INVESTIGATION OF PROTIEN COMPOSITION IN HISTORICAL AND MODERN HARD RED SPRING WHEAT CULTIVARS

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Marie Maneka Rajini Malalgoda

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

MASTER OF SCIENCE

SUPERVISORY COMMITTEE:

Dr. Senay Simsek
Chair

Dr. Steven Meinhardt

Dr. Jae-Bom Ohm

Approved:

March 29 2016
Date

Dr. Richard D. Horsley
Department Chair

ABSTRACT

Many hard red spring (HRS) wheat cultivars have been released in North Dakota with improved traits. In this study, 30 HRS wheat cultivars released between 1910 and 2013 were investigated. The objectives of this study were to determine how wheat protein chemistry changed over the last century, and if modern wheat is more toxic in terms of celiac disease in comparison to historical wheat. The gliadin and glutenin protein profiles were analyzed using HPLC, and immunogenic peptides causing celiac disease was determined using mass spectrometry. Cluster analysis was performed to evaluate how the cultivars clustered with regard to parentage and protein separation profiles. The results indicated that ω-gliadin together with glutenin proteins may have a positive contribution towards favorable dough properties, and that these cultivars can be clustered according year when parentage and protein HPLC area data are used. Additionally, immunogenic peptides were detected in both historical and modern wheat.

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GENERAL INTRODUCTION

Gluten forming proteins are responsible for the visco-elastic properties found in wheat dough (Wieser 2007). These proteins are composed of gliadin and glutenin proteins, which provide viscosity and extensibility, respectively (Delcour and Hoseney 2010a). Gliadin proteins can be divided to three major groups: α/β-, γ- and ω-. This grouping is based on the electrophoretic mobility of gliadin proteins on Acid-Polyacrylamide Gel Electrophoresis (Bonomi et al. 2013). The role of the individual gliadin proteins in dough systems have not been established thus far. Uthayakumaran et al. (1999) and MacRitchie (1987) found that gliadin proteins cause a decrease in dough strength, thus are not favorable in wheat dough systems. Similar observations were made by Barak et al. (2014) who found that gliadin proteins decrease dough stability and promote dough softening. However, some studies indicate that gliadin proteins are favorable in dough systems. Huebner and Bietz (1986) and Wrigley et al. (1981) found that certain gliadin bands improve dough quality. Ohm et al. (2009; 2010) found that ω-gliadins cause improvements in loaf volume, which is favorable.

Celiac disease is caused by gluten forming proteins found in wheat, rye, barley and possibly oat (Trier 1998). This disease is a genetically predisposed condition. Currently, 1 in 266 suffer from this disease worldwide (Niewinski 2008). Studies have found that the gliadin proteins have the highest toxicity in terms of containing peptides that initiate the immune response associated with this disease (Wieser and Kohler 2008). Gluten forming proteins are not digested completely due to high amounts of glutamine and proline, resulting in incompletely digested peptides termed ‘immunogenic peptides or toxic peptides’ (Meresse et al. 2009). Once these peptides reach the lamina propia they are further modified by tissue transglutaminase, which converts glutamine to glutamic acid. Antigen presenting cells recognize these peptides and

presents them to T- cells which gives rise to a cascade of reactions of which the end result is damage to the intestinal cell wall. Those suffering from celiac disease experience weight loss, fatigue, delayed growth and many other difficult conditions (Celiac Disease Foundation). Undiagnosed celiac disease could lead to iron deficiency, osteoporosis, nervous system disorders and infertility. At present, the only treatment for celiac disease is a gluten free diet, where food products based on wheat, rye and barley are not consumed. The toxicity in relation to celiac disease has been tested in many wheat cultivars. Some studies claim that older wheat cultivars could be less toxic to celiac disease subjects as they contain less α-gliadin, thus less immunogenic peptides (Carroccio et al. 2011; Pizzuti et al. 2006). On the contrary, some studies indicate otherwise, and claim that both historical and modern wheat are toxic (Colomba and Gregorini 2012).

North Dakota is the highest producer of hard red spring wheat in the United States (North Dakota Wheat Commission). On average 6.8 metric tons of hard red spring wheat is produced annually, which accounts for approximately half of the country's production. This type of wheat is known for its superior qualities, such as high protein content and strong gluten. Thus, some of the world's finest bakery products are made using hard red spring wheat. Breeders have produced many spring wheat cultivars with advantageous traits, and which are better adapted to conditions in the region. There have not been any studies conducted so far to determine how the protein composition of these cultivars changed over the years, and how these changes relate to quality characteristics. Moreover, the celiac disease toxicity of wheat cultivars released in the last century has not been evaluated. If less toxic cultivars are identified, this could lead the way for breeding wheat cultivars that are safe for celiac disease subjects. Thus, it would be safe for such individuals to consume wheat based products.

In this context, the main objectives of this study were as follows,

I. Evaluate the changes in wheat protein profiles, specifically gliadin proteins, to determine if wheat protein composition has changed in the wheat cultivars released over the last 100 years, and to determine how these changes relate to improvements in quality characteristics

II. Determine how historical and modern cultivars being investigated group when cluster analysis is performed with reference to pedigree information, and protein HPLC separation data. And determine how the clusters formed are different in terms of quality characteristics and the presence of reduced height genes

III. Determine if immunogenic peptides causing celiac disease can be detected in historical and modern spring wheat cultivars being analyzed using a proteomics approach, and to determine if there has been change in the amount α/β, γ and ω- gliadins over the years using reverse phase-HPLC data

CHAPTER 1. LITERATURE REVIEW

Wheat is one of the three major crops grown worldwide with over 700 million tons produced in 2013 (Food and Agriculture Organization). Studies have found that wheat was first grown as a food crop as early as 10,000 – 80,000 B.C (Wrigley 2009). Additionally, wheat among other cereal crops, contributed in the transition from the hunter-gatherer nomad to agriculture. Wheat is a unique cereal crop as it contains gluten forming proteins capable of forming elastic dough. Thus, wheat is the primary ingredient of leavened bread products. The term 'wheat' refers to many species and subspecies in the genus *Triticum* (Gooding 2009). Today, bread wheat or common wheat, *Triticum aestivum* subsp. *aestivum* accounts for more than 90% of the world wheat production.

1.1. Wheat Kernel Structure

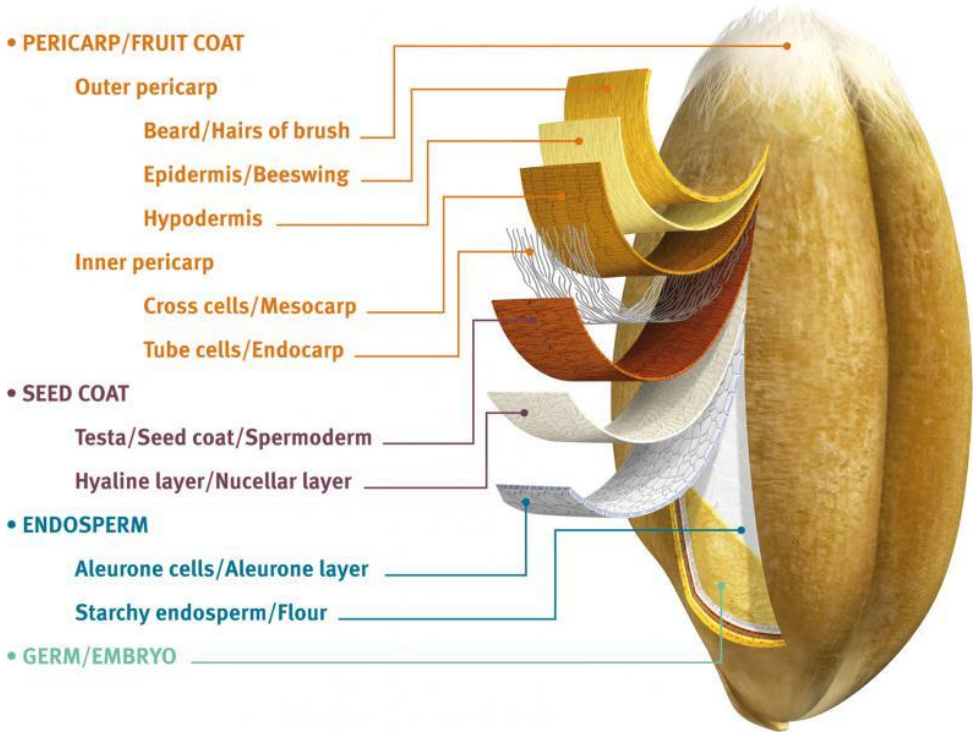

Figure 1.1. Longitudinal view of a wheat kernel (GoodMills Innovation GmbH)

The kernel size of wheat grown in North America is approximately 8 mm in length and 35 mg in weight (Delcour and Hoseney 2010a). The size varies depending on the cultivar and the location on the wheat head or spike. The three main components of a wheat kernel are endosperm, bran and germ.

The endosperm consists of an aleurone layer and a starchy core. It has the highest content of protein and starch in the wheat kernel. The aleurone layer is one cell layer thick and surrounds the entire wheat kernel. The starchy endosperm produces flour, farina or semolina when reduced to the appropriate particle size. The cells in the endosperm contain starch granules which are embedded in a protein matrix. During maturation of the wheat kernel gluten forming proteins are synthesized and deposited as protein bodies. The bran is composed of several cell layers: pericarp, seed coat and nuclear epidermis. It is the main source of flour ash during milling. The germ makes up 2.5-3.5% of the wheat kernel, and consists of the embryonic axis and the scutellum. The germ contains the highest amount of lipid and is relatively high in protein but does not contain starch.

1.2. Wheat Proteins

The key characteristic in wheat is its ability to form dough (Shewry 2009). Thus, wheat flour is used in a wide range of products such as, bread, pasta, noodles, cakes and cookies. Gluten forming proteins are responsible for these properties in wheat. These proteins are found in the endosperm of the wheat kernel as a continuous matrix around starch granules (Bonomi et al. 2013). Gluten is the rubbery mass that remains after wheat dough is washed to remove starch and water soluble constituents (Wieser 2007). The solid gluten contains 75-85% proteins and 5-10% lipids. Gluten is what provides dough visco-elastic properties which are very important in baking.

Gluten forming proteins are composed of two types of proteins, namely, gliadin and glutenin. Gliadin proteins are monomeric, while the glutenin proteins are polymeric. Both these fractions contribute towards the rheological properties of dough. Gliadins are responsible for the cohesive properties (viscosity) of wheat dough, whereas glutenins cause resistance to extension (elasticity) (Delcour and Hoseney 2010b). Extensive polymorphism found in gluten forming proteins has made the annotation of these proteins complex. Shewry et al. (1986) suggested that the genes encoding these proteins have resulted from duplication and translocation which have led to insertions and deletions of peptide sequences as well as amino acid substitutions.

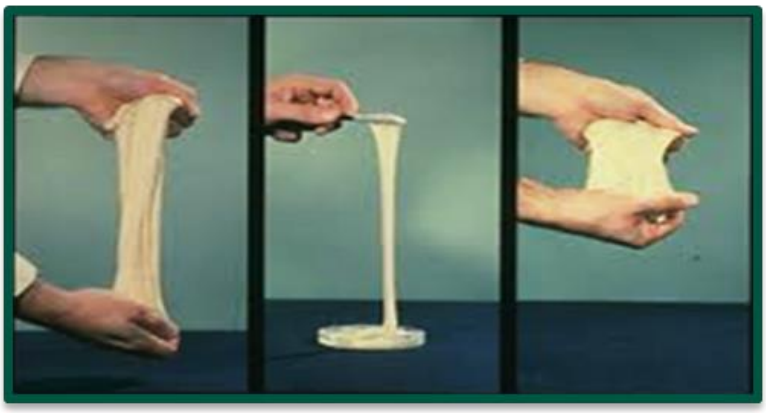

Figure 1.2. Physical properties of gluten proteins (left), gliadin (center) and glutenin (right) (Reprinted with permission from Delcour and Hoseney 2010b)

Cereal proteins are divided into four groups according to their solubility (Delcour and Hoseney 2010b). This classification is based on the work conducted by Thomas Burr Osborne in the early 20th century. Albumin proteins are soluble in water, and globulin proteins are soluble in dilute salt solutions. These two proteins are not generally considered as storage proteins and are mainly found in the germ. Gliadins and glutenins, which are storage proteins, are soluble in 70% ethanol and dilute acid or bases respectively.

1.2.1. Gliadin proteins

The molecular weight range of gliadin proteins is from 28000 to 55000 (Bonomi et al. 2013). During dough mixing, intermolecular H bonds and hydrophobic bonds occur between non-polar amino acid chains, which contribute towards the formation of the gluten network. These proteins also interact with flour lipids. Gliadins are classified into four groups, α-, β-, γ- and ω- according to their mobility on low pH electrophoresis (Wieser 2007). However, α- and β- gliadins have similar amino acid sequences and electrophoretic mobility, thus both these types are categorized as α-/β- gliadins. The three groups of gliadin proteins are also classified according to their amino acid composition and molecular weight as ω5-, ω1,2-, α-/β- and γ- by Wieser (1996).

The α-/β- and the γ- gliadins have molecular weights of 28000 to 35000 (Wieser 2007). The glutamine and the proline content of these gliadins is lower than that of ω-gliadin. The α-/β- and the γ- gliadins differ in the amount of only few amino acids, for example, tyrosine. The N termini of these proteins contains repetitive domains rich in glutamine, proline, phenylalanine and tyrosine. The repetitive domain itself is different in the two gliadin types. In the case of α-/β- gliadins, this domain is a dodecapeptide with the sequence QPQPFPQQPYP. This sequence is usually repeated five times. The sequence can be modified by the substitution of a single amino acid. The repetitive domain of γ-gliadin is QPQQPFP. This sequence is repeated sixteen times, and usually contains additional residues. The C-terminus is homologous in the two gliadin types. In comparison to the N-terminus, the C-terminus contains less glutamine and proline. Studies on the secondary structure of α-/β- and γ- gliadins have shown that the N-terminus of these proteins consists of β-turn conformation as in the case of ω-gliadins (Tatham and Shewry 1985). The molecular mass of ω-gliadins is between 44000 and 55000, which is higher than that of the other

gliadin types (Bonomi et al. 2013). The structure and composition of this type of gliadin is also different. The ω-gliadin protein lack cysteine residues and contain a single repetitive domain, which consists mainly of glutamine, glutamate, proline and phenylalanine. Due to the lack of cysteine, ω-gliadins do not contain disulfide linkages.

Pogna et al. (1990) discovered that major gliadin genes occur in clusters, which are termed blocks. The genes responsible for γ- and ω- gliadin are present at the *Gli-1* loci (*Gli-A1, Gli-B1* and *Gli-D1*) on the short arms of the homologous group 1 chromosomes. The genes for the α- and β- gliadins are located at the *Gli-2* loci (*Gli-A2, Gli-B2* and *Gli-D2*) on the short arms of the group 6 chromosomes (Payne 1987). These two loci show extensive polymorphism, leading to high diversity between wheat cultivars. These differences have been used to fingerprint different wheat cultivars (Bietz and Huebner 1995). Approximately 25-150 genes code for α-gliadins contributing to high variability in the protein group (Anderson et al. 1997).

The amount and the distribution of gliadin among the different types is dependent on the variety and growing conditions (Wieser 2007). However, studies have found that α-/β- and γ-gliadins contribute to the majority of the gliadin proteins, whereas ω-gliadin is only a minor contributor (Wieser and Kieffer 2001).

1.2.2. Glutenin proteins

As mentioned earlier, glutenin proteins are polymeric in nature. They exist as aggregates held together by disulfide bonds (Wieser 2007). The molecular weight of glutenin proteins range from 500000 to more than 10 million. Studies have found that these proteins are very important in dough systems, specifically the molecular weight distribution of these proteins is important. The largest glutenin polymers termed ‘glutenin macropolymers’ have shown to have the greatest

contribution towards favorable dough properties, such as dough strength and loaf volume. Other types include high molecular weight (HMW) and low molecular weight (LMW) glutenins.

HMW glutenin represents only 5-10% of the total glutenin content, as such it is a minor component. Researchers have found that these proteins have a positive effect on dough properties (Field et al. 1983). The long arm of the chromosomes 1A, 1B and 1D encode genes responsible for HMW glutenins. These positions are referred to as, *Glu-A1, Glu-B1* and *Glu-D1*. Two tightly associated genes, *x* and *y*, are responsible for the subunits for glutenin proteins. The molecular mass of the subunits is 83000-88000 for x-type and 67000-74000 for y-type (Payne 1987). Approximately 40 HMW glutenin genes have been found thus far (Anderson et al. 2002). Genetic analysis has determined that HMW glutenin proteins contain three domains: non-repetitive N-terminal domain, repetitive central domain and C terminal domain of 42 residues (Shewry et al. 1992). Studies have found a link between the expression of HMW glutenin genes and breadmaking quality. Feeney et al. (2003) found that the 1Dx5 subunits contain additional cysteine residues which provides higher capability of disulfide bond formation in comparison to the 1Bx20 subunit, which lacks two cysteines.

The LMW glutenins represents 20% of the glutenin content in the wheat kernel. These proteins are similar to α-/β- and γ- gliadin types based on amino acid composition and molecular weight (Wieser 2007). Based on mobility on SDS-PAGE, LMW glutenin protein can be categorized to three groups, B, C and D (Payne and Corfield 1979). The molecular mass of these groups are 42000-51000, 30000-40000 and 55000-70000 respectively. The B-type LMW glutenins are the most abundant type. These proteins contain two domains: N-terminal domain which is rich in proline and glutamine, and contains repetitive sequences such as QQQPPFS, and C-terminal domain which is homologous to the gliadin types mentioned earlier (Grosch and

Wieser 1999). LMW glutenin proteins contain eight cysteine residues. Of these, six residues contribute towards the formation of disulfide bonds, while the other two cysteine residues contribute towards the stability of the protein.

Table 1.1. Amino acid composition of the major protein fractions of wheat flour (g/100g protein)

			Soluble proteins		Gluten proteins		
	Wheat	Flour	Albumin	Globulin	Gliadin	Glutenin	Residue protein
Proportion %	-	100	3-5	6-10	40-50	30-40	6-10
Amino acid							
Asp	4.7	3.7	5.8	7.0	1.9	2.7	4.2
Thr	2.4	2.4	3.1	3.3	1.5	2.4	2.7
Ser	4.2	4.4	4.5	4.8	3.8	4.7	4.8
Glu	30.3	34.7	22.6	15.5	41.1	34.2	31.4
Pro	10.1	11.8	8.9	5.0	14.3	10.7	9.3
Gly	3.8	3.4	3.6	4.9	1.5	4.2	5.0
Ala	3.1	2.6	4.3	4.9	1.5	2.3	3.0
Val	3.6	3.4	4.7	4.6	2.7	3.2	3.6
Met	1.2	1.3	1.8	1.7	1.0	1.3	1.3
Cys	2.8	2.8	6.2	5.4	2.7	2.2	2.1
Ile	3.0	3.1	3.0	3.2	3.2	2.7	2.8
Leu	6.3	6.6	6.8	6.8	6.1	6.2	6.8
Tyr	2.7	2.8	3.4	2.9	2.2	3.4	2.8
Phe	4.6	4.8	4.0	3.5	6.0	4.1	3.8
His	2.0	1.9	2.0	2.6	1.6	1.7	1.8
Lys	2.3	1.9	3.2	5.9	0.5	1.5	2.4
Arg	4.0	3.1	5.1	8.3	1.9	3.0	3.2
Trp	1.5	1.5	1.1	1.1	0.7	2.2	2.3
NH_3	3.5	3.9	2.5	1.9	4.7	3.8	3.5

Source: Shewry et al. (2009)

The amino acid composition of the different proteins in wheat have been studied extensively. Table 1.1 shows the amount of each amino acid present in the wheat proteins discussed above. According to Table 1.1, glutamine and proline are the most abundant amino acids in wheat. This could be because of the high content of these amino acids in gluten forming proteins. The health related issues caused by these proteins will be discussed later. Other cereals, such as barley, also have similar amino acid composition with high content of glutamine and proline content (Delcour and Hoseney 2010b).

1.2.3. Wheat proteins and quality

Wheat quality is a combination of various quality characteristics attributed to kernel properties, milling parameters, flour properties, dough and baking quality. Wheat proteins have been associated with dough and baking quality by many researchers. Dough rheology measurements are done using instruments such as farinograph, extensigraph, mixograph and alveograph (Delcour and Hoseney 2010c). These instruments measure how dough deforms and flows providing numerical values for the elasticity and extensibility of wheat doughs. These characteristics are used for the characterization of wheat flour. Experimental baking tests are used to evaluate how the different properties of flour affect the end product. Standard American Association of Cereal Chemists- International (AACC-I) methods are used for this purpose. Characteristics such as loaf volume, grain and texture and crumb color are measured in the final products as parameters of baking quality.

As previously mentioned, gluten forming proteins provide visco-elasticity to dough. Many studies have been conducted to determine the role of individual glutenin and gliadin proteins in the dough system. Branlard and Dardevet (1985) found that the HMW glutenin proteins provide dough strength and extensibility which indicates that these proteins play a

prominent role in breadmaking. These findings were supported by Ng and Bushuk (1988), who found that HMW glutenins have a positive effect on baking strength and that the composition of these proteins could be used when breeding for wheat with high baking quality. Additionally, extensograph extensibility, farinograph peak time and loaf volume showed correlations with flour glutenin content. These correlations were stronger than correlations between flour protein content and quality parameters, indicating that glutenins are responsible for favorable dough and baking properties (Gupta et al. 1992).

The role of gliadin proteins in dough mixing has long been debated. During mixing of wheat dough the gliadin proteins act as plasticizer, providing viscous flow and extensibility to the dough. Hydrophobic interactions and hydrogen bonds may help during this process (Barak et al. 2015). However, addition of gliadins have been shown to decrease dough strength and also decrease mixing time and loaf volume (Uthayakumaran et al. 1999). MacRitchie (1987) made similar observations when gliadins were added to base flour to determine the influence of these proteins. Specifically, the addition of gliadins decreased mixing time and stability which were demonstrated through farinograph parameters.

Additionally, Barak et al. (2014) found that glutenins improve mixing characteristics of wheat dough, while gliadins have the opposite effect causing a decrease in dough stability and increasing dough softening. The pasting properties of dough were also studied during this experiment. They found that gliadins reduced peak viscosity more than glutenins and that gliadins improved the cohesiveness of dough while glutenin improved the hardness. In a study conducted to determine the effect of gliadins in terms of micro-structural, thermal and rheological characteristics of the gluten network, it was found that gliadins weaken the gluten network, further highlighting the role of these proteins as dough weakening agents (Khatkar et al.

2013). Experiments conducted using scanning electron microscopy showed that as the gliadin percentage increased, the compact nature of the gluten network reduced which resulted in the gluten network being more open, which is not favorable. In a study conducted to determine the inheritance pattern of LMW glutenin, it was found that the alleles at the *Gli* loci might be related to dough quality, thus can be used as genetic markers for certain LMW glutenins (Metakovsky et al. 1997).

On the contrary, some studies have shown that gliadin proteins have a favorable effect on dough quality. Huebner and Bietz (1986) separated wheat gliadins using reverse phase-HPLC and made observations supporting this. Wrigley et al. (1981) also made similar observations as certain gliadin bands separated through electrophoresis showed significant correlations with grain hardness and dough strength. Khatkar et al. (1995) found that an optimum balance between gliadins and glutenins is important in dough quality. They suggested that gliadins provide viscosity to wheat gluten whereas glutenins provide elasticity. Uthayakumaran et al. (2000) also reported that the gliadin and glutenin proteins play different, but complementary, roles in the dough system. They found that gliadin proteins contribute towards the flow properties of dough, while glutenins affect the elasticity and strength of dough.

The role of α-/β-, γ- and ω- gliadins have also been studied. Uthayakumaran et al. (2001) studied the effect of the gliadin groups on dough properties and reported that γ-gliadins caused the greatest decrease in mix time and resistance to extension when added to base flour. This was attributed to the fact that γ-gliadin proteins are more hydrophobic in nature as opposed to the other gliadin fractions. Fido et al. (1997) found that γ-gliadins cause the least effect on mix time, and that ω-gliadins cause the greatest decrease in this parameter. The α-/β- gliadins were thought to have an intermediate effect.

In a study conducted to determine the effect of γ-gliadin, fifteen transgenic wheat lines with down-regulated genes for this protein were used (Gil-Humanes et al. 2012). The results showed that the dough properties of many transgenic lines were not affected by the down-regulation of the γ-gliadin genes indicating that this protein is not a determinant of dough quality. However, the SDS sedimentation tests and Mixolab (specifically dough development time) results indicated that they may contribute towards dough strength. A previous study conducted by the same group determined that γ-gliadin proteins do not play a vital role during dough development and the breadmaking process (Piston et al. 2011).

Paananen et al. (2006) studied the interaction of α- and ω- gliadins and found that the former participates in the formation of the dough network, while the latter is responsible for viscous properties of dough. This is an indication that these proteins in fact play important roles in the dough system. Khatkar et al. (2002) also suggested that ω-gliadins are responsible for viscous properties in wheat dough. Ohm et al. (2009; 2010) found that ω-gliadin proteins may have a positive effect on loaf volume and water absorption. However, as mentioned earlier, Fido et al. (1997) suggested that ω-gliadins act primarily as dough weakening agents.

The studies referenced above show that the role of gliadin proteins and its fractions have not been established as yet. Thus, more research is needed in this area to establish the role of these protein in wheat dough systems.

1.3. Wheat Genetics and Breeding

The earliest cultivated wheat were diploid (AA genome) and tetraploid (AABB genome). Examples of such wheat varieties include, Einkorn and Emmer (Wrigley 2009). Genetic analysis has revealed that these wheat varieties originated from the south-eastern part of Turkey (Heun et al. 1997). Studies have found that the A genome of modern tetraploid and hexaploid wheat is

related to the A genome of Einkorn, the B genome is from the S genome of *Aegilops speltoides*, and the D genome of hexaploid wheat is derived from *Triticum tauschii*, also known as *Aegilops tauschii*. Thus, the origins of modern wheat goes back to ancient wheat varieties (Feldman 2001). Currently, hexaploid wheat accounts for up to 95% of the wheat grown worldwide, and the majority of the remainder is tetraploid wheat (Shewry 2009). North Dakota is the highest producer of hard red spring wheat in the United States, thus is a key player in the industry (North Dakota Wheat Commission).

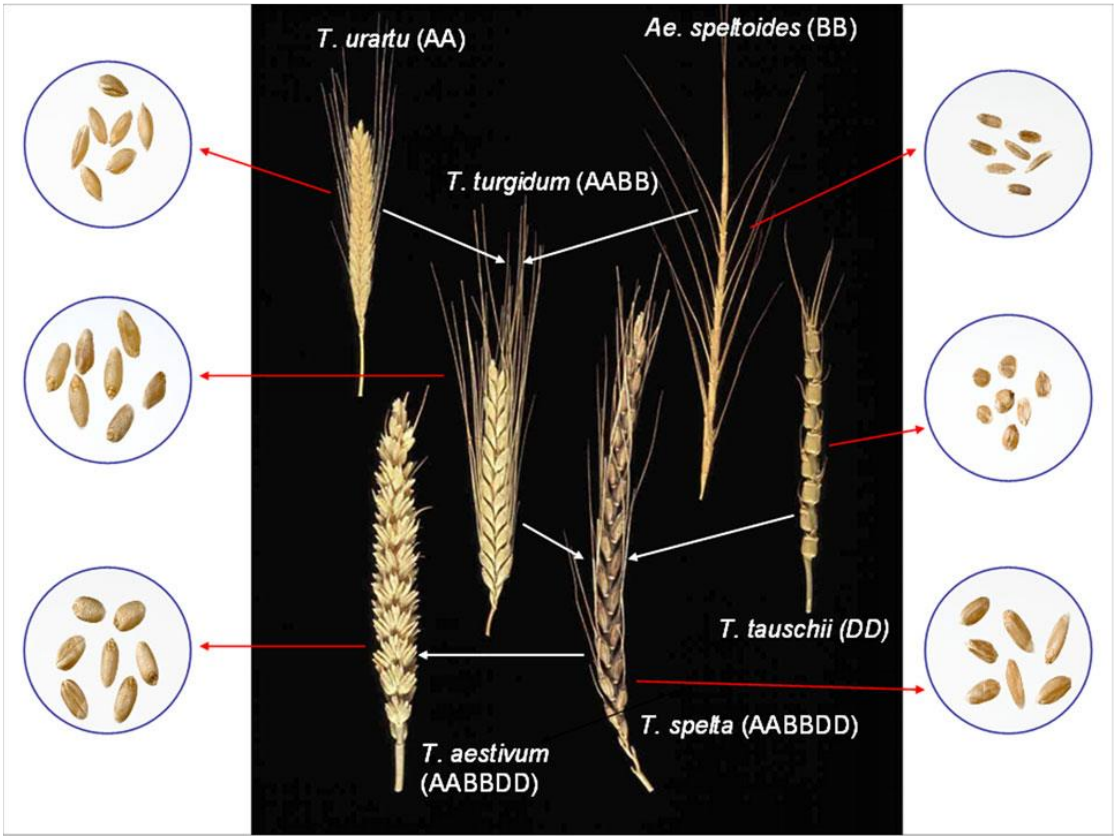

Figure 1.3. Origins of modern hexaploid wheat. (Reprinted with permission from Shewry 2009)

1.3.1. Wheat breeding

The main goal of wheat breeding is increasing yield. Traits such as disease resistance and drought resistance can also be improved through breeding for these qualities. There are two methods used in wheat breeding: I. conventional crossing during which complementary parent varieties are used to produce new genetic combinations, and II. introduction of indigenous genes from ancestral varieties of wheat or those related to modern wheat through conventional crossing so that favorable traits are incorporated (the contribution of DNA from ancestral wheat is less than 5%) (National Wheat Improvement Committee). In the state of North Dakota there have been many wheat cultivars released in the past 100 years. Examples include cultivars, Ceres (1910), Conley (1955), Olaf (1972), Reeder (1999) and Glenn (2005). Cultivars such as Prosper (2011) and Velva (2012) have been improved for high yield and resistance to diseases like stem rust and infections caused by Fusarium head blight (FHB) (Mergoum et al. 2013; 2014). Thus, breeding is important for producing wheat cultivars that are economically valuable.

1.3.1.1. Introduction of Reduced height (*Rht*) genes

Towards the late 1960s, semi-dwarf wheat varieties were released which have the phenotypic advantage of lower height to improve lodging resistance (Pearce et al. 2011). As such, most modern cultivars belong to this category. Most semi-dwarf genes have Japanese origins (Borojevic and Borojevic 2005). The Japanese wheat variety Akakomungi was the source of the *Rht8* gene and the day light insensitive gene *Ppd-D1*. The reduced height genes *Rht-B1* and *Rht-D1* also have Japanese origins, specifically they come from the variety Norin 10 (Hedden 2003). Other dwarfing genes have also been used to introduce reduced height to wheat varieties in Europe and North America. Additionally, dwarfing genes can be classified according to their sensitivity to gibberellins. The *Rht-B1* and *Rht-D1* are gibberellin insensitive genes.

These genes have a tendency to restrict coleoptile elongation and are not recommended in environmental conditions where seedling emergence could be negatively impacted, for example in low precipitation dry land regions (Ahmad and Sorrells 2002). Studies have been conducted to evaluate the performance of wheat with reduced height, mainly with the focus on yield in different environmental conditions. Butler et al. (2005) found that wheat lines with both *Rht-B1* and *Rht-D1* genes gave lower yield in under-irrigated, partially irrigated or rain fed conditions, and that semi-dwarf wheat with *Rht-B1* and wild type *Rht-D1* produced the highest grain yield in irrigated environments.

