%)	0.22	0.45	0.51
Prolamin (%)	**0.74***	0.31	0.53
Glutelin (%)	**-0.76***	-0.41	-0.62
Unextracted (%)	-0.05	-0.24	-0.41
Initial FAN content (mg/L)	-0.09	-0.26	-0.37

**Significant at $P < 0.05$.*

5.4. Conclusions

There were variations in ethanol concentrations during storage; variability trends for corn stored at ambient and refrigerated conditions were similar. Albumin, prolamin and glutelin contents varied with storage time; globulin content was generally constant during storage. Albumin content increased from wk 8 to 40 and prolamin content decreased during this period.

Initial FAN content of corn slurry had an increasing trend with storage time and FAN protein hydrolysis rates also were affected with storage time. Highest FAN protein hydrolysis rate was observed during wk 20. Storage temperatures (ambient or refrigerated) did not affect soluble protein contents and initial FAN contents. Final ethanol concentrations (72 hr) did not have any correlations with soluble protein contents and initial FAN contents. There were correlations observed between ethanol concentrations at 24 hr and glutelin content ($R^2 = 0.58$) and prolamin content ($R^2 = 0.55$); however, as the R^2 values were low, further investigation is recommended.

Chapter 6

Conclusions and Recommendations for Future work

Our goal was to quantify seasonal variation in ethanol yields during storage and evaluate the effects of different grain quality factors on ethanol yield variation. The main conclusions were:

1. Temporal trends in ethanol yields for commodity corn (from a Midwestern ethanol plant) and control corn (an identity preserved corn hybrid stored at refrigerated conditions) were similar.
2. Enzyme treatment with highest amylase and protease activity increased mean ethanol yield at a dry grind plant; this can increase plant profitability.
3. Final ethanol concentrations correlated with starch ($r = 0.47$) and total soluble sugar ($r = -0.38$) content. There were no correlations of physical grain quality factors to ethanol yields. Using multiple regression analysis, effects of combination of grain quality factors to ethanol yields were determined; however, low R^2 values were obtained. This was indicative that variation in ethanol yield over time was caused by intrinsic changes in corn kernel during storage.
4. Soluble protein classes (albumins, prolamins and glutelins) and initial FAN contents of corn varied during storage. Ethanol concentration variation was similar for corn stored at refrigerated and ambient conditions. There were no correlations of final ethanol concentrations with soluble proteins or initial FAN content. Ethanol concentrations at 24 hr had correlations with glutelin content ($r = -0.76$) and prolamin content ($r = 0.74$).

The following issues are recommended for future investigation:

1. Using effective enzyme treatments increased mean ethanol yield at a dry grind plant. Further study can be conducted to determine the optimum enzyme loading that would result in highest mean ethanol yield. Additionally, economic analyses, including the enzyme costs, should be conducted to determine if using these advanced enzymes can increase plant profitability.
2. Reduced amylose:amylopectin ratios for rice and corn stored for more than 6 mo have been reported (Labuschagne et al. 2014; Patindol et al. 2005). A study can be conducted to determine the changes in amylose:amylopectin ratios from pure starch (isolated from corn) and ground corn during storage and their relationships to ethanol yield variation. This also would reveal the effects of nonstarch components in corn during storage.
3. The positive correlation between prolamin content and ethanol yields (24 hr) was not clear. Further characterization of the soluble proteins using electrophoresis techniques could give a better insight into the protein structural features. Protein fractionation experiments need to be scaled up to recover large sample amounts for further characterization.
4. An approach used in the baking industry to reduce variability in product quality due to "new crop season phenomenon" was to store the freshly harvested wheat for 2 to 3 mo before incorporating it into the process or by mixing 5 to 15% of new wheat into the old wheat mix. Similar strategy can be explored to reduce the ethanol yield variability in the dry grind process.
5. The drastic reduction in initial FAN content of corn slurry from wk 20 to 24 and simultaneous increase in FAN content from wk 24 to 40 observed in this study was questionable. This experiment should be repeated again to confirm these results.

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Appendices

Appendix A: Chemical corn quality attributes of commodity corn (2012-14)

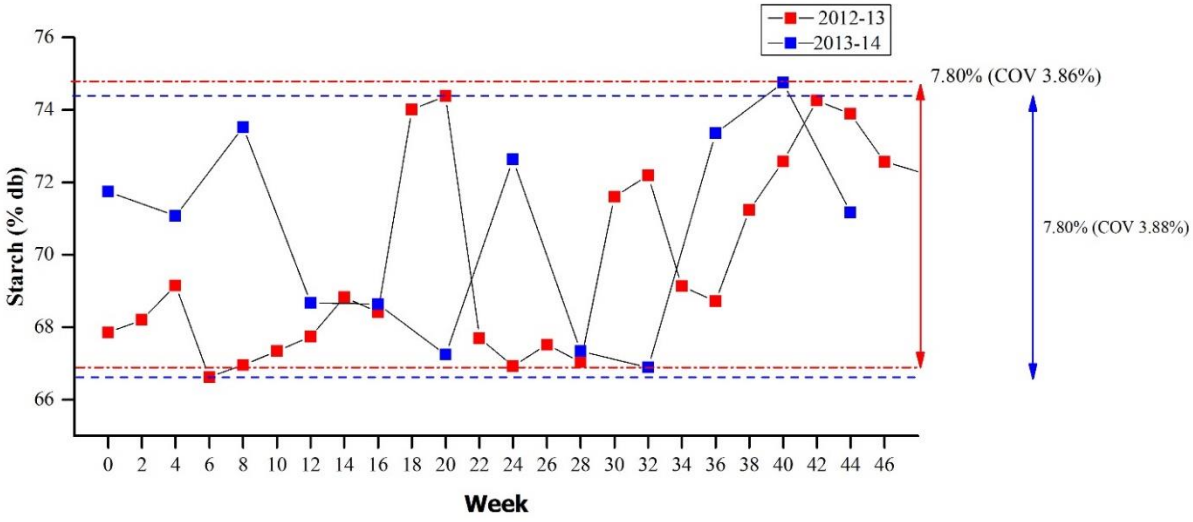

Fig. A.1. Starch content.

