HISTORICAL AND MODERN HARD RED SPRING WHEAT COMPARISON: ANALYSIS OF PROXIMATE, ENZYME ACTIVITY, PHENOLIC ACID, AND STARCH PROPERTIES

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MASTER OF SCIENCE

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ABSTRACT

Some claim modern wheat is biochemically different from historic wheat and contributes to chronic diseases. This research was necessary to determine if any significant differences existed between historic and modern hard red spring (HRS) wheat in several physical and chemical components. Thirty HRS cultivars were grown in the same year and location and underwent laboratory analysis. In relation to release year, significant ($P<0.05$) differences were found for ash content (AC), phosphorous, potassium, zinc, arabinose-to-xylose ratio, enzyme activities, and phenolic acids in whole wheat, AC, and α-amylase and xylanase activities in white flour, and total starch in bread. The remaining parameters displayed no significant ($P>0.05$) differences in correlation to release year, including starch digestibility. The majority of results indicate no significant biochemical variations between the historic and modern HRS cultivars. Parameters that demonstrated differences have the potential to affect consumer health and nutrition, but are dependent on bioavailability and consumer choice.

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LIST OF ABBREVIATIONS

AACCI.....American Association of Cereal Chemists International

AC.....Ash content

AMG.....Amyloglucosidase

AOAC.....Association of Official Analytical Chemists

AX.....Arabinoxylans

A/X.....Arabinose to xylose ratio

BU.....Brabender unit

$CaCl_2$.....Calcium chloride

CF.....Crude fat

CVD.....Cardiovascular disease

Da.....Dalton

DB.....Dry weight basis

DF.....Dietary fiber

DM.....Diabetes mellitus

DMSO.....Dimethyl sulfoxide

EDTA.....Ethylenediaminetetraacetic acid

eGI.....Estimated glycemic index

GI.....Glycemic index

GL.....Glycemic load

GOPOD.....Glucose oxidase peroxidase

HCl.....Hydrochloric acid

HI.....Hydrolysis index

HPLC .....High performance liquid chromatography

HPSEC .....High performance size exclusion chromatography

HRS.....Hard red spring

IDF .....Insoluble dietary fiber

KOH.....Potassium hydroxide

MC .....Moisture content

MOPS.....3-[*N*-morpholino]propanesulfonic acid

Mw .....Molecular weight

NaN_3.....Sodium azide

PC.....Protein content

RDS.....Rapidly digestible starch

RI.....Refractive index

RPM .....Revolutions per minute

RS.....Resistant starch

SDF .....Soluble dietary fiber

SDS .....Slowly digestible starch

TFA .....Trifluoroacetic acid

TS .....Total starch

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CHAPTER 1. INTRODUCTION

Wheat (*Triticum aestivum* L.) is a very important grain both economically and as a food source. It is the third largest produced cereal crop in the world and is unrivaled in cultivation locations (Shewry 2009). Within the United States, North Dakota is the largest producer of hard red spring (HRS) wheat, which is known for its high protein content and superior gluten strength (NDWC 2013a). In an effort to boost food security efforts, farmers and wheat breeders have sought to increase the availability of wheat by improving crop yields through the use of advanced breeding or agronomic techniques (Gooding 2009).

However, many consumers are concerned that these same agricultural practices, while increasing yields, are decreasing the end-use and nutritional quality of wheat and wheat products. In fact, a few popular doctors/authors have gone so far to say that modern wheat cultivars are genetic mutations or toxic versions of historic wheat cultivars, and, as such, should be avoided (Cordain 2011; Perlmutter and Loberg 2013; Davis 2014). Additional claims by anti-wheat advocates inaccurately cite modern wheat as the cause of the many health problems experienced by the general populous in recent years. While several experts have addressed some of the misinformation surrounding these claims (Jones 2012; Aune et al. 2013; Brouns et al. 2013; Kasarda 2013; Giacco et al. 2014; Hajihashemi et al. 2014; Peña et al. 2014), the question still remains—did selection and breeding efforts cause a significant change between historic and modern wheat cultivars?

We hypothesize that the HRS wheat cultivars selected for this research will be significantly different from each other; however, we further hypothesize that the differences observed in the experimental data will not be related sufficiently enough to release year to distinguish between the historic and modern cultivars, even when controllable environmental

variables are removed or limited. In this research study, the thirty historic and modern HRS cultivars selected were grown in the same location and year, and were then analyzed in a laboratory for differences in several physical and chemical properties. Specifically, compositional quantification was sought for the ash, protein, lipid, fiber, starch, enzyme activity, and phenolic acid components of the wheat grain. Standard experimental techniques were used during analysis, including approved AACCI and AOAC methods, as well as accepted non-standard methods. All historical and modern cultivar parameters were analyzed for statistically significant differences, in regard to genotypes. The chemical and compositional comparisons performed in this study could help to prove or disprove claims that modern wheat cultivars are substantially different from historic cultivars. Moreover, while any discoveries made concerning meaningful differences between historic and modern wheat cultivars are important for wheat research, these results could also be used to advance research in other relevant fields of study.

CHAPTER 2. LITERATURE REVIEW

2.1. Wheat grain composition

The caryopsis of wheat (*Triticum aestivum* L.) (Figure 1), known commonly as a grain, is composed of three main parts: the bran, endosperm, and germ (Posner 2000; Bechtel et al. 2009). Bran is the commercial name for the outermost portion of the caryopsis and encompasses the pericarp (fruit coat) and seed coat. The pericarp is further broken down into the outer and inner pericarp; the outer pericarp is comprised of the epidermis, hypodermis, and thin-walled cells, while the inner pericarp consists of intermediate-size cells, cross cells, and tube cells (inner epidermis). The seed coat, on the other hand, includes the testa and nucellar epidermis (hyaline layer). Furthermore, the endosperm of the caryopsis includes the aleurone layer and starchy endosperm, and constitutes more than 80% of the mass in each wheat grain. The germ (embryo), which accounts for 2.5–3.5% of the grain, contains the embryonic axis and scutellum.

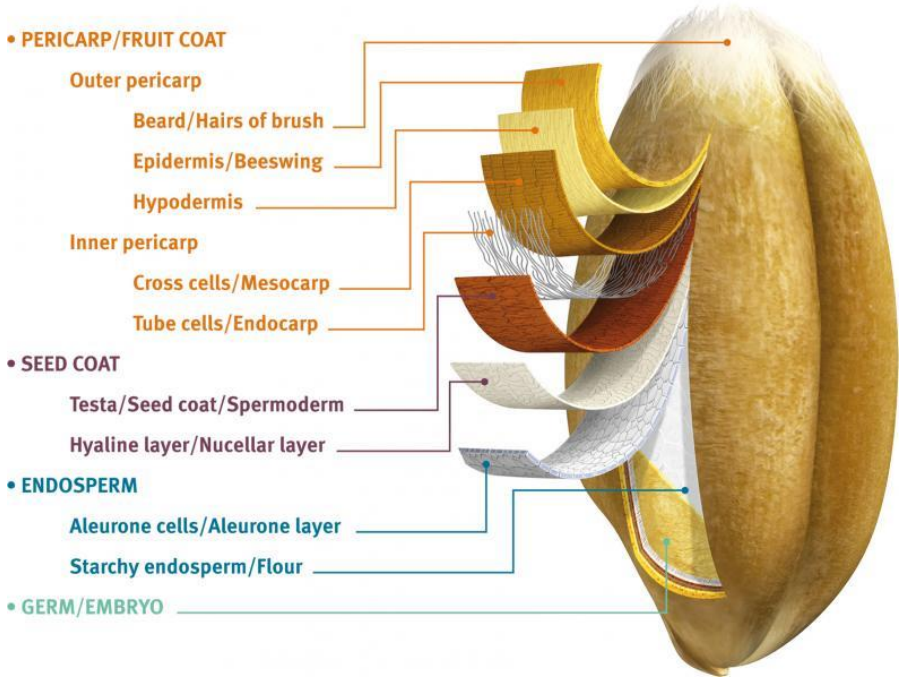


Figure 1. Wheat kernel composed of bran (pericarp and seed coat), endosperm, and germ. Reprinted from GoodMills Innovation GmbH; used with permission.

Several chemical characteristics of the caryopsis are typically evaluated prior to milling and baking—these are moisture, ash, protein, and enzymatic activity. Other components that can be measured include fats, carbohydrates (i.e. starch and cell wall polysaccharides), and phenolic compounds. The first four measurements are nearly always evaluated before milling and baking, since slight variations can greatly affect the efficiency, cost, and quality of each process. While the remaining components can certainly affect milling and baking performance, as will be overviewed below, they are usually assessed for reasons that pertain more to human health and nutrition than the production of wheat products.

Moisture content is the quantity of water in a caryopsis, where a moisture value of 14% or less is considered desirable. Moisture is an important parameter during the milling process, since it greatly affects the energy necessary to mill a batch of wheat, and the resulting flour quality (Carson and Edwards 2009). If the moisture is too high, the risk of spoilage from fungi or insects increases and the quality of the grain depreciates; this means that the flour made from that grain will also be of poorer quality. Another disadvantage to high moisture values is that the miller is paying the same price for less dry matter, when compared to the same quantity of wheat with a lower moisture value. However, if the moisture is too low, the miller must invest additional time and effort to temper the wheat to the proper moisture level to achieve the best separation of the bran and endosperm (Posner 2000).

Ash is the measurement of the mineral material in flour and is found mainly in the pericarp, aleurone, and germ layers of the caryopsis. Ash content is used to gauge mill technological performance, since the differences between the ash content in the bran, endosperm, and germ portions are used as a standard to determine the level of separation efficiency from each part (Posner 2000). Since the objective of milling is to achieve the highest flour extraction

with the lowest possible ash contamination from the bran and germ, this is a relatively important parameter for millers. Moreover, the ash content in flour can have an effect on the performance quality of baked goods. For instance, higher ash values have been found to decrease dough strength and baking performance in yeast breads, as well as make the flour color dim and drab (Carson and Edwards 2009).

Protein content consists of the amount of amino acid residues, which contain amine and carboxylic acid functional groups, found within the caryopsis. Protein content is an important parameter in baking, since the total protein content can be related to various facets of end-use quality (Shewry et al. 2009). For example, higher protein flours are capable of absorbing more water and have the potential to generate larger loaf volumes; this can result in more and bigger bread loaves produced when compared to bread loaves made from the same quantity of flour with a lower protein content (Carson and Edwards 2009). Typically, flours with both lower (7–11%) and higher (13–16%) protein contents are more expensive than flours with moderate protein values; low protein flours are used to make pastries, cookies, and crackers while high protein flours are commonly used in yeast breads, rolls, pizza crust, etc.

Enzymes are molecules (mostly proteins) that act as biological catalysts to help accelerate complex reactions. In addition, enzymatic activity refers to the quantity of active enzymes present and, as such, is dependent on the reaction conditions. There are several different types of enzymes in wheat, but the most important are the hydrolytic enzymes, which include starch-, nonstarch polysaccharide-, and protein-degrading enzymes (Brijs et al. 2009). Starch-degrading enzymes, such as α- and β-amylase, break down starch into smaller molecules, typically limit dextrins, maltotriose, maltose, and glucose (Delcour and Hoseney 2010a). α-Amylase, in particular, is very important for bakers; too little α-amylase activity and there won't be enough

sugars for fermentation, but too much α-amylase activity will produce sticky dough and loaves (Carson and Edwards 2009). Nonstarch polysaccharide-degrading enzymes, such as endoxylanase, are capable of hydrolyzing arabinoxylan into arabinoxylan oligosaccharides, xylobiose, and xylose (Delcour and Hoseney 2010b). The cell wall degradation caused by endoxylanase makes starch and protein more accessible to amylases and proteases, respectively, which can improve dough handling and bread quality. Finally, protein-degrading enzymes, such as protease and peptidase, hydrolyze proteins by breaking the peptide bond between two amino acid residues (Brijs et al. 2009; Delcour and Hoseney 2010c). Protease can be added by the baker to reduce dough strength, and improve dough handling and crumb texture (Carson and Edwards 2009). However, since gluten is essential for leavened breads, proteases are capable of seriously affecting the rheological properties of dough if the gluten proteins are overly degraded.

Lipids are made from fatty acids esterified to glycerol, and fatty acid derivatives that are soluble in organic solvents, and are concentrated primarily in the germ portion of the grain. Lipids are a minor constituent within the whole grain, only 2.5–3%, but they can affect everything from production, storage, and processing to nutrition and customer approval of wheat products (Chung et al. 2009). Since the level of free fatty acids in wheat is usually very low, if either the wheat or the flour is subjected to poor storage conditions (i.e. high temperature and/or high moisture), enzymes will degrade the native grain lipids and produce free fatty acids. As such, a free fatty acid index is considered a useful measure of the storage conditions of either the grain or the flour, since flours with higher free fatty acid index values are more susceptible to rancidity than flours with lower index values. This is of little importance in bread products, but quite important in dry products such as cookies, crackers, pretzels, etc.

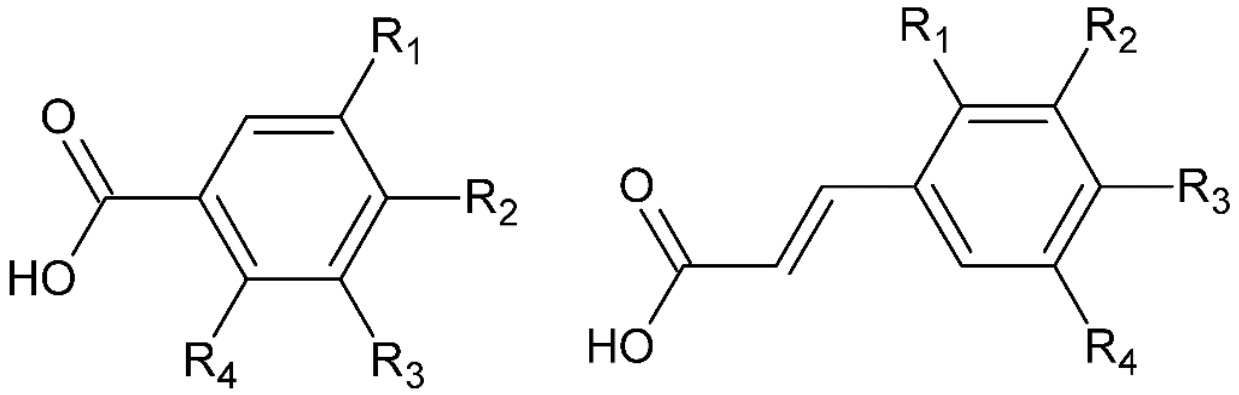
Carbohydrates are classified as polyhydroxy aldehydes or ketones, and their derivatives. Depending on their degree of polymerization, carbohydrates are also referred to as mono-, di-, oligo-, or polysaccharides, where "saccharide" simply indicates that the molecule is a sugar. In wheat, carbohydrates primarily contain starch and the cell wall polysaccharides, cellulose and arabinoxylan (Stone and Morell 2009). Starch and cellulose both have monosaccharide glucose units as their main constituent, but with differing glycosidic linkages, while arabinoxylan is made from the arabinose and xylose monosaccharides.

Starch consists of two glucose polymers, amylopectin and amylose, and is concentrated solely in the starchy endosperm portion of the caryopsis. Amylopectin, which constitutes 75% of starch, is composed of a branched structure and is digested relatively rapidly, while amylose, which constitutes 25% of starch, has a linear structure and is more slowly digested (Brouns et al. 2013). Aside from the gluten proteins in leavened goods, starch is the most important component in bread products; it absorbs water, provides yeast with additional nutrition for fermentation, gelatinizes during baking to retain the shape of the loaf, and provides glucose units for the Maillard reactions (Delcour and Hoseney 2010d). Furthermore, if starch is damaged during milling, water absorption in the dough will increase, starch molecules will be more accessible for yeast fermentation, and starch gelatinization during baking will occur more quickly (Stone and Morell 2009). While some starch damage can be desirable in most bread products, it is undesirable in dry products and pastries.

The cell wall polysaccharides, cellulose and arabinoxylan, are structural components of the caryopsis. Cellulose is very structurally similar to starch, but instead of the α-(1→4)-glycosidic bonds in starch, cellulose has β-(1→4)-glycosidic bonds. This difference in linkage allows cellulose chains to pack together tightly, which hydrogen bonds and van der Waals

interactions help to maintain, and to be insoluble in water and most organic solvents (Stone and Morell 2009). Arabinoxylans, also called heteroxylans or pentosans, are the major non-cellulose component of primary and secondary cell walls in the caryopsis. For decades, it was debated if arabinoxylan played any role during bread making; recently, it was determined that water-extractable arabinoxylan increases the viscosity of the aqueous phase within the dough and, thus, stabilizes the dough structure (Courtin and Delcour 2002; Delcour and Hoseney 2010d). Since arabinoxylan slows down the diffusion rate of carbon dioxide and aids in gas retention, this viscosity-enhancing property is especially useful in the oven, where the pressure the gases exert on the gas cells increases. Lastly, all cell wall polysaccharides contribute to the resilience and hardness of the bran and endosperm, which can impact the separation of the two during milling.