1.3.1.2. Glutenin subunit composition and dough strength

The HMW subunits are encoded by two genes: *Glu-1-1* (x-type) and *Glu-1-2* (y-type) (Shewry et al. 2009). A strong correlation between HMW glutenin and gluten strength was found by Payne et al. (1987) using SDS-sedimentation volume tests. Based on this, they also designed a scoring system for HMW glutenins and breadmaking quality based on the composition of these proteins. They found that variations at the *Glu-D1* locus resulted in great variation in the development of the gluten network. Moreover, HMW glutenin proteins were found to be indispensable in the formation of large glutenin polymers which contribute to visco-elastic properties found in wheat dough. The glutenin subunit combination 5+10 provides high visco-elastic potential resulting in high quality in comparison to the 2+12 combination (Shewry et al. 2000). The genes encoding the 5 and 10 subunits are tightly linked, therefore the contribution of each subunit to dough strength has not been established. The additional cysteine residue on subunit 5 in comparison to 2, and the high stability of subunit 10 have contributed towards the favorable dough properties caused by this subunit combination.

1.4. Wheat and Human Health: Celiac Disease

Celiac disease (CD) is an autoimmune enteropathy, which is prevalent in 0.71% (1 in 141) of adolescents and adults in the United States (Rubio-Tapia et al. 2012). The disease develops in genetically susceptible individuals as a result of ingestion of gluten forming proteins found in cereals such as wheat, rye and barley (Trier 1998). Celiac disease in principle is a T-cell mediated immunological condition (Wieser and Koehler 2008). $CD4^+$ T-cells identify peptides formed upon digestion of gluten proteins, which are presented by Major Histocompatibility Complex II (MHC II) molecules and cause an autoimmune reaction. Therefore, those who express the MHC class II Human Leukocyte Antigen (HLA) DQ2 and HLA-DQ8 would develop CD (Schuppan 2000).

1.4.1. Demographics of celiac disease

In the early days CD was thought to be a rare disease that affects children causing diarrhea and food intolerance. As of today, this disease is recognized as a multisystemic disorder. The table below shows that CD now is a wide spread disease.

Table 1.2. Estimated prevalence of CD

In the general population:	1 in 133
In symptomatic children:	1 in 322
In symptomatic adults:	1 in 105
In first-degree relatives of people with CD:	1 in 22
In second-degree relatives of people with CD:	1 in 39
In chronic disease (such as type 1 diabetes):	1 in 60
In African, Hispanic and Asian-Americans:	1 in 236
World-wide prevalence:	1 in 266

Source: Niewinski (2008)

1.4.2. Genetic aspect of celiac disease

The MHC genes account for about half of the genetic factors associated with the disease (Sollid and Lie 2005). The HLA class II alleles HLA-DQ2 and HLA-DQ8 have a very strong association with CD (Wieser and Koehler 2008). The absence of the genes is an indicator for non-celiac individuals. Thus, HLA typing is useful in diagnosing CD. However, research suggests that non-HLA genes also play a role in the onset of this disease. HLA-DQ2/8 are expressed in approximately 25% of healthy individuals, and the difference in concordance between monozygotic twins (80%) supports this premise. The contribution of other genes in the pathogenicity of CD is difficult to determine as the effect of these genes is unique to each individual (Kagnoff 2007).

1.4.3. Toxic proteins and peptides

Toxic proteins in cereals which trigger CD are gliadins, HMW-glutenin subunits and LMW-glutenin subunits. High content of glutamine and proline amino acids is a common feature in these proteins (Wieser and Koehler 2008). As a result of the high proline content, gluten forming proteins are not digested completely leading to the production of immunogenic peptides which are also known as toxic peptides or T-cell stimulatory epitopes. Studies have shown that the alcohol soluble prolamin (proline and glutamine) fraction consisting of gliadins is the most toxic component in wheat proteins. The toxicity of glutenins is classified as nontoxic, weakly toxic or not as toxic as gliadins, however, the toxicity of HMW-glutenins is said to be similar to that of gliadins.

Research has been conducted to isolate pure peptides and test for toxicity in relation to CD, and results have shown that most of the toxic sequences occur in the repetitive N-terminal domain of α-/β- gliadins which mainly consists of glutamine, proline and aromatic amino acids,

such as phenylalanine and tyrosine. In addition, T-cell stimulatory epitopes have been identified in several toxic proteins.

Table 1.3. Examples of T-cell stimulatory peptide sequences

Epitope	Other names	Sequence
DQ2.5-glia-α1a	DQ2-α-I, α9	PFPQPQLPY
DQ2.5-glia-α1b	DQ2-α-III	PYPQPQLPY
DQ2.5-glia-α2	DQ2-α-II, α2	PQPQLPYPQ
DQ2.5-glia-α3	glia-α20	FRPQQPYPQ
DQ2.5-glia-γ1	DQ2-γ-I	PQQSFPQQQ
DQ2.5-glia-γ2	DQ2-γII, γ30	IQPQQPAQL
DQ2.5-glia-ω1	DQ2-ω-I	PFPQPQQPF
DQ2.5-glia-ω2	DQ-ω-II	PQPQQPFPW
DQ8-glia-α1	DQ8-α-I	QGSFQPSQQ
DQ8.5-glia-α1	DQ-α-I	QGSFQPSQQ

Source: Sollid et al. (2012)

Some researchers claim that breeding practices might have changed wheat proteins over the years, and made modern wheat more toxic in terms of initiating the immune response related to celiac disease (van den Broeck et al. 2010b). Additionally, popular media appears to embrace the fact that modern wheat is toxic and unhealthy. In this context, it is vital that changes in wheat cultivars over the years are investigated in terms of wheat protein chemistry and other such aspects.

1.4.4. Adaptive and innate immune response

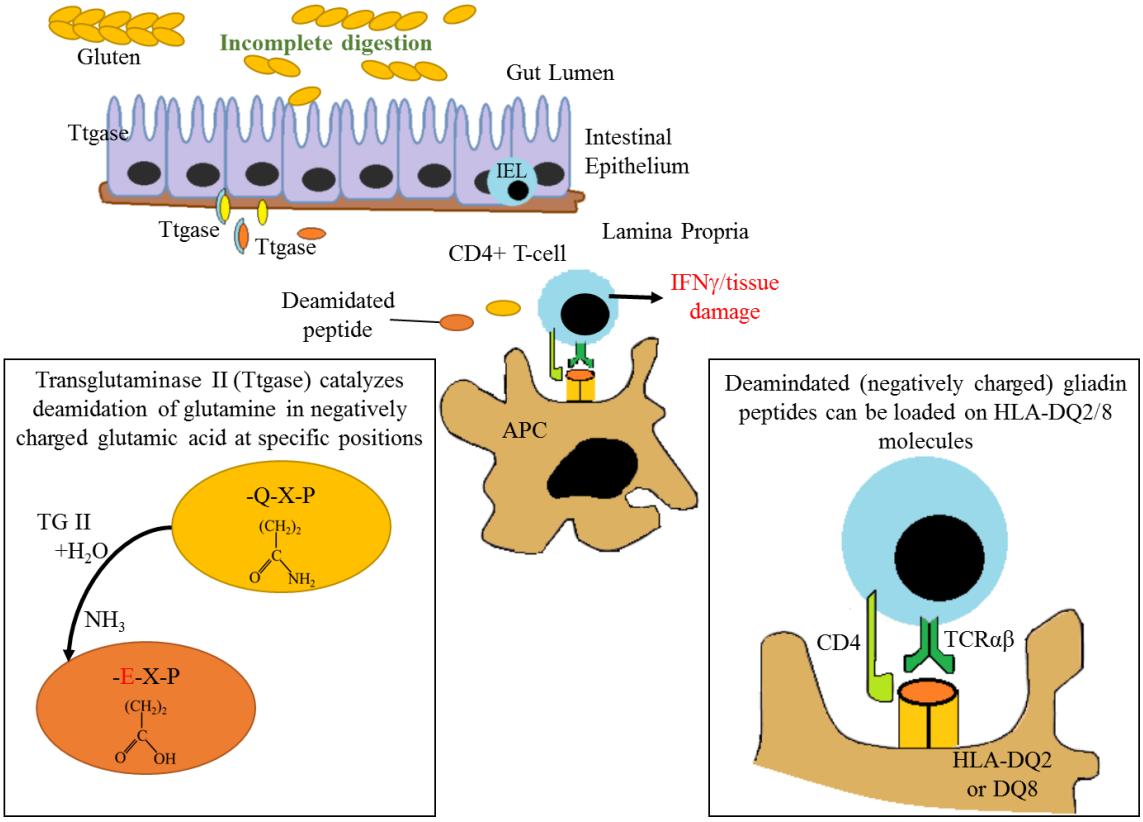

Figure 1.4. Immune response associated with celiac disease. (Adapted from Meresse et al. 2009)

As previously mentioned, CD is only prevalent in individuals predisposed with HLA-DQ2/8 expressed on the surface of Antigen Presenting Cells (APC) such as, dendritic cells, macrophages and B-cells (Wieser and Koehler 2008). The pathway for adaptive immune response is triggered when HLA-DQ2/8 bind gluten peptides and present them to T-cells, which are in the lamina propria. T-cell receptors are found in gluten-sensitive $CD4^+$ helper cells.

The deamidated form of the toxic gluten peptides have a higher affinity for HLA-class II proteins, although deamidation is not a requirement for T-cell activation. The tissue transglutaminase enzyme is a calcium-dependent transamidating enzyme, which deamidates glutamine to glutamic acid by reaction with water (Dieterich et al. 1997). This enzyme only deamidates select glutamine residues according to the enzyme's specificity, which is influenced

by the amino acids around the glutamine residue (Molberg et al. 1998). Additionally, the tissue transglutaminase enzyme catalyzes the binding of gluten peptides to extracellular matrix proteins such as collagen (Dieterich et al. 2006). This long-term immobilization mechanism is said to be linked with the intestinal inflammation associated with CD.

The toxic peptides bound to HLA-DQ2/8 are presented to T-cell receptors in gluten sensitive CD^{4+} T-cells (Wieser and Koehler 2008). Research suggests that the minimum length of a peptide should be nine amino acids to be recognized by T-cells (Sollid 2002). As a result of this interaction, cytokines Interferon (IFN) -γ, Interleukin (IL) -2 and Tumor Necrosis Factor (TNF) - α levels are increased leading to the release and activation of matrix metalloproteinases, which cause the degradation of matrix proteins, and subsequent mucosal destruction and epithelial apoptosis (Wieser and Koehler 2008). Furthermore, the activated T-cells stimulate B-cells to produce IgA and IgG antibodies against gluten peptides, tissue transglutaminase and peptide complexes formed by the activity of the enzyme.

Some toxic peptides do not stimulate the adaptive immune response, but are responsible for the innate immune response. The innate response in CD is characterized by the rapid increase in intraepithelial lymphocytes (IEL), which is a major hallmark of CD. IL-15 is a prominent player of the innate immune response. It is produced by epithelial cells and lamina propria cells. Some toxic peptides cause this reaction by directly binding to enterocytes, macrophages and dendritic cells. IL-15 stimulates IEL to express NKG2D receptor and epithelial cells to express the ligand to NKG2D. The interaction of the receptor and the ligand results in the destruction of epithelial cells. The innate response may be necessary to initiate the adaptive response.

As mentioned earlier, gluten proteins found in wheat, rye and barley are known to trigger the onset of CD (Wieser and Koehler 2008). There is still much debate as to whether oat avenins

also cause CD. All toxic grains mentioned are found in the tribe *Triticeae*, which falls under the grass family *Poaceae*. Non-toxic cereals include, corn, rice, sorghum and millet, which have a different evolutionary pathway than the cereals in the grass family.

1.4.5. Toxicity of historical and modern wheat varieties

Spaenij-Dekking et al. (2005) determined that wheat species that are low in CD causing immunogenic peptides can be found as there is high genetic variation between species. Additionally, Carroccio et al. (2010) found that wheat that is gliadin-deficient have lower CD toxicity, highlighting the importance of prolamin content in wheat. Supporting this premise Pizzuti et al. (2006) found that the ancient wheat variety *Triticum monococcum* lacks gliadin proteins that cause toxicity. Some researchers suggested that durum wheat could have lower CD toxicity, as it lacks the D genome found in tetraploid wheat (van den Broeck et al. 2010a). This hypothesis was tested using western blotting techniques on landraces, old and modern durum wheat. The results of the study showed that there are differences in terms of CD toxicity between wheat varieties, however, the study did not show that these differences could be categorized so that landraces and old wheat varieties alone could be called less-toxic.

On the contrary, Colomba and Gregorini (2012) found that two ancient durum wheat varieties in Italy (Grazielle and Kamut) are toxic to CD patients. The results showed that the ancient wheat varieties have more α-gliadin in comparison to modern wheat, thus leading to higher results in antibody tests. In another similar study, monococcum (*T. monococcum* spp. *monococcum*) wheat lines and modern wheat cultivars triggered the T-cell mediated immunological response in relation to CD (Gianfrani et al. 2012). However, as in previous studies, the toxicity varied between the varieties studied. Suligoj et al. (2013) also tested ancient wheat and suggested that they are toxic to CD patients.

Studies have shown that wheat breeding may be a cause for the increased prevalence of CD. Van den Broeck et al. (2010a) used western blotting and two-dimensional gel electrophoresis (iso-electric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis) to study historical and modern wheat in the Netherlands. The results suggested that the toxicity of modern wheat is higher in comparison to historical or ancient wheat, and that modern wheat varieties have less genetic diversity between them, suggesting that the diversity of toxic peptides is not high. In this context, it is clear that more studies in the area of historical/ ancient and modern wheat should be conducted to assess differences in CD toxicity. If less toxic varieties are identified, breeding wheat varieties that are not CD toxic is a possibility. Preservation of visco-elastic properties while producing non CD toxic wheat could be a challenge for breeders.

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CHAPTER 2. ASSOCIATION BETWEEN PROTEIN COMPOSITION AND QUALITY CHARACTERISTICS IN HARD RED SPRING WHEAT CULTIVARS RELEASED OVER 100 YEARS

2.1. Abstract

The hard red spring (HRS) wheat cultivars released in North Dakota during the last 100 years have shown improvements in characteristics, such as adaptation to growing conditions, yield, and quality traits. The objectives of this study were to identify the changes in quality characteristics that occurred over time, and to determine the association of these characteristics with the protein components in historical and modern HRS wheat. For this purpose, 30 HRS wheat cultivars released between 1910 and 2013 were analyzed for correlations between quality traits and release year, and also between quality traits and protein composition. A highly significant positive correlation ($P \leq 0.01$) appeared between release year and farinograph parameters, such as peak time and stability. Research using size exclusion-HPLC showed significant positive correlations ($P \leq 0.01$) between year of release and glutenin polymeric proteins and the ω-gliadin fraction, which also had significant positive ($P \leq 0.01$) correlations with dough characteristics. Reverse phase-HPLC was used to confirm the above findings for ω-gliadin proteins, where some peaks corresponding to these proteins showed significant positive correlations ($P \leq 0.05$) with year and dough properties. In conclusion, the quantitative increase in glutenin polymeric proteins, and certain sub-fractions of ω-gliadins could be associated with the favorable dough properties that are seen in modern HRS wheat cultivars.

2.2. Introduction

Gluten forming proteins, gliadins and glutenins, are found as a continuous matrix in the wheat endosperm (Wieser 2007). These proteins are in the form of monomers or polymers (with

inter and intra chain disulfide bridges). Previously, many studies have determined the role of the gluten forming proteins and their subgroups in the breadmaking process (Shewry et al. 1992; Barak et al. 2015; Khatkar and Schofield 1997). However, the extensive polymorphism in these proteins have resulted in gluten forming proteins varying widely in molecular mass and charge. Thus, the characterization of these proteins is a difficult task (Bonomi et al. 2013).

Glutenin proteins are divided into high molecular weight (HMW) and low molecular weight (LMW) subunits, both of which are known to form polymeric proteins by intermolecular disulfide bonds. Polymeric proteins that are mainly composed of glutenin subunits have significant but different associations with flour breadmaking quality according to their solubility in SDS buffer solution as demonstrated by Ohm et al. (2009; 2010). Size exclusion-HPLC (SE-HPLC) has been used to evaluate the relationship between the glutenin polymeric proteins and breadmaking quality, which was evaluated using farinograph characteristics and baking quality characteristics. The SDS unextractable HMW polymeric proteins were found to have positive correlations with dough mixing characteristics and loaf volume, whereas, SDS extractable polymeric proteins showed negative correlations specifically for HRS wheat.

Studies have also been performed to evaluate the role of gliadins in terms of breadmaking quality. Gliadin proteins have molecular weights ranging from 28000 to 55000, and are further divided into subgroups, α-, β-, γ- and ω-, according to their electrophoretic mobility on acid polyacrylamide gel electrophoresis (A-PAGE). These proteins do not contain disulfide bridges between polypeptide chains, thus are monomeric (Bonomi et al. 2013). Uthayakumaran et al. (2001) showed that addition of all gliadin subgroups resulted in decreased values for mixing time, peak resistance, maximum resistance to extension, and loaf height, while increasing resistance to breakdown and extensibility. When considering the separate fractions, γ-gliadin

appeared to reduce mixing time, ω-gliadin caused the greatest decrease in loaf height, and the α- and β- gliadins showed the least reduction of loaf height. In another study, gliadin as a whole, and its different subgroups, showed a negative influence on dough strength (Khatkar et al. 2002a). In this study, ω-gliadins caused the most significant decrease, whereas α-gliadin showed the least effect. The other gliadin subgroups showed an intermediate effect on dough strength. The addition of the ω-gliadin fraction had the least positive effect on peak dough resistance and loaf volume, although the addition of other gliadin fractions showed substantial improvements on the same parameters. The same group conducted studies on the influence of gliadins on the rheological properties of wheat gluten and found that the α-, β- and γ- fractions tend to interact with the gluten network more than the ω- fraction. This feature was attributed to the presence of cysteine residues in the α-, β- and γ- gliadins, as opposed to the cysteine poor ω-gliadin (Khatkar et al. 2002b). Further supporting the role of ω-gliadins as dough weakening agents, Fido et al. (1997) found that all gliadin fractions caused a decrease in dough strength and that the highest effect was caused by the ω-gliadin fraction.

On the other hand, Huebner and Bietz (1986) found that certain gliadin fractions (separated through reverse phase-HPLC) may have a positive influence on breadmaking quality. During their study, significant positive correlations were found between certain gliadin fractions of HRS wheat and quality parameters. Moreover, Park et al. (2006) showed that the subclasses of gliadin proteins fluctuate differently when the total protein content of flour is changed, and that a positive correlation appeared between gliadin levels and loaf volume in hard winter wheat. Weegels et al. (1994) also observed that hydrophobic gliadins had a beneficial effect on loaf volume, whereas the effect of hydrophilic gliadins was less. In this study gliadin proteins were separated using cation exchange chromatography. These fractions differed greatly in gliadin

protein and low molecular weight protein composition, and hydrophobicity as determined using RP-HPLC. In a study which compared closely related wheat lines differing in breadmaking quality, certain bands of ω-gliadin were related to positive effects in this parameter, whereas some γ-gliadin bands were associated with negative effects (Lookhart and Albers 1988). Size exclusion-HPLC data for the unreduced proteins also showed that gliadin proteins, primarily ω-gliadin, had positive correlations with loaf volume, and water absorption (Ohm et al. 2009; 2010). However, in another study, Vanlonkhuijsen et al. (1992) reported that γ-gliadins have a positive contribution to breadmaking, whereas the ω-gliadins have a negative contribution. In a review on ω-gliadins by Tatham and Shewry (1995), they cited that some bands of ω-gliadin correlated positively with dough strength, whereas others had negative correlations. However, they also highlighted the conflicting roles of ω-gliadin and further suggested that it is somewhat difficult to identify the role of ω-gliadins in the breadmaking process.

In light of the above information, it is clear that the role of gliadin proteins as a whole, and their different fractions in relation to breadmaking quality, has not been established, especially for hard red spring wheat. Thus, the aims of this research were to determine how quality characteristics of wheat fluctuate with year of release, and to determine the association of quality characteristics with protein components.

2.3. Materials and Methods

Thirty wheat cultivars released in North Dakota from 1910 to 2013 were used in this study. The cultivars were grown in Casselton, ND in 2013 in 3 ft x 150 ft strip plots. Plots were ordered by release year in the field. Planting was done during the first week of June and harvested during the first week of September.

Table 2.1. List of cultivars used in the study

Cultivar	Release year
Marquis	1910
Ceres	1926
Pilot	1939
Rival	1939
Vesta	1942
Mida	1944
Conley	1955
Justin	1963
Fortuna	1966
Waldron	1969
Olaf	1972
Butte	1977
Len	1979
Stoa	1984
Butte86	1986
Grandin	1989
Pioneer-2375	1990
Gunner	1995
Russ	1995
Reeder	1999
Alsen	2000
Granite	2002
Freyr	2004
Steele-ND	2004
Glenn	2005
Faller	2007
RB07	2007
Barlow	2009
Velva	2012
Elgin	2013

2.3.1. Quality analysis of wheat cultivars

American Association of Cereal Chemists International (AACC-I) approved method 39-10.10 was used to determine the nitrogen content of the wheat kernels by nitrogen combustion analysis using a Dumas nitrogen analyzer. The protein content was calculated using the determined moisture content (14%). Wheat grain ash content was measured using the AACC-I approved method 08-01.01. Test weight was determined using the AACC-I approved method 55-10.01. Thousand kernel weight was measured using a 10 g sample of cleaned wheat analyzed with an electronic seed counter (Seedburo Equipment Count-A-Pak Model 77 Totalizer). Kernel size distribution was determined using Tyler sieves (W. S. Tyler, Mentor, OH) with the following sieve openings: top sieve opening 2.92 mm, middle sieve opening 2.24 mm and the bottom sieve 1.65 mm. Kernel hardness, weight and diameter were determined using the Single Kernel Characterization System (SKCS), Perten SKCS 4100 according to the AACC-I approved method 54-31.01.

Wheat grains were cleaned and milled in house and used for analysis (HRS Wheat Quality group, Department of Plant Sciences, North Dakota State University). The wheat kernels were cleaned well and tempered to 16% moisture for 16 hours. Afterwards, an additional 0.5% of water was added 15 min prior to milling. The conditions in the milling laboratory were as follows: 68% relative humidity and 22-23°C temperature. A Buhler ML-202 laboratory mill was used to mill the samples. The straight grade flour was blended and reported as flour extraction. The blended flour was re-bolted through an 84 SS sieve to remove any foreign material. Values for break flour yield and flour yield were obtained during the milling of the wheat samples. Standard AACC-I approved methods mentioned above were used to determine flour protein content and flour ash content. The AACC-I approved method 38-12.02 was used to determine

the gluten index and the wet gluten content. In order to determine flour color characteristics, the Minolta method using a Minolta Chroma meter (Minolta CR-410) was used. The L*, a* and b* color system was used to record the readings (Wheat Marketing Center 2008).

In order to evaluate mixing characteristics of the flour, farinograph (Farinograph-E, C. W. Brabender) analysis was performed on each sample using the standard AACC-I method 54-21.02. Peak time, stability, mixing tolerance, quality number and absorption measurements were taken using the software provided by Brabender. The breadmaking quality was evaluated by preparation of 100 g pup loaves using AACC-I method 10-09.01, with some modifications. A fermentation time of 2 hours was used, fungal amylase was used in place of malt powder, instant dry yeast was used instead of compressed yeast, and ammonium phosphate (1 mL of 10% ammonium phosphate for 100 g of flour) was added to improve yeast functionality. The baked bread was evaluated for loaf volume using rapeseed displacement (AACC-I method 10-05.01) and the quality parameters were scored according to AACC-I method 10-12.01.

2.3.2. Extraction of proteins and size exclusion-HPLC

Proteins were extracted for SE-HPLC following the method of Gupta et al. (1993) with minor modifications (Ohm et al. 2009). The sodium dodecyl sulfate (SDS) soluble fraction was obtained by adding 1 ml of extraction buffer (0.5% SDS and 0.05 M sodium phosphate, pH 6.9) to 10 mg (adjusted to 14% moisture content) of flour. The mixture was stirred for 5 min using a vortex mixer (Fischer Scientific) at 2500 rpm. Afterwards, the mixture was centrifuged at 17,000 g (Eppendorf Centrifuge 5424), and the supernatant was filtered through a 0.45 μm PVDF membrane filter (Sun Sri, Rockwood, TN). Immediately after filtration, the sample was heated at 80^0C in a water bath for 2 min and allowed to cool to room temperature. The SDS insoluble fraction was obtained from the residue from the centrifugation step. The residue was

re-suspended in 1 ml of the same extraction buffer and sonicated in a probe type sonicator (Sonic Dismembrator 100, Fisher Scientific) for 30 sec at 10 W power setting, and treated the same as the SDS soluble fraction. The separation of the proteins in the SDS soluble and insoluble fractions was performed using an Agilent 1100 series narrow bore column (300 × 4.5 mm, BIOSEP SEC S4000, Phenomenex, Torrance, CA) and a guard cartridge (BIOSEP SEC S4000, Phenomenex, Torrance, CA) (Ohm et al. 2009). The injection volume was 10 μL and the eluting solution was 50% acetonitrile in water with 0.1% (v/v) trifluoroacetic acid (TFA). The flow rate for the SE-HPLC analysis was 0.5 mL/min. An Agilent 1200 photodiode array detector (Agilent Technologies, Waldbroann, Germany) was used to detect the solutes at 214 nm.

MATLAB (2015, The MathWorks, Natick, MA) functions and an in-house program were used to process SE-HPLC data (Ohm et al. 2009). UV absorbance values were interpolated to 0.002 min intervals by the 'spline' function in MATLAB. Absorbance area values were calculated for individual retention intervals of 0.01 min using the interpolated data. Linear correlation coefficients (r) were calculated between individual absorbance area values and quality traits, and shown as the continuous spectrum over SE-HPLC retention time. The SE-HPLC profiles were also divided into five fractions of which retention time intervals were 3.5–5.0 min for the first fraction (F1), 5.0–5.9 min for the second fraction (F2), 5.9–6.2 min for the third fraction (F3), 6.2–6.9 min for the fourth fraction (F4), and 6.9-8.0 min for the fifth fraction (F5) (Morel et al. 2000; Ohm et al. 2009; Park et al. 2006). The area percentage values of the individual protein fractions were converted into percentage values based on flour weight (% flour) and used in the correlation analysis.

2.3.3. Separation of protein fractions on SDS-PAGE

Total flour protein extract was obtained from the flour samples using sonication. A volume of 1 ml of the same extraction buffer used earlier was added to 10 mg of the sample and sonicated for 30 sec at a power setting of 10 W. The sample was then centrifuged, filtered and heat treated the same as the SDS solution fraction. Afterwards, the sample was separated by SE-HPLC as described in the section above. A regular column (300 x 7.80 mm, BIOSEP SEC S4000, Phenomenex, Torrance, CA) was used, with an injection volume of 20 µL, under the same conditions as previously explained. The fractions obtained from SE-HPLC were then freeze dried and separated on an SDS-PAGE (12%) gel. A Mini-PROTEAN system (Biorad) was used for this purpose. Equal volumes of the fractions were loaded on the gel and run at 100 V for approximately 1.5 hrs.

2.3.4. Extraction of gliadin proteins and reverse phase-HPLC (RP-HPLC)

Gliadin proteins were extracted and separated by RP-HPLC according to the method of Lookhart and Bean (1995), with modifications. Two different protocols were used. In the constant flour method, a constant amount of flour was used as the starting material of the extraction. A volume of 750 µL of 70% ethanol was added to 250 mg of flour and shaken at 1400 rpm for 1 h at 30^{0}C using a heating block (Eppendorf Thermomixer R). Afterwards, the mixture was centrifuged at 4550 g for 10 min (Eppendorf Centrifuge 5415 C), and filtered through a 0.45 µm nylon filter (VWR International). The samples were heated for 3 min at 80^{o}C in a heating block as the final step. In the constant protein method, the amount of protein in each extract was quantified after the extraction process. The amount of protein was measured using the Pierce^{TM} BCA assay kit (ThermoFisher Scientific). After the protein content was determined the extracts

were diluted to 13 μg/μL using water that was filtered through a 0.45 μm nylon membrane filter. The samples were then heat treated at 80°C for 3 min in a heating block.

An Agilent Zorbax 300SB-C18 (4.6 x 250 mm, 5 μm) column was used for separation of the protein extracts. Buffer A was comprised of 95% water, 5% acetonitrile and 0.1% TFA (v/v), and Buffer B was 100% acetonitrile with 0.08% (v/v) TFA. The two buffers were balanced at 210 nm with TFA, and subsequently filtered through a 0.45 μm nylon membrane prior to the analysis process. Gliadins were eluted in a linear multistep gradient at 65^{0}C at a flow rate of 1 mL/min. The injection volume was 20 μL for the constant flour method and 23 μL for the constant protein method (approximately 300 μg of protein based on the BCA assay). The signal was detected at 210 nm using an Agilent 1100 variable wavelength detector (Agilent Technologies, Waldbroann, Germany).

The elution gradient started at 75% buffer A and 25% buffer B, which was maintained for the first 5 minutes. During the next 5 min, the volumes were gradually increased so that at 10 min it was 70% buffer A and 30% buffer B. After gradually increasing again, at 60 min it was 50% of each buffer. This state was maintained until 63 min. In the next minute the gradient was changed so that at 64 min it was 100% buffer B, which was maintained for 1 min. In the next minute, the gradient was changed again so that at 66 min the percentages were brought down to the original proportions. A post time of 15 min was added after each run to ensure the complete passage of each sample through the column, and to prevent mixing of samples in the column. Prior to separation of the proteins through the column, a conditioning step was performed. That is, water and acetonitrile were flushed through the column respectively for 5 min. The column was washed with 10% methanol for storage.

The HPLC data was also processed using an in-house program coded using MATLAB (2015, The MathWorks, Natick, MA). UV absorbance values were interpolated to 0.005 min intervals by the 'spline' function in MATLAB. Absorbance area values were calculated for specific peaks using the interpolated absorbance data.

2.3.5. Statistical analysis

Statistical analysis of the results was performed using the SAS software (Version 9.3, SAS Institute, Cary, NC) and correlation analysis was done using the 'CORR' procedure in SAS.

2.4. Results and Discussion

The thirty HRS wheat cultivars that were analyzed for protein content and other quality parameters such as, farinograph peak time and stability, showed the statistical values listed below (Table 2.2). Differences could be observed with regard to quality characteristics in the wheat cultivars in question. Additionally, some characteristics showed positive correlations with release year, whereas others showed negative correlations. This shows that the variation in quality traits did not change in the same manner over the years. In general, the values obtained for the various properties fall within the range that is expected for HRS wheat.