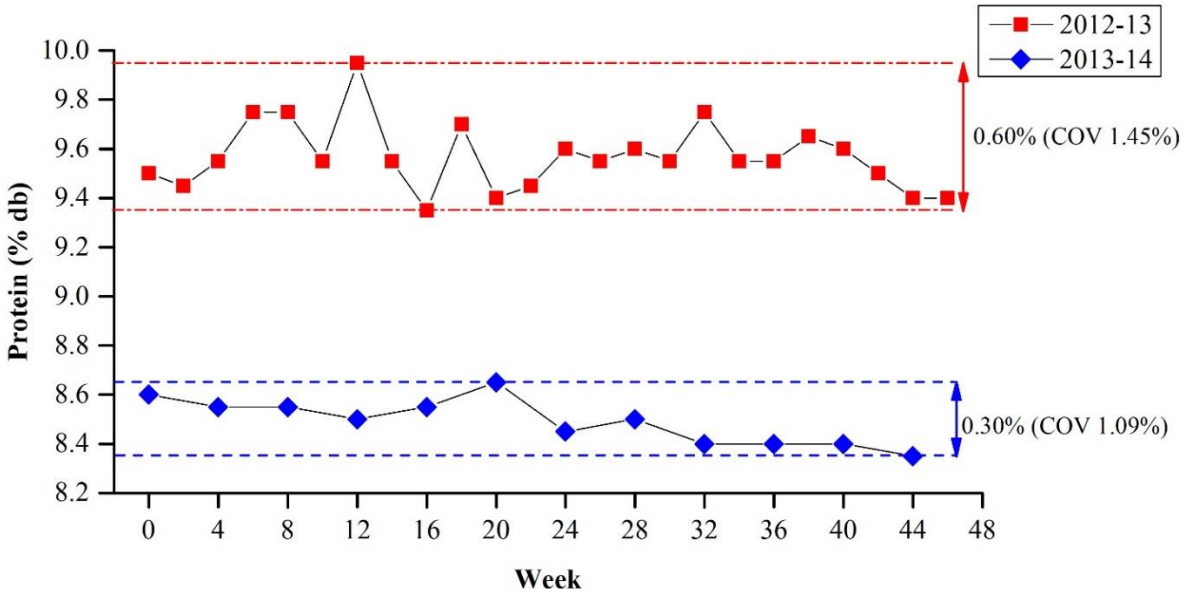

Fig. A.2. Protein content.

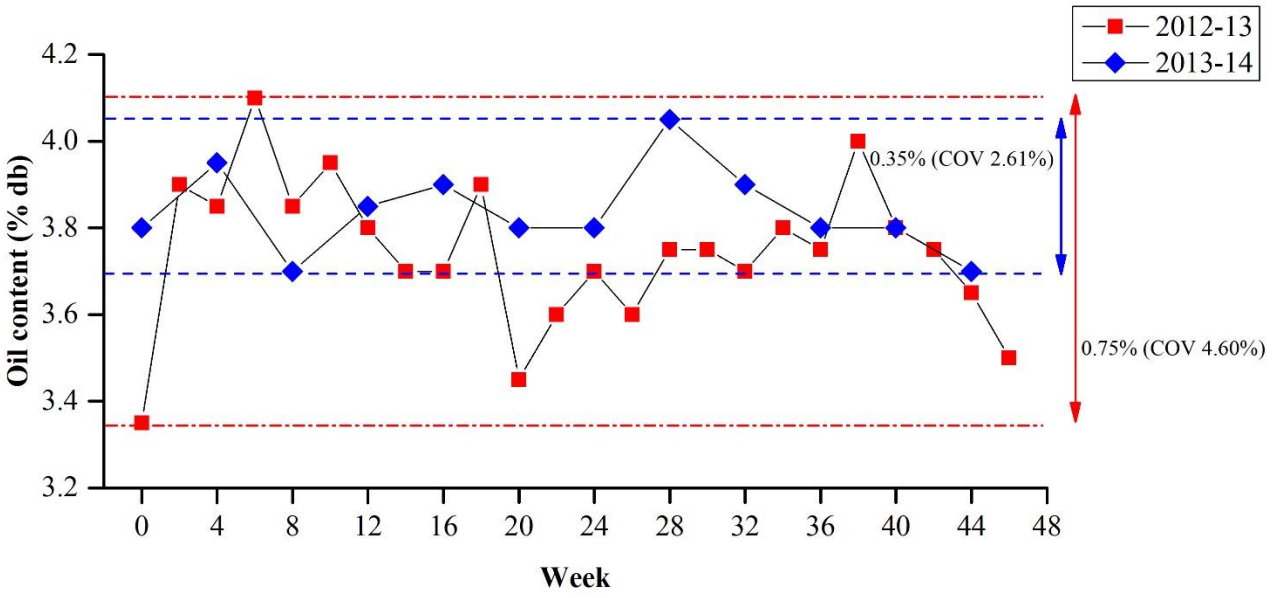

Fig. A.3. Oil content.

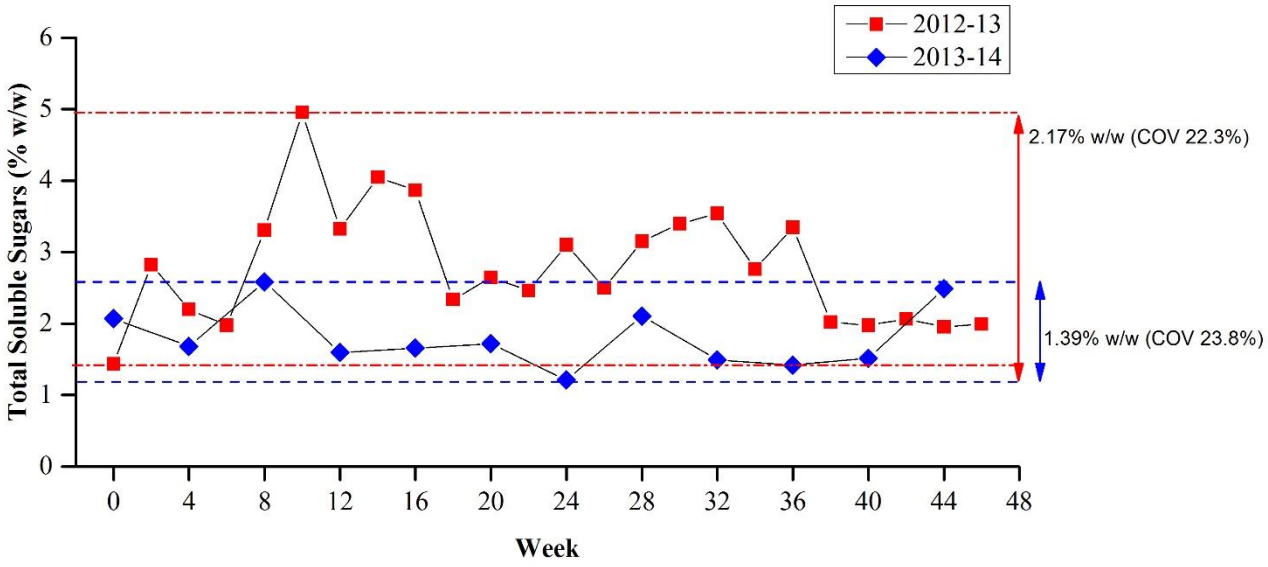

Fig. A.4. Total soluble sugar content.