Phenolic acids are a class of phenolic compounds that contain an aromatic ring with one or more hydroxyl (OH) substituents present (Piironen et al. 2009; de Lourdes Reis Giada 2013). Depending on their carbon backbone, phenolic acids can be divided into two groups: benzoic acids and cinnamic acids; their derivatives are formed by various substitutions of hydrogen (H), hydroxyl (OH), or methoxyl (OCH_3) groups on the ortho-, meta-, and para-positions of the phenol ring (Figure 2). Most phenolic acids are located in the bran portion of the caryopsis, particularly the outer and inner pericarp, and aleurone layer. Furthermore, phenolic acids are presumed to have beneficial health effects due to their antioxidant capabilities, which allegedly help to fight cancer, cardiovascular disease, hypertension, neurodegenerative disorders (e.g. Alzheimer's and Parkinson's), diabetes mellitus, and obesity; however, results are limited (Fardet 2010). Moreover, the content and quality of phenolic acids have been reported to affect the flavor and color of bread products, by producing undesirable bitter and astringent flavors, and gray, brown, or even green colors (Piironen et al. 2009).



Salicylic acid (R_1, R_2, R_3 = H; R_4 = OH)
p-Hydroxybenzoic acid (R_2 = OH; R_1, R_3, R_4 = H)
Protocatechuic acid (R_1, R_2 = OH; R_3, R_4 = H)
Vanillic acid (R_1 = OCH_3; R_2 = OH; R_3, R_4 = H)
Gallic acid (R_1, R_2, R_3 = OH; R_4 = H)
Syringic acid (R_1, R_3 = OCH_3; R_2 = OH; R_4 = H)

o-Coumaric acid (R_1 = OH; R_2, R_3, R_4 = H)
m-Coumaric acid (R_2 = OH; R_1, R_3, R_4 = H)
p-Coumaric (R_3 = OH; R_1, R_2, R_4 = H)
Caffeic acid (R_2, R_3 = OH; R_1, R_4 = H)
Ferulic acid (R_2 = OCH_3; R_3 = OH; R_1, R_4 = H)
Sinapic acid (R_2, R_4 = OCH_3; R_3 = OH; R_1 = H)

Figure 2. Structures of various benzoic (left) and cinnamic (right) acids.

2.2. Economy

Wheat (*Triticum aestivum* L.) is the third largest produced cereal crop in the world with over 700 million tonnes of wheat harvested annually (FAO 2014). However, wheat is cultivated on more land area and in more locations than any other food crop, including maize and rice, due to its unrivaled adaptability and tolerance to a wide range of growing climates (Gooding 2009; Shewry 2009). Wheat is an important food source for much of the global population, since wheat can contribute up to 19% of a person's total caloric intake (FAO 2014). Out of the five countries that produce the most wheat, the United States of America (USA) produces the third highest amounts at approximately 58 million tonnes.

Within the United States, North Dakota is the second largest producer of wheat behind Kansas, though, depending on the year's growing conditions, North Dakota has occasionally been the top producer (NDWC 2008; 2013a). Wheat is an incredibly important crop for North Dakota since it is the chief agricultural commodity of the state; it covers one-fourth of North Dakota's land mass and also accounts for approximately one-fourth of total farm revenue for the

state's economy. Currently, North Dakota leads in the national production of hard red spring (HRS) wheat, which constitutes nearly 78% of North Dakota's entire wheat crop.

HRS is specialty wheat that is well renowned for its high protein content (13–16%) and strong gluten characteristics (Carson and Edwards 2009; NDWC 2013a). The quality produced by HRS cultivars is sought after in several parts of the world due to its high yield and excellent end-use quality. These elements of HRS wheat make it the perfect flour to use when making high quality yeast breads, whole grain breads, pizza crusts, bagels, and hard rolls. In addition, there are sizable demands for HRS wheat flours for blending purposes, due to its protein and gluten properties, to improve the gluten strength of other wheat flours and the resulting wheat products. Furthermore, flours made from HRS wheat have higher water absorption values than most other flour types, especially lower protein flours, which allows for greater quantities of bread loaves to be made from a set amount of flour, and will also improve the moistness, softness, and shelf-life of the product. Finally, at least one-half of the entire national HRS wheat crop is exported to foreign countries; Asia is the largest export region at 56%, followed by Central and South America (27%), Europe (11%) and Africa (6%) (NDWC 2013a).

2.3. Yield

In order to increase the availability of wheat products, farmers and wheat breeders have been hard at work to produce wheat cultivars that are capable of delivering greater yields, whether through breeding or agronomic practices. One of the earliest advancements in wheat breeding likely occurred prior to 9,000 B.C. with the selection of wheat ears that exhibited a non-brittle or semi-tough rachis trait, caused by a mutation at the *Br* locus, to eliminate the spike from shattering at maturity (Hillman and Davies 1990; Gooding 2009; Shewry 2009). In these selected wheat plants, the ear would remain intact beyond maturation, which would allow more

grain to be harvested from the stock than previously possible. Another early advancement in wheat cultivation involved the domestication of major gene *Q*. While the *Q* gene is primarily associated with free-threshing character, the property by which the kernel is readily released from the glume, lemma, and palea also plays a role in further reducing rachis fragility so that the spike remains predominantly intact during threshing (Jantasuriyarat et al. 2003; Simons et al. 2006; Gooding 2009). The *Q* gene in wheat also affects other characteristics such as glume shape, spike length, spike emergence time, and plant height.

After initial cultivation and environmental adaptation, agronomic practices were established to further increase wheat yields; irrigation, fallowing, and the use of marl and manure as fertilizer were a few of the first techniques implemented (Evans 1980). Moreover, wheat crops supplied with fertilizers produced bigger spikes, more kernels per spike, higher kernel volume, and higher kernel weight. Unfortunately, fertilizer use also produces taller, weaker stems that are at greater risk of lodging, especially with the heavier spikes (Gooding 2009).

Aside from further agronomic progress, grain yield increases for wheat stagnated until the 1800's when farmer's realized that shorter plant heights equated to less spoilage and lodging as well as being better suited for their harvest equipment (Roberts 1847; Garnett 1883). However, it wasn't until the 1900's, after the importance of plant genetics was identified, that farmers and breeders were able to improve crop yields by reducing plant height (Gooding 2009). These modern wheat cultivars, known as semi-dwarf, have much shorter and stockier stems than their predecessors, and are much less likely to lodge, even with the heavier wheat spikes. Not only do these shorter stems reduce lodging, but they also shorten the vegetative growth phase, which causes the wheat to mature at a faster rate than the historical cultivars (Hucl and Baker 1987; Siddique et al. 1989).

2.4. Production and quality comparison

While there has always been support for producing higher yielding wheat cultivars, a necessity to increase food security, there is some concern that the practices used to increase yields have led to a decrease in product quality and nutrition. However, there are discrepancies in literature concerning the relation between grain yield, protein content, and end-use quality. Souza et al. (1993) argued that increased grain yield was correlated to decreased protein concentration in modern cultivars. DePauw et al. (2007) asserts that the protein concentration has been maintained, while Hucl et al. (2015) claims a modest increase in protein concentration with grain yield. In addition, the end-use quality in bread, i.e. the physical dough properties and loaf characteristics, tends to be conserved or improved with increased yield, even with a decrease in protein content (Cox et al. 1989; Souza et al. 1993; Morgounov et al. 2013). Many of the HRS cultivars released within the past 40 years, such as Butte, Len, Stoa, Grandin, Reeder, Steele-ND, and Glenn, demonstrate both improved yield and disease resistance while maintaining superior end-use quality traits (Underdahl et al. 2008).

Further discrepancies can be found within the mineral content and density of various wheat cultivars. For example, some modern cultivars, when compared to historic cultivars, were reported to have stable calcium concentrations (Murphy et al. 2008), while other modern cultivars exhibited declining calcium concentrations (Morgounov et al. 2013); lower calcium concentrations may result in calcium deficiencies for various populations if cereal grains are a major source of caloric intake. In regards to other minerals, significant decrease in the iron and zinc concentrations, in correspondence with higher yielding cultivars, was observed by both Garvin et al. (2006) and Fan et al. (2008); minerals like copper and magnesium also exhibited a decrease in concentration. However, while Oury et al. (2006) concluded that the concentrations

of some minerals, such as magnesium and zinc, can be controlled through cultivar selection and breeding, it was also concluded that other minerals, like iron, are influenced more by the growing environment than the specific cultivar.

Even with the disparities amongst experimental data, cultivars in the hard red market class usually demonstrate more consistent mineral nutrient concentrations, between historic and modern cultivars spanning the past century, than cultivars in the soft white market class (Murphy et al. 2008). Hard red cultivars had slightly lower zinc contents, while soft white cultivars were found to have lower copper, iron, manganese, phosphorus, selenium, and zinc contents. In the United States, commercial whole grain products that require low ash content are made using soft white cultivars. Unfortunately, when plant breeders make selections to reduce ash content, they are inadvertently reducing the mineral concentrations as well.

It is important to note that the differences in experimental conditions, such as weather, location, fertilizer, etc., could have greatly affected the results obtained by each study, except for those studies specifically monitoring the environmental effects (Syltie and Dahnke 1983). It should also be noted that the nutritional content in wheat is dependent on the bioavailability of the nutrients within each cultivar, as well as the presence of phytic acid, which can hinder the intestinal absorption of compounds through the formation of insoluble complexes (Oury et al. 2006; Delcour and Hoseney 2010b). Moreover, it has been proposed that decreasing the phytate-to-mineral concentration in wheat could help to increase mineral bioavailability (Hussain et al. 2011). Since the phytate-to-mineral ratio was not significantly related to yield, decreasing the ratio would, theoretically, lessen the need to breed cultivars with higher mineral density at the expense of current yields.

2.5. Modern and historical comparison

Many plant-based agricultural commodities, but especially cereals crops, have recently been targets of fads that tout modern crops as toxic and hazardous while lauding their historical counterparts for being healthy and safe alternatives. More and more people today believe that modern breeding and agricultural techniques have generated wheat cultivars that are dangerous hybrids, mutations, or genetic modifications of historic wheat cultivars. These beliefs are further fueled by popular anti-wheat fads, such as the Paleolithic, Grain Brain, and Wheat Belly diets, which make several claims to the detrimental health effects of modern wheat (Cordain 2011; Perlmutter and Loberg 2013; Davis 2014). Some of these claims purport that modern wheat has a different starch chemistry, which causes a more rapid conversion to blood sugar, and higher GI than historic wheat, which has led to greater incidences of diabetes mellitus (DM) and obesity. Furthermore, anti-wheat advocates claim that wheat contains additional chemicals that increase the population's susceptibility to attention-deficit/hyperactivity disorder (ADHD), autism, inflammation, cardiovascular disease (CVD), schizophrenia, rheumatoid arthritis, and even increased aging. Several experts in research fields related to wheat breeding, laboratory analysis, and health and nutrition have either directly or indirectly addressed the misleading nature of many of these claims (Yoshikawa et al. 2003; Möller et al. 2008; Guang and Phillips 2009; Jones 2012; Aune et al. 2013; Brouns et al. 2013; Kasarda 2013; Giacco et al. 2014; Hajihashemi et al. 2014; Peña et al. 2014).

CHAPTER 3. NEED STATEMENT AND OBJECTIVE

3.1. Need statement

This research study was necessary to address growing concerns from the general population that recent breeding and agricultural practices have affected the chemical composition of wheat and caused modern wheat cultivars to be more noxious than their historical counterparts. Due to misinformation spread by those who support anti-wheat diets, the general population also believes that modern cultivars are responsible for the recent spike in health issues such as celiac disease, inflammation, CVD, DM, obesity, etc. These increasingly common views not only hurt the global wheat production industry, but they also have the potential to negatively impact other cereal and agricultural commodities as well.

Furthermore, if continued, these anti-wheat trends could have serious global ramifications. Not only will these trends negatively affect the global wheat trade, they have the potential to completely undermine global food security efforts, since the growing world population has put tremendous pressure on agriculture to meet the resultant nutritional needs (Peña et al. 2014). For example, if wheat is considered unhealthy by developed countries, those countries will be much less likely to help underdeveloped countries improve their wheat production, which can cause greater malnutrition and famine than is currently experienced by impoverished nations.

Several researchers have addressed variances in chemical composition between wheat cultivars in relation to growing conditions, e.g. organic vs. conventional farming systems (Bonte et al. 2014). However, very few studies have eliminated environmental interactions to specifically determine if differences between cultivars are due to actual varietal differences or by the various environmental conditions from which the cultivar samples were obtained (Dinelli et

al. 2011; Khakimov et al. 2014). Furthermore, it is desirable to limit or remove environmental effects from research of this nature, since there are countless environmental variables that can affect the both the composition and quantity of the diverse chemicals found within wheat plants and, subsequently, the grain.

3.2. Research objective

The primary objective for undertaking this research was to determine if there were any significant differences between multiple physical and chemical characteristics of historic and modern hard red spring (HRS) wheat (*Triticum aestivum* L.) cultivars when environmental differences are limited. To do so, both the historic and modern HRS wheat samples were grown in the same location and year, under identical environmental conditions, and subjected to several experimental and analytical methods to evaluate the composition and activities of a selection of chemical properties. Computational analysis was performed on all parameters to assess whether any observed differences between cultivars were statistically significant.

CHAPTER 4. MATERIALS AND METHODS

4.1. Wheat cultivars investigated

Thirty different cultivars of hard red spring (HRS) wheat (*Triticum aestivum* L.) were obtained from the Wheat and Carbohydrate Research group at North Dakota State University, Plant Sciences Department (Table 1). The samples were all grown in 2013 in 150 ft. strip plots with no plot design at the Dalrymple Experiment Plots near Casselton, ND, where the plots were representative of the temperate climate typical for eastern North Dakota. HRS wheat planting was delayed due to wet, cool conditions in March and April and started in late April and mid-May (NDWC 2013b). The first half of the growing season consisted of conditions that had adequate to surplus levels of precipitation and cooler temperatures, while the second half of the season had warmer temperatures and drier conditions, thus limiting disease pressure. HRS harvest began in early August, two to three weeks behind average, with warm, dry conditions. Rain showers during September stalled later harvest progress and pushed final harvest into the first week of October. Even though harvest conditions varied, all research samples were harvested on the same day to help eliminate any environmental variables that may significantly alter the plants chemical composition. The HRS cultivars were divided into three sample groups with ten cultivars in each group; samples released prior to 1970 were labeled as historical, samples released between 1970 and 1999 were considered to be mostly modern, and samples released during and after 2000 were labeled as definitely modern.

Table 1. Pedigree information for hard red spring wheat cultivars evaluated in this study

Genotype	RY[a]	Released by	Pedigree
Marquis	1910	Canada[b]	Hard Red Calcutta/Red Fife
Ceres	1926	NDSU[c]	Marquis/Kota
Pilot	1939	NDSU	Hope/Ceres,USA
Rival	1939	NDSU	Ceres,USA//Hope/Florence
Vesta	1942	NDSU	Ceres,USA//Hope/Florence

Table 1. Pedigree information for hard red spring wheat cultivars evaluated in this study (continued)

Genotype	RY[a]	Released by	Pedigree
Mida	1944	NDSU	Mercury,1933/RL-625
Conley	1955	NDSU	RL-2563/Lee
Justin	1963	NDSU	Conley/ND-40-2
Fortuna	1966	NDSU	Rescue/Chinook//II-50-17
Waldron	1969	NDSU	Justin/ND-81
Olaf	1972	NDSU	Justin*3/ND-259//Conley/3/Waldron/Conley//ND-122/4/Justin
Butte	1977	NDSU	ND-480/Polk//Wisc.-261
Len	1979	NDSU	ND-499/3/Justin/RL-4205//Wisc.-261
Stoa	1984	NDSU	ND-527/(SIB) Coteau/Era
Butte-86	1986	NDSU	Butte*2/3/ND-551//Butte*2/ND-507
Grandin	1989	NDSU	Lenana//Butte*2/ND-507/3/ND-593
2375	1990	Pioneer/ NDSURF[d]	Olaf//Era,USA/Suqamuxi-68/3/Chris/ND-487/Lark,USA
Russ	1995	SDSU[e]	SD-8052/SD-2971
Gunner	1995	AgriPro[f]	Canadian CSRS/USA HRS (Composite of 49 single crosses)
Reeder	1999	NDSU	IAS-20*4/H-567.71//Stoa/3/ND-674
Alsen	2000	NDSU	ND-674/ND-2710/ND-688
Granite	2002	WPB[g]	Kent/B-564//SO/3/Kent/2*B-564//Sap/4/Hege-312-75-262/(SIB) Chat
Steele-ND	2004	NDSU	Parshall/ND-706
Freyr	2004	AgriPro	Sonja/Vance//Sumai-3/Dalen
Glenn	2005	NDSU	ND-2831 /Steele-ND
Faller	2007	NDSU	ND-2857/ND-2814
RB-07	2007	MN[h]	Norlander/HJ-98
Barlow	2009	NDSU	ND-744/ND-721
Velva	2012	NDSU	Dapps (PI-633862)/2*Reeder
Elgin-ND	2013	NDSU	Walworth/Reeder

[a] RY, release year.
[b] Dominion Canada Dept. of Agriculture and Sir C.E. Saunders Canada Dept. of Agriculture, Ottawa, Ontario, Canada.
[c] North Dakota State University, Fargo, ND, USA.
[d] Pioneer Hi-Bred International, Inc. and North Dakota State University Research Foundation, MN, USA.
[e] South Dakota State University, Brookings, SD, USA.
[f] AgriPro Biosciences, Inc., Berthoud CO, USA.
[g] Western Plant Breeders: originally developed in Germany; represented since 2002 by WestBred LLC, Bozeman, MT, in the USA.
[h] United States Dept. of Agriculture and University of Minnesota, St. Paul, MN, USA.