Table 2.2. Mean, Standard Deviation (StD), Minimum (Min) and Maximum (Max) values for quality characteristics and their correlation coefficients (r) with release year

Variable	Mean	StD	Min	Max	r^2 Value[a]
Wheat protein (%, 14% mb)	14.5	0.5	13.3	15.2	-0.205^{NS}
Wheat ash (%, 14% mb)	1.70	0.20	1.46	2.14	-0.781***
Test weight (lbs/bushel)	57.1	2.7	50.9	61.8	0.784***
Thousand kernel weight (g)	34.7	3.3	28.4	42.0	0.349^{NS}
Kernel size: Large	64.9	18.5	22.0	89.0	0.539**
Medium	39.4	21.6	10.0	74.0	-0.536**
Small	5.2	23.6	0.0	4.0	-0.597***
Hardness	78.5	6.1	65.6	92.7	0.401*
Hardness SD[b]	15.0	1.6	12.6	19.4	-0.417*
Weight (g)	34.6	3.6	27.9	42.2	0.326^{NS}
Weight SD[b] (g)	8.2	0.9	6.5	9.8	-0.270^{NS}
Diameter (mm)	2.49	0.18	2.19	2.93	0.383*
Diameter SD[b] (mm)	0.45	0.03	0.41	0.54	0.150^{NS}
Break flour yield (%)	7.9	1.0	6.0	11.9	0.054^{NS}
Flour yield (%)	70.2	1.7	66.7	73.7	0.484**
Flour protein (%, 14% mb)	12.9	0.6	11.4	13.8	-0.153^{NS}
Flour ash (%, 14% mb)	0.57	0.08	0.43	0.75	-0.830***
Gluten index (%)	94.4	6.4	69.6	99.3	0.214^{NS}
Wet gluten (%)	34.1	2.3	29.3	38.4	-0.098^{NS}
Flour color: L*	90.7	0.3	90.0	91.2	0.126^{NS}
a*	-0.9	0.2	-1.3	-0.6	-0.365*
b*	8.2	0.7	6.7	9.5	0.529**
Farinograph absorption (%)	60.6	2.2	56.6	65.1	0.583***
Farinograph peak time (min)	6.9	1.2	4.1	9.0	0.657***
Farinograph stability (min)	10.9	3.5	4.5	22.4	0.540**
Mixing tolerance index (BU)	25.6	7.9	12.3	49.5	-0.577***
Quality number (mm)	132.7	31.7	68.0	238.0	0.644***
Bake water absorption (%)	67.4	2.1	63.5	72.0	0.490**
Bake mix time (min)	4.1	0.5	3.5	5.3	0.090^{NS}
Loaf volume (cm^3)	969.3	46.8	882.5	1033.0	-0.202^{NS}
Grain and texture	7.7	0.4	6.8	8.5	-0.594***
Crumb color	7.7	0.4	7.0	8.5	-0.562**

[a]NS = non-significant, *= $P \leq 0.05$, ** = $P \leq 0.01$ and *** = $P \leq 0.001$.
[b]SD= standard deviation of single kernel characteristics estimated from 300 kernels.

According to Table 2.2, the test weight and release year showed a strong ($P \leq 0.001$) positive correlation. This suggests that the milling yield would have also increased with respect to year. The lowest test weight observed in the cultivars released after 1990 is 56.8 lbs/bushel

(cultivar ‘Elgin’), which is only slightly lower than the overall average of 57.1 lbs/bushel. Thus, improvements in cultivars released in later years have resulted positively with regard to test weight. The kernel size distribution showed a negative correlation ($P \leq 0.01$ and $P \leq 0.001$ respectively) with release year for medium and small sized kernels. As expected, the large size kernels showed a positive correlation ($P \leq 0.01$). Kernel diameter and year also showed a positive correlation ($P \leq 0.05$), further supporting the correlation between size and year. It is interesting to note that a significant correlation between year and kernel weight was not observed, despite the positive correlation between year and large size kernels.

Kernel hardness and year of release showed a positive correlation ($P \leq 0.05$). Hardness is an important factor that determines the wheat tempering level prior to milling (Doekes and Belderok 1976). Harder wheat needs to be tempered and conditioned for a longer period of time compared to wheat with lower hardness. Additionally, such wheat requires more water to make dough of a proper consistency. The positive correlation between year and hardness essentially means that cultivars released in later years require more tempering prior to milling. The highest hardness of 92.7 was shown by the cultivar ‘Glenn’, which was released in 2005, and the lowest was shown by the cultivar ‘Vesta’ released in 1942. The standard deviation of single kernel hardness determined by Single Kernel Characterization System showed a negative correlation, indicating that the uniformity of kernel hardness improved over time.

Flour yield, which is an important characteristic concerning milling quality, showed a positive correlation ($P \leq 0.01$) with year of release of cultivar. This could be due to the increase in the size of kernels over time. The cultivar ‘Faller’ released in 2007 showed the highest flour yield, whereas the cultivar ‘Velva’ released in 2012 showed the lowest flour yield. This is an interesting phenomena since both of these cultivars were released in the past decade. Flour ash

content showed a very high and negative correlation ($P \leq 0.001$) with release year of the cultivar. This suggests that the ash content of flour decreased over time, thus, enhancing flour quality. Traditionally, the purity of wheat flour is measured using ash content, and flour with low ash content is preferred (Hinton 1959). Lower ash contamination means less contamination with bran and germ. The cultivar 'Conley' released in 1955 showed the highest wheat ash content, while the cultivar 'Russ' released in 1995 showed the lowest value. In general, the cultivars released after 1990 showed a wheat ash content lower than 1.70%, which is the overall average.

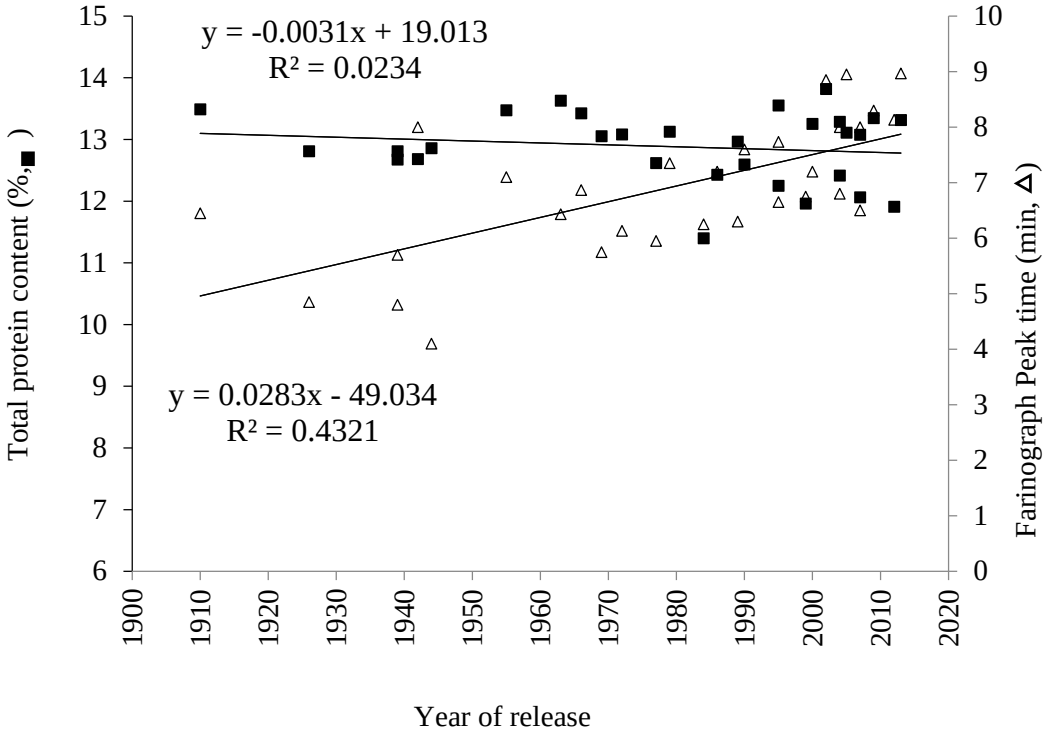

Figure 2.1. Relationship between cultivar release year vs. total protein content or Farinograph peak time

Although the flour total protein content and release year of cultivar showed a negative correlation which was not significant ($P > 0.05$) (Figure 2.1), there was a significant ($P < 0.01$) and positive correlation between release year and farinograph peak time. A similar trend was observed for farinograph absorption, and stability. This result indicates that dough properties

have improved over time, and that the cultivars released in later years have better dough making capacity. Farinograph parameters have also been linked to baking performance. In a study conducted by Confort and Johnson (1992), where the farinograph characteristics were used to predict the baking quality of chlorinated and unchlorinated flour, a strong significant correlation was found between peak time and stability in relation to baking quality. The highest values for these characteristics were found in cultivars released from the year 2005 onwards. The lowest value for farinograph peak time was noted in the cultivar 'Marquis' released in 1910, the oldest cultivar being analyzed. The cultivar 'Vesta' release in 1942 showed the lowest farinograph absorption. Hence, it is clear that newer wheat cultivars have better dough properties. In contrast, mixing tolerance index showed a strong negative correlation with year ($P \leq 0.001$). Moreover, quality number also showed a strong positive correlation with year of release, further validating the trend explained above. The average quality number for cultivars released after 1990 was 154.2, which is well above the average for the 30 cultivars investigated here. With regard to baking characteristics, bake water absorption showed a positive correlation with year ($P \leq 0.05$). Minor baking properties such as, grain and texture and crumb color, showed negative correlations with year ($P \leq 0.05$). However, loaf volume, which is an important indicator in baking quality, did not show a significant correlation ($P > 0.05$).

2.4.1. Protein size exclusion-HPLC and quality characteristics

When using SE-HPLC for the separation of SDS extractable (E) and unextractable (U) proteins distinct chromatograms were obtained. Table 2.3 shows the mean, standard deviation, minimum and maximum values for UV absorbance area of the fractions (explained in the methods section) that are indicative of quantity of individual protein fractions. The total area

(extractable + unextractable) was assumed to be 100% of flour protein percentage, and depending on the area of each fraction, and percent protein content was calculated.

Table 2.3. Mean, Standard Deviation (StD), Minimum (Min) and Maximum (Max) values for absorption area of protein fractions of size exclusion-HPLC

Protein Fraction[a]	Protein content (% flour)			
	Mean	StD	Min	Max
Ef1	1.36	0.11	1.13	1.53
Ef2	1.21	0.08	1.00	1.33
Ef3	0.73	0.08	0.61	0.89
Ef4	4.51	0.48	3.69	5.81
Ef5	1.55	0.08	1.40	1.77
Uf1	1.46	0.14	1.12	1.82
Uf2	1.14	0.09	0.93	1.34
Uf3	0.20	0.01	0.17	0.23
Uf4	0.50	0.03	0.46	0.57
Uf5	0.21	0.02	0.19	0.28

[a]Ef1-5= extractable fraction 1-5; and Uf1-5= unextractable fraction 1-5 for size exclusion HPLC

For extractable proteins, F4 showed the highest area, and for unextractable proteins, F1 showed the highest area. In order to determine the identity of the proteins that were separated by SE-HPLC, SDS-PAGE was performed for fractions obtained following separation of total proteins (both SDS extractable and unextractable) using SE-HPLC (Figure 2.3). The SDS-PAGE showed that the main component of F4 is gliadins and this result is in agreement with Larroque et al. (1997).

Table 2.4. Correlations between size-exclusion HPLC protein fraction area and release year and farinograph characteristics

Protein Fraction[a]	Release year	Farinograph characteristics				
		Absorption	Peak time	Stability	Mixing Tolerance Index	Quality number
Ef1	-0.672***	-0.247^{NS}	-0.537**	-0.658***	0.618***	-0.686***
Ef2	-0.467**	-0.198^{NS}	-0.438*	-0.445*	0.515**	-0.527**
Ef3	0.588***	0.688***	0.568**	0.278^{NS}	-0.323^{NS}	0.449*
Ef4	-0.358^{NS}	0.133^{NS}	-0.054^{NS}	-0.463*	0.372*	-0.286^{NS}
Ef5	-0.047^{NS}	0.014^{NS}	0.009^{NS}	-0.316^{NS}	0.257^{NS}	-0.206^{NS}
Uf1	0.553**	0.220^{NS}	0.652***	0.731***	-0.641***	0.738***
Uf2	0.499**	0.223^{NS}	0.589***	0.668***	-0.587***	0.664***
Uf3	0.632***	0.451*	0.695***	0.625***	-0.593***	0.709***
Uf4	0.367*	0.443*	0.517**	0.296^{NS}	-0.341^{NS}	0.471**
Uf5	0.431*	0.005^{NS}	0.425*	0.390*	-0.321^{NS}	0.429*

[a]Ef1-5= extractable fraction 1-5; and Uf1-5= unextractable fraction 1-5 for size exclusion HPLC. NS = non-significant, *= $P \leq 0.05$, ** = $P \leq 0.01$ and *** = $P \leq 0.001$.

The correlations between the different fractions and release year and quality characteristics, in terms of dough properties, are shown above (Table 2.4). Since farinograph characteristics showed strong significant correlations with year of release (Table 2.1), the study was focused on dough properties measured by farinograph. The fractions, both in E and U proteins, showed different trends for correlations with year. The extractable fractions EF1 and EF2 showed negative correlations ($P \leq 0.05$) with year of release. On the other hand, UF1-UF5 showed positive correlations with year. Of these, EF3 and UF3 showed the strongest positive correlations ($P \leq 0.001$).

For farinograph characteristics, EF3 showed a significant correlation ($P \leq 0.001$) with farinograph absorption. This fraction also showed a significant correlation with release year, therefore, it could be considered as a protein (fraction) that contributed towards the improvement of water absorption that was observed over the progression of time. Moreover, EF3 showed a significant positive correlation ($P \leq 0.05$) with peak time. However, EF1 and EF2 showed a negative correlation with the same parameter. In the SDS unextractable proteins, all fractions showed significant positive correlations with peak time. This shows that the unextractable proteins, together with EF3, positively impact peak time. Farinograph stability is a parameter that indicates dough strength. Extractable fractions 1, 2 and 4 showed a negative correlation ($P \leq 0.05$) with stability. In the SDS unextractable proteins, all fractions except UF4 showed significant positive correlations ($P \leq 0.05$).

Mixing tolerance index (MTI), is a parameter which measures the degree of softening in dough when it is subject to mixing. Fractions EF1, EF2 and EF4 showed positive correlations with MTI, whereas EF3 did not show a significant correlation. Nevertheless, fractions UF1 – UF3 showed high and negative correlations ($P \leq 0.001$) with MTI, indicating that these fractions are responsible for low MTI values, which are beneficial. In this instance as well, a small fraction of EF3 showed a significant negative correlation ($P \leq 0.05$) with MTI, suggesting that this fraction has a positive effect on mixing tolerance together with the SDS unextractable fractions. In a similar manner, quality number showed positive correlations with the SDS unextractable fractions, especially fractions 1-3, which showed highly significant correlations ($P \leq 0.001$). Fractions EF1 and EF2 showed negative correlations, while EF3, which is the fraction of interest, showed a positive correlation with quality number. With respect to minor baking

qualities, EF3 showed a positive correlation ($P \leq 0.01$) with bake water absorption. Additionally, a negative correlation was observed between this fraction and crumb color.

SDS-PAGE showed that SE-HPLC fractions 1 and 2 are mainly composed of polymers of glutenin subunits as reported by Larroque et al. (1997). The glutenin polymers were reported to have a strong impact on dough mixing properties showing significant correlations with farinograph and breadmaking characteristics for hard spring wheat (Ohm et al. 2009). Significant correlations appeared between UF1 and release year and farinograph characteristics in this experiment indicating that polymeric proteins might be mainly associated with improvements in dough characteristics that were seen over the years in hard red spring wheat breeding.

It is interesting to note that proteins in EF3 and UF3 appear to be important thus far, since they have increased in amount over the years, and appear to have a positive contribution towards dough properties, as indicated by the significant correlation between fraction 3 and release year and farinograph characteristics. Since EF3 and UF3 showed significant correlations with farinograph characteristics, correlations were also estimated between absorbance area values of the total protein SE-HPLC fractions and quality characteristics (Figure 2.2).

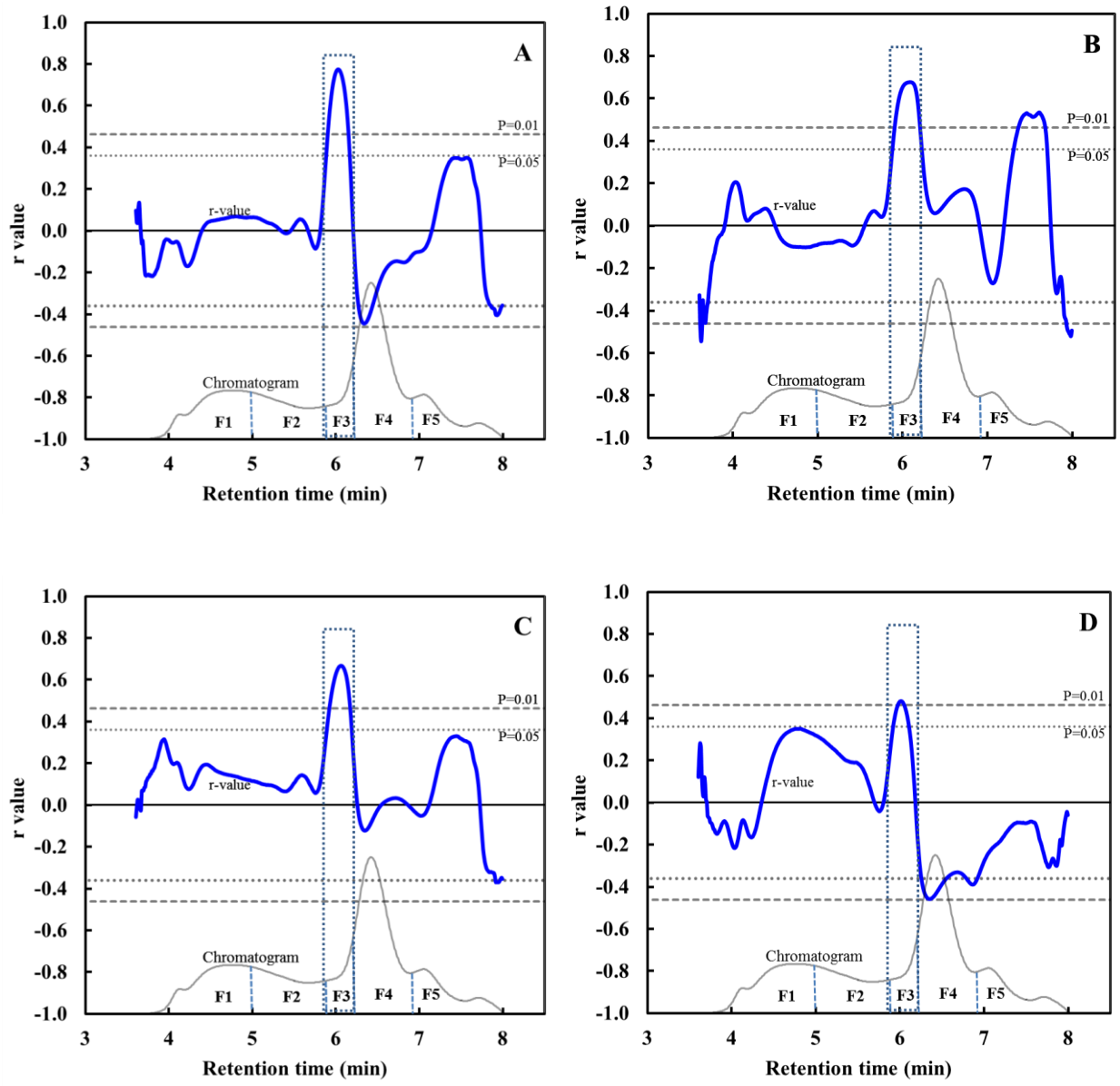

Figure 2.2. Correlations between total protein SE-HPLC fraction and release year (A), and farinograph absorption (B), peak time (C) and stability (D)

As seen previously, fraction 3 appeared to play a positive role in improving dough quality. Figure 2.2 shows that this fraction in total protein SE-HPLC has positive and significant correlations with release year ($P \leq 0.001$), farinograph absorption ($P \leq 0.001$), peak time ($P \leq 0.001$) and stability ($P \leq 0.01$). Larroque et al. (1997) reported that the range of molecular weights for ω-gliadins is 60-75 kDa. When total protein was separated using a regular SE-HPLC

column, the chromatogram shown below was obtained. As seen in Figure 2.3, the molecular weight of the most intensely stained protein in fraction 3 was determined to be 72.4 kDa, which is near that of the ω-gliadins. Thus, the results indicate that the quantitative increase of ω-gliadins might have a positive effect on the improvement of dough characteristics. The favorable effect of ω-gliadins on breadmaking quality was also reported by Lookhart and Albers (1988) and Ohm et al. (2009; 2010).

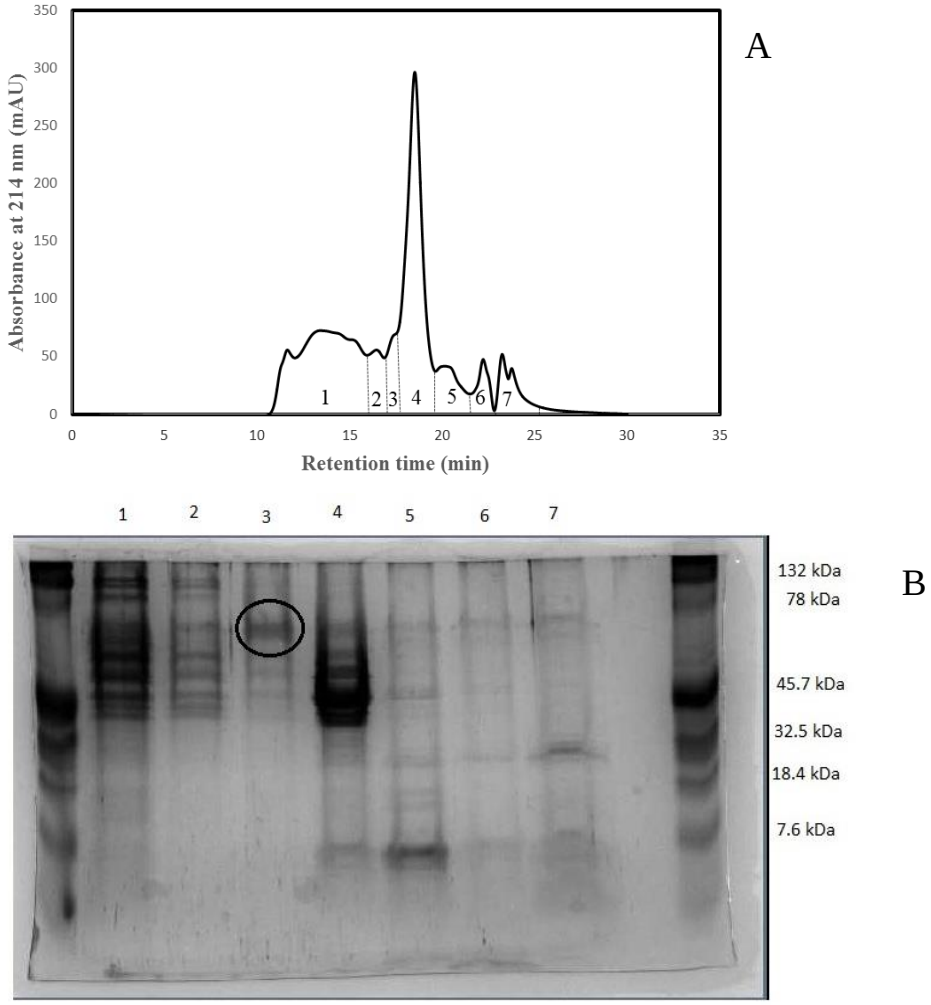

Figure 2.3. SDS-PAGE separation of total protein extracts following SE-HPLC. SE-HPLC separation of proteins (A) and SDS-PAGE of fractions (B)

2.4.2. Reverse phase-HPLC of omega-gliadins and quality

As shown above the ω-gliadin protein content has increased in relation to release year, and these proteins have a positive correlation with improved dough properties. To further confirm the role of the ω-gliadins in mixing properties, RP-HPLC was performed for extracted gliadins. Omega-gliadin peaks which showed correlations with release year were identified and further analyzed for correlations with dough properties. The RP-HPLC provided a distinct chromatogram (Figure 2.4). The regions of the chromatogram corresponding to the different gliadin types were identified according to previous studies (Gessendorfer et al. 2009; Piston et al. 2011; Wang et al. 2012).

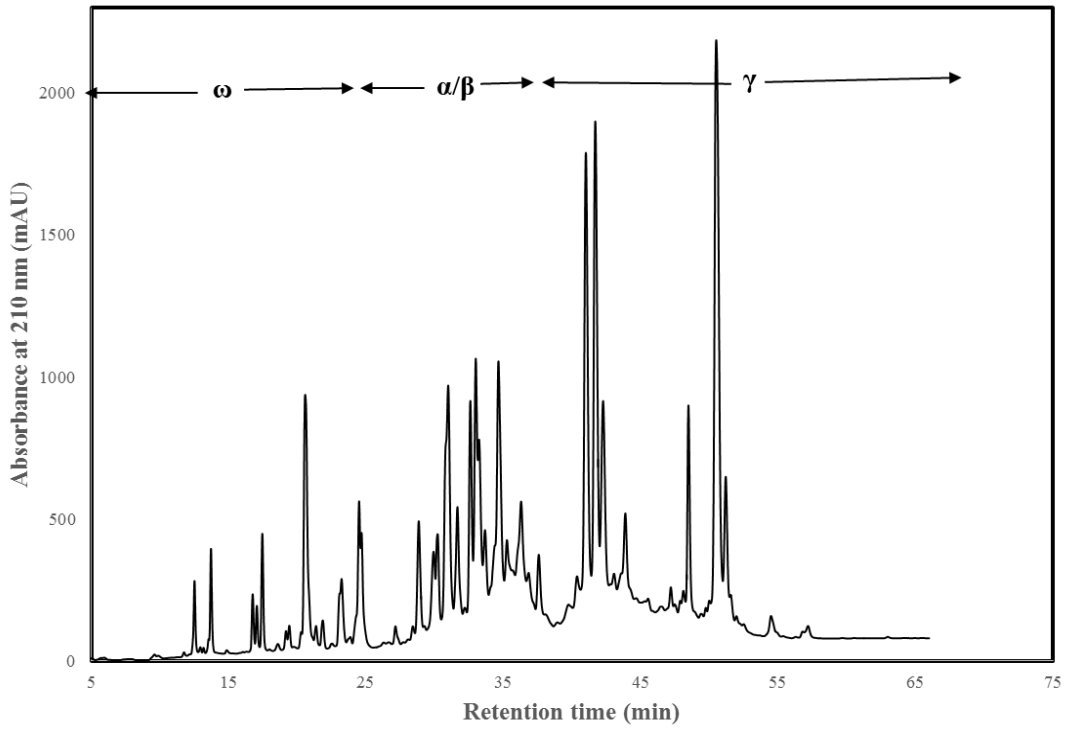

Figure 2.4. Reverse phase-HPLC chromatogram of gliadin proteins

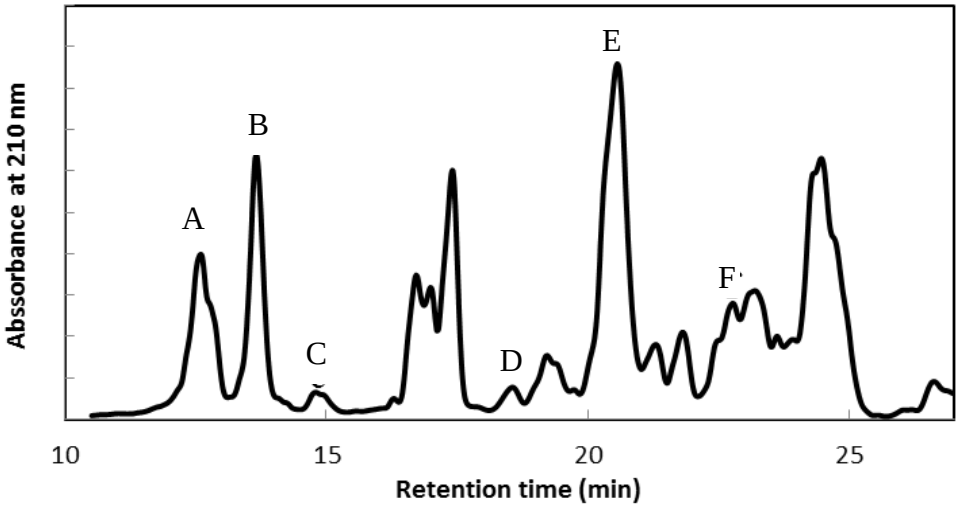

Figure 2.5. Peaks in the omega-gliadin region that were used in the analysis

As shown in Figure 2.5, six peaks in the ω-gliadin region showed significant correlations with release year and quality characteristics. Absorbance area values for the six peaks are summarized in Table 2.5.

Table 2.5. Mean, Standard Deviation (StD), Minimum (Min) and Maximum (Max) values for ω-gliadin peak areas (constant flour)

Peak[a]	Time interval (min)	Peak Area (mAu*min)			
		Mean	StD	Min	Max
A	12.1-13.0	81.8	30.1	21.8	152.9
B	13.2-14.1	88.8	45.8	18.3	191.8
C	14.6-15.2	11.9	7.5	6.4	30.1
D	18.2-18.7	11.4	6.9	6.0	35.5
E	20.1-21.0	188.2	101.5	24.0	311.6
F	21.7-22.2	39.0	32.9	2.7	123.3

[a]Refer to Figure 2.5 for HPLC peaks.

Peak E has the highest average area, while peaks C and D gave similar values. The average area of peaks A and B are similar. The average area for peak F was lower, however, it was not as low as peaks C and D. The highest peak areas for peaks A, B, C and F were found in the cultivars released after 1990. Additionally, the average peak area for these peaks in cultivars

released after 1990 was higher than the general average. This is an indication that there is a positive correlation between peaks A, B, C and F and year of release. Thus, these peaks could be responsible for the favorable dough properties associated with ω-gliadins seen in cultivars released in recent years. With regard to peaks D and E, the average peak area for these peaks in cultivars released after 1990 was lower than the cumulative average. Thus, it appears that the area of these peaks decreased in relation to year. Table 2.6 provides information about correlations between the peaks in question and various characteristics.

Table 2.6. Correlation coefficients between ω-gliadin peak areas and farinograph characteristics (constant flour)

		Farinograph characteristics[a]				
Peak[b]	Year	Absorption	Peak time	Stability	MTI	Quality number
a	0.486**	0.461*	0.299^{NS}	0.272^{NS}	-0.264^{NS}	0.314^{NS}
b	0.619***	0.343^{NS}	0.514**	0.530**	-0.411*	0.547**
c	0.478**	0.013^{NS}	0.437*	0.551**	-0.370*	0.537**
d	-0.692***	-0.507**	-0.345^{NS}	-0.315^{NS}	0.418*	-0.401*
e	-0.605***	-0.321^{NS}	-0.353^{NS}	-0.377*	0.287^{NS}	-0.435*
f	0.502**	0.405*	0.370*	0.218^{NS}	-0.239^{NS}	0.317^{NS}

[a]MTI= mixing tolerance index.
[b]Refer to Figure 2.5 for HPLC peaks.
NS = non-significant, *= $P \leq 0.05$, ** = $P \leq 0.01$ and *** = $P \leq 0.001$.

As expected, a significant and positive correlation ($P \leq 0.01$) was seen between peaks A, B, C and F and year of release. In contrast, peaks D and E showed a negative correlation ($P \leq 0.001$). Similarly, the same trend was seen between peak A and F in relation to farinograph absorption. Peaks D and E showed a negative correlation. Peaks B, C and F showed a positive correlation ($P \leq 0.05$) in relation to farinograph peak time and stability, which showed that these peaks which increased with year could be contributors of favorable dough properties.

Additionally, a negative correlation ($P \leq 0.05$) was observed between MTI and peaks B and C. However, a positive correlation was observed between peak D and MTI. In terms of quality number, peaks B and C, which appear to be important peaks for dough properties, showed a significant positive correlation ($P \leq 0.05$), while peaks D and E showed a negative correlation. The different correlations of ω-gliadin bands with breadmaking quality parameters were also reviewed by Tatham and Shewry (1995). With reference to the RP-HPLC analysis with constant protein content (Tables 2.7 and 2.8), the same trend was seen with regard to area of peaks.