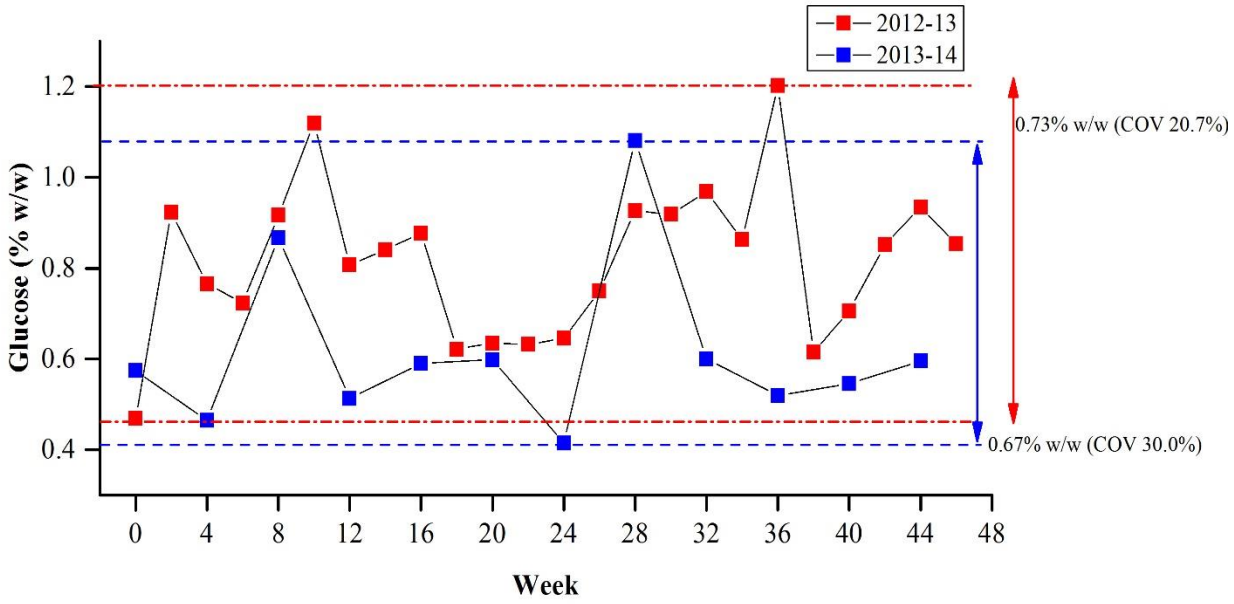

Fig. A.5. Initial glucose content.

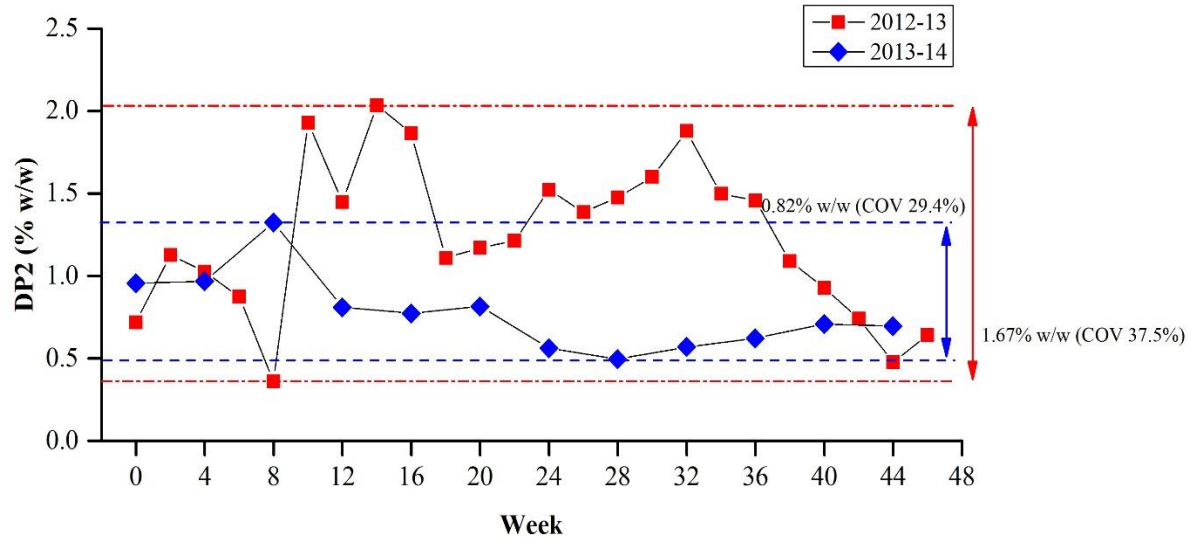

Fig. A.6. Initial DP2 content.

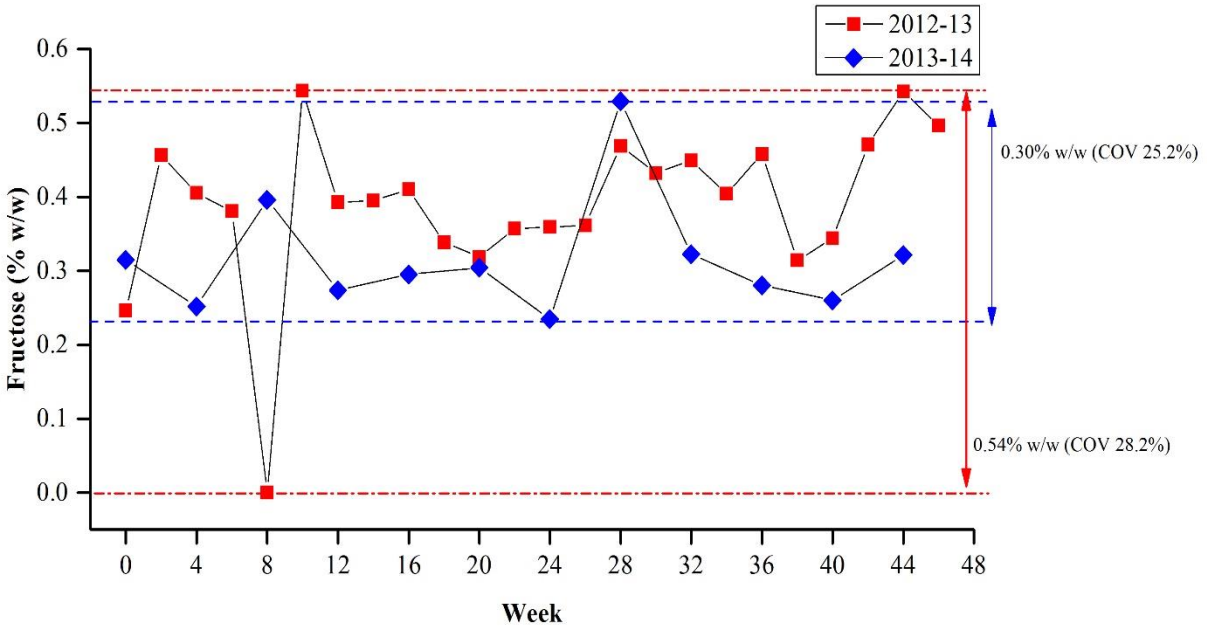

Fig. A.7. Initial fructose content.