4.2. Sample preparation

Samples were cleaned with a Carter-Day dockage tester using the official USDA dockage method (USDA 2013). The samples (2 kg) were tempered to 15.5% moisture for 16 hours before milling on a Bühler MLU-202 laboratory mill (CH-9240, Bühler Industries, Uzwil, Switzerland) to separate the straight grade flour, bran, and shorts fractions (AACC International, 1999a). The straight grade flour is a mix of flour from both the three break streams and the three reduction streams from the mill, and was blended using a cross flow blender. To remove unwanted foreign material, the straight grade flour was re-bolted over an 84 SS sieve. A portion of the coarse bran from milling and the whole grain were reduced by grinding in a falling number hammer mill with a 0.8 mm screen (Perten Instruments Springfield, IL, U.S.A.).

4.3. Experimental methods

4.3.1. Proximate data

Moisture content was determined using the AACCI approved method 44-15.02, moisture-air oven method (AACC International, 1999b). Ash was measured using the AACCI approved method 08-01.01 and expressed on a dry weight basis (AACC International, 1999c). Mineral content of the samples, which was completed by researchers from the University of Missouri Agricultural Experiment Station Chemical Laboratories, was determined using inductively coupled plasma–optical emission spectroscopy (ICP-OES) according to the AOAC Official Method 985.01 (AOAC International 2005a). Protein content was determined through the AACCI approved method 46-30.01, crude protein-combustion method, with a LECO FP 528 nitrogen/protein analyzer (LECO, St. Joseph, MI, U.S.A.) and expressed as dry weight basis (AACC International, 1999d). Crude fat content, which was also completed by researchers from the University of Missouri Agricultural Experiment Station Chemical Laboratories, was

determined by extraction of the ground sample with diethyl ether for three hours using a Soxhlet apparatus according to the AOAC Official Method 920.39 (AOAC International 2005b).

Total starch was measured using the approved AACCI method 76-13.01 and a total starch assay kit from Megazyme International (Bray, Co. Wicklow, Ireland), with some minor modifications (AACC International, 1999e). Each sample and standard was weighed (100 mg) and, to aid dispersion, 0.2 mL aqueous ethanol (80% v/v) was added, followed immediately by 3 mL Solution A (1 mL thermostable α-amylase (300 U) in 29 mL 50 m*M* MOPS buffer (pH 7.0, 5 m*M* $CaCl_2$, 0.02% NaN_3)). Tubes were incubated at 100°C in a heating block for 6 minutes, and the samples were vortexed after 2 and 4 minutes. Samples were placed in a 50°C hot water bath and allowed to sit for about 10 minutes for temperature equilibration. Next, 4 mL sodium acetate buffer (200 m*M*, pH 4.5, 0.02% NaN_3) was added, followed by the addition of 0.1 mL amyloglucosidase (AMG); tubes were vortexed after each addition. Samples were incubated at 50°C for 30 minutes, and vortexed every 10 minutes. The contents of each tube were filtered through VWR 413 grade qualitative filter paper into separate 100 mL volumetric flasks; deionized water was used to rinse each tube thoroughly. Duplicate sample and standard aliquots (0.1 mL) were transferred to large culture tubes, along with a blank (0.1 mL water) and duplicate glucose controls (0.1 mL glucose standard solution). Glucose oxidase peroxidase (GOPOD) reagent (3 mL) was added to each tube, which were incubated at 50°C for 20 minutes in a hot water bath, and the absorbance was measured at 492 nm using a microplate and microplate reader (Thermo Electron, Vantaa, Finland). Total starch was calculated using the data calculator spreadsheet provided by Megazyme International.

Total arabinoxylan (AX) and the ratio of arabinose to xylose (A/X) in the whole wheat and white flour samples were determined following acid hydrolysis and preparation of alditol

acetates as described by Blakeney et al. (1983), with modifications. Approximately 8 mg of each sample was hydrolyzed to sugars by the addition of 250 µL trifluoroacetic acid (TFA) (2.0 *M*) and heating at 121°C for 1 hour. *Myo*-inositol (75 µL) was used as an internal standard and arabinose, xylose, mannose, galactose, and glucose were used as monosaccharide standards for the standard curve. The sugars were reduced with ammonium hydroxide and sodium borohydride in dimethylsulfoxide (DMSO) with heating at 40°C for 90 minutes. A few drops of glacial acetic acid were added and then the reduced monosaccharides were acetylated by the addition of 1-methylimidazole and acetic anhydride. The reaction was stopped with about 4 mL water and the acetates were extracted with dichloromethane. The derivatized alditol acetate samples were dissolved in 1 mL acetone and analyzed on a Hewlett Packard 5890 series II gas chromatography (GC) system with a flame ionization detector (Agilent Technologies, Santa Clara, CA, U.S.A.) following the method parameters outlined by Mendis et al. (2013). A Supelco SP-2380 fused silica capillary column (30 m x 0.25 m x 2 µm) (Supelco Bellefonte, PA, U.S.A.) was used in the GC system. Helium was used as the carrier gas and the GC system settings were set as follows: flow rate, 0.8 mL/min; flow pressure, 82,737 Pa; oven temperature, 100°C; detector temperature, 250°C; and injector temperature, 230°C. AX was calculated according to Equation 4.1, as given by Henry (1986).

$$AX = (\%\ \text{arabinose} + \%\ \text{xylose}) \times 0.88 \qquad (4.1)$$

Dietary fiber was determined according to the AACCI approved method 32-07.01 by gravimetric measurement of residue after enzymatic digestion (AACC International, 1999f). Dietary fiber analysis was conducted using an ANKOM automated dietary fiber analyzer (ANKOM Technology, Macedon, NY, U.S.A.).

4.3.2. Enzyme activity

Activity levels for α-amylase, endo-protease, and xylanase were measured for both whole wheat and white flour samples. The tablet test kits used to measure α-amylase, endo-protease, and xylanase activity were T-AMZ, T-PRAK, and T-XAX, respectively, and were all purchased from Megazyme International (Bray, Co. Wicklow, Ireland).

α-Amylase activity was determined using the approved AACCI method 22-05.01 and an amylazyme kit from Megazyme International (AACC International, 1999g). Glass tubes containing 0.5 g of each whole wheat and white flour sample were pre-incubated in a heating block at 60°C for 10 minutes with stirring. The sodium maleate (100 m*M*, pH 6.0, 0.74 g $CaCl_2$, 0.02% NaN_3) buffer was also pre-incubated at 60°C, in a hot water bath, and 5 mL was added to each tube and left stirring for 5 minutes. Amylazyme tablets were added to each tube in 30 second intervals and the reaction was stopped using 6 mL of 2% trizma base after exactly 5 minutes. Each solution was filtered through VWR filter paper and the absorbance was read at 590 nm using a HACH DR/4000U spectrophotometer (Hach, Loveland, CO, U.S.A.). The α-amylase activity was calculated using Equation 4.2, as provided by Megazyme International, and reported as milliunits per gram (mU/g).

$$\text{Units/g} = [((51.6 \times \text{absorbance}) - 7)/(\text{sample weight})] \times 2 \times 0.001 \qquad (4.2)$$

Endo-protease activity was measured according to the method outlined by Ichinose et al. (2001), and using protazyme test tablets from Megazyme International. Endo-proteases were extracted from 0.5 g whole wheat or white flour samples, in glass tubes, with 5.0 mL sodium phosphate extraction buffer (100 m*M*, pH 7.0) and stirring at room temperature for 30 minutes with micro magnetic stir bars and a stir plate; samples were centrifuged (Allegra X-12 centrifuge, Beckman Coulter, Indianapolis, IN, U.S.A.) to separate crude endo-protease extract

from the sample waste. One protazyme tablet was added to 1.0 mL of the reaction buffer (sodium phosphate extraction buffer with 1% (w/v) sodium dodecyl sulfate) and stirred at 40°C for 5 minutes, followed by the addition of 1.0 mL enzyme extract and stirring for an additional 2 hours. The reaction was stopped with 10 mL of 2% (w/v) trisodium phosphate and the samples were filtered through VWR filter paper. Finally, the absorbance was read at 590 nm using a HACH DR/4000U spectrophotometer (Hach, Loveland, CO, U.S.A.); one unit of enzyme activity was defined to be the change in absorbance per gram per hour (A590/g/hr).

Xylanase activity was measured for the whole wheat and white flour samples using a Xylazyme AX assay kit and method from Megazyme International with a few modifications based on research from Courtin et al. (2005). The xylanase standards were prepared by diluting *Aspergillus niger* xylanase, supplied in the xylanase kit, with sodium acetate buffer (25 m*M*, pH 4.7, 0.02% NaN_3) to dilutions of 1000, 5000, 10000, 20000, and 40000 times less than the original concentration of 295 mU/mL. For the wheat flour samples, 2 g were weighed into plastic centrifuge tubes and 10 mL of sodium acetate buffer was added. For the white flour samples, only 1 g of sample and 5 mL of sodium acetate buffer was used for the extraction, due to a limited amount of sample. Samples were shaken on a MaxQ 4000 A-class benchtop orbital shaker (Thermo Scientific, Marietta, OH, U.S.A.) at 6°C and 150 RPM for 1 hour. After centrifuging at 3,273 *g* for 15 minutes (Allegra X-12, Beckman Coulter, Indianapolis, IN, U.S.A.), 1.0 mL of the sodium acetate buffer (for two blanks), xylanase standard dilutions, and supernatant were placed in glass tubes and pre-incubated at 40°C for 10 minutes. One xylazyme tablet was then added to each tube, except for one of the two blanks. The samples were incubated at 40°C for 17 hours, and the reaction was stopped using 10 mL of 1.0% trizma base. The samples were filtered through VWR filter paper, the absorbance was read at 595 nm using a

microplate reader (Thermo Electron, Vantaa, Finland), and the concentrations, defined as the change in absorbance per gram per hour (A590/g/hr), were calculated from the line equation generated by the absorbance of the standard curve dilutions.

4.3.3. Phenolic content

Phenolic content in the samples was determined using the method outlined by Nardini et al. (2002), with modifications to optimize the procedure for whole wheat flour. Ground samples (0.5 g) were extracted using 10 mL of 80% methanol for 2 hours in a reciprocal shaker (Eberbach, Ann Arbor, MI, U.S.A.) on a low speed setting. The extracts were centrifuged (Allegra X-12, Beckman Coulter, Indianapolis, IN, U.S.A.) at 9,000 *g* for 10 minutes, decanted, and analyzed for total free phenolic acids.

For the determination of bound phenolic acids, each sample residue was washed three times with water, and then extracted using 4 *M* NaOH containing 1% ascorbic acid (w/v) and 10 m*M* ethylenediaminetetraacetic acid (EDTA) and shaken for 4 hours using a reciprocal shaker (Eberbach, Ann Arbor, MI, U.S.A.) on low speed. Ascorbic acid and EDTA were added to prevent the loss of phenolic acids during alkaline hydrolysis (Nardini et al. 2002). The extracts were centrifuged (Beckman Coulter, Indianapolis, IN, U.S.A.) at 9,000 *g* and decanted; they were then acidified with ice-cold HCl (6 *M*) to pH 1.5–2.0 and washed three times with ethyl acetate. The ethyl acetate fractions were collected, pooled, and evaporated. The dried extracts were dissolved in 2 mL 80% methanol, and filtered with a 0.2 µm nylon syringe filter (Whatman, Maidstone, UK) prior to HPLC analysis.

A 0.1 mL aliquot of the methanol extract was diluted with 1.1 mL water and reacted with 0.5 mL diluted (10-fold with water) Folin-Ciocalteu reagent and 1.0 mL ethanolamine (0.5 *M*). The mixture was reacted for 20 minutes and the absorbance was read at 750 nm. Ferulic acid

was used as the standard and results were expressed as milligram ferulic acid equivalents per gram of sample (mg FAE/g).

Bound phenolic extracts were analyzed on a Shimadzu Nexera-i LC-2040C equipped with a photodiode array detector (Shimadzu Scientific Instruments, Inc., Columbia, MD, U.S.A.). Phenolic acids were separated using a Kinetex C18 column (150 x 2.1 mm, 2.6 um) from Phenomenex (Torrance, CA, U.S.A.). Column temperature was maintained at 30°C and injection volume was 1 µL. The mobile phase consisted of 2% formic aid in water (v/v, Solvent A) and acetonitrile (Solvent B); solvent flow rate was 0.4 mL/min. Phenolic acids were separated using the following gradient: 1–2 min, 7% B; 2–15 min, 7–21.4% B; 15–20 min, 21.4–75% B; 20–23 min, 75% B; gradient was then returned to 7% B and the column was equilibrated for 5 minutes at 7% B before the next run. Protocatechuic, vanillic, and syringic acids were detected at 280 nm. Caffeic, *p*-coumaric, ferulic, and sinapic acids were detected at 320 nm; *p*-hydroxybenzoic acid was detected at 255 nm. Quantification of each phenolic acid was accomplished by comparing peak areas with that of a calibration curve of each standard. Data was processed using LabSolutions version 5.73 (Shimadzu Scientifica Instruments, Inc.).

4.3.4. Starch properties

Starch properties of the white flour and bread were ascertained through starch characterization, where the amounts of amylose and amylopectin were determined, and the bread samples underwent starch digestibility tests to measure the starch fractions. Starch was isolated from the white flour and bread samples through defatting and precipitating the starch. Approximately 30 to 40 mg of each sample was defatted by the addition of 2.5 mL methanol and heating at 100°C for 30 minutes, followed by centrifugation at 931 *g* for 5 minutes (Allegra X-12, Beckman Coulter, Indianapolis, IN, U.S.A.), decanting the supernatant waste, and drying the

pellet in an oven at 55°C. Starch extraction was performed by the addition of 2 mL potassium hydroxide/urea solution (4.5 mL 1.0 *M* KOH with 0.5 mL 6.0 *M* urea) to the pellet and heating at 100°C for 15–25 minutes. The extracted starch was precipitated by the addition of 6 mL 95% ethanol, in two batches of 3.0 mL, followed by centrifugation at 931 *g* for 5 minutes (Beckman Coulter, Indianapolis, IN, U.S.A.), and drying.

Starch samples were prepared for high performance size exclusion chromatography (HPSEC) analysis by treatment with KOH and urea, as described by Grant et al. (2002). The starch (~30 mg) was solubilized from the addition of 4.5 mL KOH (1.0 *M*) and 0.5 mL urea (6.0 *M*) and heating at 100°C, under nitrogen, for 110 minutes. After heating, 1.0 mL aliquots were neutralized with 1.0 *M* hydrochloric acid (HCl) and filtered through a hydrophilic 0.45 µm nylon syringe filter prior to HPSEC analysis. The samples were run using an Agilent 1200 series high performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA, U.S.A.), equipped with an auto sampler, a refractive index (RI) detector, and a Wyatt Dawn Helios-II multi-angle light scattering (MALS) detector. A Waters Ultrahydrogel guard column (6 µm, 6 mm X 40 mm), and Ultrahydrogel 1000 (12 µm, 7.8 mm X 300 mm) and linear size exclusion columns (10 µm, 7.8 mm X 300 mm) (Waters, Milford, MA, U.S.A.) were used for starch separation. The columns and detector temperature was set to 40°C, and the mobile phase, HPLC-grade water, was pumped at a 0.4 mL/min flow rate, and the injection volume was 20 µL.

A PC with ChemStation (HP ChemStation for LC Rev. A.04.01) was used for control and integration to determine the percent of amylose and amylopectin. Further analysis and calculation of molecular weights (Mw) for amylose and amylopectin were performed on the Astra 6.0.5 data processing software (Wyatt Technology Corporation, Santa Barbara, CA, U.S.A.), with a dn/dc value, defined as the proportional change in RI with change in

concentration, of 0.146 (You et al. 1999). Pullulan standards were used to normalize all data before baseline corrections and peak alignments, and the Debye model, with a fit degree of two and a first-order polynomial fit, was applied for the molar mass calculations.