Table 2.7. Mean, Standard Deviation (StD), Minimum (Min) and Maximum (Max) values for ω-gliadin peak areas analyzed on constant protein basis

	Peak Area (mAu*min)			
Peak[a]	Mean	StD	Min	Max
A	137.8	61.3	39.8	297.3
B	137.3	81.2	25.7	341.6
C	16.3	12.8	7.2	50.6
D	13.1	7.1	8.3	34.3
E	225.5	130.9	29.7	470.7
F	47.8	39.9	6.2	130.5

[a]Refer to Figure 2.5 for HPLC peaks.

Table 2.8. Correlation coefficients between farinograph and characteristics ω-gliadin peak areas determined on constant protein basis

		Farinograph Characteristics[a]				
Peak[b]	Year	Absorption	Peak Time	Stability	MTI	Quality Number
A	0.472^{**}	0.327^{NS}	0.364^{*}	0.456^{*}	-0.329^{NS}	0.462^{*}
B	0.633^{***}	0.271^{NS}	0.577^{***}	0.650^{***}	-0.463^{**}	0.648^{***}
C	0.491^{**}	-0.027^{NS}	0.461^{*}	0.597^{***}	-0.381^{*}	0.575^{***}
D	-0.649^{***}	-0.393^{*}	-0.334^{NS}	-0.335^{NS}	0.446^{*}	-0.387^{*}
E	-0.466^{**}	-0.155^{NS}	-0.201^{NS}	-0.344^{NS}	0.251^{NS}	-0.349^{NS}
F	0.672^{***}	0.380^{*}	0.521^{**}	0.359^{NS}	-0.350^{NS}	0.466^{**}

[a]MTI= mixing tolerance index.
[b]Refer to Figure 2.5 for HPLC peaks.
NS = non-significant, *= $P \leq 0.05$, ** = $P \leq 0.01$ and *** = $P \leq 0.001$.

Using a constant amount of protein in the analysis appears to have enhanced some of the correlation values. For instance, the significant positive correlation ($P \leq 0.001$) between peak F and release year seems to have improved. The same is true for peak F and farinograph peak time. Similarly, correlations between peaks A and B and farinograph characteristics were enhanced. Also, in this analysis there is a positive correlation ($P \leq 0.05$) between peak A and farinograph peak time, stability and quality number. This significant correlation was not observed in the constant flour method. However, for some parameters this analysis gave non-significant results, for example, the correlation between peak A and farinograph absorption. Also, the significant negative correlation between peak E and year has become weaker. As such, the data suggests that the constant flour method and the constant protein method are suitable for correlation studies, however, the constant protein method appears to enhance some positive correlations.

Overall, this experiment demonstrates that individual ω-gliadin peaks have different (positive or negative) correlations with flour mixing characteristics, although total quantity of ω-

gliadin might contribute to the development of favorable dough properties, as indicated by the significant correlation that appeared between mixing characteristics and SE-HPLC absorbance around fraction 3 (Figure 2.2). The ω-gliadins have been known to negatively influence breadmaking quality when investigated by reconstitution studies (Tatham and Shewry 1995). The rationale as to why some studies show positive correlations is cited as the tight genetic linkage of gliadins and LMW glutenin subunits (Tatham and Shewry 1995; Gianibelli et al. 2001). Therefore, the positive correlations observed between ω-gliadin quantity and farinograph characteristics in the current research might be due to the accumulation of ω-gliadins that are closely linked to LMW glutenin subunits which appear to have favorable effect on mixing quality.

2.5. Conclusions

The results indicate that the total protein content of wheat cultivars released from 1910 to 2013 did not change significantly over the years. However, positive correlations were observed between certain quality parameters, such as farinograph peak time and stability and release year of cultivar. It is widely accepted that gluten forming proteins play a vital role in breadmaking properties of wheat. In this context, it is possible that certain fractions of proteins found in wheat have increased over the years, leading to favorable qualities that are found in the cultivars released in recent years. Polymeric proteins analyzed by SE-HPLC showed significant correlations with cultivar release year and farinograph characteristics. As such, a positive correlation was seen between cultivar release year and the ω-gliadin fraction analyzed by SE-HPLC. Furthermore, this fraction showed positive correlations with farinograph peak time and stability, indicating that the quantitative increase of ω-gliadins may have contributed towards the favorable dough properties seen in hard red spring wheat cultivars released in later years. The

RP-HPLC analysis was performed to further investigate the role of ω-gliadins. Certain peaks corresponding to the ω-gliadin fractions showed positive correlations with farinograph parameters, while other peaks that had negative correlations were also identified. Given that there are conflicting ideas regarding the role of glutenin and gliadin in terms of dough properties, these results give a clear indication that polymeric proteins and ω-gliadin proteins may be highly associated with the improvement of favorable dough properties that are seen in hard red spring wheat released in recent years.

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CHAPTER 3. CLUSTER ANALYSIS BASED ON PARENTAGE AND PROTEIN HPLC IN HISTORICAL AND MODERN HARD RED SPRING WHEAT CULTIVARS

3.1. Abstract

There have been countless breeding efforts in North Dakota to produce wheat cultivars that are well adapted, in terms of weather conditions and disease resistance. In this study, 30 hard red spring (HRS) wheat cultivars released between 1910 and 2013 were analyzed with regard to how they cluster in terms of parentage relationships and protein data, analyzed by reverse phase-HPLC (RP-HPLC) of gliadin proteins and size exclusion-HPLC (SE-HPLC) of unreduced proteins, and how these clusters differ with respect to quality characteristics and the semi-dwarf characteristic. In terms of parentage, the clusters formed were grouped according to the release year of cultivar and semi-dwarf characteristics. Also, some farinograph characteristics showed significant differences between clusters, indicating improvement of mixing characteristics as a result of breeding efforts. In the dendrograms based on peak binary data of gliadin RP-HPLC, the clusters were not grouped according to release year and mixing characteristics. However, the cluster groups based on absorbance area data of RP-HPLC of gliadins and SE-HPLC of unreduced proteins showed significant differences for release year, semi-dwarf characteristics and some farinograph parameters. Overall, the cluster analysis indicated that dough mixing and breadmaking quality traits have been enhanced by the introduction of reduced height genes and changes in protein content in HRS wheat breeding for the last 100 years.

3.2. Introduction

North Dakota is the nation's highest producer of hard red spring (HRS) wheat. Thus, there have been many wheat cultivars released during the past century. In this study, thirty wheat cultivars released during the past 110 years were investigated. Wheat cultivars have been

modified for traits such as high yield and drought resistance. For example, cultivars Prosper and Velva, released in 2011 and 2012 respectively, have high yield and resistance to diseases such as stem rust and infections caused by Fusarium head blight (FHB) (Mergoum et al. 2013; 2014).

Several studies have been performed concerning historical and modern hard red spring wheat grown in North Dakota. In a study conducted by Underdahl et al. (2008a), 33 HRS wheat cultivars released after 1968 were analyzed for quality traits. The results of the study showed that there was no decline in end-use quality and that a significant improvement was observed for factors such as crumb grain appearance. Additionally, it was found that Mixogram characteristics were maintained during this time. As such, this study showed that improvement in yield has not had a negative effect on wheat quality. Another study conducted on the same wheat cultivars also showed that a decline of quality is not apparent over the 40 years and, as expected, there was a significant increase in yield, grain volume yield, and thousand kernel weight. Moreover, the results also indicated that there was an improvement in disease resistance during this time (Underdahl et al. 2008a). Cultivars Steele-ND, Alsen and Glenn are reported to have the highest scab resistance with respect to cultivars released until 2008 (Mergoum et al. 2007).

As mentioned earlier, many breeding programs have produced many wheat cultivars suitable for North Dakota. However, one study focusing on wheat grown in North Dakota showed that genetic diversity in hexaploid HRS is significantly lower in comparison with other hexaploid wheat, and that the reason for this is the fact that the ‘foundation germplasm’ is somewhat limited for this type of wheat (Chen et al. 1994).

Towards the late 1960s, semi-dwarf wheat varieties were released which had the phenotypic advantage of lower height. As such, most modern cultivars belong to this category. The reduced height (*Rht*) alleles have helped improve lodging resistance in wheat over the years

(Pearce et al. 2011). In a study conducted by Sherman et al. (2014), the results showed that an *Rht* gene, *Rht-D1b* allele, had positive effects on grain yield and tiller number while having negative effects on grain protein, seed weight and test weight. Studies have also been performed to determine the effect of these genes on various factors. Wojciechowski et al. (2009) suggested that there is a direct link between the dwarfing alleles in wheat cultivars and root growth during the seedling stage. In this study *Rht-B1b, Rht-D1b, Rht-B1c, Rht-8c, Rht-D1c* and *Rht-12* alleles were studied. Another study found that the gibberellin-responsive dwarfing genes have the potential to increase grain number and yield, without any impact on the biomass or coleoptile length in bread wheat (Rebetzke et al. 2012). On the same note, Nenova et al. (2014) showed that gibberellin-insensitive dwarfing genes have an effect on modifying the reaction of wheat under water stress conditions at the early seedling stage. It was suggested that the advantageous behavior of dwarf lines under water stress could be attributed to morpho-anatomical and metabolic characteristics of such lines. Additionally, in research conducted to determine the effect of *Rht-B1b* and *Rht-D1b* alleles on FHB susceptibility, it was determined that the choice of the dwarfing gene has an effect on the resistance to FHB, as under high disease pressure the presence of both of these alleles decreased resistance shown by the wheat cultivars that were examined (Srinivasachary et al. 2009).

In this study, wheat proteins were separated using SE-HPLC and RP-HPLC. The major component of wheat proteins is gluten forming proteins (Bonomi et al. 2013; Wieser 2007). These proteins are mainly found in the endosperm of the wheat kernel. Gluten forming proteins have two major constituents, glutenin and gliadin. Glutenin proteins are divided into high molecular weight and low molecular weight subunits. These proteins contain disulfide bonds between the protein chains, thus are polymeric proteins. Gliadins can be divided into three

groups, α-/β-, γ- and ω-. The α-/β- gliadins are grouped together as they have similar electrophoretic mobility on polyacrylamide gel electrophoresis. Many studies have been conducted to evaluate the effect of these proteins on baking quality, and the results suggest that high molecular weight glutenin protein polymers play an important role in dough development (Ohm et al. 2009; 2010). However, the role of the individual gliadin proteins has not been established as yet.

In many studies, cluster analysis has been used as a method to assess genetic relationships among germplasm of interest. In one study this type of analysis was used to assess the genetic diversity of winter wheat cultivars, and the results indicated that the clusters formed differentiates the differences for the yield trait. The authors suggested that cultivars grouped in the same clusters with high values for yield parameters could be used in breeding programs to produce elite wheat cultivars (Desheva and Kyosev 2015). This type of analysis could be performed to determine factors which influence grouping of cultivars with reference to various factors (heat stress tolerance for example) and to determine which cultivars have favorable features. Nagar et al. (2015) performed such a study and identified heat tolerant wheat cultivars. As such, it appears that cluster analysis using Ward's method is suitable to identify genetic variation among cultivars as demonstrated in many studies (Ajmal et al. 2013; Khodadadi et al. 2011; Shen et al. 2002; Talebi and Fayyaz 2012). Thus, Ward's method was used for the cluster analysis based on the parentage information, together with Principle component analysis, to analyze SE-HPLC and RP-HPLC data.

In this context, the goal of this study was to use cluster analysis as a method to group wheat cultivars released over a period of 110 years, and to determine the differences between

these clusters in terms of quality characteristics. As mentioned earlier, cluster analysis would be based on the genetic makeup of the cultivars and protein analysis data.

3.3. Materials and Methods

Thirty wheat cultivars released in North Dakota from 1910 to 2013 were used in this study. The cultivars were grown in Casselton, ND in 2013 in 150 ft x 6 ft strip plots. Planting was done during the first week of June and harvested during the first week of September.

Table 3.1. List of cultivars used in the study

Cultivar	Release year
Marquis	1910
Ceres	1926
Pilot	1939
Rival	1939
Vesta	1942
Mida	1944
Conley	1955
Justin	1963
Fortuna	1966
Waldron	1969
Olaf	1972
Butte	1977
Len	1979
Stoa	1984
Butte86	1986
Grandin	1989
Pioneer-2375	1990
Gunner	1995
Russ	1995
Reeder	1999
Alsen	2000
Granite	2002
Freyr	2004
Steele-ND	2004
Glenn	2005
Faller	2007
RB07	2007
Barlow	2009
Velva	2012
Elgin	2013

3.3.1. Analysis of quality characteristics of wheat cultivars

Wheat grains were cleaned and milled by the HRS Wheat Quality group, Department of Plant Sciences, North Dakota State University. A Buhler ML-202 laboratory mill was used to mill the samples. Nitrogen combustion analysis was used to determine the flour nitrogen content using a Dumas nitrogen analyzer according to the American Association of Cereal Chemists International (AACC-I) approved method 46-30. Protein content of the flour was calculated taking the moisture content (14%) into consideration. The standard AACC-I method 08-01.01 was used to determine flour ash content. Wet gluten content and gluten index were measured using the standard AACC-I method 38-12.02.

The mixing characteristics of the flour were determined using farinograph (Farinograph E- C.W. Brabender) analysis according to the Standard AACC-I method 54-21. Peak time, stability, mixing tolerance, quality number and absorption measurements were taken using the software provided by Brabender. Pup loaves (100 g) were made according to the standard AACC-I method 10-09.01, with some modifications to evaluate the breadmaking quality of the flour. A fermentation time of 2 hours was used, fungal amylase was used in place of malt powder, instant dry yeast was used instead of compressed yeast, and ammonium phosphate (1 mL of 10% ammonium phosphate for 100 g of flour) was added to improve yeast functionality. The baked bread was evaluated for loaf volume using rapeseed displacement (AACC-I method 10-05.01) and the quality parameters were scored according to AACC-I method 10-12.01.

3.3.2. Pedigree data analysis

In order to obtain the pedigree information of the cultivars in question several resources were used. The primary source of information was the Genetic Resources Information System for Wheat and *Triticale* (www.wheatpedigree.net). Once all the pedigree details were collected, a

binary data set was created depending on the presence or absence of a certain parent wheat variety in the wheat cultivars under consideration. Using this data, a dendrogram was created according to Ward's method using SAS software (Version 9.3, SAS Institute, Cary, NC).

3.3.3. Gliadin reverse phase-HPLC and data analysis

Gliadin proteins were extracted and separated using RP-HPLC according to the method of Lookhard and Bean (1995), with modifications. A sample of 250 mg of flour was used as the starting material, to which 750 µL of 70% ethanol was added and kept shaking at 1400 rpm for 1 h at 30^0C using a heating block (Eppendorf Thermomixer R). Afterwards, the mixture was subjected to centrifugation at 4550 g for 10 min (Eppendorf Centrifuge 5415 C), and membrane filtered through a 0.45 µm nylon filter (VWR International). Following this step, each sample was place in 80°C for 3 min in a heating block. An Agilent Zorbax 300SB-C18 (4.6 x 250 mm, 5 µm) column was used for analysis of the extracted gliadin proteins. Buffer A was comprised of 95% water, 5% acetonitrile and 0.1% trifluoroacetic acid (TFA,v/v), and the buffer B was 100% acetonitrile with 0.08% (v/v) TFA. The two buffers were balanced at 210 nm with TFA, and subsequently filtered through a 0.45 µm nylon membrane prior to running through the instrument. The gliadin proteins were eluted in a linear multistep gradient at 65^0C at a flow rate of 1 mL/min, with an injection volume of 20 µL. A variable wavelength detector (Agilent Technologies, Waldbroann, Germany) was used at 210 nm to collect the signal data.

The elution gradient started at 75% buffer A and 25% buffer B, which was maintained for the first 5 minutes. During the next 5 min, the volumes were gradually increased so that at 10 min it was 70% buffer A and 30% buffer B. After gradually increasing again, at 60 min it was 50% of each buffer. This state was maintained until 63 min. In the next minute the gradient was changed so that at 64 min it was 100% buffer B, which was maintained for 1 min. In the next

minute, the gradient was changed again so that at 66 min the percentages were brought down to the starting percentages. A post time of 15 min was added after each run. Prior to separation of the proteins through the column, a conditioning step was performed. That is, water and acetonitrile were flushed through the column respectively for 5 min. The column was washed with 10% methanol for storage.

Two separate dendrograms were produced using the RP-HPLC data. The first was based on the presence or the absence of a peak at a certain time point. This binary data set was created after considering peaks at a certain time point. Using this binary data a dendrogram was created using Ward's method in the SAS software (Version 9.3, SAS Institute, Cary, NC). The second was based on absorbance areas data from RP-HPLC. The HPLC absorbance data was processed using an in-house program coded using MATLAB (2015, The MathWorks, Natick, MA). UV absorbance values were interpolated to 0.005 min intervals by the 'spline' function in MATLAB and then absorbance area values were calculated for 0.025 min intervals using the interpolated absorbance data. The 1900 absorbance area data of RP-HPLC was converted to 15 principal component scores using the PLS_Toolbox software (Version 8.02, Eigenvector Research, Inc., Wenatchee, WA). The 15 principal components explained 89.4% of the variance found for the total absorbance area data. Cluster analysis was performed using principal component scores as variables according to Ward's method in SAS software (Version 9.3, SAS Institute, Cary, NC).

3.3.4. Size exclusion-HPLC of unreduced proteins and data analysis

Proteins were extracted for SE-HPLC following the method of Gupta et al. (1993) with minor modifications (Ohm et al. 2009). A sodium dodecyl sulfate (SDS) based buffer was used to isolate SDS soluble and insoluble proteins from wheat. In order to obtain the SDS soluble fraction, 1 mL of extraction buffer (0.5% SDS and 0.05 M sodium phosphate, pH 6.9) was added

to 10 mg (adjusted to 14% moisture content) of flour, then stirred for 5 min using a vortex mixer (Fischer Scientific) at 2500 rpm. Afterwards, the mixture was centrifuged at 17,000 g (Eppendorf Centrifuge 5424), and the supernatant was filtered through a 0.45 μm PVDF membrane filter (Sun Sri, Rockwood, TN). Following the filtration process, the sample was heated at 80^0C for 2 min in a water bath and cooled to room temperature. The SDS insoluble fraction was obtained from the residue of the filtration step. The residue was re-suspended in 1 mL of the same extraction buffer and sonicated for 30s at 10 W power setting in a probe type sonicator (Sonic Dismembrator 100, Fisher Scientific), which was followed by centrifugation, filtration and heating the same as for the SDS soluble fraction. The separation of the proteins in the SDS soluble and insoluble fractions was performed using an Agilent 1100 series narrow bore column (300 × 4.5 mm, BIOSEP SEC S4000, Phenomenex, Torrance, CA) and a guard cartridge (BIOSEP SEC S4000, Phenomenex, Torrance, CA) in Agilent 1100 Chromatograph (Agilent Technologies, Waldbroann, Germany) (Ohm et al. 2009). The injection volume was 10 μL and the eluting solution was 50% acetonitrile in water with 0.1% (v/v) TFA. The flow rate for the SE-HPLC analysis was 0.5 mL/min. An Agilent 1200 photodiode array detector (Agilent Technologies, Waldbroann, Germany) was used to detect the solutes at 214 nm.

MATLAB (2015, The MathWorks, Natick, MA) functions and an in-house program were used to process SE-HPLC data (Ohm et al. 2009). UV absorbance values were interpolated to 0.002 min intervals by the 'spline' function in MATLAB. The SE-HPLC profiles were divided into five fractions, of which retention time intervals were 3.5–5.0 min for the first fraction (F1), 5.0–5.9 min for the second fraction (F2), 5.9–6.2 min for the third fraction (F3), 6.2–6.9 min for the fourth fraction (F4), and 6.9-8.0 min for the fifth fraction (F5) (Morel et al. 2000; Ohm et al. 2009; Park et al. 2006). The main proteins in the fractions were identified as high molecular

weight polymeric proteins for F1, low molecular weight polymeric proteins for F2, ω-gliadins for F3, α-/β-, γ- gliadins for F4, and albumin and globulins for F5 (Morel et al. 2000; Ohm et al. 2009). Absorbance area was calculated for individual fractions and area percentage values were also calculated based on total absorbance area (Ohm et al. 2009; Park et al. 2006). The cluster analysis procedure with Ward's method in the SAS software (Version 9.3, SAS Institute, Cary, NC) was performed using absorbance area and area percentage data of individual HPLC fractions.

3.3.5. Identification of markers for glutenin subunits and *Rht1/ Rht2*

DNA markers developed for HMW glutenin subunits encoded by *Glu-1D* locus, and plant height encoded by *Rht 1* (*Rht-B1*) gene on chromosome 4B, and *Rht 2* (*Rht-D1*) gene on chromosome 4D responsible for the dwarf traits introduced into wheat varieties released in the late 1960s onwards, were used to analyze the thirty spring wheat cultivars. Genotyping of the STS markers, UMN25 and 26, designed specifically from 1Dx and 1Dy coding sequences (Liu et al. 2008), was carried out using PCR, followed by separating fluorescent dye labeled PCR fragments on a ABI 3130xl Genetic Analyzer (Life Technologies) following the protocols previously described (Chao et al. 2007). Genotyping of the SNP markers developed from the semi-dwarfing genes *Rht-B1b (Rht1)* and *Rht-D1b (Rht2)* (Ellis et al. 2002) followed KASP assay method developed by LGC Genomics.

3.4. Results and Discussion

When looking at the pedigree information for thirty cultivars regarding their sources of parents, it was interesting to note that Hard Red Calcutta and Red Fife are common to all cultivars in question. This supports the findings of Mercado et al. (1996), who found that Hard Red Calcutta and Red Fife contributed 13% and 18%, respectively, for the formation of the gene

pool of North American hard spring wheat cultivars. Other contributors included various spring wheat, winter wheat, *Triticum turgidum* var. *durum* L. and *T. turgidum* var. *emmer* L. and Turkey Red. Wheat varieties Kota and Yaroslav Emmer appeared to be the second most common parents. Other parental wheat cultivars included, Kanred, Redman, Exchange, and many more. The dendrogram displayed in Figure 3.1 was developed based on the parentage information of the cultivars in question. The pedigree information of cultivar Gunner (released by AgriPro) could not be obtained, thus was not included in the analysis.

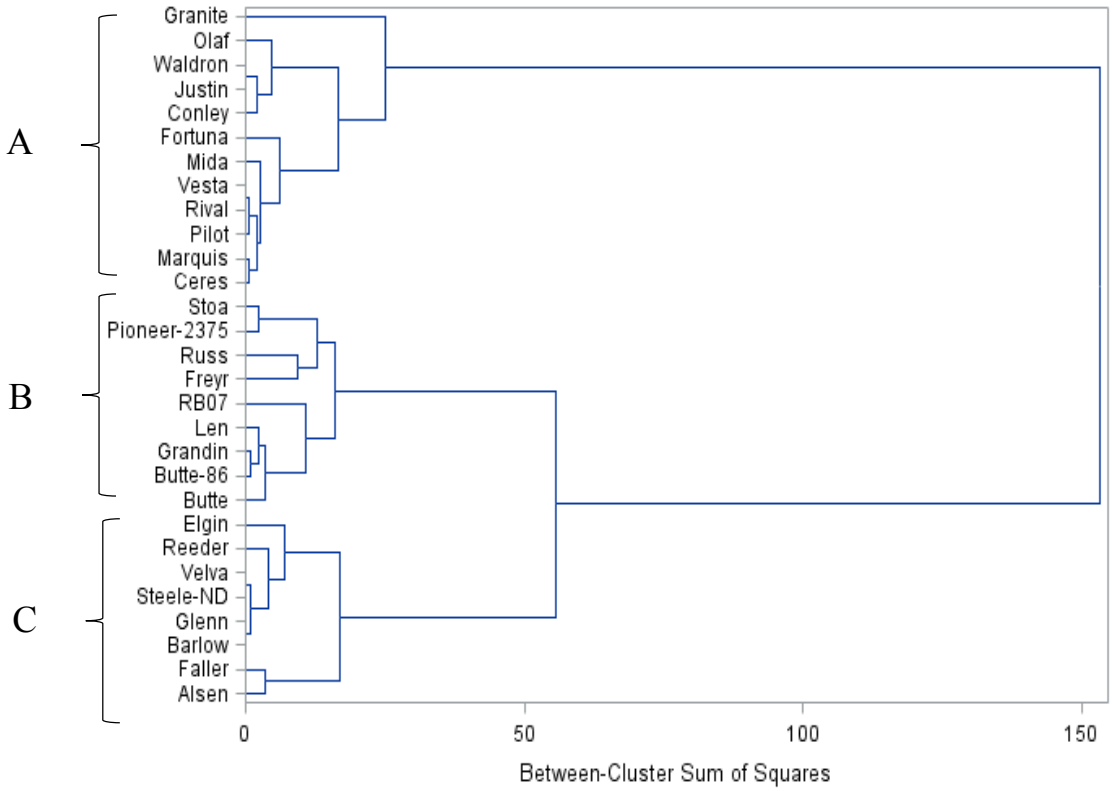

Figure 3.1. Dendrogram based on pedigree information of wheat cultivars

At similarity cutoff of 50, three clusters were observed for the thirty wheat cultivars. Cluster A contains 12 cultivars that were released in the years ranging from 1910 to 1972, except for cultivar Granite which was released in 2002, and is considered as an outlier. It is interesting to note that Granite was in a different branch from the other 11 cultivars in this cluster, further

highlighting its role as an outlier. Cluster B contains nine cultivars, mostly released after 1972 but before 2000, with the exception of Freyr and RB07, released in 2004 and 2007, respectively. Cluster C contains eight cultivars that were all released after the year 1999, thus these can be considered as 'modern wheat'. Given that the dendrogram contains three clusters that grouped cultivars based on the release years, it is possible to conclude that the historical and modern spring wheat cultivars could be separated with respect to their release year when parentage information is taken into consideration. The Analysis of Variance (ANOVA) done on the clusters also showed that there is a significant genetic difference ($P \leq 0.05$) between the clusters with respect to release year. To further compare the similarities and dissimilarities for the bread making quality traits between the clusters, ANOVA tests were performed based on the three clusters determined earlier (Table 3.2).

Table 3.2. Quality parameters with respect to wheat cultivars in the same ward cluster in dendrogram based on parentage[a]

Quality parameters	Ward A (n=12)	Ward B (n=9)	Ward C (n=8)
Flour protein content (%)			
Mean	13.2a	12.7a	12.7a
Min	12.7	11.4	11.9
Max	13.8	13.3	13.3
SD	0.4	0.6	0.6
Flour ash content (%)			
Mean	0.64a	0.52b	0.49b
Min	0.50	0.49	0.43
Max	0.74	0.61	0.54
SD	0.07	0.04	0.03
Gluten index			
Mean	92.6a	93.5a	97.8a
Min	69.5	78.5	95.5
Max	98.1	99.2	99.3
SD	7.9	6.5	1.2
Wet gluten			
Mean	34.8a	33.7a	33.3a
Min	32.0	29.3	30.2
Max	38.4	37.2	35.8
SD	2.1	2.7	2.2

Table 3.2. Quality parameters with respect to wheat cultivars in the same ward cluster in dendrogram based on parentage[a] (continued)

Quality parameters	Ward A (n=12)	Ward B (n=9)	Ward C (n=8)
Farinograph absorption (%)			
Mean	59.3b	61.26a	61.8a
Min	56.5	58.2	58.9
Max	62.8	64.8	65.1
SD	1.87	2.14	2.13
Farinograph peak time (min)			
Mean	6.3b	7.1ab	7.7a
Min	4.1	5.9	6.5
Max	8.9	8.0	8.9
SD	1.3	0.8	1.0
Farinograph stability (min)			
Mean	9.0b	10.4b	14.3a
Min	4.4	7.2	10.4
Max	12.9	14.9	22.3
SD	2.3	2.4	3.9
Loaf volume (cm^3)			
Mean	987.3a	944.2a	967.5a
Min	902.5	882.5	890.0
Max	1032.5	1027.5	1027.5
SD	40.9	49.8	46.3
Bake water absorption (%)			
Mean	66.5a	67.9a	67.8a
Min	63.5	65.6	64.7
Max	70.4	72.0	70.5
SD	2.0	2.0	2.0
Grain and texture			
Mean	7.9a	7.6ab	7.3b
Min	7.0	7.0	6.7
Max	8.5	8.2	7.7
SD	0.4	0.4	0.4

[a] Means with the same letter in the same row are not significantly different at the 5% level. Min=minimum, Max=maximum, and SD=standard deviation

Results showed that the quality characteristics of each cluster can be analyzed in order to determine the trend on how quality has changed during the last century of spring wheat cultivar breeding. The first factor under consideration is flour protein content. When looking at the mean protein content, it appears that there was a slight decrease. However, ANOVA showed no significant ($P \geq 0.05$) difference with regard to protein content in the three Ward clusters

identified. As such, it is possible to conclude that protein content did not change significantly over the years. The cultivar with the highest protein content was cultivar Glenn in cluster C released in 2005. This cultivar is known for its superior baking quality, and is among the best wheat cultivars adapted to the growth conditions in North Dakota. The cultivar with the lowest protein content was Ceres in cluster A, which was released in 1926. Granite appeared to have high protein content and is considered as an anomaly in this analysis. Flour ash content measures the amount of minerals found in flour. High ash content shows high bran contamination, and is an indication of low flour quality (Hinton 1959). Thus, flour with low ash content is preferred. The analysis showed that there is a significant difference between the mean ash content of the three clusters. This means that the ash content decreased over the years judging from the average values for this parameter. The average ash content of cluster C is 0.15 units lower than that of cluster A, and 0.03 units lower than that of cluster B. Therefore, it is clear that there is a pronounced decrease in ash content for cultivars in cluster C that were released after 1990 as compared to those in cluster A that were released prior to 1972. The F-protected LSD further confirmed that the mean ash content of cluster A was significantly different from those of clusters B and C (The mean ash content of cluster A is higher than those of clusters B and C). A significant difference was not found among the clusters for the wet gluten content and gluten index, and is in agreement with the results for flour protein content.

Farinograph analysis is one of the most common methods of measuring dough quality, thus, farinograph related parameters for cultivars in three clusters were analyzed in order to determine their differences. Farinograph absorption mean value for cluster A was significantly different than that of clusters B and C. Generally, for bread production, high peak time is preferred, as this indicates stronger dough. The farinograph peak time was significantly different

between the clusters, indicating that dough development quality has improved over the years. Farinograph stability is another important parameter which indicates how stable or how tolerant the dough is during mixing. Same as for peak time, higher values of stability are preferred, and significant differences between the three clusters were also observed for this parameter. The average value for stability in each cluster has increased over the years. Thus, the improved dough quality should lead to a better baking performance. Loaf volume and bake water absorption did not show significant differences between clusters, which indicates that these two factors did not change significantly in relation to release year. However, grain and texture, which is considered as a minor baking quality parameter, showed significant differences. This result indicates that cluster A, containing the older wheat cultivars, and cluster C, containing the modern wheat are significantly different for grain and texture. Previously, Underdahl et al. (2008a) indicated that there was no decline in quality over the period of 1968-2006, however, our results suggests that quality has improved gradually since 1910. This could be due to the fact that Underdahl et al. (2008b) studied cultivars released for a period of 40 years, whereas the current study includes cultivars from the past 110 years.