Appendix B: Physical corn quality attributes for commodity corn (2012-14)

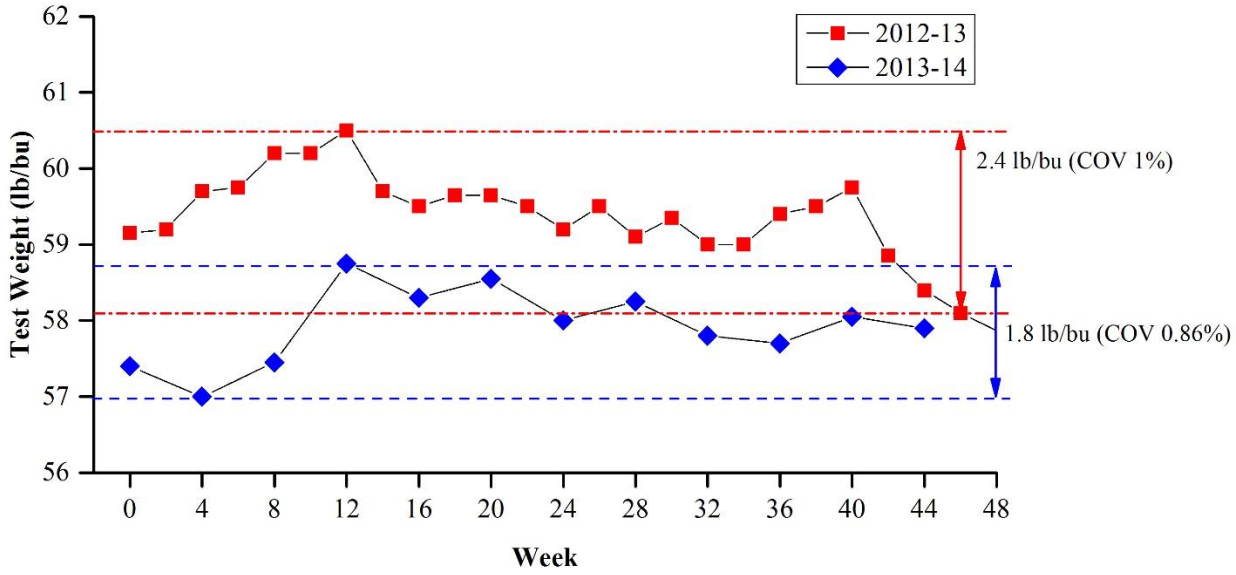

Fig. B.1. Test weight.

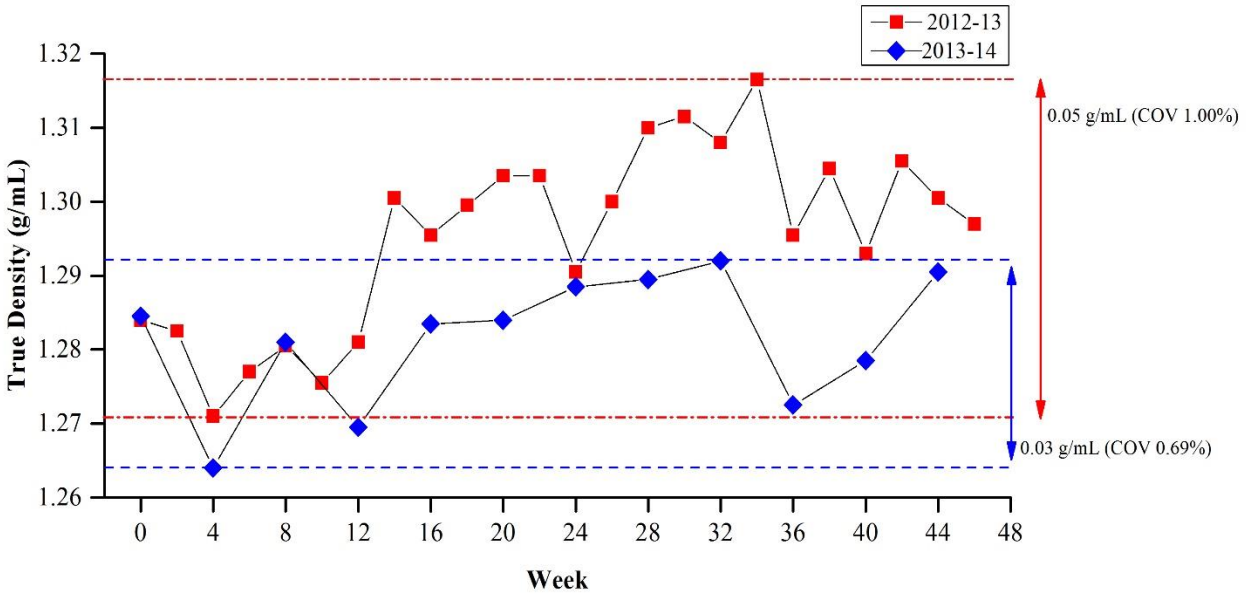

Fig. B.2. True density.