4.3.5. Starch digestibility

In vitro starch digestibility of the bread starch was analyzed according to the method described by Englyst et al. (1992). The sodium acetate buffer (0.1 *M*, pH 5.2) and glucose standard (5 mg/mL) was prepared the day prior to the digestion analysis, while the enzyme solutions were prepared fresh. Amyloglucosidase (140 AGU/mL, Megazyme International, Bray, Co. Wicklow, Ireland) was brought to 25 mL, from 1.07 mL, with deionized water. Next, 60 mg invertase (Sigma I-4504) was added to 8 mL deionized water. Finally, 18 g pancreatin (Sigma P-7545) was evenly distributed between six separate centrifuge tubes and dispersed with 20 mL deionized water in each tube. Tubes were shaken in a MaxQ 4000 shaker (Thermo Scientific, Marietta, OH, U.S.A.) at 4°C and 150 RPM for 10 minutes, and centrifuged (Allegra X-12, Beckman Coulter, Indianapolis, IN, U.S.A.) at 3,000 *g* for 10 minutes. From these three enzyme solutions, approximately 108 mL pancreatin supernatant was mixed with 12 mL amyloglucosidase and 8 mL invertase to create the final enzyme solution mixture.

Sodium acetate buffer (20 mL) was added to the samples (300 mg), white bread standard (300 mg), glucose standard (50 mg), and blank (10 mL H_2O), which were placed in a 37°C water bath with reciprocal agitation at 200 strokes per minute. Guar gum (50 mg) and five glass marbles were added to each tube and allowed to agitate for ten minutes to disperse contents. The enzyme solution mixture (5 mL) was added to each tube at one minute intervals. Every 20 minutes, for 180 minutes total, 0.5 mL aliquots were taken and placed in tubes containing 5 mL absolute ethanol (prepared in advance). Tubes were stored overnight (approx. 16 hours) at 4°C.

Samples were centrifuged (Beckman Coulter, Indianapolis, IN, U.S.A.) at 1,500 *g* for 10 minutes. Aliquots (0.1 mL) were taken from the sample supernatants, each glucose standard solution (0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 mg/mL), and the blank (H_2O). The glucose content was measured using a GOPOD assay kit from Megazyme International; 3.0 mL GOPOD reagent was added to each tube, incubated in a water bath at 50°C for 20 minutes, and the absorbance was read at 492 nm using a microplate reader (Thermo Electron, Vantaa, Finland). The absorbance values were used to generate a standard curve to calculate the glucose content released, and the rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) were determined from the glucose values at 20 and 120 minutes; RDS is the hydrolyzed starch portion from 0 to 20 minutes, SDS is the hydrolyzed starch portion between 20 to 120 minutes, and RS is the remaining starch after 120 minutes of digestion (Englyst et al. 1992). Furthermore, the hydrolysis index (HI) was obtained by dividing the area under the hydrolysis curve of the sample by the area obtained for the white bread standard (0 to 180 min), and the estimated glycemic index (eGI) was determined using Equation 4.3, as described by Granfeldt et al. (1992), where the white bread standard used was a generic store brand.

$$eGI = 8.198 + (0.862 \times HI) \quad (4.3)$$

4.4. Statistical analysis

The experimental results were analyzed using a SAS statistical analysis software package for Windows (version 9.3, SAS Institute, Cary, NC, U.S.A.). Analysis of variance (ANOVA) was performed using a completely random design (CRD) and the mean separation was conducted by Tukey's honest significant difference test ($P<0.05$). Hierarchical cluster analysis was performed using Ward's minimum variance method.

CHAPTER 5. RESULTS AND DISCUSSION

5.1. Proximate analysis of whole wheat and white flour

Moisture is the amount of water present in the caryopsis. It is desirable for moisture values to be 14% or less, or milling efficiency and quality are compromised, and the risk of spoilage increases (Carson and Edwards 2009). In whole wheat, the range in moisture values was 10.3–12.6% (Table 2), while 13.1–15.1% was observed in the white flour (Table 3). The five-year moisture average for all HRS whole wheat was 12.2%; moisture for white flour is not typically measured (NDWC 2013b). Significant differences ($P<0.001$) in moisture content were observed between genotypes for both whole wheat and white flour fractions. However, the correlation between moisture and release year was not determined.

Ash consists of the mineral material in wheat grain. Ash helps miller's gauge the separation efficiency of the mill equipment, since the miller's objective is to achieve the highest flour extraction with the lowest possible ash contamination (Posner 2000). Ash is also important during baking, since it can negatively affect dough strength and baking performance (Carson and Edwards 2009). Ash values ranged from 1.69–2.49% and 0.53–0.87% (db) in whole wheat and white flour, respectively (Tables 2–3). The white flour results were within the typical published range for ash, however, the whole wheat values were a little higher than usual (Simsek et al. 2011a; NDWC 2013b). These differences between the whole wheat and white flour results confirm that the ash is mainly concentrated in the pericarp, aleurone, and germ portions of the caryopsis, which are mostly removed during flour milling. The ash content between genotypes was significantly different ($P<0.001$) for both whole wheat and white flour fractions. Furthermore, these differences in whole wheat ($r = -0.78$) and white flour ($r = -0.81$) were significantly ($P<0.001$) related to release year (Table 4).

Table 2. Proximate analysis of hard red spring wheat whole wheat flour[a]

Genotype	RY	MC (%)	AC (db)	PC (db)	CF (db)	TS (db)	AX (db)	A/X	IDF (db)	SDF (db)
Marquis	1910	10.38	2.27	16.74	2.33	56.22	6.57	0.65	12.22	4.30
Ceres	1926	10.31	2.25	15.98	1.28	62.75	7.32	0.66	11.26	3.79
Pilot	1939	10.34	2.10	16.09	1.88	57.99	7.50	0.65	11.77	3.79
Rival	1939	10.92	2.22	16.12	1.02	58.55	7.35	0.61	10.89	3.09
Vesta	1942	11.12	2.25	16.42	2.31	62.88	5.74	0.58	10.86	2.76
Mida	1944	10.88	2.47	16.50	2.19	58.95	6.01	0.61	9.93	2.64
Conley	1955	11.32	2.49	16.85	1.53	60.64	7.30	0.66	12.52	2.99
Justin	1963	11.44	2.13	16.81	2.05	53.97	6.93	0.69	11.57	3.39
Fortuna	1966	11.43	1.95	16.51	2.13	61.37	7.87	0.64	10.56	2.65
Waldron	1969	10.84	2.18	17.20	2.41	60.29	4.81	0.65	11.10	2.86
Olaf	1972	10.92	2.28	16.79	2.00	59.18	6.04	0.67	11.84	3.26
Butte	1977	11.99	1.96	16.17	1.06	64.51	6.75	0.77	10.91	4.20
Len	1979	11.09	1.99	16.39	2.03	58.80	5.99	0.70	10.63	3.26
Stoa	1984	11.55	1.85	14.99	1.01	61.80	6.01	0.73	11.08	3.28
Butte-86	1986	12.03	1.71	15.73	1.09	64.95	7.50	0.69	10.63	4.04
Grandin	1989	10.29	1.73	16.32	2.02	52.47	5.57	0.67	11.59	2.84
2375	1990	12.60	1.73	15.57	1.28	67.29	7.92	0.72	10.53	4.23
Russ	1995	11.53	1.69	15.91	0.72	62.18	6.94	0.72	11.02	3.56
Gunner	1995	11.20	1.93	16.81	1.81	58.49	6.94	0.67	10.76	3.94
Reeder	1999	11.30	1.81	15.15	1.31	62.13	6.19	0.63	9.98	2.54
Alsen	2000	11.66	1.77	16.61	1.23	61.25	5.27	0.86	10.41	3.57
Granite	2002	11.02	1.72	17.20	2.15	56.99	5.31	0.66	11.18	2.98
Steele-ND	2004	10.94	1.79	15.91	0.58	73.86	7.43	0.70	11.00	3.76
Freyr	2004	11.90	1.74	16.41	2.13	60.37	6.60	0.67	11.75	4.03
Glenn	2005	11.13	1.86	16.18	1.96	58.87	5.56	0.68	10.86	3.54
Faller	2007	10.47	1.78	14.85	2.00	61.75	6.11	0.68	10.50	3.46
RB-07	2007	11.43	1.98	16.72	0.93	57.55	6.66	0.69	11.57	3.90
Barlow	2009	11.61	1.88	16.58	0.93	59.82	6.46	0.73	10.75	4.24
Velva	2012	11.42	1.84	15.69	1.16	58.18	7.57	0.65	11.23	3.22
Elgin-ND	2013	11.59	1.92	16.45	2.22	54.90	6.41	0.65	11.20	4.24
HSD[b]		9.0E-08	0.08	0.31	0.80	2.15	0.22	0.02	0.73	0.62

[a] RY, release year; MC, moisture content; AC, ash content; PC, protein content; CF, crude fat; TS, total starch; AX, arabinoxylans; A/X, arabinose to xylose ratio; IDF, insoluble dietary fiber; SDF, soluble dietary fiber; db, dry basis.
[b] Tukey's honest significant difference (P<0.05).

Table 3. Proximate analysis of hard red spring wheat white flour[a]

Genotype	RY	MC (%)	AC (db)	PC (db)	TS (db)	AX (db)	A/X
Marquis	1910	14.16	0.77	15.54	74.72	1.93	0.85
Ceres	1926	13.52	0.76	14.89	78.70	1.64	0.76
Pilot	1939	13.14	0.68	14.74	72.79	2.46	0.65
Rival	1939	14.66	0.85	14.89	80.57	2.15	0.77
Vesta	1942	14.08	0.77	14.75	78.92	2.63	0.94
Mida	1944	13.97	0.87	14.95	75.51	2.46	0.82
Conley	1955	13.06	0.83	15.67	68.53	1.94	0.90
Justin	1963	14.53	0.70	15.85	72.99	2.01	0.74
Fortuna	1966	13.55	0.69	15.61	73.27	1.83	0.83
Waldron	1969	13.97	0.75	15.18	75.87	2.25	0.93
Olaf	1972	13.37	0.70	15.21	72.15	2.36	0.63
Butte	1977	13.56	0.57	14.67	75.60	2.06	0.85
Len	1979	14.19	0.71	15.26	69.90	1.56	0.72
Stoa	1984	14.04	0.58	13.26	70.52	1.74	0.83
Butte-86	1986	13.78	0.57	14.45	80.40	2.41	0.94
Grandin	1989	13.61	0.57	15.08	65.65	1.81	0.95
2375	1990	14.87	0.61	14.76	74.67	1.85	0.74
Russ	1995	14.66	0.63	14.24	73.46	2.21	0.80
Gunner	1995	13.58	0.67	15.76	71.90	2.14	0.76
Reeder	1999	15.06	0.56	13.91	79.38	2.68	0.71
Alsen	2000	14.43	0.61	15.48	72.83	1.82	0.90
Granite	2002	13.49	0.59	16.07	63.20	1.89	0.71
Steele-ND	2004	14.26	0.59	14.44	81.49	1.99	0.70
Freyr	2004	13.88	0.60	15.45	74.96	2.46	0.78
Glenn	2005	13.24	0.61	15.25	71.67	2.31	0.65
Faller	2007	14.13	0.63	14.03	76.36	2.11	0.76
RB-07	2007	13.22	0.63	15.21	76.78	2.15	0.75
Barlow	2009	14.00	0.57	15.52	65.25	1.79	0.95
Velva	2012	14.17	0.53	13.85	75.87	2.12	0.76
Elgin-ND	2013	14.47	0.56	15.48	67.98	1.80	0.92
HSD[b]		4.8E-08	0.09	0.29	1.92	0.18	0.09

[a] RY, release year; MC, moisture content; AC, ash content; PC, protein content; TS, total starch; AX, arabinoxylans; A/X, arabinose to xylose ratio; db, dry basis.
[b] Tukey's honest significant difference ($P<0.05$).

Table 4. Correlation coefficients (r) between year, chemical composition, and enzymatic activities for hard red spring whole wheat and white flour[a,b,c]

		RY	AC	AS	PS	XS	CMA	CAF
Whole wheat	AC	-0.781 ***	1.000	0.512 **	-0.487 **	0.817 ***	0.772 ***	0.693 ***
	Ca	-0.313 NS	0.453 *	0.184 NS	0.199 NS	0.440 *	0.235 NS	0.329 NS
	P	-0.774 ***	0.957 ***	0.509 **	0.311 NS	0.810 ***	0.783 ***	0.638 ***
	K	-0.750 ***	0.940 ***	0.393 *	0.132 NS	0.831 ***	0.762 ***	0.730 ***
	Na	0.177 NS	-0.214 NS	0.172 NS	-0.072 NS	-0.266 NS	-0.229 NS	-0.201 NS
	Zn	-0.695 ***	0.853 ***	0.467 **	0.145 NS	0.711 ***	0.641 ***	0.564 **
	PC	-0.212 NS	0.413 *	0.045 NS	-0.221 NS	0.174 NS	0.329 NS	0.428 *
	CF	-0.289 NS	0.337 NS	-0.095 NS	-0.142 NS	0.210 NS	0.353 NS	0.290 NS
	TS	0.108 NS	-0.210 NS	0.023 NS	0.122 NS	-0.183 NS	-0.390 *	-0.376 *
	AX	-0.164 NS	0.015 NS	0.250 NS	0.090 NS	0.134 NS	0.168 NS	-0.052 NS
	A/X	0.404 *	-0.447 *	-0.237 NS	-0.120 NS	-0.544 **	-0.459 *	-0.198 NS
	IDF	-0.281 NS	0.323 NS	-0.054 NS	-0.149 NS	0.202 NS	0.514 **	0.603 ***
	SDF	0.168 NS	-0.269 NS	-0.044 NS	0.090 NS	-0.279 NS	-0.091 NS	-0.135 NS
White flour	AC	-0.810 ***	0.881 ***	0.500 **	0.162 NS	0.768 ***	0.736 ***	0.553 **
	PC	-0.131 NS	0.262 NS	0.015 NS	-0.217 NS	0.050 NS	0.276 NS	0.343 NS
	TS	-0.254 NS	0.122 NS	0.209 NS	0.325 NS	0.242 NS	0.106 NS	-0.188 NS
	AX	-0.060 NS	0.125 NS	-0.030 NS	0.063 NS	0.076 NS	-0.042 NS	-0.114 NS
	A/X	-0.055 NS	0.044 NS	-0.138 NS	-0.106 NS	-0.079 NS	-0.091 NS	0.162 NS

[a] RY, release year; AC, ash content; AS, amylase; PS, endo-protease; XS, xylanase; CMA, *p*-coumaric acid; CAF, caffeic acid; Ca, calcium; P, phosphorus; K, potassium; Na, sodium; Zn, zinc; PC, protein content; CF, crude fat; TS, total starch; AX, arabinoxylan; A/X, arabinose to xylose ratio; IDF, insoluble dietary fiber; SDF, soluble dietary fiber.
[b] All column titles, excluding RY, correspond to hard red spring whole wheat parameters.
[c] NS, not significant; *, **, and *** Significant at 0.05, 0.01, and 0.001 probability levels, respectively.

Protein content is a measure of the amino acid residues found within the caryopsis. Protein content is important for determining the type of products made from a given samples of flour (e.g. breads require more protein and pastries require less protein), as well as the end-use quality of the wheat product (Carson and Edwards 2009; Shewry et al. 2009). In the whole wheat, the range in protein values was 14.85–17.20% db (Table 2), while 13.26–16.07% db was observed in the white flour (Table 3). These values are within the range of published results, which average 16% db for whole wheat and 15% db for white flour (NDWC 2013b; Simsek et al. 2014a). Moreover, these results demonstrate that much of the protein content in the caryopsis is located in the endosperm and germ portions. While the protein content among genotypes was

significant (P<0.001) for both whole wheat and white flour fractions, these differences were not significantly (P>0.05) related to release year (Table 4).

Moisture, ash, and protein values for white flour from the year 1938 were found for the cultivars Marquis and Ceres (Harris and Sanderson 1938). Moisture for Marquis averaged 13.92%, while Ceres averaged 13.61%; accounting for differences in experimental equipment (i.e. present day scales are more accurate); these values are very similar to the moisture values acquired for this research (Table 3). Furthermore, moisture values should be relatively equal since the grain is tempered, or cold-conditioned, prior to milling (Jones and Ziegler 1964).

Ash means reported for white flour from the Marquis and Ceres cultivars were 0.57% db and 0.56% db, respectively (Harris and Sanderson 1938). Historic ash values for Pilot, Rival, Vesta and Mida white flour were also reported at 0.44, 0.51, 0.49, and 0.45% db, respectively (Harris et al. 1945). Ash values for Conley (0.47% db) and Justin (0.44% db) white flour were also found (Sibbitt and Gilles 1962). When compared to current results (Table 2), these values are distinctly different, which is likely a result of differences in equipment, as well as the environmental conditions during the growing season, as established previously (Oury et al. 2006). Moreover, while differences in current results between flour ash and release year were negatively correlated (r = -0.81, P<0.001) (Table 4), the above historic values may indicate fewer differences than originally ascertained, since they are closer in value to the modern cultivars than their respective cultivar.