The dwarfing genes were introduced to wheat breeding programs around the late 1960s. The DNA markers associated with the plant height genes located on chromosomes 4B and 4D were used to determine if these genes are present in the cultivars grouped in the three clusters (Figure 3.1). The marker data were also compared with the semi-dwarf characteristic (phenotype) of the cultivars. Results showed that it was possible to determine which *Rht* gene was responsible for the dwarf phenotype (Table 3.3).

Table 3.3. Genetic marker analysis of *Rht* genes[a]

Cultivar	Release year	*Rht-B1 (Rht1)*	*Rht-D1 (Rht2)*	Semi-dwarf phenotype
Marquis	1910	0	0	0
Ceres	1926	0	0	0
Pilot	1939	0	0	0
Rival	1939	0	0	0
Vesta	1942	0	0	0
Mida	1944	0	0	0
Conley	1955	0	0	0
Justin	1963	0	0	0
Fortuna	1966	0	0	0
Waldron	1969	0	0	1
Olaf	1972	1	0	1
Butte	1977	0	0	0
Len	1979	1	0	1
Stoa	1984	0	0	0
Butte-86	1986	0	0	0
Grandin	1989	0	0	1
Pioneer-2375	1990	0	0	1
Gunner	1995	0	0	1
Russ	1995	0	0	0
Reeder	1999	1	0	1
Alsen	2000	1	0	1
Granite	2002	0	0	1
Freyr	2004	0	1	1
Steele-ND	2004	1	0	1
Glenn	2005	0	0	1
Faller	2007	1	0	1
RB07	2007	1	0	1
Barlow	2009	1	0	1
Velva	2012	1	0	1
Elgin	2013	0	0	0

[a]1= positive, 0= negative

The results indicate that the dwarfing genes are not found in cultivars released prior to the late 1960s. The *Rht-B1* allele was detected in most cultivars with semi-dwarf characteristics. The *Rht-D1* was only detected in cultivar Freyr. Table 3.3 also shows that other genes that were not analyzed in this study could also cause the semi-dwarf phenotype.

Based on the phenotype data, there were significant differences ($P \leq 0.05$) between clusters A and C. Cluster A contains older wheat cultivars released between 1910 and 1972, whereas cluster C contained newer cultivars released after 1990, which also possibly contain dwarfing genes. The marker data for *Rht-B1* showed a significant difference between the same clusters. This difference is because older cultivars in cluster A do not contain dwarfing genes (as seen in Table 3.2), and modern cultivars in cluster C contain these genes.

The historical and modern hard red spring wheat cultivars were further grouped into clusters based on RP-HPLC data. In the protein related dendrogram, the presence or absence of a peak at a certain time point was considered to produce a binary set of data, from which a dendrogram was produced (Figure 3.2).

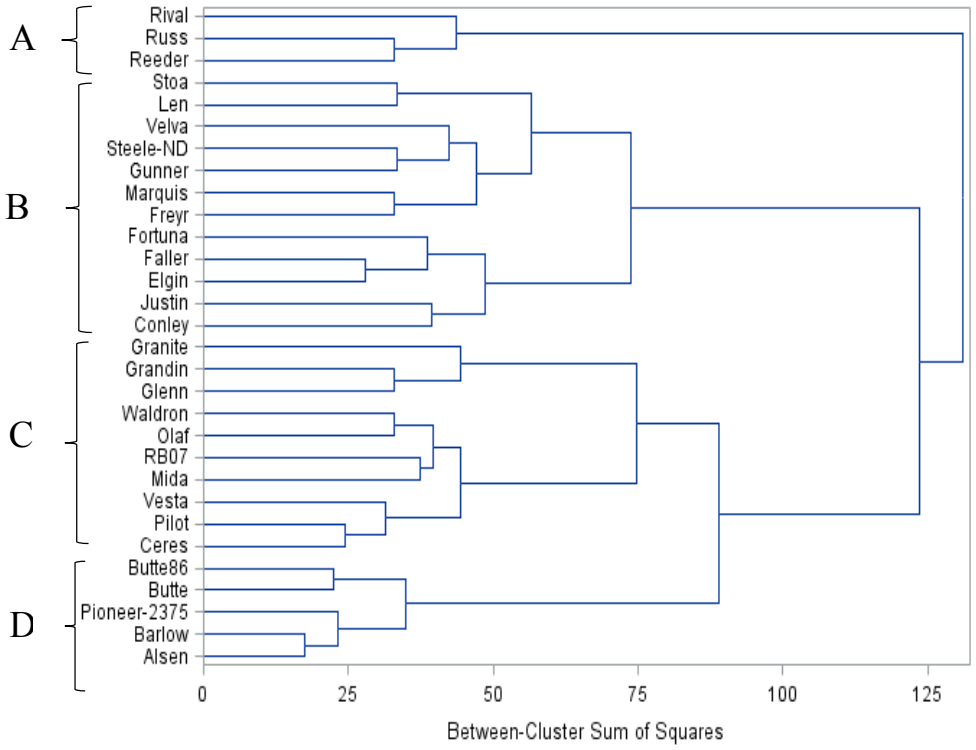

Figure 3.2. Dendrogram based on gliadin RP-HPLC binary data

The dendrogram in Figure 3.2 shows that the clustering of cultivars with regard to gliadin RP-HPLC binary data is more random compared with the dendrogram developed using the

pedigree information. At cutting point 75, four clusters were identified. However, as the cutting point is reduced further more clusters can be seen. For instance, at 50, seven clusters could be identified. Cluster A in this dendrogram contains one cultivar released in 1939 and two other cultivars released in the late 1990s. Cluster B, which is larger than cluster A in terms of the number of cultivars, contains one of the oldest cultivars in question (Marquis released in 1910), and few of the newer cultivars, for example, Velva and Elgin, released in 2012 and 2013 respectively. Cluster C is also diverse in terms of the release years of the wheat cultivars in this cluster. This too contains cultivars spanning a time frame from 1926 to 2007. As for cluster D, the same diversity can be observed, where clusters with release years of 1977 to 2009 are seen. Table 3.4 provides a more detailed analysis of these clusters where various quality parameters were taken into consideration. The purpose of this analysis is to determine if these clusters have differences in terms of quality characteristics.

Table 3.4. Quality parameters with respect to wheat samples in the same ward cluster in dendrogram based on gliadin reverse phase-HPLC binary data[a]

Quality parameters	Ward A (n=3)	Ward B (n=12)	Ward C (n=10)	Ward D (n=5)
Flour protein content (%)				
Mean	12.3a	12.9a	13.0a	12.8a
Min	11.9	11.4	12.7	12.4
Max	12.8	13.6	13.8	13.3
SD	0.4	0.7	0.3	0.4
Flour ash content (%)				
Mean	0.58a	0.56a	0.59a	0.51a
Min	0.48	0.43	0.49	0.49
Max	0.72	0.71	0.74	0.53
SD	0.13	0.08	0.08	0.02
Gluten index				
Mean	97.1a	96.3a	91.1a	94.8a
Min	96.1	91.8	65.5	89.3
Max	98.4	99.2	99.3	97.7
SD	1.2	2.6	9.8	3.5

Table 3.4. Quality parameters with respect to wheat samples in the same ward cluster in dendrogram based on gliadin reverse phase-HPLC binary data[a] (continued)

Quality parameters	Ward A (n=3)	Ward B (n=12)	Ward C (n=10)	Ward D (n=5)
Wet gluten				
Mean	32.1a	33.9a	34.7a	34.6a
Min	31.1	29.3	32.0	33.0
Max	33.0	37.4	38.4	35.7
SD	0.9	2.9	1.9	1.2
Farinograph absorption (%)				
Mean	61.0ab	60.4b	59.5b	63.0a
Min	59.8	57.3	56.5	61.7
Max	62.7	64.8	62.8	65.0
SD	1.5	2.0	2.2	1.4
Farinograph peak time (min)				
Mean	6.4a	7.1a	6.6a	7.2a
Min	4.8	6.2	4.1	5.9
Max	7.7	8.9	8.9	8.3
SD	1.5	0.8	1.7	0.8
Farinograph stability (min)				
Mean	11.1a	11.7a	10.1a	10a
Min	7.5	9.0	4.4	7.3
Max	14.3	17.0	22.3	11.4
SD	3.3	2.6	4.9	1.7
Loaf volume (cm^3)				
Mean	952.5ab	985.6a	981.2a	916.0b
Min	915.0	935.0	902.5	882.5
Max	1010.0	1032.5	1027.5	1010.0
SD	50.5	31.6	42.3	53.8
Bake water absorption				
Mean	66.6a	67.2a	67.3a	68.6a
Min	64.7	63.5	63.9	66.6
Max	69.0	72.0	70.5	70.2
SD	2.2	2.4	2.1	1.3
Grain and texture				
Mean	7.4a	7.8a	7.7a	7.6a
Min	7.0	6.7	7.0	7.2
Max	8.0	8.5	8.2	8.0
SD	0.5	0.5	0.5	0.3

[a] Means with the same letter in the same row are not significantly different at the 5% level. Min=minimum, Max=maximum, and SD=standard deviation

The clusters in the gliadin-based dendrogram (Figure 3.2) did not group cultivars according to release year. This means that cultivars released in different years can have similar gliadin compositions. This was further highlighted in the ANOVA test where a significant difference was not found between clusters for release year. The flour protein content did not show a significant difference between the clusters. Gluten index and wet gluten content also did not show significant differences between the clusters in the same way as protein content. As such, the presence of peaks in the gliadin RP-HPLC profile is independent of flour protein content, and thus has no connection to that parameter. As per flour ash content, the same trend was seen, where there was no significant difference between the clusters. In this analysis, farinograph parameters were also taken into consideration in order to examine dough quality differences between the clusters. The results indicated that significant differences were not found between the four clusters in terms of farinograph peak time and stability. However, cluster D showed significant differences ($P \leq 0.05$) with clusters B and C for the absorption parameter. Since two of the three farinograph characteristics did not show significant differences it appears that there is no significant difference with reference to dough quality. This means categorization of cultivars based on gliadin RP-HPLC profiles will not be useful to assess the dough quality. Loaf volume, which is one of the most important baking quality parameters, showed significant differences between clusters, that is, cluster D was significantly different from clusters B and C. This result is similar to that of farinograph absorption. However, other baking quality parameters were not significantly different between the clusters.

Some studies have suggested the breeding practices have changed wheat protein chemistry over the years, and that breeding has caused increased celiac disease toxicity in modern wheat (van den Broeck et al. 2010). As per the first section, analysis was also performed

based on *Rht1* and *Rht2* genes to determine if there is a significant difference between clusters in the dendrogram based on gliadin RP-HPLC binary data. Significant differences were not observed between clusters for both phenotypic and the *Rht1* gene. As such, the clusters based on gliadin profiles are not important with regard to the dwarfing genes. To analyze the gliadin RP-HPLC data further, a dendrogram was constructed based on the HPLC area data of the chromatogram.

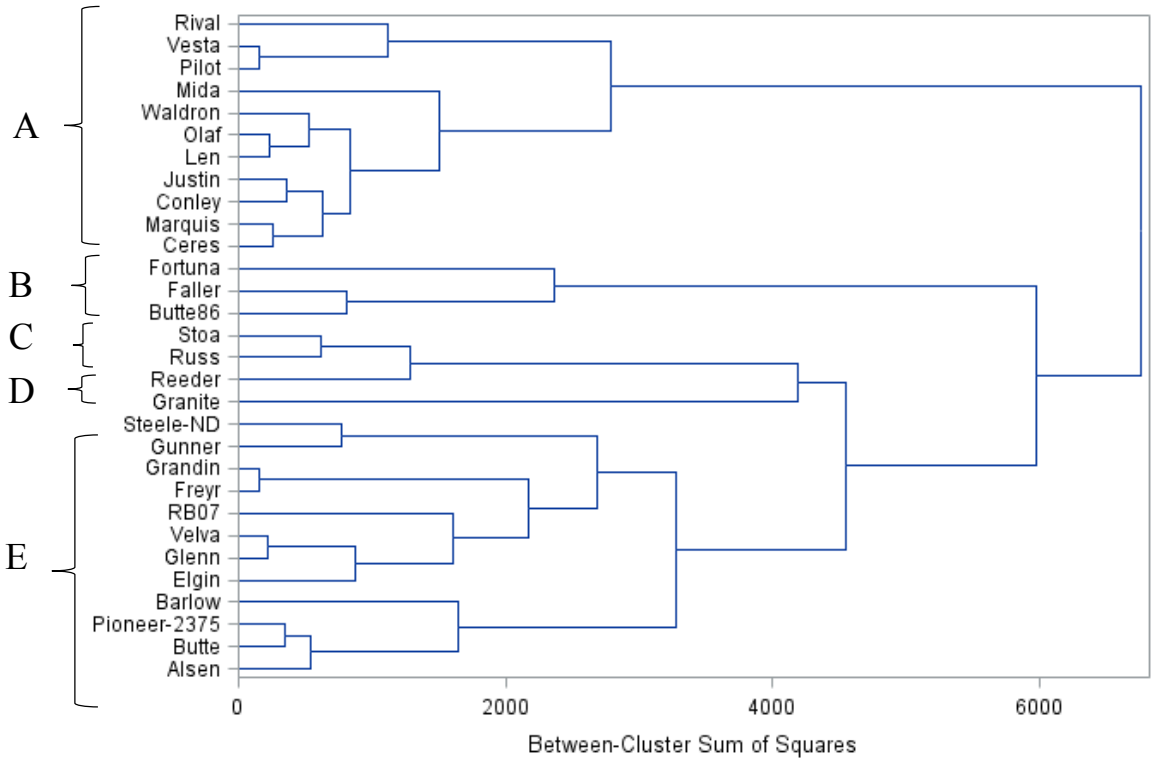

Figure 3.3. Dendrogram based on gliadin RP-HPLC chromatogram area

As shown in Figure 3.3, five clusters were identified when cultivars were grouped according to the area of gliadin RP-HPLC chromatograms. These clusters were found at a cut point of 4000. Cluster A contains cultivars released between 1910 and 1979, as such, this cluster contained somewhat historical wheat cultivars. Cluster B, on the other hand, is more variable as two of the cultivars in this cluster were released prior to 1990, and one in 2007. Cluster C also

has a cultivar released in 1984 and is grouped with two other cultivars released in 1995 and 1999 respectively. Cluster D only contains cultivar Granite. This cultivar appeared as an anomaly in the dendrogram based on pedigree information, and also stood out as a separate branch in the dendrogram based on gliadin RP-HPLC binary data. As such, given that the behavior of Granite is unique, this cultivar appears to be an outlier considering the thirty cultivars in question. Cluster E in the dendrogram (Figure 3.3) contains twelve cultivars. The release years of the cultivars ranged from 1977 to 2013. Thus, it contains cultivars that were released after the semi-dwarf genes were introduced. Table 3.5 provides information on the quality aspects of each of the clusters discussed above.

Table 3.5. Quality parameters with respect to wheat samples in the same ward cluster in dendrogram based on gliadin reverse phase-HPLC absorbance area data[a]

Quality parameter	Ward A (n=11)	Ward B (n=3)	Ward C (n=3)	Ward D (n=1)	Ward E (n=12)
Flour protein content (%)					
Mean	13.1ab	12.6bc	11.8c	13.8a	12.9b
Min	12.6	12.1	11.4	-	11.9
Max	13.6	13.4	12.2	-	13.5
SD	0.34	0.70	0.43	-	0.47
Flour ash content (%)					
Mean	0.66a	0.54b	0.51b	0.50b	0.51b
Min	0.59	0.49	0.48	-	0.43
Max	0.75	0.60	0.54	-	0.57
SD	0.05	0.05	0.03	-	0.03
Gluten index					
Mean	93.7a	94.7a	97.9a	87.3a	94.6a
Min	69.6	91.8	96.6	-	78.6
Max	99.2	96.6	98.8	-	99.3
SD	8.3	2.5	1.2	-	5.8
Wet gluten					
Mean	33.9bc	34.9ab	31.2c	38.4a	34.6bc
Min	30.4	33.0	29..3	-	30.3
Max	36.0	37.4	33.0	-	37.2
SD	1.9	2.3	1.8	-	2.1

Table 3.5. Quality parameters with respect to wheat samples in the same ward cluster in dendrogram based on gliadin reverse phase-HPLC absorbance area data[a] (continued)

Quality parameter	Ward A (n=11)	Ward B (n=3)	Ward C (n=3)	Ward D (n=1)	Ward E (n=12)
Farinograph absorption (%)					
Mean	58.9b	60.9ab	60.2ab	62.8a	62.1a
Min	56.5	60.1	58.2	-	58.5
Max	61.4	62.3	62.7	-	65.0
SD	1.5	1.2	2.3	-	1.9
Farinograph peak time (min)					
Mean	6.0b	6.8b	6.9b	8.8a	7.7ab
Min	4.1	6.5	6.2	-	5.9
Max	8.0	7.2	7.7	-	8.9
SD	1.1	0.3	0.7	-	0.9
Farinograph stability (min)					
Mean	9.0a	9.6a	13.6a	10.2a	12.2a
Min	4.4	9.0	11.6	-	7.2
Max	12.9	10.4	14.9	-	22.3
SD	2.4	0.7	1.7	-	4.1
Loaf volume (cc)					
Mean	991.1a	955.0a	927.5a	902.5a	968.7a
Min	912.5	882.5	915.0	-	885.0
Max	1032.5	1022.5	935.0	-	1027.5
SD	31.5	70.1	10.9	-	50.4
Bake water absorption (%)					
Mean	66.1a	67.5ab	66.5b	70.4a	68.6ab
Min	63.5	66.5	64.7	-	66.1
Max	69.6	69.2	69.0	-	72.0
SD	1.7	1.5	2.3	-	1.7
Grain and texture					
Mean	8.0a	7.7ab	7.2bc	7.0b	7.6ab
Min	7.5	7.5	7.0	-	6.7
Max	8.5	8.0	7.5	-	8.2
SD	0.3	0.3	0.3	-	0.5

[a] Means with the same letter in the same row are not significantly different at the 5% level. Min=minimum, Max=maximum, and SD=standard deviation

As explained earlier, it appears that the clusters could be differentiated according to the release years of the cultivars. That is, cluster A: older varieties, clusters B, C and D: intermediate to modern varieties, and cluster E: modern cultivars. To further evaluate these differences, results from statistical analysis found a significant difference with respect to release year between the clusters. Statistical analysis showed that cluster A (containing older cultivars) is significantly

different from the other clusters indicating that the amount of gliadin proteins changed over the years. Therefore, although gliadin peaks profile did not change with release year (as seen in the previous analysis), the amount of gliadin proteins changed. As per the quality characteristics, a significant difference was found with regard to flour protein content between clusters. Overall, when looking at the flour protein content, it has somewhat decreased from cluster A, containing older cultivars, to cluster E, containing newer cultivars. Clusters C, D and E showed significant differences for protein content. The same trend was seen for flour ash as well. Significant differences in ash content were found between cluster A and the rest of the clusters. The values for ash content suggests that the ash content decreased over the years, as the highest value can be seen in cluster A containing older cultivars and the lowest in cluster E containing modern cultivars. This agreed with the dendrogram analysis based on pedigree information (discussed earlier), where there was a significant difference between clusters with respect to both year and ash content. This could be due to the fact that in these two analyses it was possible to cluster the cultivars according to release year, with some clusters having older cultivars and others having modern cultivars. Wet gluten content, which is a reflection of protein content, showed significant differences between clusters C and D, which is in agreement with the findings for protein content. However, there was no significant difference for gluten index.

With regard to the farinograph quality parameters of the clusters, significant differences between clusters were found for farinograph absorption and peak time. Mean absorption of cluster A was significantly different than clusters D and E, and cluster D was significantly different than clusters B, C, and D with regard to peak time. However, significant differences were not found for stability. This means that these clusters are significantly different from each other with respect to dough quality. As for baking quality, bake water absorption and grain and

texture showed significant differences between clusters. Cluster A and C were significantly different for both qualities. Loaf volume was not significantly different between the clusters. As in the previous analysis, both phenotype and the *Rht* gene data did not show significant differences between the clusters. SE-HPLC area data was also used for the construction of a dendrogram as explained above.

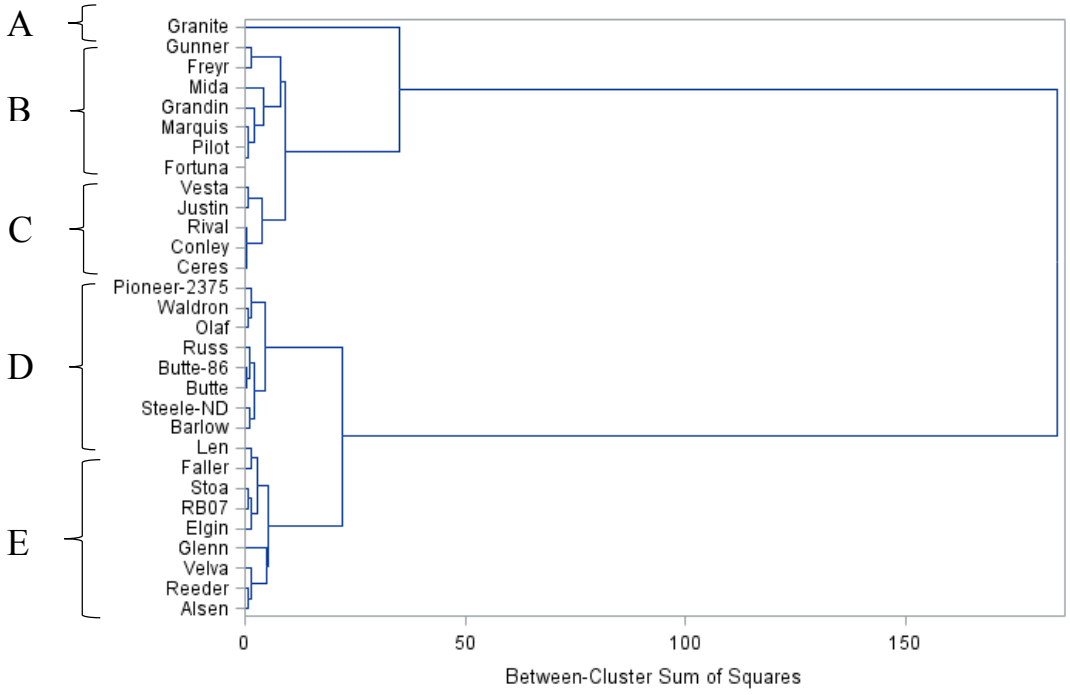

Figure 3.4. Dendrogram based on SE-HPLC chromatogram area

The thirty historical and modern spring wheat cultivars were clustered into five groups when a dendrogram was constructed using SE-HPLC area data for SDS extractable and unextractable proteins. In this case, a cut point of 10 was taken into consideration. As in the dendrogram for gliadin RP-HPLC area data, in this analysis too Granite stood out as an outlier. As such, cluster A only contains this cultivar. Cluster B, on the other hand, contains seven cultivars. The release year of the cultivars in this cluster appear to be variable ranging from 1910 to 2004. As per cluster C, this contains mainly older varieties, with the latest release year being

1963. As such, this cluster contains cultivars that were released prior to when semi-dwarf genes were first introduced. Cluster D contains mid-range and modern cultivars. The oldest cultivar in this cluster is cultivar Waldron, which was released in 1969. Cluster E contains modern cultivars, with the majority of cultivars released after the year 2000. As per the previous dendrograms, the wheat samples in each of the clusters were analyzed for differences in quality traits.

Table 3.6. Quality parameters with respect to wheat samples in the same ward cluster in dendrogram based on protein size exclusion-HPLC absorbance area data[a]

Quality parameters	Ward A (n=1)	Ward B (n=7)	Ward C (n=5)	Ward D (n=8)	Ward E (n=9)
Flour protein content (%)					
Mean	13.8a	13.2a	13.1a	12.7a	12.5a
Min	-	12.6	12.6	12.2	11.4
Max	-	13.5	13.6	13.3	13.3
SD	-	0.34	0.43	0.38	0.73
Flour ash content (%)					
Mean	0.50b	0.60ab	0.68a	0.54b	0.52b
Min	-	0.49	0.60	0.49	0.43
Max	-	0.75	0.73	0.64	0.61
SD	-	0.09	0.05	0.06	0.05
Gluten index					
Mean	87.3b	87.7b	96.6a	95.5ab	98.1a
Min	-	69.5	94.8	89.3	95.5
Max	-	96.1	98.1	97.8	99.4
SD	-	9.8	1.3	2.8	1.2
Wet gluten					
Mean	38.4a	36.4ab	33.8bc	33.6c	32.6c
Min	-	35.3	32.1	30.9	29.3
Max	-	37.4	35.7	35.8	35.3
SD	-	0.7	1.6	1.7	2.3
Farinograph absorption (%)					
Mean	62.8a	60.4a	59.5a	61.5a	60.4a
Min	-	57.3	56.5	57.1	58.2
Max	-	64.8	61.3	65.1	64.0
SD	-	2.6	1.8	2.3	2.0

Table 3.6. Quality parameters with respect to wheat samples in the same ward cluster in dendrogram based on protein size exclusion-HPLC absorbance area data[a] (continued)

Quality parameters	Ward A (n=1)	Ward B (n=7)	Ward C (n=5)	Ward D (n=8)	Ward E (n=9)
Farinograph peak time (min)					
Mean	8.8a	6.3a	6.2a	6.9a	7.6a
Min	-	4.1	4.8	5.7	6.2
Max	-	8.0	8.0	8.3	8.9
SD	-	1.1	1.4	0.9	1.0
Farinograph stability (min)					
Mean	10.2ab	8.4b	9.6ab	10.5ab	13.9a
Min	-	4.4	6.6	7.3	10.4
Max	-	10.5	12.9	14.8	22.3
SD	-	2.1	2.7	2.3	3.8
Loaf volume (cc)					
Mean	902.5a	980.7a	1001.0a	950.9a	966.4a
Min	-	912.5	970.0	882.5	890.0
Max	-	1022.5	1032.5	1015.0	1027.5
SD	-	38.6	24.8	54.7	47.4
Bake water absorption (%)					
Mean	70.4a	67.6a	66.5a	68.1a	66.9a
Min	-	63.5	63.9	65.7	64.7
Max	-	72.0	69.6	70.2	70.5
SD	-	2.8	2.3	1.5	1.8
Grain and texture					
Mean	7.0c	8.1a	7.9a	7.6ab	7.4bc
Min	-	7.7	7.5	7.3	6.7
Max	-	8.3	8.5	8.0	8.0
SD	-	0.2	0.4	0.3	0.4

[a] Means with the same letter in the same row are not significantly different at the 5% level. Min=minimum, Max=maximum, and SD=standard deviation

Significant differences were found between the clusters with reference to release year. As explained earlier, the clusters in the dendrogram can be grouped according to year in most cases. For example, cluster E was composed of modern wheat cultivars. Cluster A was significantly different than clusters B and C, cluster E was significantly different than clusters B and C, and cluster D and C were significantly from each different. Thus, the amount of unreduced wheat proteins changed over the years. There were no significant differences in flour protein content in the clusters that were analyzed. As such, the average value for this parameter did not vary

significantly over the clusters. Table 3.6 shows that cluster A has the highest average flour protein content, while cluster E has the lowest. However, it is important to note that cluster A is only composed of Granite, which appears to be an outlier. As per flour ash content, the results indicated that there is a significant difference between the clusters. The flour ash content appears to have a decreasing trend from cluster B to cluster E. The highest ash content was found in cluster C, which is composed of older cultivars, and the lowest in cluster E, which is composed of modern wheat cultivars. As such, this is a clear indication that modern spring wheat has lower ash content in comparison with historical wheat cultivars. The ash content of cluster C appeared to be significantly different than that of clusters A, D and E. Although there was no significant difference between clusters in terms of protein content, gluten index and wet gluten content showed significant differences. As for gluten index, cluster E was significantly different than clusters A and B, and for wet gluten content, cluster A was significantly different than clusters C, D and E.

In terms of farinograph quality parameters, farinograph absorption and peak time did not show significant differences between clusters. Farinograph stability was significantly different between clusters B and E. As for baking quality, loaf volume and bake water absorption did not show significant differences between clusters. However, significant differences between clusters were found for grain and texture.

The results showed that as per phenotypic information, there were significant differences between cluster A (only Granite), and clusters B and C, and clusters E, and B and C. The fact that Granite stands out as an outlier could be the reason for this difference. As per *Rht* genetic information, significant differences were found between clusters E and clusters A, B and C. Cluster E contains modern cultivars, and clusters A, B and C intermediate to old cultivars.

Therefore, the difference could be due to the fact that older cultivars do not contain dwarfing genes.

Polymeric proteins analyzed using SE-HPLC have been identified as main components that influence mixing and breadmaking characteristics in HRS wheat cultivars (Ohm et al. 2009; 2010). Therefore, it is most likely that polymeric proteins are mainly associated with clustering of wheat cultivars using the SE-HPLC data. The results also indicate that polymeric proteins have played an important role in improving quality in HRS wheat breeding. Polymeric proteins consist of glutenin subunits that are linked by disulfide bonds. As early as 1987, Payne et al. (1987) found that the presence of certain HMW-glutenin subunits have a positive influence on gluten strength. It was also found that allelic variation at the *Glu-D1* gene had a greater influence than the other glutenin gene alleles. Studies have found that the subunit combination 5+10 at the *Glu-D1* locus results in stronger gluten, thus good quality dough (Rogers et al. 1989; Payne et al. 1987). The subunit combination 2+12 was associated with poor gluten strength. Buonocore et al. (1996) found that the superior characteristics of *Glu-D1* (5+10) is due to an extra cysteine residue on the Dx-5 subunit in comparison to the Dx-2 subunit. This leads to the formation of polymers of larger distribution. Genetic marker analysis for the glutenin subunits showed that only cultivar Ceres (released in 1926) contains subunit 2+12, which is not favorable for dough and baking properties. All other cultivars contain glutenin subunits 5+10, which is favorable. As such, this could be a contributing factor for Ceres having low dough and baking quality.

3.5. Conclusion

In this study thirty hard red spring wheat cultivars released between 1910 and 2013 were analyzed using dendrograms. These dendrograms were constructed using parentage information of the cultivars, and also separation of gliadins through RP-HPLC and unreduced proteins

through SE-HPLC. The results indicate that the cultivars in question can be clustered into three groups when parentage information is considered, and that the clusters differ significantly with regard to year. As such, when these cultivars were clustered using parentage information, the clusters formed were grouped by release year of cultivar. Another aspect of this study was to determine how the presence of dwarfing genes changes between clusters. In the dendrogram based on parentage information, significant differences were found between some clusters regarding *Rht* marker information. Also, the results show that ash content, farinograph characteristics and bread crumb grain and texture are significantly different between the clusters. On the other hand, when wheat cultivars were clustered according to the presence or absence of peaks at a certain time point in RP-HPLC, different observations were made. In this case, no significant differences appeared between the clusters with regard to release year, semi-dwarf characteristics, protein and ash content, and most farinograph and baking quality characteristics. However, in the dendrogram constructed using the RP-HPLC chromatogram area data that could be associated with quantitative variation in addition to qualitative variation in gliadins, significant difference appeared between the clusters in terms of release year. Additionally, significant differences between clusters were observed for flour protein content, ash content, wet gluten, farinograph absorption and peak time, bake water absorption and grain and texture, demonstrating that these clusters are different in terms of dough and baking quality parameters. As per the dendrogram produced using SDS extractable and unextractable SE-HPLC data, significant difference was found between the clusters with reference to release year, semi-dwarf characteristics, ash content, gluten index, wet gluten content, farinograph stability and bread crumb grain and texture. Overall, the results indicate that clusters produced using the different sources of information are similar to each other in some aspects, while being significantly

different to each other with respect to other properties. The information obtained from cluster analyses generally indicates that wheat quality traits have been improved since the introduction of dwarf genes in HRS wheat. However, the introduction of *Rht* genes was not likely to accompany prominent changes in gliadin composition, such as addition of new gliadin proteins in modern wheat cultivars, including toxic proteins that might cause celiac disease, since clusters constructed using gliadin peak data did not show a significant difference for release year and semi-dwarfism. Also, high molecular weight glutenin subunit composition did not appear to be largely different between historical and modern cultivars, as all the wheat cultivars carried markers for 5+10 for *Glu-D1* locus, except for one cultivar in the current research. Moreover, significant differences were found for release year, semi-dwarf characteristics, and quality characteristics between clusters grouped using absorbance area data of RP-HPLC of gliadins and SE-HPLC of unreduced proteins as pointed out above. These results indicate that quantitative changes in specific protein components including polymeric proteins might be mainly associated with quality improvement in modern HRS wheat breeding in North Dakota.