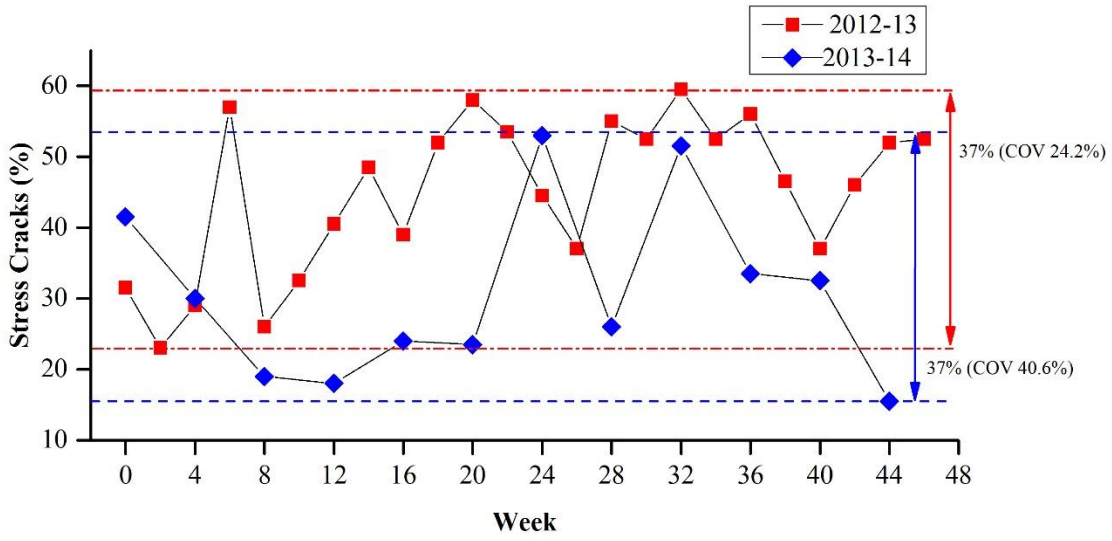

Fig. B.3. Stress cracks percentage.

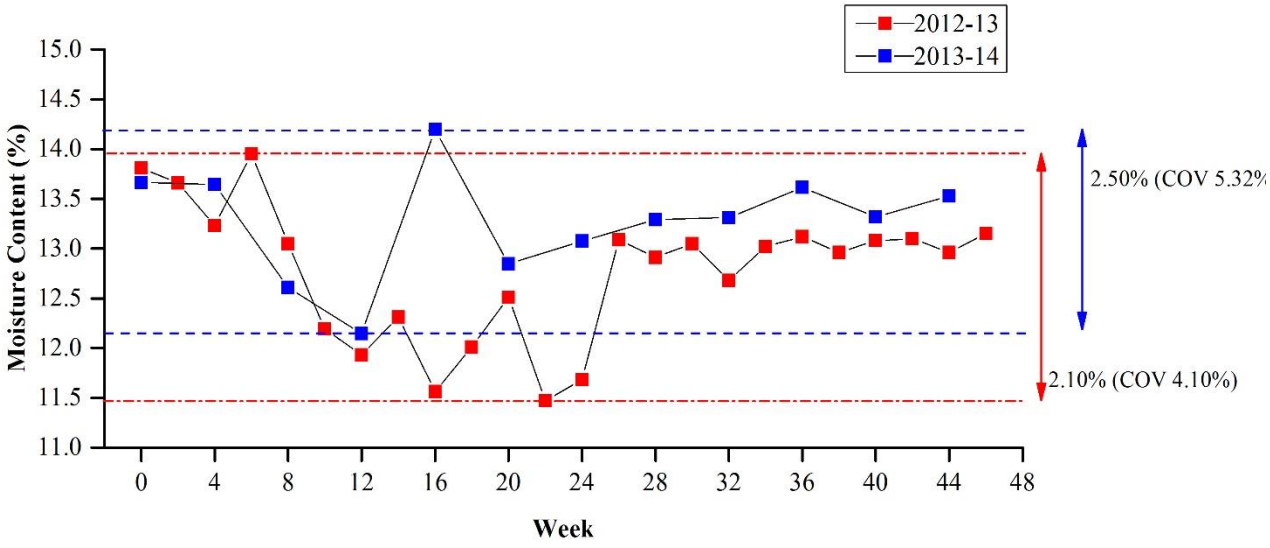

Fig. B.4. Moisture content.

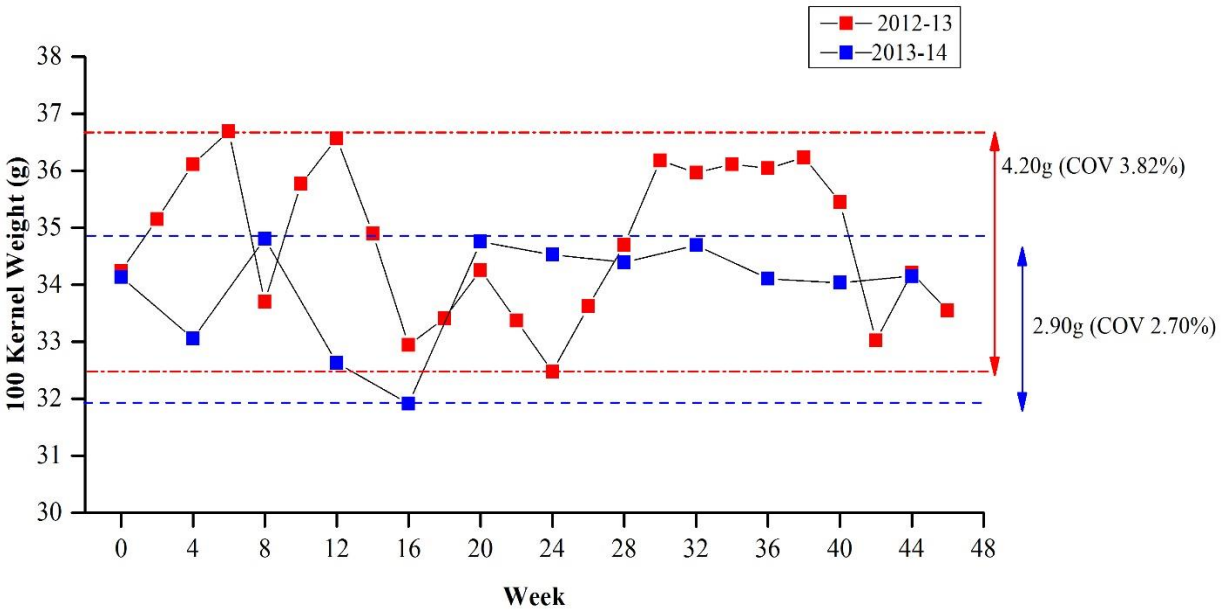

Fig. B.5. Kernel weight.