Protein in white flour from Marquis averaged 14.10%, while Ceres averaged 14.84% (Harris and Sanderson 1938); again, accounting for differences in equipment, the historic and current values for white flour from Ceres are quite similar, but the values for Marquis are comparatively different (Table 3). The observed differences for the Marquis values can largely

be attributed to the different growing years and locations, as demonstrated by Waldron et al. (1942). Furthermore, historic protein values for Pilot, Rival, Vesta and Mida white flour were also discovered and reported at 14.91, 15.61, 15.26, and 15.72% db, respectively (Harris et al. 1945). While similar in value, these historic results are slightly higher than the current results for protein content in white flour. Additional results were found for Justin (16.98% db), Fortuna (15.58% db), and Waldron (16.98% db) white flour (Lebsock et al. 1966; Sibbitt 1971). The historic values for Justin and Waldron are higher than the currently reported values, while the historic value for Fortuna is lower than currently reported. It should be noted that all of the historic ash and protein results are averages that vary in regards to growing year, location, and the number of samples included in each set.

Crude fat (CF) refers to the total precipitated lipid content in wheat grain. While lipids are only a small portion of the caryopsis, they affect the production, storage, and processing of wheat products (Chung et al. 2009). Since lipids are present primarily in the germ, CF was only measured in the whole wheat and not the white flour samples. For the whole wheat, the CF values were 0.58–2.41% db, with an average of 1.62% db (Table 2), and the genotypes exhibited significant ($P<0.001$) differences. However, like the protein content results, the differences were not significantly ($P>0.05$) related to release year (Table 4). These results for CF are typical for whole wheat flour made from hard wheat cultivars (Prabhasankar and Haridas Rao 2001). Fat values for historic HRS wheat cultivars released prior to 1942 were found to be 2.2% db for whole wheat; these values are higher than current results for all cultivars, both historic and modern, except Marquis, Vesta, Waldron, and Elgin-ND (Bechtel et al. 1964).

Total starch (TS) is a measure of the combined total amount of amylopectin and amylose in a sample. Starch is important in bread products, since it absorbs water, provides yeast with

some nutrition, gelatinizes during baking, and participates in Maillard reactions (Delcour and Hoseney 2010d). Total starch values for the whole wheat were 52.47–73.86% db, with a mean of 60.3% db (Table 2), and the values for white flour were 63.20–81.49% db, with a mean of 73.73% db (Table 3). Similar to all previous results, significant differences ($P<0.001$) between genotypes were observed in both the whole wheat and white flour, but none of the differences were significantly ($P>0.05$) correlated to release year (Table 4). Typical values for TS in HRS wheat range from 56.2% db to 79.6% db, for different cultivars and growing locations (Simsek et al. 2011a; Simsek et al. 2011b). Furthermore, TS values for historic HRS wheat cultivars were found to average 61.27% db for whole wheat and, depending on the type, 65.09–77.11% db for white flour (Bechtel et al. 1964). These values fall within the range of the current results for all cultivars, and are very close in value to the historic cultivars released prior to 1942.

Arabinoxylans (AX), which are the major non-cellulose component of the cell walls in the caryopsis, impact bran and endosperm separation during milling and can also stabilize dough structure (Courtin and Delcour 2002). AX content in the whole wheat averaged 6.55% db (Table 2), while the AX content of the white flour averaged 2.09% db (Table 3); the published results for AX content averaged 1.29% for white flour, and 3.46% for whole wheat (Simsek et al. 2011b). Since AX are a nonstarch, cell wall polysaccharide, the concentration should be higher in the whole grain than just the endosperm portion (Delcour and Hoseney 2010d). Moreover, even though there were significant differences ($P<0.001$) between genotypes, those differences were not significantly related to release year (Table 4).

What are currently referred to as AX today, was previously referred to as pentosans. Pentosan values for HRS whole wheat averaged 6.01% db and white flour, from different mill fractions, ranged from 1.85% db to 3.01% db (Bechtel et al. 1964). The historic whole wheat

values were within the range of current results for all cultivars (Tables 2 and 3), though the historic values are lower than the current values for cultivars released prior to 1942. Moreover, the historic values for white flour are slightly higher than most of the currently reported values, but are close in value to the currently reported results for cultivars released prior to 1942.

AX consists of a backbone of β-(1,4)-linked xylose residues, which are substituted with arabinose residues on the C(O)-2 and/or C(O)-3 position (Dornez et al. 2009). Since AX is mainly composed of the pentose sugars xylose and arabinose, the arabinose-to-xylose ratio (A/X) was measured to determine the degree of substitution in AX; higher values indicate a higher level of arabinose substitution in AX. The mean value for whole wheat was 0.68 (68% arabinose substitution) (Table 2) and the mean value for white flour was 0.80 (80% arabinose substitution) (Table 3). There were significant differences ($P<0.001$) in A/X ratio among genotypes, which indicates that variations in substitution pattern in AX are genotype dependent, and these differences were significantly correlated ($r = 0.40$, $P<0.05$) to release year (Table 4). Published values for A/X average 0.75 for white flour and 0.78 for whole wheat (Simsek et al. 2011b).

Historic results for A/X in HRS wheat were reported at 0.57–0.76 (50–76%) across different white flour fractions (Medcalf et al. 1968). These average values were lower than the mean result obtained for the white flour, but were close to the mean of whole wheat samples (Tables 2 and 3). These variances could have been caused by differences in milling practices, as well as experimental method differences, i.e. advanced chromatographic equipment was not readily available in most laboratories at that time.

Dietary fiber (DF) consists primarily of non-starch polysaccharides, such as arabinoxylans and cellulose (Stone and Morell 2009). Furthermore, DF can be broken down into two categories—insoluble dietary fiber (IDF) and soluble dietary fiber (SDF)—of which both are

defined as undigested cellulose and noncellulosic polysaccharides, in the form of soluble fiber and cell wall residues, that pass through the gut to the large intestine to be fermented by microorganisms (Carson and Edwards 2009). However, the rate and efficiency of fermentation differs between fractions; SDF, which absorbs water, is readily fermented in the colon, whereas IDF, which does not absorb water, is more resistant to fermentation. Genotypes were significantly different ($P<0.001$) for both IDF and SDF, but the differences were not significantly ($P>0.05$) related to release year (Table 4). Cluster analysis was performed on the fiber fractions, AX, IDF, and SDF, but the cluster separation was not significant ($P>0.05$) (Figure A1). In whole wheat, IDF and SDF values were 9.93–12.52% db and 2.54–4.30% db, respectively. The USDA (2016) reports an average of 12.2% total dietary fiber, comprised of both insoluble and soluble fiber, for HRS wheat; while this mean is lower than the combined mean for whole wheat SDF and IDF (14.55%), the USDA states that the weights were for the edible portion and, moreover, the experimental procedures were not reported and could not be compared to the dietary fiber method presented in this paper (Carson and Edwards 2009). Historically, crude fiber in white flour was reported to be in the range of 2.33–3.14% db, but since crude fiber was not commonly used in wheat quality evaluations, it was seldom reported for HRS wheat (Zeleny 1964). Moreover, the nutritional effects of dietary fiber were not fully understood at the time (Hegsted et al. 1954).

Presently, the role of dietary fiber in human nutrition is much more understood, and consists of fecal bulking, enhanced colon function, and the production of short-chain fatty acids to be used as an energy source for cells in the colon (Patil 2008; Stone and Morell 2009). Further health benefits include decreased glycaemia, through rate delays in gastric emptying and glucose absorption, improved insulin response, increased satiety effects, and delivery of bound

phenolic acids to the colon, which all have the potential to help prevent or treat DM, obesity, CVD, and colon cancer (Fardet 2010). However, even though dietary fiber has many health benefits, it has been discovered that fiber may reduce absorption and bioavailability of many minerals and trace elements, due to the higher levels of phytic acid present in whole grain wheat products (Piironen et al. 2009).

Cluster analysis was performed on all proximate data, including enzyme activities and minerals for the whole wheat (Figure 3). Cluster 1, which constitutes the cultivars Waldron to Ceres, had release years of 1910–1972; cluster 2, with cultivars Granite through Elgin-ND, had the largest range of release years 1939–1966, and 1979–2013; and cluster 3, which contained cultivars Stoa through Pioneer-2375, exhibited release years of 1977–2009. Clusters 2 and 3 were not significantly ($P>0.05$) separated. In contrast, cluster 1 was significantly ($P<0.05$) separate from clusters 2 and 3. Even though the dendogram demonstrates some clustering based on release year, it cannot yet be concluded if this separation is based solely on genotype differences.

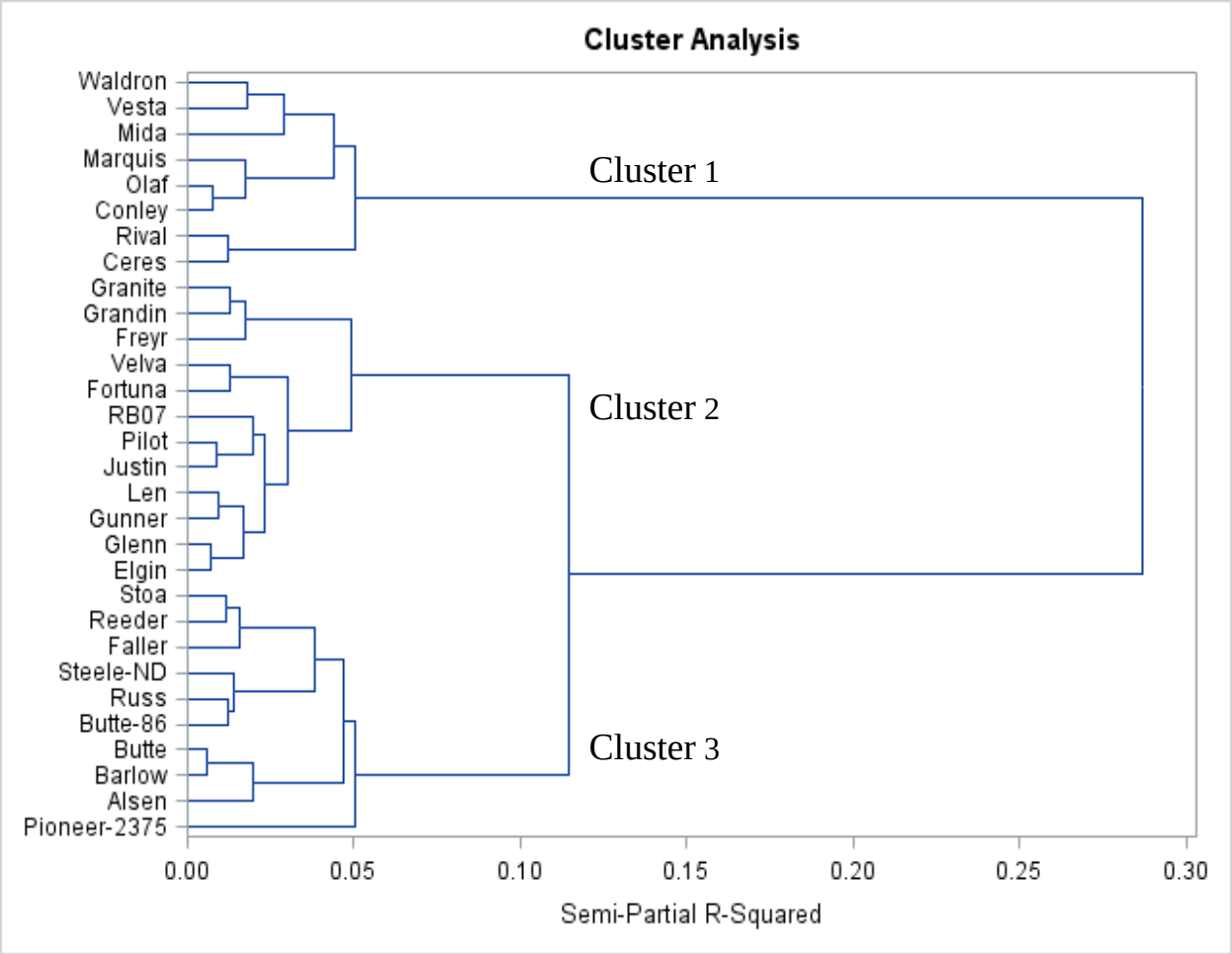


Figure 3. Cluster analysis for all hard red spring whole wheat quality parameters (including minerals); Cluster 1 (Waldron–Ceres) 1910–1972, Cluster 2 (Granite–Elgin-ND) 1939–1966 and 1979–2013, Cluster 3 (Stoa–2375) 1977–2009.

Cluster analysis was also performed on all white flour proximate data, including the enzyme activity (Figure 4). Cluster 1, which constitutes the cultivars Len to Butte, had the largest range of release years 1939, and 1977–2012; cluster 2, with cultivars Glenn through Alsen, exhibited release years of 1989, and 2000–2013; and cluster 3, which contained cultivars Waldron through Pioneer-2375, had release years of 1910–1972, and 1990. No significant (P>0.05) separation occurred in clusters 1 and 2; however, cluster 3 had significant (P<0.05) separation. Again, it cannot be determined if this separation is based solely on genotype differences.

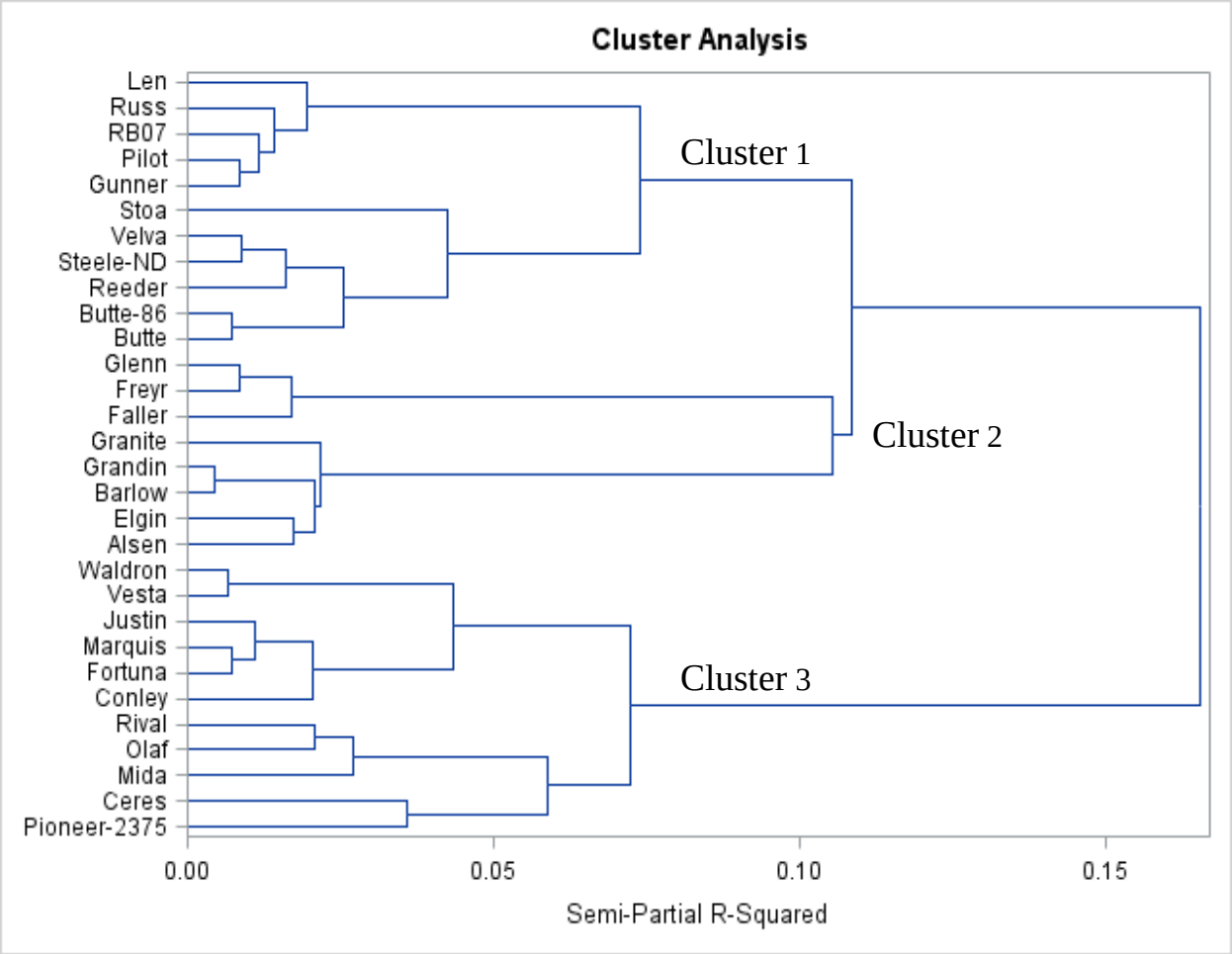


Figure 4. Cluster analysis for all hard red spring white flour quality parameters; Cluster 1 (Len–Butte) 1939, 1977–2012, Cluster 2 (Glenn–Alsen) 1989, and 2000–2013, Cluster 3 (Waldron–2375) 1910–1972, and 1990.