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CHAPTER 4. DETECTION OF IMMUNOGENIC PEPTIDES CAUSING CELIAC DISEASE IN HISTORICAL AND MODERN HARD RED SPRING WHEAT CULTIVARS

4.1. Abstract

Celiac disease (CD) is prevalent in 0.5 to 1.26% of adolescents and adults. The disease develops in genetically susceptible individuals as a result of ingestion of gluten forming proteins found in cereals such as, wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.) and barley (*Hordeum sativum* L.). In wheat, gluten forming protein is a storage protein that is found in the endosperm. Gluten is composed of glutenin and gliadin proteins. Gliadins are further divided into α-/β-, γ- and ω- gliadins. Studies suggest that the majority of toxic peptides are produced by α-gliadin proteins. It is unknown if all wheat cultivars cause the same reaction or if the intensity of the reaction is dependent on the type of wheat ingested. There are claims that breeding practices have changed wheat protein chemistry over the years, and this has resulted in modern wheat being more toxic in terms of CD as opposed to historical wheat. In this context, the aim of the study was to detect celiac-disease-initiating peptides of the gliadin proteins in historical and modern spring wheat cultivars grown in North Dakota during the last 110 years. For this purpose, gliadin proteins were extracted from wheat and then used in mass spectrometric analysis. The results indicate that immunogenic peptides causing CD are detected in both historical and modern spring wheat cultivars irrespective of release year.

4.2. Introduction

Celiac disease is a genetically predisposed autoimmune disease, which is one of the most common food intolerances in the world (Wieser and Koehler 2008). It may be defined as an inflammatory disease, which causes damage to the upper small intestine. CD is most prevalent in

Europeans and those with European ancestry (Gallagher et al. 2004). In the Unites States, it is estimated that 1 in 111 people suffer from CD, which accounts for approximately 1% of the general population.

The inflammation is caused by the ingestion of cereals, such as wheat, barley, rye and, possibly, oat products (Wieser and Koehler 2008). The key factor which causes the immune reaction is gluten forming proteins and homologous proteins found some cereals. Gluten forming proteins are composed of gliadin and glutenin proteins. These proteins act as antigens in genetically susceptible individuals and trigger an immune response to these proteins. In normal physiological conditions, gastric, pancreatic and brush-border enzymes digest proteins into small peptides and amino acids. However, toxic peptides in terms of CD are high in proline content, thus, are resistant to digestion. As a result of non-digestion, proline and glutamine rich fragments accumulate in the small intestine. These peptides are referred to as immunogenic peptides/ toxic peptides/ T-cell epitopes. The pathological conditions associated with CD begins with the alteration of the barrier function of the intestinal mucosa, allowing dietary gluten peptides to reach the subepithelial lymphatic tissue. Recent studies have demonstrated that zonulin protein is responsible for causing increased permeability of the intestinal mucosa in CD patients. Gluten peptides that have reached the lamina propia are able to trigger the adaptive and innate immune response associated with CD. The final outcomes of the immune response include degradation of matrix proteins, and subsequent mucosal destruction, production of antibodies against gluten peptides, tissue transglutaminase and peptide complexes formed by the activity of the enzyme.

The molecular weight range of gliadin proteins is 28000- 55000 (Bonomi et al. 2013). These proteins are involved in forming the gluten network during mixing of dough. Gliadins are divided into subgroups, namely, α-/β-, γ- and ω-. This grouping is based on the mobility of the

different proteins on Acid- Polyacrylamide Gel Electrophoresis (A-PAGE). The primary structure of α- and β- gliadins are similar in terms of electrophoretic mobility, therefore, these two types of gliadins are referred to as α-/β- type gliadins. Gliadins are soluble in aqueous alcohols. Glutenins are much larger proteins than gliadins, and the molecular weight of these proteins range from 60000 to more than 10 million. After the reduction of intra-chain disulfide bonds, glutenin subunits become soluble in aqueous alcohol solutions. Glutenin proteins are divided into High Molecular Weight (HMW) and Low Molecular Weight (LMW) proteins, according to their mobility on A-PAGE. Studies have shown that the alcohol soluble prolamin (proline and glutamine) fraction consisting of gliadins is the most toxic component in wheat proteins. The toxicity of glutenins is classified as nontoxic, weakly toxic or not as toxic as gliadins, however, the toxicity of HMW-glutenins is said to be similar to that of gliadins (Wieser and Koehler 2008).

Wheat breeding practices have been in use for many decades, and one of the main purposes of wheat breeding is the introduction of favorable traits such as high yield, disease resistance and drought tolerance. Currently, tetraploid and hexaploid wheat varieties are commonly used for pasta and bread production respectively. These varieties have been developed from older varieties though breeding practices. Some researchers claim that breeding for higher yield has resulted in changes in wheat proteins, and this has caused an increase in the amount of immunogenic peptides in modern wheat. Thus, modern wheat is said to be more toxic than historical wheat in terms of initiating the immune response associated with CD.

A study conducted by Spaenij-Dekking et al. (2005) determined that there is high genetic variation among different wheat species, and that it is possible to identify wheat accessions that are low in the amount of T-cell stimulatory epitope sequences. The study was conducted through

determining the presence of T-cell stimulatory epitopes in gluten protein sequences in databases, and by using T-cell and antibody based assays to determine the gliadin and glutenin levels of diploid, tetraploid and hexaploid wheat varieties. To further confirm the premise that wheat varieties low in gliadin are less toxic to CD patients, in vitro assays have been performed using wheat lines lacking gliadin and glutenin subunits, and wild-type wheat (Carroccio et al. 2011). The results indicated that gliadin-deficient wheat has lower toxicity in terms of causing an immunogenic reaction, thus the prolamin content has an effect on the innate immune response related to CD.

Tetraploid wheat (durum wheat), which is mainly used for pasta production, lacks the D genome, which is present in bread wheat. Therefore, it is possible that this type of wheat could have reduced levels of T-cell stimulatory epitopes that trigger the onset of CD (van den Broeck et al. 2010a). Immunoblotting using antibodies produced against different T-cell stimulatory gliadin epitopes has been used to test this hypothesis with regard to landraces, old, modern and domesticated tetraploid wheat accessions. The results suggested that some varieties of tetraploid wheat have lower toxicity compared to others. On the contrary, in another study conducted to determine the toxicity of ancient wheat varieties, it was found that the two ancient varieties in question were toxic to CD patients (Colomba and Gregorini 2012). The study compared the immunogenic properties of ancient durum wheat varieties found in Italy (Graziella and Kamut) to modern accessions using monoclonal antibodies produced against toxic α-gliadin peptides. The results show that the ancient wheat varieties have more α-gliadin in general. This study clearly indicates that the ancient wheat varieties in question are definitely not suitable for individuals with CD.

Studies have shown that wheat breeding may be a cause for the increased prevalence of CD. Monoclonal antibodies developed against toxic α-gliadin peptides, QPFPQPQ and RPQQPYP, have been used to compare different wheat varieties, in order to identify accessions that are of lower toxicity (van den Broeck et al. 2010b). Additionally, two-dimensional electrophoresis (iso-electric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis) was used in the process. The results indicate that, in general, the toxicity of modern wheat has increased, and that the genetic diversity among these varieties has decreased. This suggests that CD patients may be faced with a less diverse group of toxic proteins.

The primary objective of this study is to identify and quantify CD causing α-gliadin proteins in historical wheat varieties grown in the United States, specifically in the state of North Dakota, during the last 110 years. Thus, the toxicity of the different varieties can be compared. Toxicity, in this context, refers to the ability of wheat proteins to trigger the onset of CD. For this purpose, 30 wheat cultivars released in North Dakota were analyzed in terms of the presence of immunogenic peptides in gliadin proteins, which is the main gluten protein that causes the immune reaction associated with CD.

4.3. Materials and Methods

Thirty wheat cultivars released in North Dakota from 1910 to 2013 were used in this study. The cultivars were grown in Casselton, ND in 2013 in 3ft x 150 ft strip plots. The plots were ordered by release year in the field. The wheat cultivars were planted during the first week of June and harvested during the first week of September.

Table 4.1. List of cultivars used in the study

Cultivar	Release year
Marquis	1910
Ceres	1926
Pilot	1939
Rival	1939
Vesta	1942
Mida	1944
Conley	1955
Justin	1963
Fortuna	1966
Waldron	1969
Olaf	1972
Butte	1977
Len	1979
Stoa	1984
Butte86	1986
Grandin	1989
Pioneer-2375	1990
Gunner	1995
Russ	1995
Reeder	1999
Alsen	2000
Granite	2002
Freyr	2004
Steele-ND	2004
Glenn	2005
Faller	2007
RB07	2007
Barlow	2009
Velva	2012
Elgin	2013

4.3.1. Reverse-phase HPLC (RP-HPLC) analysis of gliadin proteins

Gliadin proteins were extracted and separated by RP-HPLC according to the method of Lookhart and Bean (1995), with modifications. A volume of 750 μL of 70% ethanol was added to 250 mg of flour and kept shaking at 1400 rpm for 1 h at 30^0C in a heating block (Eppendorf

Thermomixer R). The mixture was then centrifuged at 4550 g for 10 min (Eppendorf Centrifuge 5415 C), and filtered through a 0.45 µm nylon filter (VWR International). The Pierce™ BCA assay kit (ThermoFisher Scientific) was used to measure the protein content in each extract. After the protein content was determined, each extract was diluted to 13 µg/µL using water filtered through a 0.45 µm nylon membrane filter. The samples were then heat treated at 80°C for 3 min in a heating block prior to running through the HPLC.

An Agilent Zorbax 300SB-C18 (4.6 x 250 mm, 5 µm) column was used for separation of the protein extracts. Two buffer solutions, A and B, were used in the analysis. Buffer A: 95% water, 5% acetonitrile and 0.1% Trifluoroacetic acid (TFA; v/v), and buffer B: 100% acetonitrile with 0.08% (v/v) TFA. The two buffers were balanced at 210 nm with TFA, and subsequently filtered through a 0.45 µm nylon membrane prior to running through the instrument. Gliadins were eluted in a linear multistep gradient at 65^{0}C at a flow rate of 1 mL/min. The injection volume was 23 µL (approximately 300 µg of protein based on the BCA assay). The signal was detected at 210 nm using an Agilent 1100 variable wavelength detector (Agilent Technologies, Waldbroann, Germany).

The elution gradient started at 75% buffer A and 25% buffer B, which was maintained for the first 5 minutes. For the next 5 min, the volumes were gradually increased so that at 10 min it was 70% buffer A and 30% buffer B. After gradually increasing again, at 60 min it was 50% of each buffer. This state was maintained until 63 min. In the next minute the gradient was changed so that at 64 min it was 100% buffer B, which was maintained for 1 min. In the next minute, the gradient was changed again so that at 66 min the percentages were brought down to the original. A post time of 15 min was added after each run. Prior to separation of the proteins through the

column, a conditioning step was performed. That is, water and acetonitrile were flushed through the column respectively for 5 min. The column was washed with 10% methanol for storage.

The HPLC data was also processed using an in-house program coded using MATLAB (2015, The MathWorks, Natick, MA). UV absorbance values were interpolated to 0.005 min intervals by the 'spline' function in MATLAB. Absorbance area values were calculated for specific peaks using the interpolated absorbance data. The SAS software (Version 9.3, SAS Institute, Cary, NC) was used for the statistical analysis of the results, and correlation analysis was done using the 'CORR' procedure in SAS.

4.3.2. Gliadin protein analysis using liquid chromatography-mass spectrometry (LC-MS)

4.3.2.1. Preparation of gliadin proteins

Gliadin proteins were extracted as previously mentioned, and the amount of protein in each extract was quantified using a Pierce™ BCA assay kit. Afterwards, each sample was mixed with sample buffer (0.5M Tris-HCl, pH 6.8, glycerol, 10% SDS, 2-mercaptoethanol, and 0.05% bromophenol blue) so that each sample would contain 40 µg of protein. Afterwards, the samples were boiled for 5 min. An 8% Tris-Tricine gel was used to run the samples (Schagger and von Jagow 1987). The gel was run for 15 min at 55 V so that the proteins would be incorporated in the gel. For staining the gels, a protocol adapted from the European Molecular Biology Laboratory (EMBL) was used. The gel was washed briefly with water, then fixed in a solution containing 45% methanol, 45% water and 10% acetic acid for 15 min. Afterwards, the gel was washed again with water and fixed in the same solution for another 15 min, after which the gel was washed again. The gel was stained in Colloidal Coomassie blue overnight. The following day the individual gel bands were cut using a sterile razor blade and stored at -20°C in 1.5 ml Eppendorf tubes for mass spectrometric analysis

4.3.2.2. Digestion of gliadin proteins using the chymotrypsin enzyme

This method was also adapted from the EMBL method for in-gel tryptic digestion of proteins. A volume of 75 µL of 1:1 100 mM ammoniumbicarbonate: acetonitrile solution was added to Eppendorf tubes containing the gel pieces, vortexed briefly and incubated for 15 min at room temperature. Afterwards, the solution was removed and this step was repeated again. Next, 75 µL of 100% acetonitrile was added to each tube. Once the gel pieces had shrunk and turned white the acetonitrile was removed. The reduction and the alkylation of the extracts were then performed.

The gel pieces were rehydrated using 75 µL of 10 mM dithiothreitol (DTT) in 50 mM ammonium bicarbonate for 1 hour at 56°C in a water bath. The tubes were then spun down, and the DTT solution was removed. Then 75 µL of 55 mM iodoacetamide in 50 mM ammonium bicarbonate was added to the tube and incubated for 30 min at room temperature in a dark room. Afterwards, the iodoacetamide solution was removed by centrifugation. The gel slices were washed again twice with 75 µL of 1:1 acetonitrile: 100 mM ammonium bicarbonate solution as previously stated. After the washing solution was removed, 75 µL of 100% acetonitrile was added, and kept in the tubes until the gel slices had shrunk and turned opaque white. Once the acetonitrile was removed the digestion step was performed using the chymotrypsin enzyme.

The gel pieces were rehydrated in a digestion buffer at 4°C (on ice) for 15 min. This buffer was composed of 50 mM NH_4HCO_3, 5 mM $CaCl_2$ and 5 ng/µg chymotrypsin. Then the supernatant was removed and replaced with 70 µL of 50 mM NH_4HCO_3, 5mM $CaCl_2$ and incubated overnight at room temperature.

The following day, peptides were extracted from the digested samples. The tubes were spun down briefly in a centrifuge at low speed and the supernatant was retained. The supernatant

of each tube was transferred to new Eppendorf tubes. Solutions of 50% acetonitrile and 0.3% formic acid were added to the tubes so that the gel pieces were covered and kept for 15 min. The supernatant from each tube was then obtained and transferred to the corresponding Eppendorf tubes. The same volume (as in the previous step) of 80% acetonitrile and 0.3% formic acid was added again and incubated for 15 min. As previously done, the supernatant was recovered and transferred to new corresponding tubes. The pooled extracts were kept at -80°C for 30 min, then freeze dried in a speed vacuum. The samples were stored at -80°C until mass spectrometric analysis was performed.

4.3.2.3. Desalting of samples using STAGE (Stop And Go Extraction) tip desalting procedure

This method was adapted from Rappsilber et al. (2003). The pH of the samples were checked, and acidified to pH 4 or less. The stage tips were assembled prior to the procedure. The Empore reversed-phase extraction disks from 3M (SDB-XC reversed-phase material, 3M product number 2240/2340) were placed flat on a clean hard surface. An 18 gauge blunt ended syringe needle was pressed into the disk to core out a piece of the filter material. This was done twice. Then the needle was placed into a 200 µL pipette tip and the cored disk pieces were pushed into the tip using a fused silica tube with 0.5 µm internal diameter. The filter material was packed into the end of the pipette tip.

To assemble the stage tip a 1.5 mL Eppendorf tube was used. The cap was cut off, and a hole was made into the center of the cap. The cap was then snapped onto a new 1.5 mL Eppendorf tube. The pipette tip prepared earlier was placed in the hole of the cap. For each digested sample such tubes were prepared.

For the desalting procedure three solvents were freshly prepared. The wash solvent was composed of 98: 2: 0.1% water: acetonitrile: trifluoroacetic acid (TFA). The wetting solvent was composed of 80: 20: 0.1% acetonitrile: water: TFA., and the elution solvent was prepared with 60: 40: 0.1% acetonitrile: water: TFA.

First the samples were reconstituted with 60 µL of wash solvent, vortexed for 45 sec and then centrifuged at 3000 g for 1 min. The pH of the samples were then checked using pH strips. If the pH was not below 3, a solution containing 10% TFA was used to make the samples acidic. Then 60 µL of the wetting solvent was added onto the stage tip/tube assembly prepared earlier, and centrifuged at 450 g for 2 min. Afterwards, 60 µL of the wash solvent was added to the assembly and centrifuged again under the same conditions. The liquid at the bottom of the Eppendorf tube was then discarded, and the tubes and tips were reassembled. The sample was pipetted into the stage tip, and then centrifuged at 450 x g for 2 min. This was done for a longer time if the solvent did not wash through after 2 min. Then 60 µL of wash solvent was added into the Stage tip/ tube assembly and centrifuged at 450 x g for 2 min. This step was done twice. The cap/Stage tip assembly was placed onto a new Eppendorf tube with a name corresponding with the sample. A volume of 60 µL of elution solvent was added onto the Stage tip/ tube assembly and centrifuged at 450 x g for 2 min. Finally the desalted peptide mixture was dried using a speed vacuum system.

4.3.2.4. Liquid chromatography-mass spectrometry and data analysis

Approximately 1.5 µg of the chymotrypsin digested peptide mixtures were used for LC-MS analysis on an Orbitrap Velos system (Thermo Fisher Scientific, Waltham, MA) as previously described by Lin-Moshier et al. (2013). Identical LC conditions were used and the following modifications were made to the MS acquisition settings: the MS1 scan range was 360

– 1800 *m/z*; the minimum abundance for MS/MS trigger was 10000 counts; lock mass was not selected; dynamic exclusion settings were: list size 200 values, duration 30 seconds, exclusion mass tolerance +/- 10 ppm.

The PEAKS 7.0 software (Bioinformatics Solutions, Waterloo, ON, Canada) was used to analyze the .RAW data files against the database nr_triticinae 1648030_20150930_cRAP123 downloaded from NCBI. The data was used to determine the proteins which are present in each gliadin extract, and if immunogenic peptides can be detected in the historical and modern wheat cultivars being investigated. Additionally, a binary dataset was created according to the presence or absence of immunogenic peptides and this information was used to produce a dendrogram according to Ward's method using SAS software (Version 9.3, SAS Institute, Cary, NC).

4.4. Results and Discussion

The peaks identified in the RP-HPLC chromatogram were analyzed for correlations with release year to determine which peaks increased/ decreased in amount in the last 100 years. The regions of the chromatogram corresponding to the different gliadin types were identified according to previous studies (Gessendorfer et al. 2009; Piston et al. 2011; Wang et al. 2012).

4.4.1. Reverse phase-HPLC data analysis

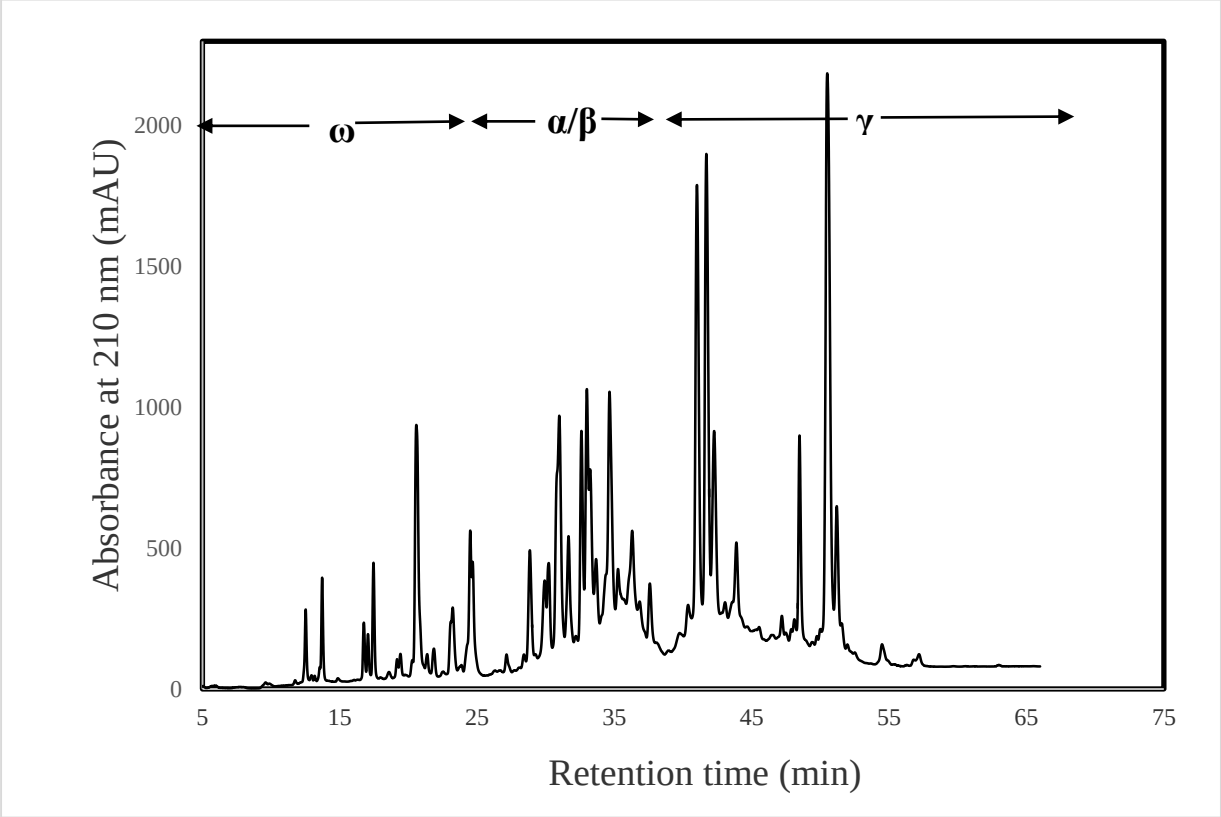

Figure 4.1. Regions of RP-HPLC chromatogram corresponding to different gliadin types

As shown in Figure 4.1, the RP-HPLC chromatogram was used for the identification of the different gliadin proteins. Table 4.2 shows the correlation between the regions corresponding to the different gliadin types and release year.

Table 4.2. Correlations between reverse phase-HPLC chromatogram area corresponding to different gliadin types and release year

Gliadin region	Time interval (min)	Mean	Min	Max	StD	Correlation with year
ω-gliadin	10.5-26	1218	876	1666	175	0.420*
α/β- gliadin	26-38.5	2272	1719	3128	350	0.206^{NS}
γ-gliadin	38.5-58	1930	1274	3049	411	0.186^{NS}

*= $P \leq 0.05$

The results of the correlation study indicate that only the RP-HPLC chromatogram area corresponding to ω-gliadin proteins showed significant positive correlations ($P \leq 0.05$) with release year. Thus, the amount of these proteins is higher in modern wheat in comparison to historical wheat. The α-/β- and γ- gliadin proteins did not show significant correlations ($P \leq 0.05$) with year, therefore, the amount of these proteins in historical and modern wheat is not significantly different. As mentioned earlier, the α-/β- gliadin proteins have been associated with initiating the immune reaction in CD subjects. Some studies suggest that decreased amounts of these proteins could mean decreased CD toxicity (Carroccio et al. 2011). Since the amount of the proteins did not change significantly over the years, there may not be a difference in terms of CD toxicity in historical and modern wheat.

These gliadin regions were also analyzed for correlations with dough and baking quality characteristics. The regions corresponding to ω-gliadin showed significant positive correlations ($P \leq 0.01$) with farinograph peak time, indicating that this quality parameter improved with increasing amounts of ω-gliadin. This gliadin also showed significant positive correlations with farinograph quality number. However, significant negative correlations ($P \leq 0.05$) were found between the ω-gliadin region and minor baking quality parameters such as crumb color. The α/β- and γ- gliadins did not show significant correlations with the quality parameters explained above. Thus, the results indicate that ω-gliadin proteins may contribute towards improvements in dough properties, while the α/β- and γ- gliadins may not play a role in this regard.

Several peaks in the ω-gliadin and γ-gliadin regions of the chromatogram showed significant correlations ($P \leq 0.01$) with release year. Thus, these regions of the chromatogram varied somewhat between the cultivars being investigated. As for peaks in the α/β-gliadins, significant correlations with year were not found. This is an indication that the amount of α/β-

gliadin proteins did not vary significantly over the years, as previously noted. The peaks that showed correlations are shown in Figure 4.2 and the quantitative values are shown in Table 4.3.

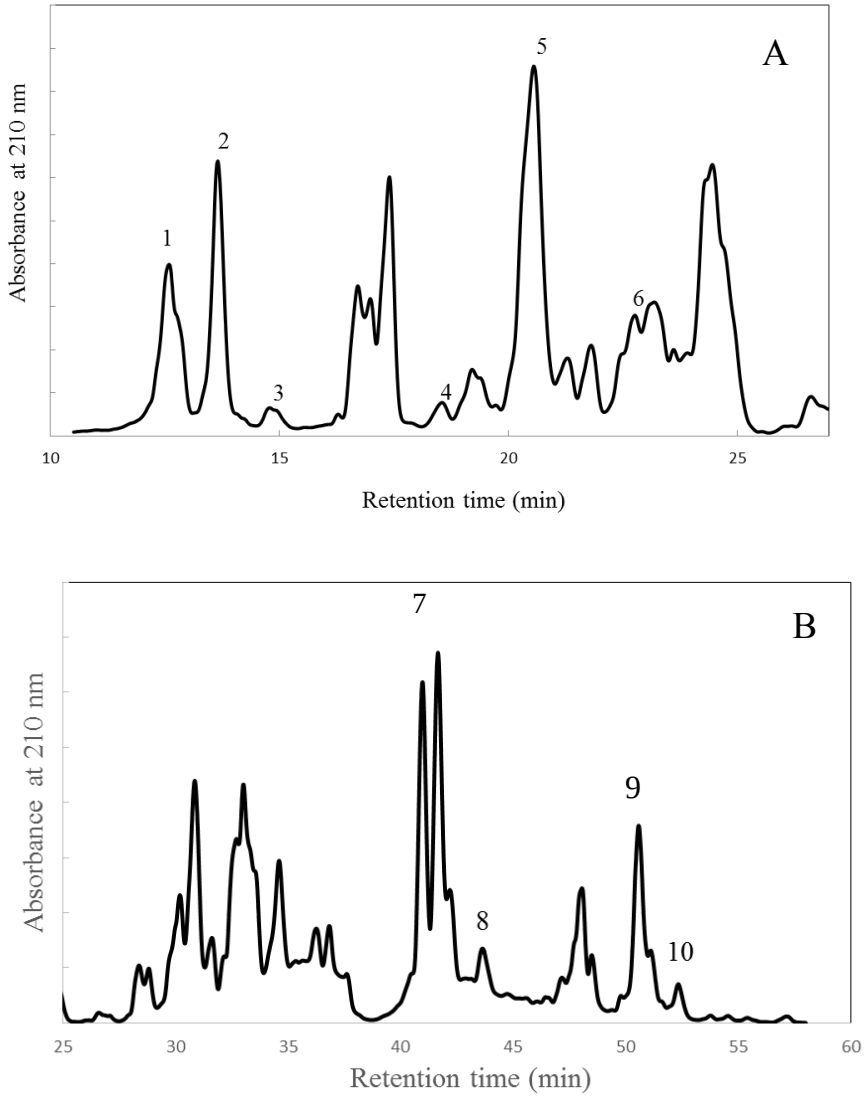

Figure 4.2. Peaks in the ω- and γ- gliadin regions that showed significant correlations with year. (A) The ω-gliadins peaks, (B) γ-gliadin peaks

Six ω-gliadin peaks and four γ-gliadin peaks showed significant correlations with release year. As shown in Table 4.3, some peaks showed positive correlations, whereas other showed negative correlations. This means that the amount of some ω- and γ- gliadin proteins increased with year, while the amount of α-/β- gliadins did not change significantly.

Table 4.3. Correlations between area of ω- and γ- gliadin peaks with release year

Gliadin type	Peak	Retention time (min)	Mean	Min	Max	StD	Correlation with year
ω-gliadin	1	12.1-13.0	137.8	39.8	297.3	61.3	0.472**
	2	13.2-14.1	137.3	25.7	341.6	81.2	0.633***
	3	14.6-15.2	16.3	7.2	50.6	12.8	0.491**
	4	18.2-18.7	13.1	8.3	34.3	7.1	-0.649***
	5	20.1-21.0	225.5	29.7	470.7	130.9	-0.466**
	6	21.7-22.2	47.8	6.2	130.5	39.9	0.672***
γ-gliadin	7	40.2-40.8	327.5	217.6	504.1	56.7	0.474**
	8	42.9-43.4	68.8	7.6	144.1	32.2	0.578***
	9	50.0-50.6	198.0	5.2	579.6	200.9	-0.545**
	10	51.8-52.3	31.1	2.9	80.6	22.6	0.714***

** = $P \leq 0.01$ and *** = $P \leq 0.001$.

In the ω-gliadin region, peaks 1, 2 and 3 showed significant positive correlations ($P \leq 0.01$) with release year, whereas peaks 4 and 5 showed significant negative correlations ($P \leq 0.01$). As for the γ-gliadins, peaks 7, 8 and 10 showed significant correlations with year ($P \leq 0.01$), whereas peak 9 showed a negative correlation. If a connection between the increase in gliadin content and celiac disease toxicity is established, the proteins in the peaks that increased with year could be important, as they may contain high amounts of immunogenic peptides.

4.4.2. Liquid chromatography-mass spectrometry data analysis

Following mass spectrometric analysis of the samples, the PEAKS software was used for the sorting and exporting of the data. Databases were created for the peptides and proteins that were identified. In this shot gun experiment the following proteins were detected (Table 4.4).