Appendix C: Corn protein quality attributes and ethanol concentrations during storage (2013-14)

Table C.1. Crude composition of corn grain during storage

Storage time (wk)	Starch content (% db)		Protein content (% db)		Oil content (% db)	
	Ref[1]	Amb[2]	Ref	Amb	Ref	Amb
0	72.3 a	72.3 ab	9.30 a	9.30 b	3.50 ab	3.50 ab
4	73.5 a	73.5 ab	9.20 a	9.20 c	3.25 abc	3.25 bc
8	73.5 a	72.8 ab	9.25 a	9.30 b	3.25 abc	3.25 bc
12	70.3 a	72.0 ab	9.20 a	9.25 bc	3.15 bc	3.15 c
16	72.5 a	73.7 ab	9.20 a	9.20 c	3.15 bc	3.65 a
20	70.1 a	70.4 b	9.25 a	9.25 bc	3.60 a	3.25 bc
24	71.9 a	73.2 ab	9.25 a	9.30 b	3.30 abc	3.40 abc
28	70.4 a	70.9 b	9.20 a	9.20 c	3.20 bc	3.10 c
32	70.3 a	70.6 b	9.20 a	9.20 bc	3.20 bc	3.40 abc
36	71.2 a	70.3 b	9.25 a	9.40 a	3.25 bc	3.15 bc
40	74.3 a	74.5 a	9.15 a	9.25 bc	3.20 bc	3.20 bc
44	71.0 a	72.6 ab	9.15 a	9.20 c	3.05 c	3.10 c
LSD	4.30	3.66	0.17	0.89	0.37	0.30

[1]Refrigerated, [2]Ambient. Values are means of two replicates. Means with the same lowercase letter within a column were not different.

Table C.2. Albumin contents of corn during storage[1]

	Albumin content (% of total protein)	
Storage time (wk)	**Amb**	**Ref**
0	14.6 b,A	14.6 b,A
8	32.1 a,A	31.3 a,A
16	32.6 a,A	28.7 a,A
24	**18.2 b,A**	**14.7 b,B**
40	18.1 b,A	16.0 b,A

[1] Values are reported with respect to the total crude protein content of the sample. All values are means of two replicates. Means with the same lowercase letter within a column were not different. Means with the same uppercase letter within a row were not different.

Table C.3. Globulin contents of corn during storage[1]

	Globulin content (% of total protein)	
Storage time (wk)	**Amb**	**Ref**
0	11.7 a,A	11.7 a,A
8	12.4 a,A	15.7 a,A
16	10.4 a,A	9.02 a,A
24	12.5 a,A	12.7 a,A
40	**13.6 a,B**	**16.1 a,A**

[1] Values are reported with respect to the total crude protein content of the sample. All values are means of two replicates. Means with the same lowercase letter within a column were not different. Means with the same uppercase letter within a row were not different.

Table C.4. Prolamin contents of corn during storage[1]

	Prolamin contents (% of total protein)	
Storage time (wk)	**Amb**	**Ref**
0	17.6 c,A	17.6 d,A
8	26.1 b,A	24.7 c,A
16	19.0 bc,A	25.8 c,A
24	40.0 a,A	42.4 a,A
40	36.0 a,A	35.7 b,A

[1] Values are reported with respect to the total crude protein content of the sample. All values are means of two replicates. Means with the same lowercase letter within a column were not different. Means with the same uppercase letter within a row were not different.

Table C.5. Glutelin contents of corn during storage[1]

	Glutelin contents (% of total protein)	
Storage time (wk)	**Amb**	**Ref**
0	27.6 a,A	27.6 a,A
8	18.9 c,A	20.0 bc,A
16	25.7 a,A	24.9 ab,A
24	19.1 c,A	18.1 c,A
40	22.2 b,A	24.8 ab,A

[1] Values are reported with respect to the total crude protein content of the sample. All values are means of two replicates. Means with the same lowercase letter within a column were not different. Means with the same uppercase letter within a row were not different.

Table C.6. FAN production (mg/L) profiles[1] for corn stored at refrigerated conditions

	Time after protease incubation (hr)				
Storage time (wk)	**0**	**1**	**2**	**4**	**6**
0	69.0 g	104.4 f	146.0 e	182.5 i	217.2 e
4	83.6 g	161.8 de	201.8 d	273.5 ef	282.0 d
8	63.6 g	102.2 f	145.3 e	209.5 h	233.9 e
12	103.6 ef	186.1 d	214.5 d	284.9 e	294.7 d
16	175.1 c	296.7 bc	319.8 bc	396.4 c	354.6 c
20	235.3 b	416.4 a	493.7 a	595.3 a	613.3 a
24	96.8 ef	136.3 ef	147.0 e	185.1 i	215.2 e
28	110.3 e	173.5 de	180.5 de	230.3 gh	268.8 d
32	131.2 d	208.7 d	215.9 d	250.2 fg	269.8 d
36	167.6 c	266.0 c	291.9 c	346.9 d	367.2 bc
40	277.7 a	295.7 bc	301.3 c	336.7 d	362.0 bc
44	174.1 c	326.0 b	356.4 b	431.1 b	391.5 b
LSD	20.2	49.7	41.3	24.3	33.2

[1]*Values are means of two replicates. Means with the same lowercase letter within a column were not different.*

Table C.7. FAN production (mg/L) profiles[1] for corn stored at ambient conditions

	Time after protease incubation (hr)				
Storage time (wk)	**0**	**1**	**2**	**4**	**6**
0	69.0 h	104.4 h	146.0 f	182.5 g	217.2 g
4	82.4 gh	139.3 fgh	193.7 e	253.7 ef	260.9 ef
8	77.2 h	112.7 gh	144.3 f	227.8 f	234.8 fg
12	106.1 f	151.8 fg	206.8 e	265.5 e	274.6 e
16	159.0 cd	290.9 cd	315.8 c	381.7 bc	399.6 bc
20	233.0 b	408.3 a	488.4 a	577.4 a	625.5 a
24	96.6 fg	137.1 fgh	149.2 f	190.6 g	225.7 g
28	110.3 ef	174.1 ef	196.2 e	249.7 ef	289.7 e
32	124.7 e	207.8 e	216.1 e	260.1 e	279.6 e
36	149.6 d	263.2 d	278.0 d	323.5 d	349.9 d
40	301.6 a	308.2 bc	355.4 b	354.1 c	379.7 cd
44	172.7bc	332.0 b	363.7 b	408.9 b	431.1 b
LSD	17.3	39.8	30.6	29.1	31.1

[1]*Values are means of two replicates. Means with the same lowercase letter within a column were not different.*