The minerals calcium (Ca), phosphorous (P), potassium (K), sodium (Na), and zinc (Zn) were each measured individually from the ash residue. For all minerals, there were significant differences ($P<0.001$) between genotypes. Furthermore, P (r = -0.77), K (r = -0.75), and Zn (r = -0.70) were the only minerals that were significantly ($P<0.001$) correlated to release year (Table 4). Ca and Na were not significantly ($P>0.05$) related to release year (Table 4), which helps to confirm that Ca concentration does not decrease over time (Murphy et al. 2008). Moreover, Murphy et al. (2008) indicated that Zn concentrations in hard red wheat displayed a slightly negative trend in relation to release year. Conversely, Murphy et al. (2008) also observed a

neutral or slightly positive trend for P in hard red wheat, in relation to release year, which does not agree with the results obtained in this study.

Table 5. Mineral content for hard red spring whole wheat[a]

Genotype	RY	Ca (ppm)	P (%w/w)	K (%w/w)	Na (ppm)	Zn (ppm)
Marquis	1910	465.27	0.58	0.52	15.12	40.84
Ceres	1926	638.88	0.52	0.50	11.93	39.14
Pilot	1939	346.86	0.48	0.45	15.34	33.91
Rival	1939	457.48	0.53	0.45	11.28	37.61
Vesta	1942	532.17	0.55	0.48	7.09	39.60
Mida	1944	444.89	0.61	0.53	16.66	45.95
Conley	1955	529.98	0.56	0.54	15.79	40.20
Justin	1963	379.98	0.52	0.46	17.73	39.35
Fortuna	1966	519.36	0.47	0.43	15.13	34.49
Waldron	1969	390.33	0.54	0.48	13.29	41.67
Olaf	1972	515.25	0.55	0.52	14.76	37.38
Butte	1977	562.46	0.44	0.39	21.53	33.52
Len	1979	395.91	0.48	0.47	25.70	29.30
Stoa	1984	420.58	0.41	0.42	17.86	25.04
Butte-86	1986	376.25	0.41	0.36	15.80	28.13
Grandin	1989	373.96	0.42	0.36	9.42	29.87
2375	1990	294.62	0.42	0.37	47.20	31.06
Russ	1995	339.67	0.40	0.38	10.06	22.32
Gunner	1995	278.73	0.46	0.42	19.48	24.04
Reeder	1999	459.97	0.40	0.38	17.81	27.00
Alsen	2000	444.28	0.43	0.38	26.20	28.41
Granite	2002	260.73	0.42	0.35	15.28	25.68
Steele-ND	2004	469.36	0.43	0.39	9.88	28.97
Freyr	2004	215.66	0.42	0.33	8.91	22.76
Glenn	2005	478.24	0.44	0.38	14.63	32.13
Faller	2007	418.27	0.41	0.39	33.23	31.55
RB-07	2007	348.30	0.49	0.38	19.98	27.89
Barlow	2009	535.15	0.43	0.41	16.29	32.81
Velva	2012	538.50	0.43	0.42	9.77	32.46
Elgin-ND	2013	484.69	0.47	0.42	11.76	35.74
HSD[b]		45.627	0.02	0.02	18.59	3.81

[a] RY, release year; Ca, calcium; P, phosphorus; K, potassium; Na, sodium; Zn, zinc; ppm, parts per million; %w/w, percent weight per weight.
[b] Tukey's honest significant difference ($P<0.05$).

Historic mineral values were reported at 500 ppm db Ca, 0.44% db P, 0.24% db K, and 100 ppm db Zn for the whole wheat samples of the cultivar Marquis (Sullivan and Near 1927). The historical Ca value falls within the range for all cultivars, but is slightly higher than the currently reported Marquis value (Table 5). The historical P value is also within the currently reported cultivar range, though it is positioned on the lower end and is also lower than the currently reported Marquis value. Furthermore, the historic K value is almost 10% lower than the lowest reported value for all cultivars, and is more than 25% lower than the reported Marquis value. On the other hand, the historic Zn value is more than twice the value of the highest reported result, as well as the value reported for Marquis; these results appear to confirm the observations of Garvin et al. (2006) and Fan et al. (2008), who observed decreasing Zn concentrations with increased yield. However, considerations must be made for differences in growing locations, soil mineral density, and the fertilizer type used (Syltie and Dahnke 1983).

Since both the total ash and three of the minerals were significantly ($P<0.001$) different between genotypes, it was important to investigate the differences further using cluster analysis (Figure 5). Cluster 1, which constitutes the cultivars Mida to Ceres, had release years of 1910–1972; cluster 2, with cultivars Fortuna through Barlow, had the range of years 1966–1977, and 2004–2013; and cluster 3, which contained cultivars Pilot through Pioneer-2375, had release years of 1939, and 1979–2007. Similar to the whole wheat dendogram, clusters 2 and 3 had no significant ($P>0.05$) separation, while cluster 1 was significantly ($P<0.05$) separated from clusters 2 and 3. The grouping for ash and mineral was based primarily on release years, minus a couple outliers.

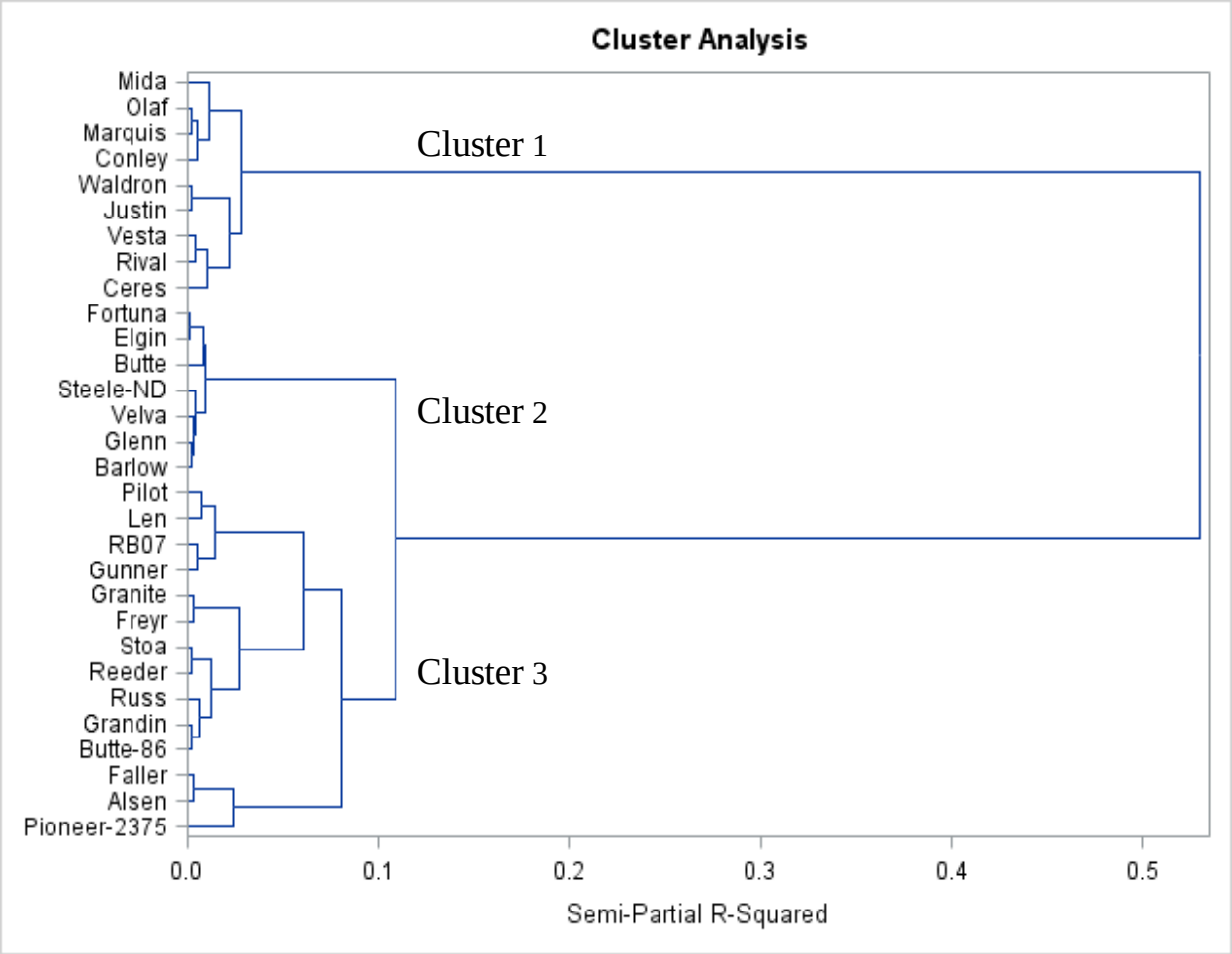


Figure 5. Cluster analysis for ash and mineral content in hard red spring whole wheat; Cluster 1 (Mida–Ceres) 1910–1972, Cluster 2 (Fortuna–Barlow) 1966–1977, and 2004–2013, Cluster 3 (Pilot–2375) 1939, and 1979–2007.

The internet and media are filled with people who have voiced concerns that whole grain wheat flours and products produced by millers and manufacturers are not as whole as the industry claims. The concern is that millers are not properly recombining or reconstituting mill streams, which would lead to a loss of whole grain components and nutritional value, or that producers are purchasing separate grain components from different classes of wheat (e.g. hard wheat endosperm and soft wheat bran and germ), and combining them in a way that seeks to improve the baking quality and sensory appeal of their whole wheat products, especially for consumers that prefer white flour products (Jones et al. 2015). If practices like this are

occurring, consumers purchasing whole wheat products may not be receiving the health benefits they would expect, such as higher mineral concentrations, from whole grain foods. Furthermore, wheat products produced using these shady methods could lead to claims that wheat products made from modern wheat cultivars are "less healthy" than historic wheat products (Perlmutter and Loberg 2013; Davis 2014).

5.2. Enzyme activity in whole wheat and white flour

Enzyme activity describes the quantity of active enzymes present in a given media. In breadmaking, the activity of hydrolytic enzymes is especially important because it affects the starch, nonstarch polysaccharide, and protein components in wheat flour. Changes in these components can seriously affect dough rheology, strength, and handling, which, in turn, can impact bread quality (Brijs et al. 2009; Carson and Edwards 2009). Historically, the first known role of enzymes in wheat, in regards to milling and baking, came about from studies on ungerminated and malted barley, where it was determined that there are two main types of starch degrading enzymes, α-amylase and β-amylase (Geddes 1946). Studies on protease activity, on the other hand, originated in the fields of physiology and dairy science during the early twentieth century, before being applied to cereals, and xylanase activity was not fully understood until the mid-twentieth century (Brijs et al. 2009). While the role of these enzymes in bread quality was fairly well understood, the experimental methods used to measure their activity relied heavily on difference measurements, i.e. the compositional difference of the sample material from the start of the procedure to the end, which greatly depended on the enzyme concentration, substrate concentration, and substrate hydrolysis susceptibility (Reed and Thorn 1964). Due to the differences in experimental procedures and units, a direct value-to-value comparison of any historic values with those presented in this paper cannot be made.

For α-amylase, which breaks down starch, the whole wheat activities ranged from 84.44–307.39 mU/g, with an approximate mean 151 mU/g, and the white flour range was 45.71–211.75 mU/g, with a mean 97 mU/g (Table 6); clearly, the results indicate that the α-amylase activity is present in more than the endosperm portion of the wheat caryopsis (Every et al. 2002). Moreover, significant differences ($P<0.001$) between genotypes was observed for α-amylase activity, and the differences were significantly related ($r = -0.47$, $P<0.01$) to release year (Table 7). While most of the α-amylase values fall within the typical range, some of the cultivars, such as Ceres, Mida, Pioneer-2375, and RB-07, have higher α-amylase values that may indicate the start of pre-harvest sprouting (Simsek et al. 2014a). Since pre-harvest sprouting can occur when harvest conditions are wet or very humid, it is possible that the rain showers that caused delays during harvest also contributed to higher α-amylase values, especially for cultivars susceptible to pre-harvest sprouting (McCrate et al. 1981; Rugg 2012).

Endo-protease, which hydrolyzes proteins, values were 1.58–2.44 A590/g/h for whole wheat and 1.24–1.75 A590/g/h for white flour (Table 6), which signifies that the endo-protease activity is higher in the whole grain and not just the endosperm. Like α-amylase, these activity results were significantly different ($P<0.001$) between genotypes and the differences in whole wheat were significantly correlated ($r = -0.49$, $P<0.01$) to release year (Table 7). Again, similar to the α-amylase values, the results are slightly higher than the typical range for endo-protease activity in wheat, which would also indicate possible pre-harvest sprouting (Simsek et al. 2014a).

Table 6. Enzyme activity for hard red spring whole wheat and white flour[a]

		Whole wheat			White flour		
Genotype	RY	AS (mU/g)	PS (A590/g/h)	XS (A590/g/h)	AS (mU/g)	PS (A590/g/h)	XS (A590/g/h)
Marquis	1910	135.35	2.16	7.25	88.24	1.40	2.51
Ceres	1926	289.93	2.44	7.92	129.44	1.75	4.07
Pilot	1939	140.10	2.09	3.35	76.56	1.41	1.65
Rival	1939	212.93	2.25	6.52	110.49	1.49	2.90
Vesta	1942	187.29	2.15	4.87	119.27	1.42	2.19
Mida	1944	307.39	1.88	7.84	207.05	1.42	4.69
Conley	1955	150.15	1.63	5.06	87.68	1.42	2.59
Justin	1963	184.10	1.79	3.18	131.63	1.32	0.85
Fortuna	1966	143.70	1.66	2.09	104.55	1.39	1.87
Waldron	1969	107.58	1.63	2.83	81.96	1.41	1.41
Olaf	1972	175.45	1.82	5.81	136.85	1.47	2.84
Butte	1977	145.29	2.12	0.86	111.03	1.50	0.45
Len	1979	111.81	1.83	3.77	73.51	1.34	1.66
Stoa	1984	160.57	2.13	1.88	111.62	1.24	0.61
Butte-86	1986	110.00	2.17	1.82	78.99	1.51	1.04
Grandin	1989	106.40	1.58	1.78	87.35	1.32	0.77
2375	1990	268.51	1.77	1.62	211.75	1.49	1.00
Russ	1995	133.83	1.60	1.40	88.54	1.36	0.57
Gunner	1995	103.03	1.96	2.24	62.57	1.39	1.06
Reeder	1999	107.02	2.08	2.89	80.58	1.46	1.32
Alsen	2000	84.44	1.93	0.73	55.47	1.51	0.23
Granite	2002	138.07	2.09	1.84	75.62	1.40	0.62
Steele-ND	2004	98.11	1.77	1.55	72.29	1.27	0.47
Freyr	2004	91.01	1.62	0.84	45.71	1.47	0.34
Glenn	2005	114.30	1.87	1.00	59.49	1.44	0.28
Faller	2007	92.45	1.76	0.88	56.80	1.42	0.52
RB-07	2007	252.69	1.66	1.46	139.69	1.39	0.66
Barlow	2009	144.33	1.82	1.66	83.60	1.44	0.51
Velva	2012	114.51	1.86	4.74	62.12	1.36	1.71
Elgin-ND	2013	118.92	1.75	1.69	79.37	1.50	0.60
HSD[b]		7.42	0.20	0.37	10.48	0.15	0.16

[a] RY, release year; AS, α-amylase; PS, endo-protease; XS, xylanase.
[b] Tukey's honest significant difference (P<0.05).

Xylanase, which dismantles the cell wall polysaccharide arabinoxylan, had an apparent activity range of 0.73–7.92 A590/g/h in whole wheat and 0.23–4.69 A590/g/h in white flour

(Table 6). Again, these results suggest that xylanase activity is present in the germ and bran portion of the caryopsis, as well as the endosperm (Mendis et al. 2013). Similar results have been observed in previous studies, which attribute most of the xylanase activity in wheat to the outer layers of the grain (Gys et al. 2004; Dornez et al. 2006; Gebruers et al. 2010). Furthermore, there were significant differences (P<0.001) between genotypes and the differences were significantly related (r = -0.79, P<0.001) to release year (Table 7), more so than α-amylase and endo-protease. However, it should be noted that wheat contains endogenous xylanase inhibitors and, since the xylanase/xylanase inhibitor complexes are not measured by the assay, the measured xylanase activities must be regarded as apparent and not actual (Courtin et al. 2005; Simsek et al. 2011a).