Table 4.4. Number of unique proteins detected in each sample during LC-MS

		Number of unique proteins detected in each cultivar					
Cultivar	Release year	α/β-gliadin	γ-gliadin	ω-gliadin	HMW glutenin	LMW glutenin	Alpha amylase inhibitor
Marquis	1910	0	0	0	0	4	1
Ceres	1926	5	2	0	5	1	1
Pilot	1939	3	1	0	0	0	0
Rival	1939	3	2	0	0	7	4
Vesta	1942	4	2	0	2	7	0
Mida	1944	4	2	0	0	5	1
Conley	1955	4	2	5	0	5	4
Justin	1963	1	2	0	0	0	0
Fortuna	1966	4	2	1	4	5	0
Waldron	1969	2	1	0	2	1	1
Olaf	1972	3	1	0	2	5	1
Butte	1977	10	1	0	2	12	0
Len	1979	1	1	0	3	6	10
Stoa	1984	4	3	1	2	3	2
Butte-86	1986	4	2	0	2	11	1
Grandin	1989	0	0	0	0	4	0
Pioneer-2375	1990	2	1	0	2	3	0
Gunner	1995	3	1	0	2	8	0
Russ	1995	5	3	1	2	16	4
Reeder	1999	3	2	1	3	3	0
Alsen	2000	15	1	0	2	10	2
Granite	2002	5	3	2	2	6	1
Freyr	2004	1	0	0	3	3	2
Steele-ND	2004	0	1	0	0	8	4
Glenn	2005	5	7	0	0	3	0

Table 4.4. Number of unique proteins detected in each sample during LC-MS (continued)

Cultivar	Release year	Number of unique proteins detected in each cultivar					
		α/β-gliadin	γ-gliadin	ω-gliadin	HMW glutenin	LMW glutenin	Alpha amylase inhibitor
Faller	2007	5	2	0	0	0	2
RB07	2007	3	0	1	3	6	2
Barlow	2009	3	2	1	1	2	2
Velva	2012	3	2	0	3	8	0
Elgin	2013	1	4	0	1	5	3
Total number of detected proteins		816	406	59	303	1057	269

The extraction procedure used for this experiment was specifically for the extraction of gliadin proteins. However, a large number of HMW/ LMW glutenins and alpha-amylase inhibitor proteins were also detected in the samples. The highest number of proteins detected were LMW glutenins and α-gliadins. As per the gliadin proteins, the highest number of proteins was detected for the α-gliadins, and the lowest for the ω-gliadins. Proteins such as avenin like proteins, β-purothionin, grain softness proteins, puroindoline A and serpin proteins were also detected.

The lack of specificity in the extraction method could be because the 70% ethanol extraction not only dissolves gliadin proteins, but also completely or partially dissolves glutenins and other proteins. As such, it might have been advantageous to extract other proteins such as albumins, globulins and glutenins using the traditional Osborne fractionation method prior to extracting the gliadins. Modified extraction protocols can also be used for this purpose. In a study conducted by van den Broeck et al. (2009), an extraction solution containing 50%

isopropanol, 50 mM Tris-HCl (pH 7.5) and dithiothreitol (DTT) was found to be the most suitable for the extraction of gluten proteins. This extraction method is based on the sequential extraction of gliadins and glutenins and then combining these protein fractions to produce a 'gluten extract'. The authors claim that this is a successful method, as the albumin and globulin proteins are not removed prior to the extraction process, thus reducing protein loss. From this perspective, the extraction protocol used in this study also can be advantageous. However, the non-gliadin proteins found in the extraction shows that the method is not specific for isolation of gliadins only.

In a study conducted to quantify immunogenic peptides causing CD, an untargeted experiment was performed at first to identify what proteins are present in the sample. In this experiment, the extraction protocol of van den Broeck et al. (2009) was used. The data was searched against the Uniprot *Viridiplantae* database to determine the identity of the proteins present. The proteins detected included, α/β-gliadins, γ-gliadins, ω-gliadins, HMW and LMW glutenins, progesterone binding protein, ω-secalin, and a few uncharacterized and predicted proteins (van den Broeck et al. 2015). Thus, it appears that the method of van den Broeck et al. (2009) is suitable for this experiment too, however, the extraction of gluten is not needed for the current study as the purpose was to look at gliadin proteins only.

The LC-MS results were also analyzed to determine the presence of immunogenic peptides causing celiac disease (Table 4.4). The peptides identified by Sollid et al. (2012) was used in this section.

Table 4.5. Immunogenic peptides detected in historical and modern spring wheat cultivars

Cultivar	Release year	α/β-gliadin					γ-gliadin								ω-gliadin	
		FRPQQPYPQ	PFPQPQLPY	PYPQPQLPY	PQPQLPYPQ	QGSFQPSQQ	PQQSFPQQQ	IQPQQPAQL	QQPQQPYPQ	SQPQQQFPQ	PQPQQQFPQ	QQPQQPFPQ	PQPQQPFCQ	QQPFPQQPQ	PFPQPQQPF	PQPQQPFPW
Marquis	1910	✓	✓	✓	✓	✓	✓	✓			✓	✓		✓		
Ceres	1926	✓	✓		✓	✓	✓	✓						✓		
Rival	1939	✓	✓		✓	✓	✓	✓				✓		✓	✓	
Pilot	1939	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓		✓		
Vesta	1942	✓	✓	✓	✓		✓	✓	✓		✓	✓		✓	✓	✓
Mida	1944	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓		✓		
Conley	1955	✓	✓		✓	✓	✓	✓						✓	✓	
Justin	1963	✓	✓	✓	✓	✓	✓	✓			✓	✓		✓	✓	✓
Fortuna	1966	✓	✓			✓	✓	✓						✓	✓	
Waldron	1969	✓	✓	✓	✓		✓	✓				✓		✓	✓	
Olaf	1972	✓	✓		✓	✓	✓	✓						✓	✓	
Butte	1977	✓	✓	✓	✓	✓	✓	✓				✓		✓	✓	✓
Len	1979	✓	✓	✓	✓	✓	✓	✓			✓	✓		✓	✓	
Stoa	1984	✓	✓		✓	✓	✓	✓				✓		✓	✓	
Butte-86	1986	✓	✓	✓	✓	✓	✓	✓	✓			✓		✓	✓	✓
Grandin	1989	✓	✓	✓	✓	✓	✓	✓				✓		✓	✓	✓

Table 4.5. Immunogenic peptides detected in historical and modern spring wheat cultivars (continued)

Cultivar	Release year	α/β-gliadin					γ-gliadin								ω-gliadin	
		FRPQQPYPQ	PFPQPQLPY	PYPQPQLPY	PQPQLPYPQ	QGSFQPSQQ	PQQSFPQQQ	IQPQQPAQL	QQPQQPYPQ	SQPQQQFPQ	PQPQQQFPQ	QQPQQPFPQ	PQPQQPFCQ	QQPFPQQPQ	PFPQPQQPF	PQPQQPFPW
Pioneer-2375	1990	✓	✓	✓	✓	✓	✓	✓				✓		✓	✓	✓
Gunner	1995	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓		✓	✓	✓
Russ	1995	✓	✓		✓	✓	✓	✓						✓	✓	
Reeder	1999	✓	✓	✓	✓		✓	✓				✓		✓	✓	
Alsen	2000	✓		✓	✓	✓	✓	✓				✓		✓	✓	✓
Granite	2002	✓	✓	✓	✓		✓	✓				✓		✓	✓	
Freyr	2004	✓	✓	✓	✓	✓	✓	✓				✓		✓	✓	✓
Steele-ND	2004	✓	✓	✓		✓	✓	✓				✓		✓		
Glenn	2005	✓	✓		✓	✓	✓	✓				✓				
Faller	2007	✓	✓	✓	✓	✓	✓	✓	✓			✓		✓	✓	
RB07	2007	✓	✓		✓	✓	✓	✓						✓	✓	
Barlow	2009	✓	✓	✓	✓	✓	✓	✓	✓			✓		✓	✓	✓
Velva	2012	✓	✓	✓	✓	✓	✓	✓			✓	✓		✓	✓	
Elgin	2013	✓	✓		✓	✓	✓	✓						✓	✓	

Table 4.4 shows that immunogenic or toxic peptides which trigger the immune reaction related to CD are found in both historical and modern spring wheat cultivars. All five peptides belonging to the α/β-gliadin proteins were found in a majority of the cultivars. It is interesting to note that the peptide PFPQPQLPY was detected in all cultivars except cultivar Alsen released in 2000. Two peptides (SQPQQQFPQ and PQPQQPFCQ) found in the γ-gliadins were not detected in any cultivar. As per the peptides in ω-gliadin, these two peptides were detected randomly across the cultivars that were investigated. Thus, the LC-MS experiment shows that immunogenic peptides are present in both historical and modern spring wheat cultivars irrespective of release year. To analyze the relationship between the presence of these peptides and release year cluster analysis was performed according to Ward's method.

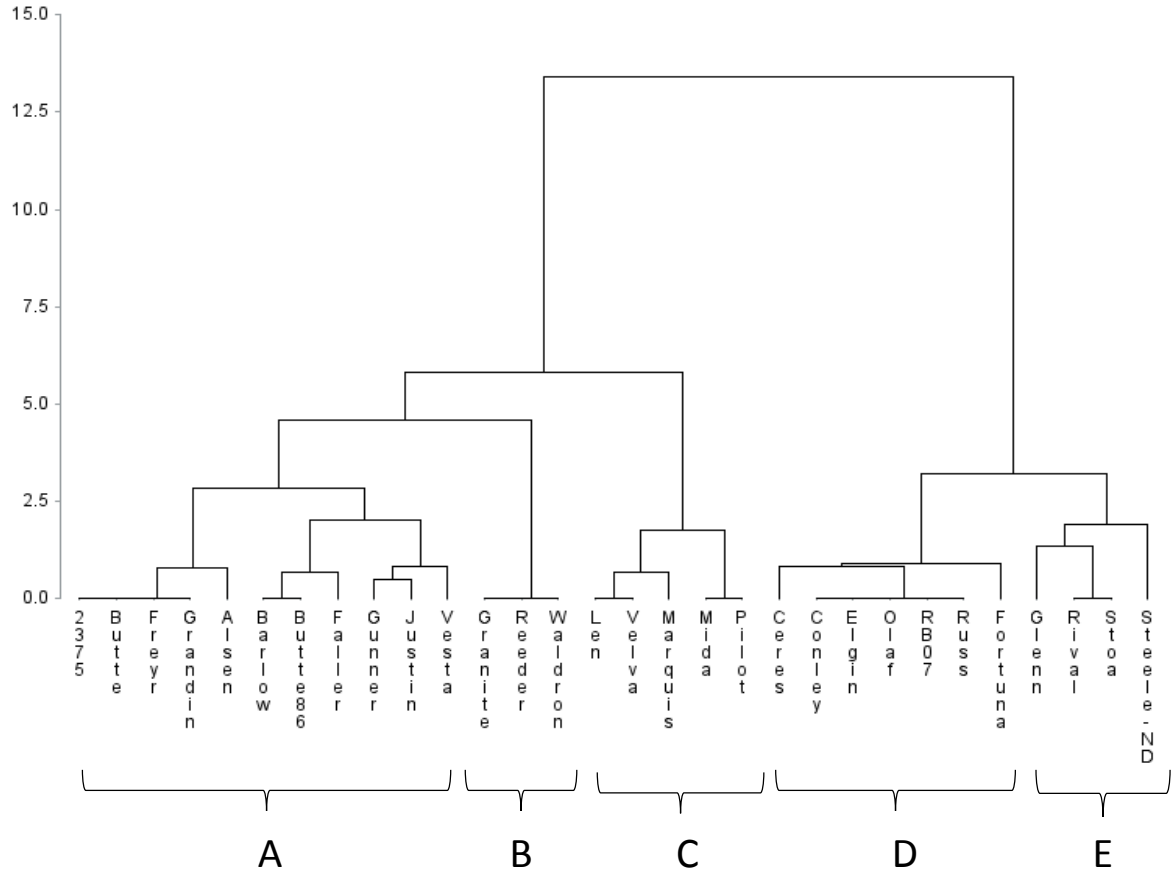

Figure 4.3. Dendrogram based on the presence of immunogenic peptides

At cutting point 2.7, the cluster analysis produced 5 clusters. The release year of the cultivars in the different clusters is as follows, cluster A: 1942 – 2009, B: 1969 – 2002, C: 1936 – 2012, D: 1926 – 2013, E: 1939 – 2005. As such, in this dendrogram the cultivars did not cluster according to release year. The cultivars in any of the clusters formed do not represent only modern or historical wheat cultivars. The ANOVA test showed that there is no significant difference ($P \geq 0.05$) between clusters regarding release year. Thus, the results suggest that the presence or absence of immunogenic peptides is independent of release year, indicating that historical and modern wheat may not be different in terms of CD toxicity.

The ANOVA test showed significant differences ($P \geq 0.05$) between clusters with regard to the area of peak 7 (γ- gliadin peak). That is, cluster B was significantly different than the other clusters. The immunogenic peptide QGSFQPSQQ was not detected in four of the thirty cultivars that were analyzed, and three of these cultivars are present in cluster B. This could be the cause for the significant difference that was observed. Thus, further studies could be performed on this peak to determine its protein composition.

As discussed earlier in the RP-HPLC analysis of the gliadin proteins, several peaks in the ω- and γ- gliadin regions increased with release year, however, the results indicated that the amount of α/β-gliadin proteins did not vary significantly through the years. Thus, some ω- and γ-gliadin immunogenic peptides may have increased in amount over the years. Therefore, although the qualitative analysis gives some indication about immunogenic peptides in historical and modern wheat, quantitative analysis should be performed to determine changes in CD toxicity more accurately.

Some peptides may not have been detected due to limitations in the instrument or the method used for the analysis. Therefore, non-detection of a peptide does not imply that the

peptides are absent in the gliadin extract. In order to address this issue, the analysis method can be modified and also the sample could be run on a different mass spectrometer. Additionally, different modes could be used, for example Parallel Reaction Monitoring (PRM) or Multiple Reaction Monitoring (MRM) modes could be tested.

One factor that could contribute towards determining which peptides are detected is the enzyme used in the digestion of the extracted protein sample. Studies have found that trypsin does not produce peptides with appropriate mass, compared to chymotrypsin, for detection through mass spectrometry, specifically matrix-assisted laser/desorption/ ionization time-of-flight (MALDI-TOF) (Salplachta et al. 2005). This is because cereal proteins are low in lysine and arginine, thus trypsin has limited cleavage sites. Chymotrypsin cleavage sites are C-terminal to phenylalanine, tryptophan and tyrosine, if the next amino acid is not proline. Chymotrypsin also cleaves at leucine, methionine, alanine, aspartic acid and glutamic acid at a lower rate (van den Broeck et al. 2015). Moreover, Mitea et al. (2010) showed that a single substitution of P to S in the peptide sequence could result in the inactivation of a certain peptide, removing its toxicity.

In a study conducted by van den Broeck et al. (2015), synthetic peptides corresponding to peptides of interest were used to quantify the amount of immunogenic peptides. The first two α/β-gliadin peptides were quantified in this experiment (PFPQPQLPY and RPQQPYPQ). The results showed that a modern hexaploid wheat cultivar ‘Toronto’ contained a higher amount of the immunogenic peptides in comparison to an older hexaploid ‘Minaret’ and a tetraploid landrace ‘Dibillik’. Thus, these results indicate that modern wheat is more toxic than historical wheat. However, only three wheat varieties were investigated in this experiment, thus it is not possible to draw broad conclusions.

Immunoblotting studies have also been conducted to determine difference in CD toxicity in historical and modern wheat varieties. Van den Broeck et al. (2010b) used monoclonal antibodies against two CD epitopes (PFPQPQLPY and FRPQQPYPQ) to determine differences in toxicity in 36 modern European wheat varieties and 50 landraces (representing wheat varieties from a century ago). The results indicated that the presence of the PFPQPQLPY peptide is higher in modern wheat in comparison to landraces raising questions as to whether breeding practices have contributed towards increasing CD toxicity in wheat. However, the amount of the peptides was low in some modern wheat varieties, and such samples could be used for breeding low CD toxic wheat. Monoclonal antibodies against the above mentioned peptides were also used by Mitea et al. (2008) and Spaenij-Dekking et al. (2004) in similar studies with the goal of detecting T-cell stimulatory immunogenic peptides.

Prandi et al. (2014) conducted a study to determine celiac disease toxicity in durum wheat varieties using an isotopically labelled internal standard for peptide PFPQPQLPY. In this experiment pepsin, trypsin and chymotrypsin were used for the digestion of gliadin proteins extracted using the 70% ethanol method. The results indicated that there is considerable variability in the amount of immunogenic peptides among the wheat cultivars that were investigated, and that environment has very low effect in comparison to genetic factors. In a similar study done by the same group, the CD toxicity of *Triticum durum* and *Triticum aestivum* were compared (Prandi et al. 2012). It was found that the content of pathogenic peptides have positive correlations with total protein content and the amount of gliadin. Additionally, the amount of immunogenic peptides in *Triticum durum* was found to be lower than that of *Triticum aestivum*, and high variability was observed in the different durum samples that were analyzed.

Thus, these studies indicate that the quantitation of immunogenic peptides is important in determining the CD toxicity of wheat cultivars.

Furthermore, Gregorini et al. (2009) also attempted to determine the difference in toxicity in historical and modern durum wheat. In this study, two methods were used: enzyme linked immunosorbent assay (ELISA) and Western blot. Two monoclonal antibodies for α-gliadins were used in this process. They found that the ancient wheat is toxic, and that this could be because ancient wheat contain a higher amount of protein than modern wheat. Interestingly, the higher amounts of α-gliadin were detected in the ancient wheat varieties that were analyzed. Additionally, the results suggested that both modern and ancient wheat share common immunological features and molecular characteristics.

Cellular assays have also been used to determine the CD toxicity of wheat cultivars. Gianfrani et al. (2012) conducted a study to evaluate the CD toxicity of two diploid wheat cultivars (*Triticum monococcum* ssp. *monococcum*) using in vitro assays to measure the production of interferon-γ, and also organ cultures of jejunal biopsies from CD patients to determine the innate and adaptive immune response produced by enzymatically digested (pepsin and trypsin) gliadin extracts. The results indicated that the two diploid wheat cultivars were toxic to CD patients, however, one of the varieties was concluded to be less toxic than the other since it did not trigger the innate immune response. Therefore, this is an indication that ancient diploid wheat and modern wheat derived from these less toxic varieties could have less CD toxicity overall. Thus, the quantification of the immunogenic peptides is vital in drawing conclusions.

In the same manner, intestinal biopsies from CD patients have been used to assess toxicity of ancient and modern wheat. Suligoj et al. (2013) found that wheat varieties trigger the T-cell response associated with CD irrespective of their origin (ancient/ modern) or ploidy.

Additionally, they found that response to these peptides is heterogeneous among CD patients. Thus, this result brings another complicated aspect to this study, as each CD patient has a unique response when immunogenic peptides are introduced. Therefore, to fully understand if a certain wheat variety is toxic multiple T-cell lines from different CD patients should be used.

4.5. Conclusions

In this experiment the toxicity in relation to CD was tested in historical and modern hard red spring wheat cultivars released in North Dakota from 1910 to 2013. The objective of the study was to determine if modern wheat is more toxic than historical wheat. Two methods were used to reach this objective. First, RP-HPLC was used to determine how the gliadin profiles changed in relation to release year. And second, mass spectrometry analysis was performed to determine if immunogenic peptides causing celiac disease are present in the wheat cultivars being investigated. The RP-HPLC experiments showed that certain peaks corresponding to ω-gliadin and γ-gliadin showed positive correlations ($P \leq 0.01$) with release year, whereas peaks corresponding to α/β-gliadins did not show any significant correlation. Thus, the amount of ω- and γ- gliadins varied over the years, while the amount of α/β-gliadins did not vary significantly. As per the LC-MS experiment, immunogenic peptides were detected in both historical and modern spring wheat cultivars irrespective of release year. Thus, the results indicate that peptides causing CD can be detected in historical and modern hard red spring wheat cultivars irrespective of release year indicating that there might be no difference in terms of CD toxicity between historical and modern spring wheat.

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CHAPTER 5. GENERAL CONCLUSIONS

This study was focused on gluten forming proteins found in wheat. Gluten forming proteins are composed of gliadin and glutenin proteins. Thirty HRS wheat cultivars were investigated in terms of differences in their protein profile, specifically gliadin protein profiles, and how these differences are related to release year. The relationship between the protein profiles and quality characteristics was also analyzed. For this purpose, protein separation methods, such as SE-HPLC and RP-HPLC were used, and standard AACC-I methods were used to assess quality parameters (kernel, milling, flour, dough and baking quality characteristics). Additionally, cluster analysis was performed to determine how the 30 HRS wheat cultivars are clustered when parentage and protein separation data are considered. Two *Rht* genes, *Rht-B1* and *Rht-D1* were also considered in this analysis. The clusters were also analyzed for differences in quality characteristics. The final component of the study looked at immunogenic peptides causing celiac disease. The presence or absence of 15 immunogenic peptides was tested in the HRS wheat cultivars using a proteomics approach.

The total protein content of the wheat cultivars investigated did not show a significant correlation with release year. Thus, the amount of protein in the kernels did not change significantly over the years. However, quality parameters, such as farinograph peak time and stability, improved with year indicating that the quality of proteins improved over the years resulting in better dough handling properties. The region corresponding to glutenins and ω-gliadin in SE-HPLC showed significant positive correlations ($P \leq 0.05$) with release year and also with dough quality characteristics. Thus, these proteins increased in amount over the years, and also may have contributed towards favorable dough characteristics. Additionally, 3 peaks in the ω-gliadin region of RP-HPLC showed significant positive correlations ($P \leq 0.05$) with year,

and of these two peaks showed significant positive correlations ($P \leq 0.05$) with dough properties as well. Therefore, polymeric glutenin proteins and the ω-gliadin proteins in these peaks could be responsible for improvements in dough properties that are observed in modern HRS wheat.

The cluster analysis showed that the clusters formed are significantly different ($P \leq 0.05$) with reference to release year when pedigree data and HPLC (SE-HPLC and RP-HPLC) area data are considered. In the dendrogram based on pedigree information, significant differences ($P \leq 0.05$) were found regarding the *Rht* genes, ash content and farinograph characteristics. The clusters based on the gliadin RP-HPLC profile did not show significant difference ($P \leq 0.05$) with year indicating that the profile itself did not vary in relation to year. However, the clusters based on the HPLC area data showed significant differences ($P \leq 0.05$) with year which indicated that the amount of the gliadin proteins changed with year. Farinograph characteristics also showed the same trend. Overall, the results show that the clusters formed based on different characteristics vary in several aspects. Additionally, it appears that the introduction of the *Rht* genes did not change the gliadin protein composition.

The mass spectrometric analysis showed that immunogenic peptides causing CD are present in both historical and modern spring wheat. The RP-HPLC analysis of the gliadin proteins indicated that the ω-gliadins showed significant positive correlations ($P \leq 0.05$) with release year, whereas the α-/β- and γ- gliadins did not. The cluster analysis based on the presence or absence on immunogenic peptides showed that there is no significant difference ($P \leq 0.05$) between the clusters formed in relation to year. Therefore, the presence of these peptides is not related to year, which indicates that there may not be a difference in terms of CD toxicity in historical and modern wheat.

CHAPTER 6. FUTURE WORK

Statistical analysis of SE-HPLC and RP-HPLC area data showed that the amount of ω-gliadin proteins increased with release year, and that these proteins could be associated with favorable dough properties that are seen in modern wheat cultivars. As future work, mass spectrometric analysis would be used to determine which proteins are present in the band corresponding to ω-gliadin in the SDS-PAGE separation of the SE-HPLC freeze dried protein fractions. In the same manner, peaks corresponding to ω-gliadins in RP-HPLC which showed significant correlations with release year would also be analyzed. These experiments would enable the identification of the proteins in these specific fractions more accurately.

Specific genetic markers were used to detect the presence of the dwarfing genes *Rht-B1* and *Rht-D1* in this study. However, other reduced height genes, such as the *Rht-8* gene, could be responsible for semi-dwarf phenotypes. Thus, as next steps, specific markers for *Rht-8* would be used in the genetic analysis of the wheat cultivars being investigated. This analysis would help broaden the study and draw better conclusions about the genes responsible for the semi-dwarf characteristics. The data could also be used the assess the effect of the different dwarfing genes *Rht-B1, Rht-D1* and *Rht-8* on wheat quality parameters, such as farinograph characteristics and baking quality parameters such as, loaf volume, grain and texture and crust color.

The quantitative analysis of the immunogenic peptides is needed to firmly establish differences in toxicity in historical and modern spring wheat. The quantification experiment would be focused on immunogenic peptides originating from α-gliadin proteins due to several reasons. First, studies have shown that α-/β-gliadin proteins are more abundant than the other gliadin types. And second, high sequence variability of the ω- and γ- sequences leads to the formation of many different peptides which makes quantitation a difficult task. Thus, two

immunogenic epitopes of the α-/β-gliadin proteins, PFPQPQLPY and FRPQQPYPY, would be quantified using heavy labelled peptides as internal standards in future experiments. The total amount of α-/β-gliadins would also be quantified using a peptide sequence common in these gliadins. Following the quantification of the α-/β-gliadin proteins and the two immunogenic peptides, the toxicity of the wheat cultivars could be assessed in numerical terms. Thus, it would be possible to conclude if there is a difference in terms of celiac disease toxicity between historical and modern wheat. If less toxic wheat cultivars are identified, these findings could be used by breeders to produce wheat cultivars which are less or non-toxic to celiac disease subjects.

APPENDIX

Table A1. Pedigree information about the wheat varieties used in the study

	Wheat line	Release year	Released by	Pedigree
1	Marquis	1910	Canada	Hard red culcutta, Red fife
2	Ceres	1926	NDSU	Hard red culcutta, Red fife, Kota
3	Pilot	1939	NDSU	Hard red culcutta, Red fife, Kota, Yaroslav emmer
4	Rival	1939	NDSU	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Alpha 1888
5	Vesta	1942	NDSU	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Alpha 1888
6	Mida	1944	NDSU	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Kanred, Iumillo, Florence AUS
7	Conley	1955	NDSU	Hard red culcutta, Red fife, Yaroslav emmer, Kanred, Iumillo, Timstein, Redman, Exchange, McMurachy
8	Justin	1963	NDSU	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Kanred, Iumillo, Florence AUS, Timstein, Redman, Exchange, McMurachy, Kenya Farmer
9	Fortuna	1966	NDSU	Hard red culcutta, Red fife, Yaroslav emmer, Kanred, Iumillo, Kenya-58, Mentana, Fronteira, S-615-11, S-615 CAN
10	Waldron	1969	NDSU	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Kanred, Iumillo, Florence AUS, Timstein, Redman, Exchange, McMurachy, Kenya Farmer

Table A1. Pedigree information about the wheat varieties used in the study (continued)

	Wheat line	Release year	Released by	Pedigree
11	Olaf	1972	NDSU	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Kanred, Iumillo, Florence AUS, Timstein, Redman, Exchange, McMurachy, Kenya Farmer, Kenya 338AA, Maria escobar, Brevor, Tohuku-34, Mayo-48, Egyptian-101
12	Butte	1977	NDSU	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Kanred, Iumillo, Florence AUS, Timstein, Redman, Exchange, McMurachy, Kenya Farmer, Kenya-58, Mentana, Fronteira, Maria escobar, Brevor, Tohuku-34, IL-1, Red-resaca, Webster, Kenya-73, P-54. Supresa, Kenya, Marroqui
13	Len	1979	NDSU	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Kanred, Iumillo, Florence AUS, Timstein, Redman, Exchange, McMurachy, Kenya Farmer, Kenya-58, Mentana, Fronteira, Kenya 338AA, Maria escobar, Brevor, Tohuku-34, IL-1, Red-resaca, Webster, Kenya-73, P-54, Red-Egyptian, Selkirk, Transfer
14	Stoa	1984	NDSU	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Kanred, Iumillo, Florence AUS, Timstein, Redman, Exchange, McMurachy, Kenya Farmer, Kenya-58, Mentana, Fronteira, Kenya 338AA, Maria escobar, Brevor, Tohuku-34, Mayo-48, Supresa, Kenya, Marroqui, Selkirk, Centana, Kenya 177-A, Peru, Egyptian-101, Estanzuela-Dakuru, TR.TI, Agen

Table A1. Pedigree information about the wheat varieties used in the study (continued)

	Wheat line	Release year	Released by	Pedigree
15	Butte86	1986	NDSU	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Kanred, Iumillo, Florence AUS, Timstein, Redman, Exchange, McMurachy, Kenya Farmer, Kenya-58, Mentana, Fronteira, Kenya 338AA, Maria escobar, Brevor, Tohuku-34, IL-1, Red-resaca, Webster, Kenya-73, P-54, Supresa, Kenya, Marroqui, Red-Egyptian, Selkirk, Transfer, Estanzuela-Dakuru
16	Grandin	1989	NDSU	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Kanred, Iumillo, Florence AUS, Timstein, Redman, Exchange, McMurachy, Kenya Farmer, Kenya-58, Mentana, Fronteira, Kenya 338AA, Maria escobar, Brevor, Tohuku-34, IL-1, Red-resaca, Webster, Kenya-73, P-54, Supresa, Kenya, Marroqui, Red-Egyptian, Selkirk, Transfer, Aguilera-8
17	2375	1990	NDSURF	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Kanred, Iumillo, Florence AUS, Timstein, Redman, Exchange, McMurachy, Kenya Farmer, Kenya-58, Mentana, Fronteira, Kenya 338AA, Maria escobar, Brevor, Tohuku-34, Mayo-48, Supresa, Kenya, Marroqui, Selkirk, Centana, Kenya 177-A, Egyptian-101, Aguilera-8

Table A1. Pedigree information about the wheat varieties used in the study (continued)

	Wheat line	Release year	Released by	Pedigree
18	Russ	1995	SD	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Kanred, Iumillo, Florence AUS, Timstein, Redman, Exchange, McMurachy, Kenya Farmer, Kenya-58, Mentana, Fronteira, Kenya 338AA, Maria escobar, Brevor, Tohuku-34, Mayo-48, IL-1, Red-resaca, Webster, Kenya-73, P-54, Supresa, Kenya, Marroqui, Red-Egyptian, Selkirk, Transfer, Centana, Kenya 177A, Egyptian-101, Aguilera-8, Gen. urquiza, Gabo, Klein-rendidor, ND-441, Argento, Parker, Cheyenne, Warrior, II-21031
19	Gunner	1995	Agripro	(Not available)
20	Reeder	1999	NDSU	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Kanred, Iumillo, Florence AUS, Timstein, Redman, Exchange, McMurachy, Kenya Farmer, Kenya-58, Mentana, Fronteira, Kenya 338AA, Maria escobar, Brevor, Tohuku-34, Mayo-48, IL-1, Red-resaca, Webster, Kenya-73, P-54, Supresa, Kenya, Marroqui, Red-Egyptian, Selkirk, Transfer, Centana, Kenya 177A, Peru, Egyptian-101, Estanzuela-Dakuru, TR.TI, Agen, Aguilera-8, FA-15-3, C.R, C.O, Santa Catalina, S-948-A-1, Colonias

Table A1. Pedigree information about the wheat varieties used in the study (continued)

	Wheat line	Release year	Released by	Pedigree
21	Alsen	2000	NDSU	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Kanred, Iumillo, Florence AUS, Timstein, Redman, Exchange, McMurachy, Kenya Farmer, Kenya-58, Mentana, Fronteira, S-615-11, S-615 CAN, Kenya 338AA, Maria escobar, Brevor, Tohuku-34, IL-1, Red-resaca, Webster, Kenya-73, P-54, Supresa, Kenya, Marroqui, Red-Egyptian, Selkirk, Transfer, Centana, Kenya 177A, Aguliera-8, Gen. urquiza, Gabo, C.R, C.O, Santa Catalina, S-948-A-1, Colonias, Sumai3, Queretaro, Mediterranean, Sinvalocho, Poole, Klein-record, L.S.31, Klein-titan, T.taushiischal, Stewart, Suwon-28-1
22	Granite	2002	WPB	Hard red culcutta, Red fife, Yaroslav emmer, Kanred, Iumillo, Timstein, McMurachy, Kenya-58, Mentana, Fronteira, Maria escobar, Brevor, Tohuku-34, Supresa, Marroqui, Aguliera-8, Gen. urquiza, Gabo, Klein-rendidor, C.R, C.O, Mediterranean, Klein-33, Lutescens-17, Neuzucht-14-44, HEGE-312-75-262, SAP, SO, Ladoga, Garant, Riga-M, Weihenstephaner-KJ, Hussar