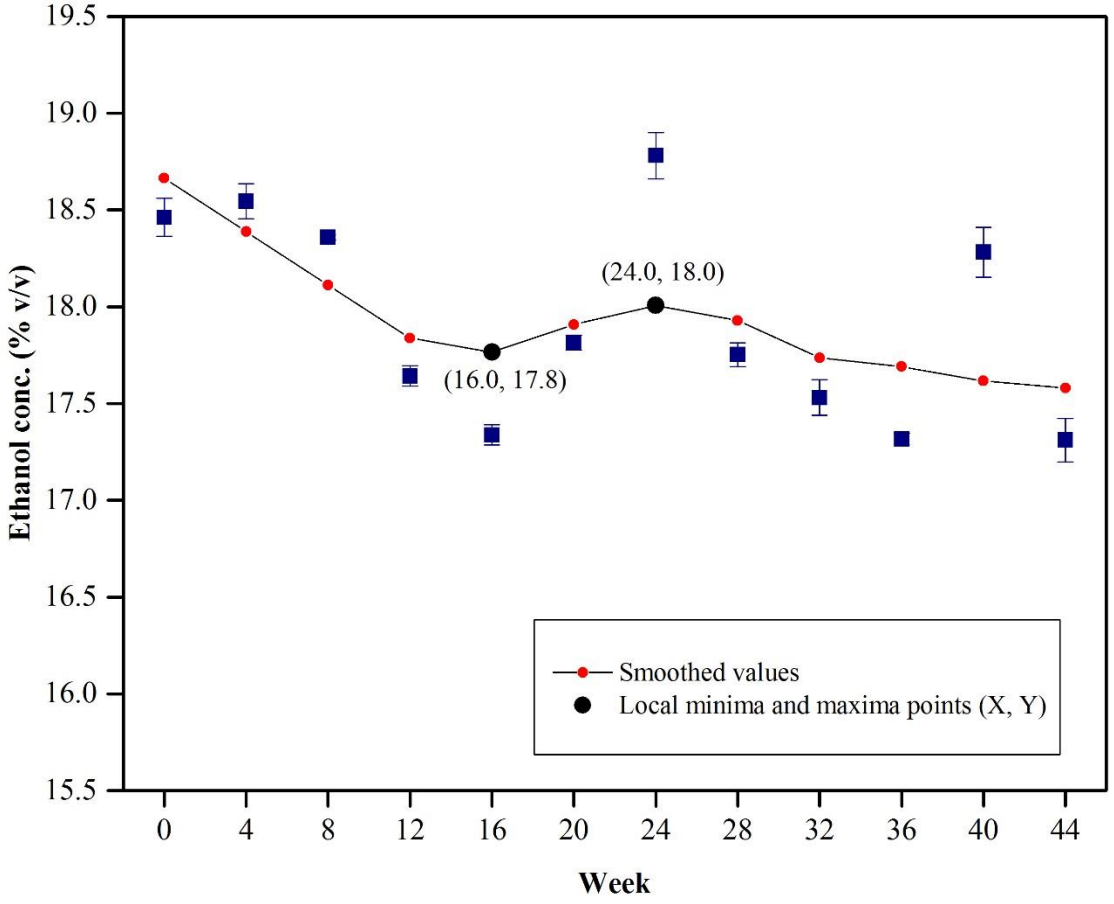

Fig. C.1. Ethanol concentration variation with time for corn stored at refrigerated conditions. Two replicates were run for each corn sample. Error bars indicate standard deviations.

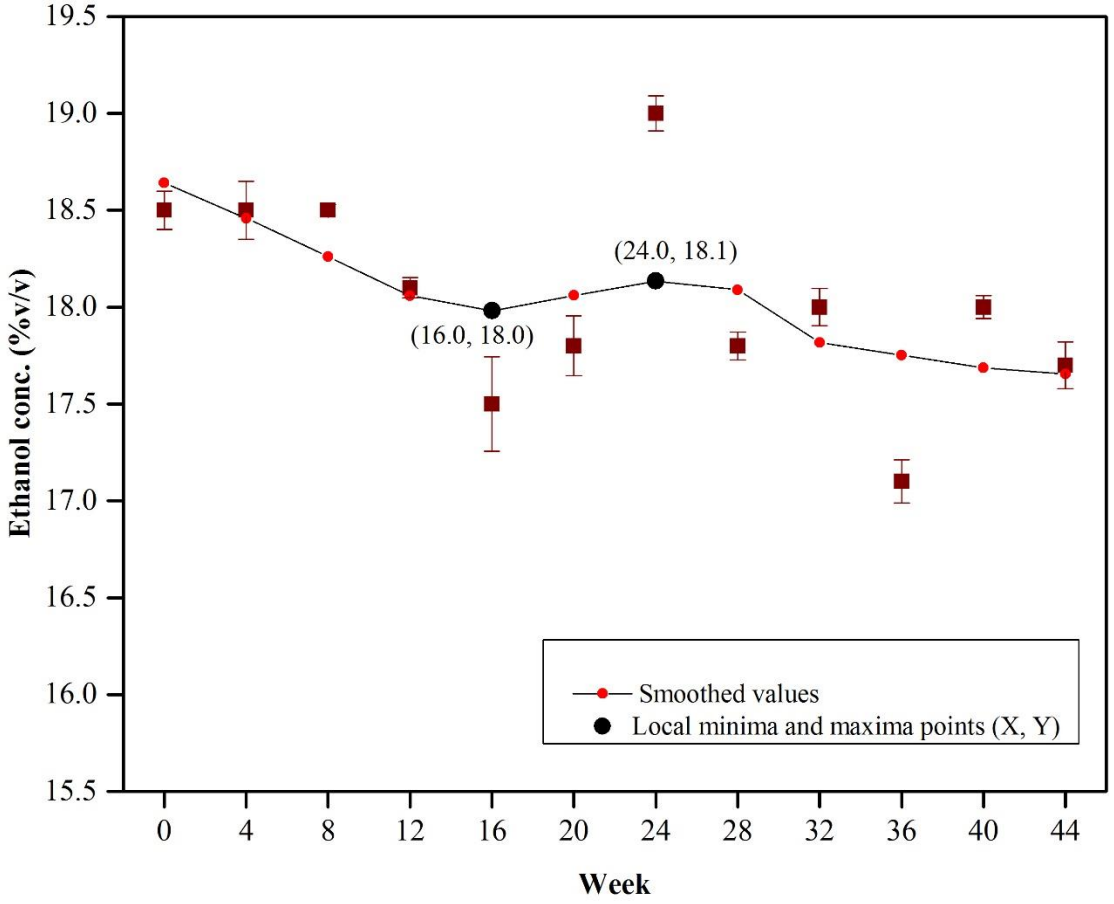

Fig. C.2. Ethanol concentration variation with time for corn stored at ambient conditions. Two replicates were run for each corn sample. Error bars indicate standard deviations.