Table 7. Correlation coefficients (r) between enzymatic activities and year, chemical composition, and enzymatic activities for hard red spring whole wheat and white flour[a,b,c]

		RY	AC	AS	PS	XS	CMA	CAF
Whole wheat	AS	-0.472 **	0.512 **	1.000	0.258 NS	0.555 **	0.374 *	-0.032 NS
	PS	-0.487 **	0.199 NS	0.258 NS	1.000	0.438 *	0.352 NS	0.102 NS
	XS	-0.792 ***	0.817 ***	0.555 **	0.438 *	1.000	0.843 ***	0.534 **
White flour	AS	-0.378 *	0.414 *	0.897 ***	0.079 NS	0.406 *	0.212 NS	-0.059 NS
	PS	-0.230 NS	0.176 NS	0.335 NS	0.437 *	0.269 NS	0.162 NS	-0.029 NS
	XS	-0.766 ***	0.814 ***	0.634 ***	0.359 NS	0.946 ***	0.712 ***	0.402 *

[a] RY, release year; AC, ash content; AS, α-amylase; PS, endo-protease; XS, xylanase; CMA, *p*-coumaric acid; CAF, caffeic acid.
[b] All column titles, excluding RY, correspond to hard red spring whole wheat parameters.
[c] NS, not significant; *, **, and *** Significant at 0.05, 0.01, and 0.001 probability levels, respectively.

Using genotype as the independent variable and the enzyme activities as the dependent variables, a dendogram was created to aid in analysis of the significant differences between genotypes (Figure 6). Cluster 1, which constitutes the cultivars Vesta to Ceres, had release years of 1910–1944; cluster 2, with cultivars Reeder through Alsen, had release years of 1939, and 1977–2005; and cluster 3, which contained cultivars Velva through Pioneer-2375, had the largest range of release years from 1955 to 2013. Clusters 2 and 3 were not significantly (P>0.05)

separated, while cluster 1 was significantly (P<0.05) separated from clusters 2 and 3. However, even though the dendograms demonstrated some grouping based on release year, it cannot be concluded that this separation is based solely on genotype differences.

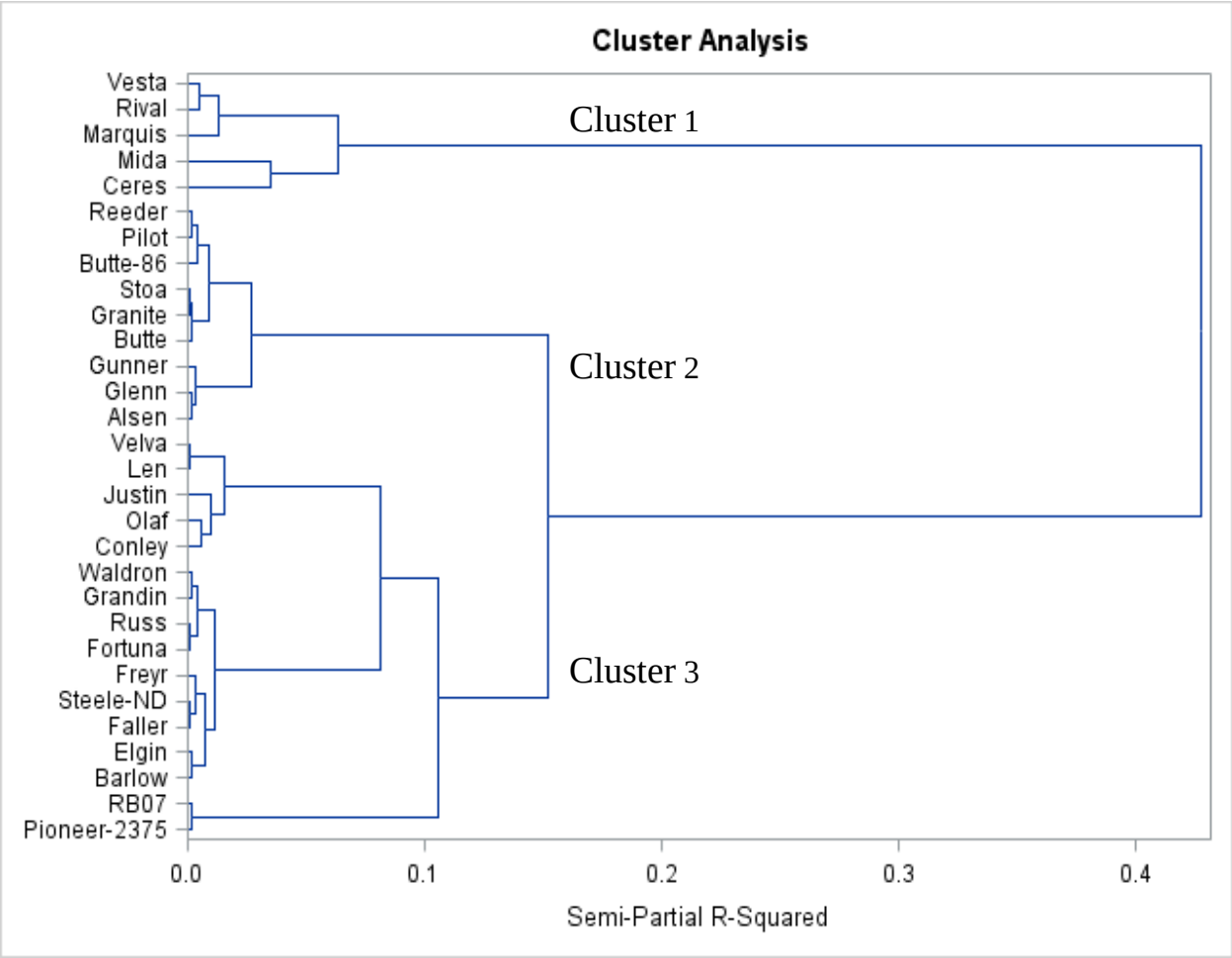


Figure 6. Cluster analysis for enzyme activity in hard red spring whole wheat; Cluster 1 (Vesta–Ceres) 1910–1944, Cluster 2 (Reeder–Alsen) 1939, and 1977–2005, Cluster 3 (Velva–2375) 1955–2013.

Fungal enzymes, such as those derived from the fungus *Aspergillus oryzae*, are added to flour at the mill or bakery to improve dough quality, but have been found to be an allergen source in people who suffer from baker's asthma (Brisman and Belin 1991; Quirce et al. 1992; Smith and Smith 1998; Sander et al. 2000). While fungal α-amylase is usually considered the primary allergen source, some study participants have demonstrated allergic reactions to

xylanase as well (Tarvainen et al. 1991; Baur et al. 1998). Furthermore, fungal enzymes are not the only allergen source; several other wheat proteins, including bacterial enzymes and enzyme inhibitors, also have allergenic properties (Elms et al. 2006; Tatham and Shewry 2008). However, due to the high temperatures during the baking phase of bread making, all native and added fungal enzymes are denatured, which reduces the allergenicity of these proteins by destroying most of the active sites recognized by the antibodies (Davis and Williams 1998; Delcour and Hoseney 2010d). Therefore, except for those with baker's asthma and who regularly handle flour with added fungal enzymes, these results indicate that it is improbable that any residual enzymes in bread products would negatively affect the consumer's health, and, as such, any changes in enzyme activity would mostly impact dough and bread quality.

5.3. Phenolic acids in whole wheat

Phenolic acids have gained popularity over recent years due to their antioxidant properties in cereals and cereal products (Fardet 2010; Khakimov et al. 2014). However, phenolic acids have been reported to produce undesirable bitter and astringent flavors in bread products, as well as gray, brown, or green colors (Piironen et al. 2009). For all phenolic acids measured, including the total free and total bound, significant differences ($P<0.001$) were observed between genotypes. However, the significant differences observed were not correlated to release year, except for vanillic acid, *p*-coumaric acid, caffeic acid, and syringic acid (Table 9). *p*-Coumaric ($r = -0.84$) and caffeic acid ($r = -0.57$) were related more significantly ($P<0.001$) to release year than either vanillic ($r = -0.43$, $P<0.05$) or syringic acid ($r = 0.36$, $P<0.05$). Moreover, since Sosulski et al. (1982) observed higher phenolic acid concentrations in fresh wheat than in wheat stored for six months or more, it would reason that the values given in this

paper would have been higher (except for the cis-isomer of ferulic acid) if the samples had been processed immediately, but the ratios should have remained the same.

The profiling of phenolic acids, and metabolomics in general, is a fairly recent endeavor, due to the advanced, state-of-the-art technology required for accurate analysis (Khakimov et al. 2014). While a couple early cereal studies attempted to analyze phenolic acids in wheat, the experimental methods used had many limitations, and the focus of early phenolic acid analysis was mainly concerned with their effects on plant growth and maturation, instead of human health and nutrition (Bardinskaya and Shubert 1962; Guenzi and McCalla 1966). Moreover, as researchers continue to investigate phenolic acids in wheat, and their antioxidant capabilities, it has been discovered that environmental conditions influence the phenolic acid concentration more than genotype (Mpofu et al. 2006; Khakimov et al. 2014).

Due to differences in extraction methods and units, which can be reported as ppm, µg GAE/ 100 g (gallic acid equivalent), or mg FAE/g (ferulic acid equivalent), a direct comparison of published phenolic acid results to the results given in the paper was difficult without a conversion factor. However, some observations from the results in this thesis can be confirmed by published results (Table 8). First, it was observed that total bound phenolic acids were present in greater quantities than free phenolic acids (Dinelli et al. 2011; Leoncini et al. 2012). Second, ferulic acid accounts for 70–90% of the total phenolic acids present in wheat (Naczk and Shahidi 2006; Dinelli et al. 2011). And third, the most common phenolic acids detected were ferulic, vanillic, syringic, *p*-coumaric, sinapic, and caffeic acids (Mpofu et al. 2006; Dinelli et al. 2011); protocatechuic and *p*-hydroxybenzoic acids have also been reported (Piironen et al. 2009).

Table 8. Phenolic acid content for hard red spring whole wheat[a]

		Free	Bound								
Genotype	RY	Total[b]	HYB	PRT	VNC	CMA	CAF	SYG	FRL	SNP	Total
Marquis	1910	1.32	2.49	4.36	8.99	31.63	14.65	4.52	669.84	14.06	750.53
Ceres	1926	1.19	1.73	3.20	4.66	26.02	9.32	2.69	546.20	8.97	602.79
Pilot	1939	1.29	1.69	3.42	4.88	20.89	9.51	2.81	527.48	9.22	579.89
Rival	1939	1.23	1.88	3.51	5.96	23.63	10.56	2.34	503.26	10.27	561.40
Vesta	1942	1.24	1.63	3.11	5.07	18.35	8.63	2.94	470.65	9.88	520.26
Mida	1944	1.42	2.07	3.53	5.38	18.92	7.60	2.48	520.95	8.52	569.46
Conley	1955	1.25	2.28	4.71	6.38	22.18	15.60	2.96	678.36	13.41	745.87
Justin	1963	1.24	2.22	5.57	6.66	23.82	11.78	3.75	643.71	12.24	709.75
Fortuna	1966	1.47	1.72	3.52	4.75	13.23	8.27	3.22	507.89	9.02	551.63
Waldron	1969	1.42	1.96	3.16	5.88	15.99	11.35	2.98	562.30	11.78	615.39
Olaf	1972	1.37	2.59	3.32	6.18	18.76	12.11	4.04	637.06	16.05	700.11
Butte	1977	1.44	1.88	3.67	5.69	11.60	8.67	3.30	624.31	13.01	672.12
Len	1979	1.24	2.31	4.10	5.44	18.08	9.28	3.60	572.06	14.29	629.16
Stoa	1984	1.22	1.66	3.34	4.88	11.58	8.74	2.89	587.70	12.83	633.63
Butte-86	1986	1.38	1.99	4.07	4.91	9.20	7.67	3.08	521.73	12.94	565.59
Grandin	1989	1.34	2.15	3.40	5.45	13.48	9.00	2.83	557.75	10.36	604.43
2375	1990	1.32	1.74	2.89	4.60	10.87	5.53	3.73	495.58	12.66	537.60
Russ	1995	1.13	2.01	3.71	5.34	10.46	6.85	3.29	500.93	10.62	543.22
Gunner	1995	1.28	1.43	3.33	4.45	15.70	9.45	4.28	516.54	10.71	565.89
Reeder	1999	1.24	1.78	3.43	4.86	12.07	8.69	2.94	591.63	10.20	635.60
Alsen	2000	1.23	1.70	3.42	4.83	9.59	8.03	3.32	501.32	9.68	541.89
Granite	2002	1.69	2.15	3.52	7.03	13.33	8.10	5.47	543.93	19.11	602.65
Steele-ND	2004	1.19	1.88	3.33	4.68	9.49	6.90	3.27	512.31	11.60	553.47
Freyr	2004	1.27	1.53	3.12	5.16	14.08	6.36	4.78	471.53	10.92	517.49
Glenn	2005	1.21	2.00	3.59	5.63	9.10	7.41	3.97	548.87	11.99	592.55
Faller	2007	1.40	1.57	3.12	4.08	11.51	8.02	3.76	470.12	11.08	513.28
RB-07	2007	1.36	1.51	3.43	5.22	13.30	7.28	3.93	461.80	9.94	506.41
Barlow	2009	1.30	1.92	4.03	5.23	10.80	9.64	4.54	597.39	14.83	648.38
Velva	2012	1.20	2.18	3.44	5.82	17.04	8.11	3.62	675.91	12.13	728.25
Elgin-ND	2013	1.33	1.68	3.49	4.64	12.30	8.48	2.85	532.75	8.34	574.53
HSD[c]		0.05	0.61	1.05	1.42	1.99	1.66	0.44	65.78	1.52	72.16

[a] RY, release year; HYB, p-hydroxybenzoic; PRT, protocatechuic; VNC, vanillic; CMA, p-coumaric; CAF, caffeic; SYG, syringic; FRL, ferulic; SNP, sinapic.

[b] Expressed as mg ferulic acid equivalent per g of sample

[c] Tukey's honest significant difference ($P<0.05$).

Table 9. Correlation coefficients (r) between phenolic acids and year, chemical composition, and enzymatic activities for hard red spring whole wheat[a,b]

	RY	AC	AS	PS	XS	CMA	CAF
TFP	0.033 NS	-0.014 NS	0.049 NS	-0.027 NS	-0.113 NS	-0.105 NS	-0.008 NS
HYB	-0.279 NS	0.336 NS	-0.019 NS	-0.060 NS	0.442 *	0.405 *	0.565 **
PRT	-0.231 NS	0.287 NS	-0.050 NS	-0.055 NS	0.156 NS	0.385 *	0.580 ***
VNC	-0.428 *	0.388 *	0.016 NS	0.106 NS	0.421 *	0.601 ***	0.656 ***
CMA	-0.838 ***	0.772 ***	0.374 *	0.352 NS	0.843 ***	1.000	0.719 ***
CAF	-0.574 ***	0.693 ***	-0.032 NS	0.102 NS	0.534 **	0.719 ***	1.000
SYG	0.363 *	-0.328 NS	-0.257 NS	-0.166 NS	-0.299 NS	-0.095 NS	-0.032 NS
FRL	-0.212 NS	0.345 NS	-0.108 NS	0.085 NS	0.353 NS	0.420 *	0.697 ***
SNP	0.130 NS	-0.096 NS	-0.176 NS	0.053 NS	-0.053 NS	0.004 NS	0.285 NS
TBP	-0.278 NS	0.399 *	-0.078 NS	0.110 NS	0.408 *	0.496 **	0.746 ***

[a] RY, release year; AC, ash content; AS, α-amylase; PS, endo-protease; XS, xylanase; CMA, *p*-coumaric acid; CAF, caffeic acid; TFP, total free phenolic; HYB, *p*-hydroxybenzoic acid; PRT, protocatechuic acid; VNC, vanillic acid; SYG, syringic acid; FRL, ferulic acid; SNP, sinapic acid; TBP, total bound phenolic.
[b] NS, not significant; *, **, and *** Significant at 0.05, 0.01, and 0.001 probability levels, respectively.

A dendogram was prepared to analyze the significant differences in phenolic acids between genotypes and to determine if the clusters were separated based on historic and modern cultivars (Figure 7). Cluster 1, which constitutes the cultivars Marquis to Conley, had release years of 1910–1963; cluster 2, with cultivars Waldron through Ceres, had release years of 1926–1969, 1989, and 2013; and clusters 3 and 4, which contained cultivars Velva through Alsen and Granite to Pioneer-2375, respectively, had the largest range of release years 1972–2012. Clusters 1 and 2 were significantly (P>0.05) similar, and clusters 3 and 4 were significantly (P>0.05) similar as well; however, both clusters 1 and 2 were significantly (P<0.05) separate from clusters 3 and 4. The observed separations indicate that the cluster grouping was based mostly on historic (1910–1969) or modern (1970–2013) release years, aside from the 1989 and 2013 outliers seen in cluster 2.