Table A1. Pedigree information about the wheat varieties used in the study (continued)

	Wheat line	Release year	Released by	Pedigree
23	Steele-ND	2004	NDSU	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Kanred, Iumillo, Florence AUS, Timstein, Redman, Exchange, McMurachy, Kenya Farmer, Kenya-58, Mentana, Fronteira, S-615-11, S-615 CAN, Kenya 338AA, Maria escobar, Brevor, Tohuku-34, , Mayo-48, IL-1, Red-resaca, Webster, Kenya-73, P-54, Supresa, Kenya, Marroqui, Red-Egyptian, Selkirk, Transfer, Centana, Kenya 177A, Peru, Egyptian-101, Estanzuela-Dakuru, TR.TI, Agen, Aguilera-8, FA-15-3, C.R, C.O, Santa Catalina, S-948-A-1, Colonias, T.taushiischal, Stewart, Suwon-28-1
24	Freyr	2004	Agripro	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Kanred, Iumillo, Florence AUS, Timstein, Redman, Exchange, McMurachy, Kenya Farmer, Kenya-58, Mentana, Fronteira, Kenya 338AA, Maria escobar, Brevor, Tohuku-34, Mayo-48, IL-1, Red-resaca, Webster, Kenya-73, P-54, Supresa, Kenya, Marroqui, Selkirk, Centana, Kenya 177A, Egyptian-101, Aguilera-8, Gen. urquiza, Gabo, Sumai3, Queretaro, Mediterranean, Sinvalocho, Klein-titan, Klein-33, HS-80-0401, IRN-46, Heines-VII, HS-81-0074

Table A1. Pedigree information about the wheat varieties used in the study (continued)

	Wheat line	Release year	Released by	Pedigree
25	Glenn	2005	NDSU	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Kanred, Iumillo, Florence AUS, Timstein, Redman, Exchange, McMurachy, Kenya Farmer, Kenya-58, Mentana, Fronteira, S-615-11, S-615 CAN, Kenya 338AA, Maria escobar, Brevor, Tohuku-34, Mayo-48, IL-1, Red-resaca, Webster, Kenya-73, P-54, Supresa, Kenya, Marroqui, Red-Egyptian, Selkirk, Transfer, Centana, Kenya 177A, Peru, Egyptian-101, Estanzuela-Dakuru, TR.TI, Agen, Aguilera-8, FA-15-3, C.R, C.O, Santa Catalina, S-948-A-1, Colonias, Sumai3, T.taushiischal, Stewart, Suwon-28-1
26	Faller	2007	NDSU	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Kanred, Iumillo, Florence AUS, Timstein, Redman, Exchange, McMurachy, Kenya Farmer, Kenya-58, Mentana, Fronteira, S-615-11, S-615 CAN, Kenya 338AA, Maria escobar, Brevor, Tohuku-34, Mayo-48, IL-1, Red-resaca, Webster, Kenya-73, P-54, Supresa, Kenya, Marroqui, Red-Egyptian, Selkirk, Transfer, Centana, Kenya 177A, Peru, Egyptian-101, Estanzuela-Dakuru, TR.TI, Agen, Aguilera-8, Gen. urquiza, Gabo, C.R, C.O, Santa Catalina, S-948-A-1, Colonias, Sumai3, Queretaro, Mediterranean, Sinvalocho, Klein-record, L.S.31, Klein-titan, T.taushiischal, Stewart, Suwon-28-1

Table A1. Pedigree information about the wheat varieties used in the study (continued)

	Wheat line	Release year	Released by	Pedigree
27	RB07	2007	MN	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Kanred, Iumillo, Florence AUS, Timstein, Redman, Exchange, McMurachy, Kenya Farmer, Kenya-58, Mentana, Fronteira, S-615-11, S-615 CAN, Maria escobar, Brevor, Tohuku-34, IL-1, Red-resaca, Webster, Kenya-73, P-54, Supresa, Kenya, Marroqui, Selkirk, Centana, Kenya 177A, Aguilera-8, Sinvalocho, T.taushiischal, Stewart, Suwon-28-1, W-8814, Century, CO-695552
28	Barlow	2009	NDSU	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Kanred, Iumillo, Florence AUS, Timstein, Redman, Exchange, McMurachy, Kenya Farmer, Kenya-58, Mentana, Fronteira, S-615-11, S-615 CAN, Kenya 338A, Maria escobar, Brevor, Tohuku-34, Mayo-48, IL-1, Red-resaca, Webster, Kenya-73, P-54, Supresa, Kenya, Marroqui, Red-Egyptian, Selkirk, Transfer, Centana, Kenya 177A, Peru, Egyptian-101, Estanzuela-Dakuru, TR.TI, Agen, Aguilera-8, FA-15-3, C.R, C.O, Santa Catalina, S-948-A-1, Colonias, Sumai3, T.taushiischal, Stewart, Suwon-28-1

Table A1. Pedigree information about the wheat varieties used in the study (continued)

	Wheat line	Release year	Released by	Pedigree
29	Velva	2012	NDSU	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Kanred, Iumillo, Florence AUS, Timstein, Redman, Exchange, McMurachy, Kenya Farmer, Kenya-58, Mentana, Fronteira, S-615-11, S-615 CAN, Kenya 338A, Maria escobar, Brevor, Tohuku-34, Mayo-48, IL-1, Red-resaca, Webster, Kenya-73, P-54, Supresa, Kenya, Marroqui, Red-Egyptian, Selkirk, Transfer, Centana, Kenya 177A, Peru, Egyptian-101, Estanzuela-Dakuru, TR.TI, Agen, Aguilera-8, FA-15-3, C.R, C.O, Santa Catalina, S-948-A-1, Colonias, T.taushiischal, Stewart, Suwon-28-1
30	Elgin	2013	NDSU	Hard red culcutta, Red fife, Kota, Yaroslav emmer, Kanred, Iumillo, Florence AUS, Timstein, Redman, Exchange, McMurachy, Kenya Farmer, Kenya-58, Mentana, Fronteira, S-615-11, S-615 CAN, Kenya 338A, Maria escobar, Brevor, Tohuku-34, Mayo-48, IL-1, Red-resaca, Webster, Kenya-73, P-54, Supresa, Kenya, Marroqui, Red-Egyptian, Selkirk, Transfer, Centana, Kenya 177A, Peru, Egyptian-101, Estanzuela-Dakuru, TR.TI, Agen, Aguilera-8, Parker, Cheyenne, Warrior, II-21031, FA-15-3, C.R, C.O, Santa Catalina, S-948-A-1, Colonias, Sinvalocho, T.taushiischal, Stewart, Suwon-28-1, Pioneer-W-6744, ARG-5, Maraita

Table A2. Kernel quality characteristics of wheat samples (I)

Variety	Year	Wheat protein (%)	Wheat ash (%)	Test weight (lbs/bushel)	1000 kernel weight (g)	Sizing		
						Large	Medium	Small
2375	1990	14.0212	1.4902	58.9	40.7	77	22	1
Alsen	2000	14.6581	1.5191	60.1	34.7	73	26	0
Barlow	2009	14.6708	1.6205	59.7	33.1	59	40	0
Butte	1977	14.2638	1.6827	57.9	34.0	68	32	1
Butte86	1986	14.0362	1.4708	58.9	38.8	82	17	0
Ceres	1926	14.1445	1.9321	54.5	28.4	22	74	4
Conley	1955	14.9274	2.1427	50.9	29.9	44	51	1
Elgin	2013	14.5731	1.6552	56.8	32.3	58	41	1
Faller	2007	13.4619	1.5306	59.4	35.8	78	22	0
Fortuna	1966	14.6516	1.6808	55.7	36.8	50	49	2
Freyr	2004	14.7886	1.4930	59.7	39.8	77	23	0
Glenn	2005	14.6875	1.6011	61.8	33.9	62	38	0
Grandin	1989	14.5553	1.4887	58.5	36.5	75	25	1
Granite	2002	15.1514	1.4754	60.6	33.7	65	34	1
Gunner	1995	14.9381	1.6591	58.9	33.3	54	45	1
Justin	1963	14.8467	1.8281	53.5	30.5	56	43	1
Len	1979	14.6079	1.7111	56.1	35.5	62	37	0
Marquis	1910	14.9740	1.9503	54.5	28.9	47	52	2

Table A2. Kernel quality characteristics of wheat samples (I) (continued)

Variety	Year	Wheat protein (%)	Wheat ash (%)	Test weight (lbs/bushel)	1000 kernel weight (g)	Sizing Large	Medium	Small
Mida	1944	14.6444	2.1263	54.8	39.8	81	19	0
Olaf	1972	14.8706	1.9593	53.2	32.2	50	49	1
Pilot	1939	14.3812	1.8027	54.6	32.5	48	50	2
RB07	2007	14.7921	1.6996	58.7	33.4	54	45	1
Reeder	1999	13.7639	1.5527	59.3	35.8	67	32	1
Rival	1939	14.5783	1.9054	53.3	33.8	53	45	1
Russ	1995	14.0000	1.4557	59.0	42.0	89	10	0
Steele-ND	2004	14.1133	1.5373	60.3	37.0	80	20	0
Stoa	1984	13.2626	1.5941	57.2	33.8	62	38	1
Velva	2012	13.9227	1.5845	57.3	33.2	66	33	1
Vesta	1942	14.4037	1.9347	54.6	37.0	60	40	1
Waldron	1969	14.8693	1.8709	55.2	33.7	67	34	1

Table A3. Kernel quality characteristics of wheat samples (II)

Variety	Year	Hardness	Hardness StD	Weight (g)	Weight StD (g)	Diameter (mm)	Diameter StD (mm)
2375	1990	72.2444	13.8730	40.8556	9.2651	2.7283	0.4835
Alsen	2000	84.8327	14.2570	36.5682	7.7221	2.6657	0.4963
Barlow	2009	85.1283	14.4861	32.4951	7.4802	2.3715	0.4548
Butte	1977	71.3541	13.6775	33.7250	7.2790	2.4077	0.4350
Butte86	1986	73.7915	13.7226	37.8754	7.7321	2.6666	0.4465
Ceres	1926	76.0944	17.1157	29.6587	8.7704	2.1988	0.4284
Conley	1955	85.1348	19.3623	28.9845	8.0898	2.2380	0.4420
Elgin	2013	76.4215	17.4178	32.4338	8.7836	2.3714	0.4576
Faller	2007	79.2137	14.6859	35.8435	8.7444	2.6595	0.5049
Fortuna	1966	77.8533	17.1860	32.9398	9.4152	2.3650	0.5158
Freyr	2004	74.7709	12.5861	38.2554	7.1411	2.5492	0.4542
Glenn	2005	92.7010	14.0781	34.2404	7.4626	2.5439	0.4373
Grandin	1989	83.3135	13.5041	37.5333	6.4550	2.6976	0.4110
Granite	2002	79.6402	14.7858	33.2030	9.7883	2.4599	0.4854
Gunner	1995	84.3104	15.4010	33.0987	7.6321	2.4150	0.4388
Justin	1963	72.7787	15.5932	30.3380	7.5848	2.3268	0.4495
Len	1979	85.5144	15.2914	30.0034	8.2194	2.3040	0.4493
Marquis	1910	78.9596	15.7029	27.8723	7.7842	2.1913	0.4209

Table A3. Kernel quality characteristics of wheat samples (II) (continued)

Variety	Year	Hardness	Hardness StD	Weight (g)	Weight StD (g)	Diameter (mm)	Diameter StD (mm)
Mida	1944	68.6527	15.2878	41.0565	9.1269	2.7561	0.4514
Olaf	1972	80.3911	16.1283	31.5595	8.1516	2.3688	0.4516
Pilot	1939	67.3413	13.3560	33.8091	9.0947	2.4265	0.4380
RB07	2007	78.8864	14.0067	34.3387	6.4864	2.4330	0.4071
Reeder	1999	81.1614	14.4796	35.3534	8.4175	2.4749	0.4762
Rival	1939	81.5301	17.1491	33.1517	7.9299	2.4154	0.4646
Russ	1995	77.2115	13.5344	42.2292	9.2362	2.9342	0.5443
Steele-ND	2004	76.9781	13.1670	39.0372	8.0758	2.7818	0.4172
Stoa	1984	83.9920	14.6159	35.4450	7.8467	2.5156	0.4139
Velva	2012	82.7508	13.9448	32.9794	7.6104	2.3884	0.4204
Vesta	1942	65.5854	13.9006	38.4840	9.7533	2.5976	0.4628
Waldron	1969	75.6787	17.0049	33.8083	8.3584	2.4169	0.4481

Table A4. Milling and flour quality characteristics of wheat samples

Variety	Year	Flour yield (%)	Break flour yield (%)	Flour protein (%)	Flour ash (%)	Gluten index (%)	Wet gluten (%)	Color analysis		
								L*	a*	b*
2375	1990	71.1459	7.1723	12.5948	0.5242	89.2612	33.9790	90.2558	-0.7940	7.9043
Alsen	2000	71.8423	6.9187	13.2530	0.5289	97.7679	34.6790	90.0965	-1.0512	8.7899
Barlow	2009	72.6537	7.5243	13.3482	0.4918	96.8760	35.7875	90.6108	-1.0549	8.6584
Butte	1977	71.2408	8.2991	12.6182	0.4945	93.3795	35.7470	90.6908	-1.0549	8.1363
Butte86	1986	71.4402	8.6249	12.4306	0.4915	96.6352	33.0070	90.6858	-0.9969	8.3180
Ceres	1926	70.2419	7.5806	12.8096	0.6506	96.4326	34.7880	90.4554	-1.0220	8.3170
Conley	1955	68.4509	7.8670	13.4743	0.7129	94.8077	35.7365	89.9628	-0.5674	7.3601
Elgin	2013	71.5097	7.7922	13.3157	0.4851	98.1678	35.3115	90.6812	-1.0668	9.0966
Faller	2007	73.7261	7.8822	12.0638	0.5432	95.5514	34.1595	90.9216	-0.5833	7.0999
Fortuna	1966	70.4397	7.8990	13.4244	0.5955	91.8426	37.4450	91.0439	-1.0049	8.1253
Freyr	2004	71.9442	11.8950	13.2871	0.5117	92.2202	37.1655	90.7187	-1.2929	9.4839
Glenn	2005	71.9256	8.0097	13.1117	0.5279	99.3480	34.2465	90.9348	-0.9114	8.1530
Grandin	1989	71.4512	8.4060	12.9657	0.4922	78.5557	36.8550	90.4774	-0.7910	8.3861
Granite	2002	69.4805	8.6851	13.8199	0.5037	87.2885	38.4280	90.5783	-0.9899	8.6934
Gunner	1995	71.4751	7.4980	13.5534	0.5722	96.1419	36.2405	90.8738	-0.9967	8.3718
Justin	1963	67.5697	6.6932	13.6294	0.6015	98.0655	34.1015	91.2257	-0.8369	7.4809
Len	1979	70.9574	5.9533	13.1259	0.6123	99.2202	30.3740	90.6259	-0.9678	8.7803
Marquis	1910	66.9565	8.3794	13.4898	0.6920	94.1954	36.0255	91.0562	-0.8138	7.4809

Table A4. Milling and flour quality characteristics of wheat samples (continued)

Variety	Year	Flour yield (%)	Break flour yield (%)	Flour protein (%)	Flour ash (%)	Gluten index (%)	Wet gluten (%)	Color analysis		
								L*	a*	b*
Mida	1944	70.4564	7.6861	12.8603	0.7454	69.5564	36.0310	90.2276	-0.5743	6.7226
Olaf	1972	68.5110	7.4858	13.0833	0.6037	97.7549	32.0000	90.2866	-0.7007	8.0037
Pilot	1939	69.1874	8.5278	12.6746	0.5870	91.3376	35.2830	90.9389	-0.8876	7.3249
RB07	2007	70.5546	6.6069	13.0791	0.5466	96.9945	33.6920	91.1945	-0.7600	6.9365
Reeder	1999	68.2965	7.8864	11.9636	0.4820	98.4574	31.1270	91.0948	-1.0090	8.6364
Rival	1939	69.9683	7.1315	12.8095	0.7284	96.1145	32.1230	90.2405	-0.9739	8.2214
Russ	1995	70.0161	8.4405	12.2493	0.5408	96.6108	33.0080	90.7817	-1.2397	9.1537
Steele-ND	2004	71.4056	7.8715	12.4157	0.5105	97.7908	30.9525	90.7890	-1.1172	9.2343
Stoa	1984	70.3824	7.5671	11.3994	0.4961	98.7761	29.3095	91.0094	-1.0361	8.1988
Velva	2012	66.7478	7.9481	11.9101	0.4302	98.6509	30.3275	91.0562	-1.1115	9.0423
Vesta	1942	69.0380	8.8925	12.6841	0.6898	97.6897	32.3250	91.1011	-0.8030	7.1898
Waldron	1969	68.2220	6.8383	13.0552	0.6443	95.6614	33.9645	90.6318	-0.8580	8.0267

Table A5. Farinograph characteristics of wheat samples

Variety	Year	Farinograph absorption (%)	Farinograph peak time (min)	Farinograph stability (min)	MTI (BU)	Quality number (mm)
2375	1990	61.800	7.600	11.100	26.000	138.500
Alsen	2000	64.050	7.200	11.100	24.500	135.500
Barlow	2009	65.067	8.300	11.467	20.667	153.000
Butte	1977	61.750	5.950	7.350	29.500	110.000
Butte86	1986	62.350	7.200	9.050	31.500	121.500
Ceres	1926	59.600	4.850	6.650	34.000	91.500
Conley	1955	61.367	7.100	9.000	31.000	119.000
Elgin	2013	62.400	8.967	13.000	18.333	162.000
Faller	2007	60.133	6.500	10.433	22.667	124.667
Fortuna	1966	60.233	6.867	9.533	29.333	123.667
Freyr	2004	64.800	8.000	10.300	24.500	140.500
Glenn	2005	62.500	8.950	22.350	14.000	238.000
Grandin	1989	61.700	6.300	7.200	37.333	103.667
Granite	2002	62.850	8.850	10.200	22.000	155.000
Gunner	1995	61.900	6.650	10.500	21.500	136.500
Justin	1963	59.333	6.433	12.000	17.000	144.333
Len	1979	59.450	7.350	10.750	29.000	124.500
Marquis	1910	57.300	6.450	9.000	30.000	110.500
Mida	1944	59.450	4.100	4.450	49.500	68.000

Table A5. Farinograph characteristics of wheat samples (continued)

Variety	Year	Farinograph absorption (%)	Farinograph peak time (min)	Farinograph stability (min)	MTI (BU)	Quality number (mm)
Olaf	1972	59.400	6.133	10.233	26.667	116.333
Pilot	1939	57.600	5.700	7.850	33.000	100.500
RB07	2007	58.550	8.000	11.100	28.000	140.500
Reeder	1999	59.800	6.750	14.300	14.500	164.500
Rival	1939	60.650	4.800	7.550	31.500	93.500
Russ	1995	62.767	7.733	11.600	23.000	137.333
Steele-ND	2004	61.700	6.800	14.867	12.333	162.333
Stoa	1984	58.200	6.250	14.950	14.500	149.500
Velva	2012	58.900	8.133	17.000	19.000	171.333
Vesta	1942	56.567	8.000	12.967	23.667	145.000
Waldron	1969	57.150	5.750	8.700	30.500	101.000

Table A6. Baking quality characteristics of wheat samples

Variety	Year	Bake water absorption (%)	Bake mixing time (min)	Dough Score	Loaf volume (cm3)	Grain and Texture	Crumb Color
2375	1990	66.60	4.125	9.0	912.5	8.00	8.00
Alsen	2000	68.70	4.375	10.0	890.0	7.25	7.00
Barlow	2009	70.20	3.750	9.5	1010.0	7.50	7.25
Butte	1977	68.55	3.500	8.5	885.0	7.75	7.75
Butte86	1986	69.20	3.625	9.0	882.5	7.50	7.50
Ceres	1926	67.60	4.000	9.0	982.5	8.00	8.00
Conley	1955	69.65	4.250	9.5	970.0	7.75	8.00
Elgin	2013	67.50	4.250	9.5	1027.5	7.75	7.50
Faller	2007	66.50	4.375	10.0	960.0	7.75	8.00
Fortuna	1966	66.85	4.250	10.0	1022.5	8.00	7.75
Freyr	2004	72.00	3.625	8.5	942.5	7.75	7.25
Glenn	2005	70.50	5.250	9.0	967.5	7.25	7.25
Grandin	1989	68.70	3.500	8.5	1000.0	8.25	8.00
Granite	2002	70.40	3.750	9.0	902.5	7.00	7.50
Gunner	1995	69.70	3.750	9.5	992.5	8.25	8.25
Justin	1963	64.90	4.500	10.0	1032.5	8.50	8.25
Len	1979	65.60	5.000	10.0	980.0	8.00	8.00

Table A6. Baking quality characteristics of wheat samples (continued)

Variety	Year	Bake water absorption (%)	Bake mixing time (min)	Dough Score	Loaf volume (cm3)	Grain and Texture	Crumb Color
Marquis	1910	63.50	3.875	9.0	995.0	8.25	8.00
Mida	1944	66.50	3.500	8.0	912.5	8.00	8.25
Olaf	1972	67.10	4.000	10.0	995.0	7.75	8.25
Pilot	1939	66.25	3.500	9.0	1000.0	8.25	7.50
RB07	2007	66.55	4.000	9.5	1027.5	7.00	7.00
Reeder	1999	64.70	4.500	10.0	915.0	7.00	7.25
Rival	1939	66.30	4.750	9.5	1010.0	8.00	7.75
Russ	1995	69.00	4.250	9.5	932.5	7.25	7.50
Steele-ND	2004	68.20	4.250	9.5	975.0	7.25	7.50
Stoa	1984	65.70	4.625	10.0	935.0	7.50	8.00
Velva	2012	66.10	4.625	9.5	995.0	6.75	7.50
Vesta	1942	63.90	5.000	10.0	1010.0	7.50	8.50
Waldron	1969	65.70	3.500	10.0	1015.0	8.00	8.00

Table A7. Size-exclusion-HPLC area data

Variety	Year	Protein content (% flour)									
		Ef1	Ef2	Ef3	Ef4	Ef5	Uf1	Uf2	Uf3	Uf4	Uf5
2375	1990	1.3577	1.2625	0.7355	4.1870	1.6370	1.3466	1.1288	0.2066	0.5124	0.2206
Alsen	2000	1.3914	1.3119	0.8936	4.2224	1.5428	1.6623	1.2620	0.2232	0.5321	0.2114
Barlow	2009	1.3167	1.1820	0.8462	4.6411	1.6139	1.5427	1.2225	0.2178	0.5468	0.2186
Butte	1977	1.3511	1.2366	0.7447	4.3065	1.5664	1.4120	1.1192	0.1948	0.4898	0.1971
Butte86	1986	1.3264	1.2215	0.7416	4.1992	1.4897	1.4131	1.1401	0.2013	0.4965	0.2013
Ceres	1926	1.5216	1.2095	0.6279	4.6154	1.5956	1.3232	1.0679	0.1824	0.4790	0.1871
Conley	1955	1.5330	1.2962	0.7057	4.8471	1.6499	1.4067	1.1359	0.1961	0.5010	0.2028
Elgin	2013	1.3184	1.2065	0.8584	4.3827	1.6257	1.6531	1.2738	0.2238	0.5457	0.2276
Faller	2007	1.3338	1.1643	0.7212	3.8665	1.5873	1.4474	1.1035	0.1907	0.4552	0.1940
Fortuna	1966	1.4898	1.2749	0.6323	4.9647	1.6229	1.4216	1.0910	0.1894	0.5255	0.2123
Freyr	2004	1.2675	1.1325	0.8417	5.0844	1.4272	1.4343	1.1295	0.2063	0.5466	0.2172
Glenn	2005	1.2135	1.1440	0.7840	4.2851	1.5014	1.8241	1.3366	0.2295	0.5631	0.2304
Grandin	1989	1.3650	1.1825	0.8216	4.9142	1.5261	1.2771	1.0292	0.1841	0.4697	0.1962
Granite	2002	1.2070	1.0045	0.7921	5.8087	1.7747	1.3423	0.9818	0.1850	0.5188	0.2051
Gunner	1995	1.4377	1.1465	0.7747	5.0613	1.4842	1.5161	1.1506	0.2034	0.5667	0.2122
Justin	1963	1.4720	1.2905	0.7707	4.9331	1.5374	1.5102	1.1690	0.2036	0.5301	0.2128
Len	1979	1.4688	1.3169	0.6947	4.2508	1.5676	1.6716	1.2547	0.2060	0.4916	0.2030
Marquis	1910	1.4798	1.2949	0.7376	5.0555	1.5680	1.3372	1.1068	0.1948	0.5071	0.2080
Mida	1944	1.4450	1.3167	0.6629	4.9725	1.5798	1.1244	0.9315	0.1663	0.4648	0.1965

Table A7. Size-exclusion-HPLC area data (continued)

Variety	Year	Protein content (% flour)									
		Ef1	Ef2	Ef3	Ef4	Ef5	Uf1	Uf2	Uf3	Uf4	Uf5
Olaf	1972	1.4805	1.3282	0.6751	4.3848	1.6686	1.4703	1.1528	0.1970	0.5026	0.2234
Pilot	1939	1.3938	1.2207	0.6305	4.7038	1.5334	1.2851	1.0404	0.1806	0.4842	0.2022
RB07	2007	1.3415	1.2206	0.6644	4.3324	1.5803	1.6145	1.2644	0.2206	0.5575	0.2829
Reeder	1999	1.2026	1.1708	0.7932	3.7955	1.4869	1.4638	1.1580	0.2022	0.4752	0.2154
Rival	1939	1.4906	1.2574	0.6349	4.6650	1.5071	1.3528	1.0645	0.1796	0.4579	0.1998
Russ	1995	1.3171	1.2322	0.7256	4.1121	1.4025	1.4691	1.1388	0.1924	0.4609	0.1988
Steele-ND	2004	1.2675	1.1665	0.6613	4.2594	1.4511	1.5078	1.1663	0.2034	0.5176	0.2149
Stoa	1984	1.1269	1.0714	0.6178	3.7191	1.4610	1.3963	1.1239	0.1913	0.4734	0.2183
Velva	2012	1.1773	1.1170	0.8146	3.6928	1.5154	1.5566	1.1696	0.2005	0.4577	0.2087
Vesta	1942	1.3543	1.1618	0.6481	4.6580	1.4034	1.4890	1.1165	0.1849	0.4724	0.1957
Waldron	1969	1.4847	1.2884	0.6144	4.4974	1.6474	1.4804	1.1433	0.1905	0.4926	0.2161

Table A8. Reverse-phase-HPLC area data

Variety	Year	Constant flour Peak absorbance area (mAu x min)						Constant protein Peak absorbance area (mAu x min)					
		Peak A	Peak B	PeakC	Peak D	Peak E	Peak F	Peak A	Peak B	PeakC	Peak D	Peak E	Peak F
2375	1990	87.15	90.92	10.56	9.51	190.86	10.10	88.16	95.92	9.71	11.63	291.89	24.07
Alsen	2000	90.96	180.58	23.57	7.34	311.59	7.92	95.96	192.48	26.34	9.88	470.69	15.26
Barlow	2009	131.64	131.06	10.04	7.87	57.33	74.36	216.82	217.60	11.46	9.78	60.23	91.32
Butte	1977	83.86	80.21	9.22	8.26	252.73	8.18	88.26	94.37	9.04	11.45	318.69	19.28
Butte-86	1986	93.88	89.64	7.41	11.90	191.13	59.13	139.03	113.78	10.01	9.94	214.11	48.05
Ceres	1926	65.89	46.60	9.20	23.24	221.99	6.92	98.76	70.28	10.22	30.26	289.65	19.11
Conley	1955	53.29	70.25	7.51	9.98	304.15	7.78	77.69	83.39	9.07	11.86	313.65	9.11
Elgin	2013	105.49	191.77	25.28	7.53	135.17	64.64	139.49	280.40	37.78	10.61	163.53	77.73
Faller	2007	111.11	87.32	6.52	14.65	50.61	75.77	181.38	146.30	10.62	12.11	56.47	99.54
Fortuna	1966	58.48	52.18	6.47	13.75	214.58	49.50	129.99	97.28	9.42	11.28	307.98	28.28
Freyr	2004	152.94	106.88	6.43	6.80	45.10	123.34	297.28	196.56	8.49	9.74	52.85	130.54
Glenn	2005	100.58	142.49	22.41	6.00	24.01	61.00	239.50	290.47	38.52	8.61	29.73	84.05
Grandin	1989	146.93	97.41	8.84	6.87	39.45	96.62	257.75	150.36	11.02	8.59	42.02	99.05
Granite	2002	21.79	18.29	7.44	8.47	268.63	22.76	39.82	25.67	8.98	14.82	435.62	64.31
Gunner	1995	92.76	55.70	8.71	7.01	49.41	86.72	153.26	73.01	10.64	8.46	55.94	90.73
Justin	1963	85.55	74.41	8.20	8.48	238.78	53.24	128.42	92.05	10.04	9.40	237.16	45.76
Len	1979	54.44	75.21	11.23	7.34	280.91	11.21	115.21	146.44	17.05	10.09	362.74	18.28
Marquis	1910	57.05	51.08	7.56	23.88	262.40	15.75	116.42	85.78	9.47	23.08	342.08	14.63

Table A8. Reverse-phase-HPLC area data **(**continued)

Variety	Year	Constant flour Peak absorbance area (mAu x min)						Constant protein Peak absorbance area (mAu x min)					
		Peak A	Peak B	PeakC	Peak D	Peak E	Peak F	Peak A	Peak B	PeakC	Peak D	Peak E	Peak F
Mida	1944	50.11	47.12	6.40	26.36	237.42	4.19	75.79	56.20	7.95	32.75	267.63	6.19
Olaf	1972	55.56	65.49	9.80	8.96	256.85	15.11	104.81	103.11	12.39	11.50	301.12	24.43
Pilot	1939	45.60	43.52	8.35	9.38	273.40	22.04	95.77	76.15	10.86	11.62	323.78	12.08
RB07	2007	76.52	129.01	30.12	6.43	39.20	63.93	143.26	231.08	50.57	8.80	49.91	118.87
Reeder	1999	76.49	165.77	25.71	7.29	254.93	2.72	141.17	266.63	38.83	8.65	273.36	7.80
Rival	1939	79.83	60.33	8.38	12.02	287.40	34.37	112.51	73.57	10.33	10.93	250.94	6.80
Russ	1995	64.65	86.47	6.92	10.25	283.72	13.41	121.80	183.10	11.54	13.94	384.46	17.85
Steele-ND	2004	80.59	56.79	8.13	7.36	52.81	65.48	154.97	84.76	10.52	9.01	58.46	91.65
Stoa	1984	88.53	71.26	9.87	10.42	238.88	11.47	156.56	125.59	13.82	13.19	260.03	22.23
Velva	2012	111.01	183.97	29.56	7.60	49.84	68.29	245.83	341.64	48.76	8.33	58.71	108.40
Vesta	1942	88.72	61.18	8.24	35.48	290.82	25.84	127.19	77.82	8.82	34.34	290.96	24.97
Waldron	1969	43.64	50.60	7.78	11.50	240.59	9.29	49.69	47.82	7.15	9.27	200.94	14.82