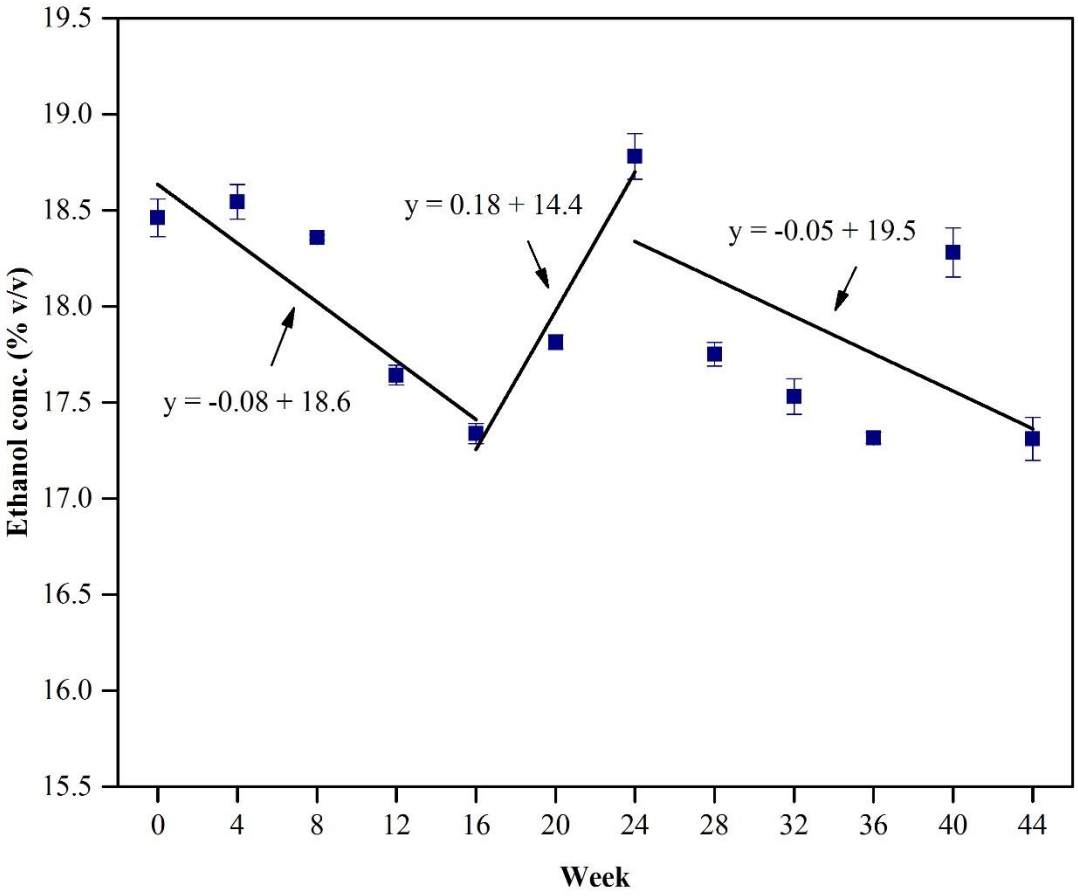

Fig. C.3. Corn stored at refrigerated conditions (least square regression lines fitted in the three chosen time segments). Slopes in time segments 0 (0 to 16 wk) and 2 (16 to 24 wk) were different from 0.

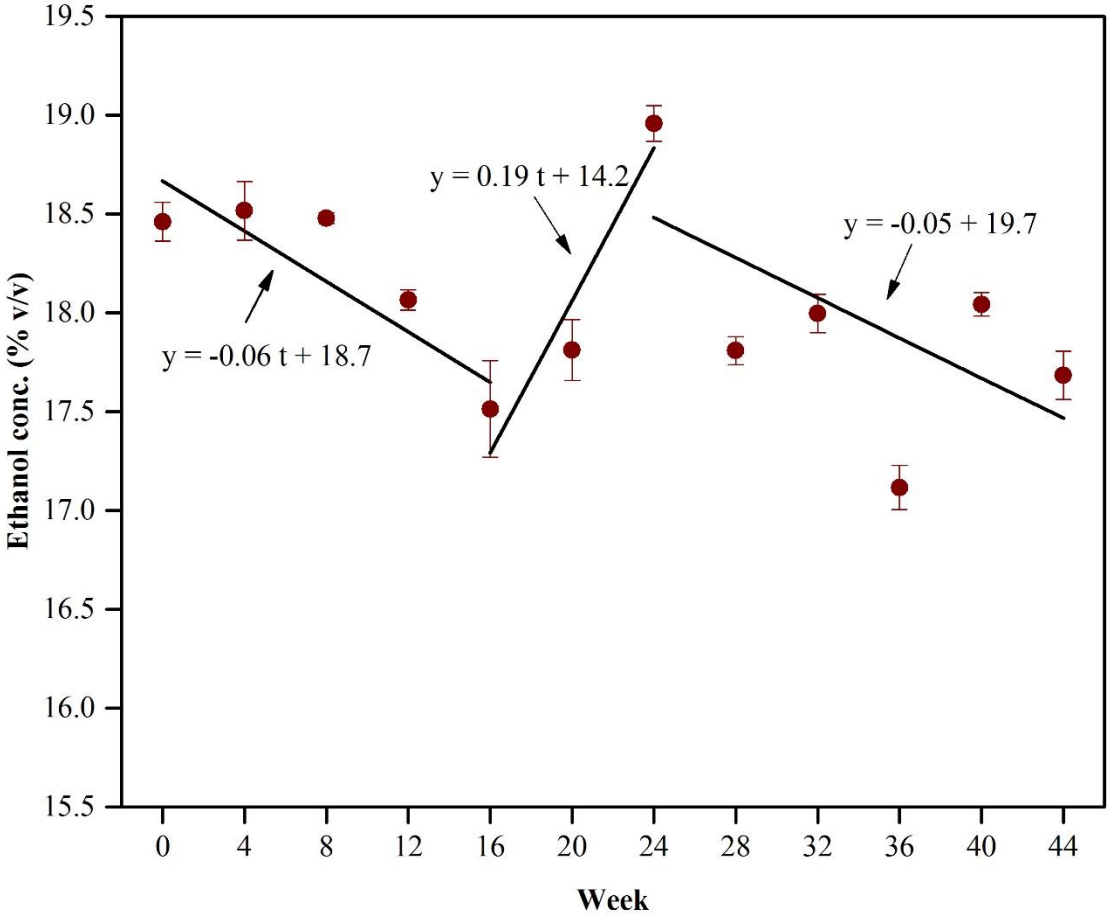

Fig. C.4. Corn stored at ambient conditions (least square regression lines fitted in the three chosen time segments). Slopes in time segments 0 (0 to 16 wk) and 2 (16 to 24 wk) were different from 0.