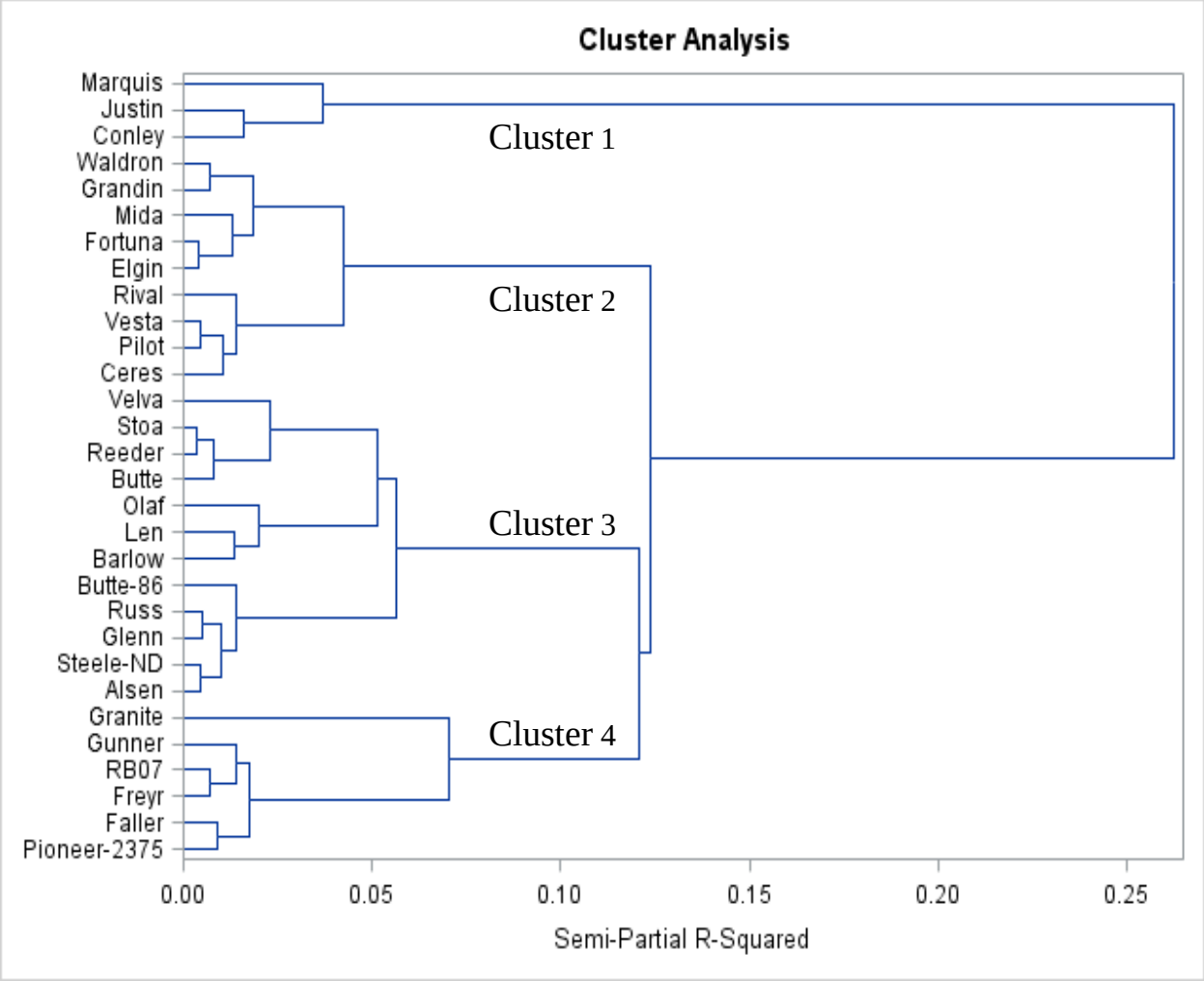


Figure 7. Cluster analysis for phenolic acids in hard red spring whole wheat; Cluster 1 (Marquis–Conley) 1910–1963, Cluster 2 (Waldron–Ceres) 1926–1969, 1989, and 2013, Cluster 3 (Velva–Alsen) 1972–2012, Cluster 4 (Granite–2375) 1990–2007.

In addition to antioxidant properties, there is evidence to support additional health benefits of phenolic acids, including anti-microbial, anti-inflammatory, blood cholesterol- and glucose-lowering effects, as well as UV absorption, vascular relaxation (reduces blood pressure), tumor growth suppression, and enzyme modulation (Fardet 2010). Theoretically, any decrease in phenolic acid concentration would decrease these potential health benefits. However, in actuality, the potential benefits of phenolic acids depends more on their bioavailability than on their concentration, which can vary depending on how the wheat product was processed or

prepared (Liu 2007). Furthermore, research has indicated that only free and soluble phenolic acids are absorbed, and that the antioxidative activities are different for each phenolic acid, with ferulic acid being the most potent of the phenolic acids in wheat (Piironen et al. 2009).

In view that the results presented in this paper do not indicate any significant (P>0.05) differences in the total free phenolic and ferulic acid concentrations, in regards to release year (Table 9), it can be concluded that, while there are differences between genotypes, the phenolic acid levels have not substantially changed in HRS wheat cultivars over the last century. Moreover, the amount of phenolic acids consumed depends entirely on each consumer's food choices; since phenolic acids are concentrated mainly in the bran, only whole grain wheat products will supply phenolic acids to consumer's diets (Piironen et al. 2009). Furthermore, the type of foods consumed in conjunction with the whole wheat product can also affect the bioavailability of the phenolic acids; the exact details of these interactions are yet unknown, due to the large number of phenolic chemicals and biological effects involved (Fardet 2010).

5.4. Starch characterization in white flour and bread

As mentioned previously, starch consists of two glucose polymers, amylopectin and amylose, and is concentrated in the starchy endosperm of the caryopsis (Stone and Morell 2009). In breadmaking, starch gelatinization is crucial for shape retention in bread loaves after baking, and starch hydrolysis can provide the necessary reducing sugar units, specifically glucose, required for the Maillard reactions (Delcour and Hoseney 2010d). For all parameters measured, significant differences (P<0.001) were observed between genotypes for both the white flour and bread results. Furthermore, except for the total starch (r = 0.38) values in bread, which were significantly (P<0.05) related, none of the other starch values were significantly (P>0.05) correlated with release year (Table 11).

Table 10. Hard red spring wheat amylose content in white flour and bread

Genotype	RY[a]	Flour (%)	Bread (%)
Marquis	1910	25.23	16.40
Ceres	1926	25.85	16.50
Pilot	1939	25.86	20.99
Rival	1939	24.54	21.58
Vesta	1942	25.87	19.67
Mida	1944	25.12	16.90
Conley	1955	25.37	20.21
Justin	1963	24.90	18.88
Fortuna	1966	24.98	17.46
Waldron	1969	25.93	22.64
Olaf	1972	24.44	22.83
Butte	1977	25.46	18.27
Len	1979	25.84	16.80
Stoa	1984	25.82	20.60
Butte-86	1986	25.65	20.49
Grandin	1989	25.64	20.62
2375	1990	25.79	19.28
Russ	1995	25.44	25.00
Gunner	1995	25.90	20.75
Reeder	1999	25.77	20.15
Alsen	2000	25.65	22.58
Granite	2002	24.98	19.43
Steele-ND	2004	25.44	15.06
Freyr	2004	25.01	19.15
Glenn	2005	24.39	16.02
Faller	2007	24.16	18.66
RB-07	2007	25.85	25.46
Barlow	2009	25.75	20.13
Velva	2012	25.64	16.21
Elgin-ND	2013	24.58	20.12
HSD[b]		0.47	0.56

[a] RY, release year.
[b] Tukey's honest significant difference ($P<0.05$).

In the HRS wheat samples analyzed, it was confirmed that amylose constitutes approximately 25% of starch in flour, while amylopectin constitutes approximately 75%; the amylopectin values are not presented since they are simply the difference between 100 and the

amylose values (Table 10). During the baking process in bread making, the amount of amylose decreases due to the formation of amylose-lipid complexes and starch modifications, which transpire as a result of amylase activity during baking and usually occur from the beginning of starch gelatinization until the enzymes are denatured (Delcour and Hoseney 2010d). Anti-wheat advocates argue that the amylopectin in wheat is the most digestible form of amylopectin found in foods or that the starch chemistry is different in modern wheat and, as such, is very efficiently converted to blood sugar, which causes dramatic elevations in blood sugar levels (Cordain 2011; Perlmutter and Loberg 2013; Davis 2014). While there exists mutants of wheat that have extremely high (>90%) amylopectin amounts, known as waxy starches, these varieties are not produced commercially and, furthermore, wheat breeding efforts have focused on increasing the amount of amylose in wheat starch, which would help to decrease the rate of starch digestion after consumption (Delcour and Hoseney 2010a; Jones 2012; Brouns et al. 2013).

The difference in amounts of amylose and amylopectin in wheat starch have been well known for over 50 years (Medcalf and Gilles 1965). However, the determination of the molecular weights was not very accurate initially. Historically, the linear portion of starch—amylose—was reported to have an approximate weight of 87,000 to 140,000 g/mole, depending on if the periodate oxidation or osmotic pressure method was used, respectively, while the branched portion—amylopectin—was reported at 4 million g/mole (Bechtel et al. 1964). For reference, one gram per mole is the equivalent of one Dalton (Da). Nonetheless, as technology has improved, the determination of amylose and amylopectin molecular weights has as well, since current methods are more accurate than the experimental techniques used previously.

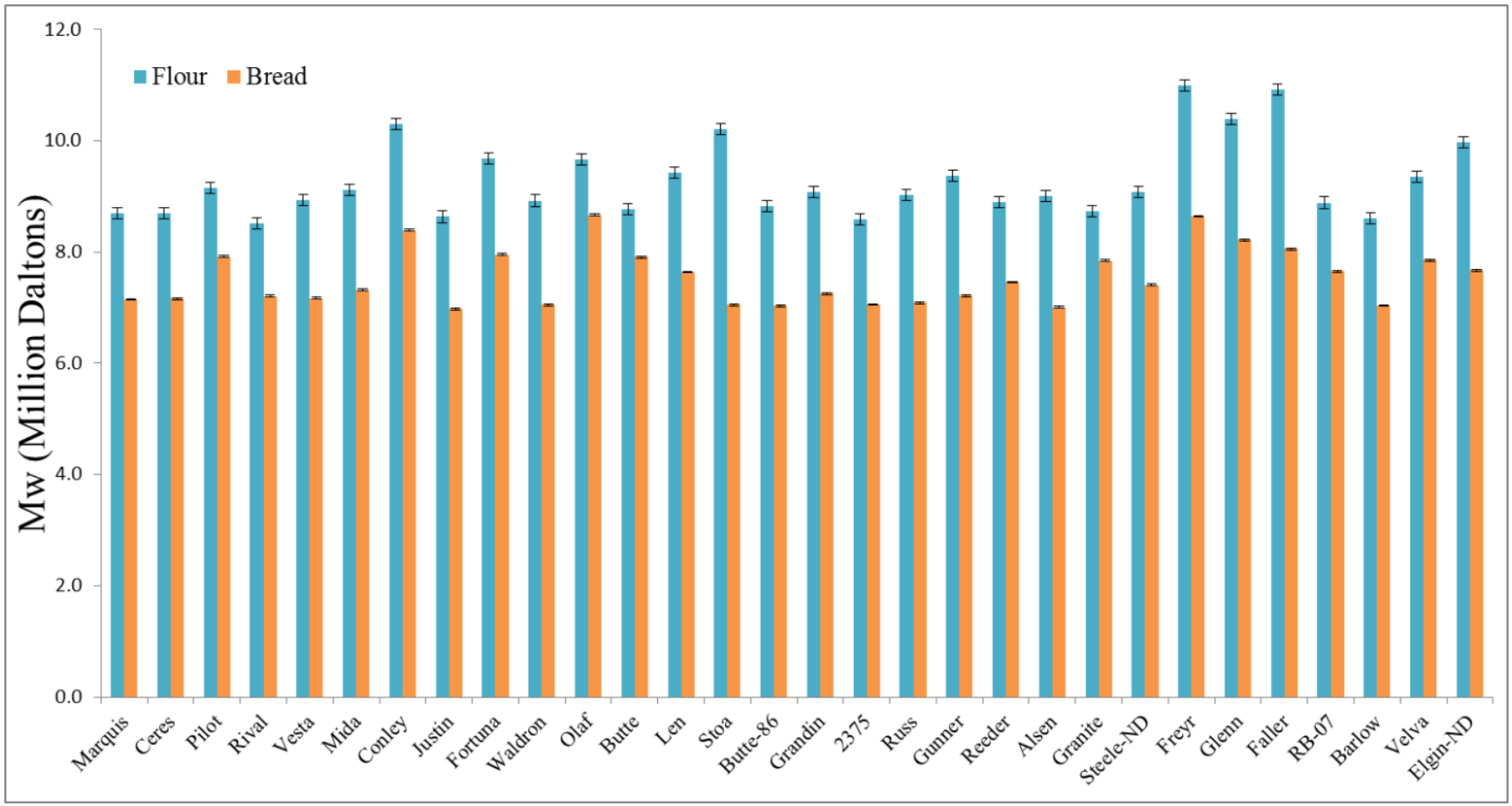


Figure 8. Amylopectin molecular weights in hard red spring wheat white flour and bread[a,b]

[a] White flour average is 9.3×10^6 Da and bread average is 7.5×10^6 Da.

[b] Error bars represent ± Tukey's HSD ($P<0.05$): flour = 1.01×10^5; bread = 1.70×10^4.

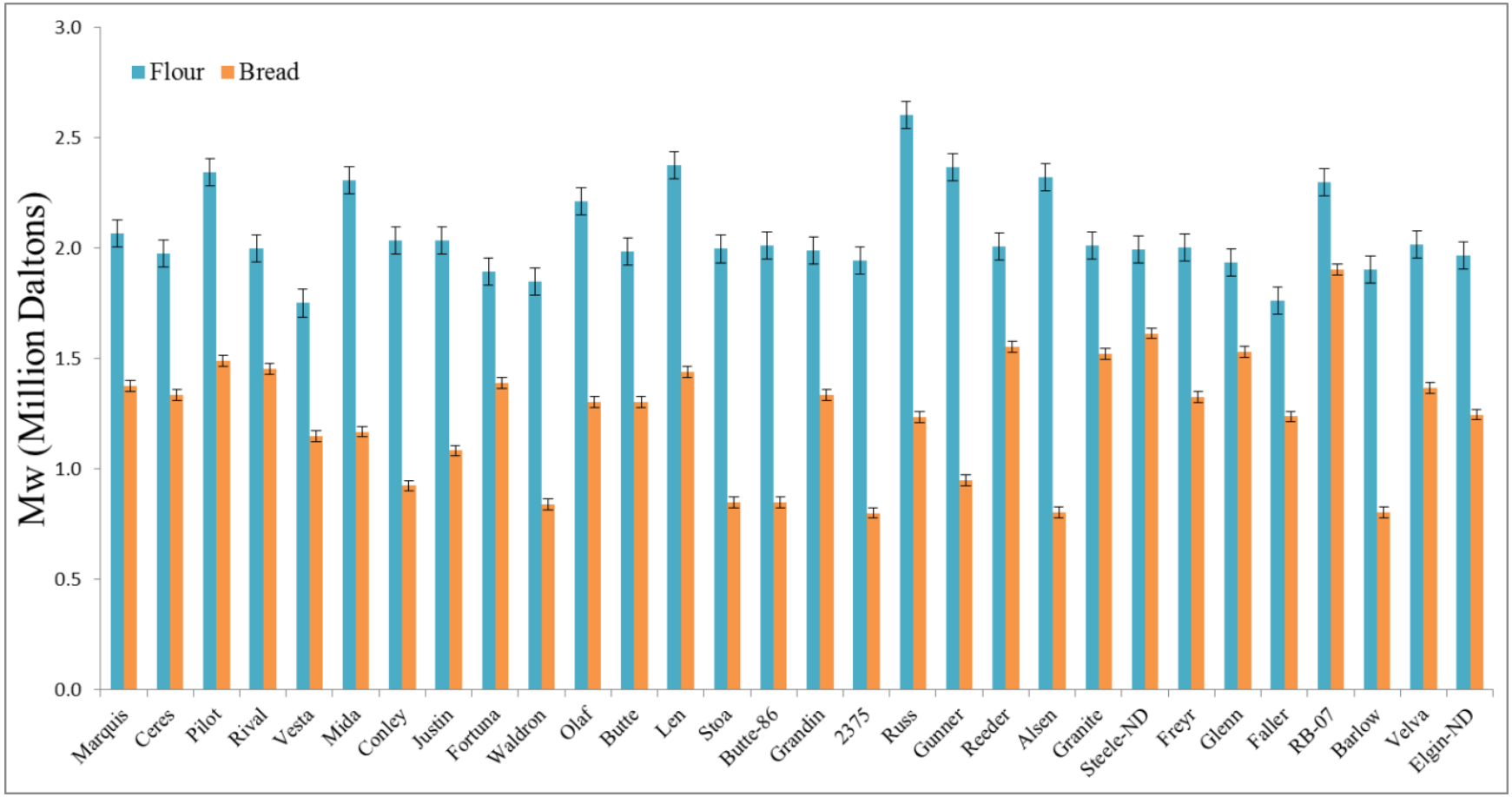


Figure 9. Amylose molecular weights in hard red spring wheat white flour and bread[a,b]

[a] White flour average is 2.1×10^6 Da and bread average is 1.2×10^6 Da.

[b] Error bars represent ± Tukey's HSD ($P<0.05$): flour = 6.15×10^4; bread = 2.39×10^4.

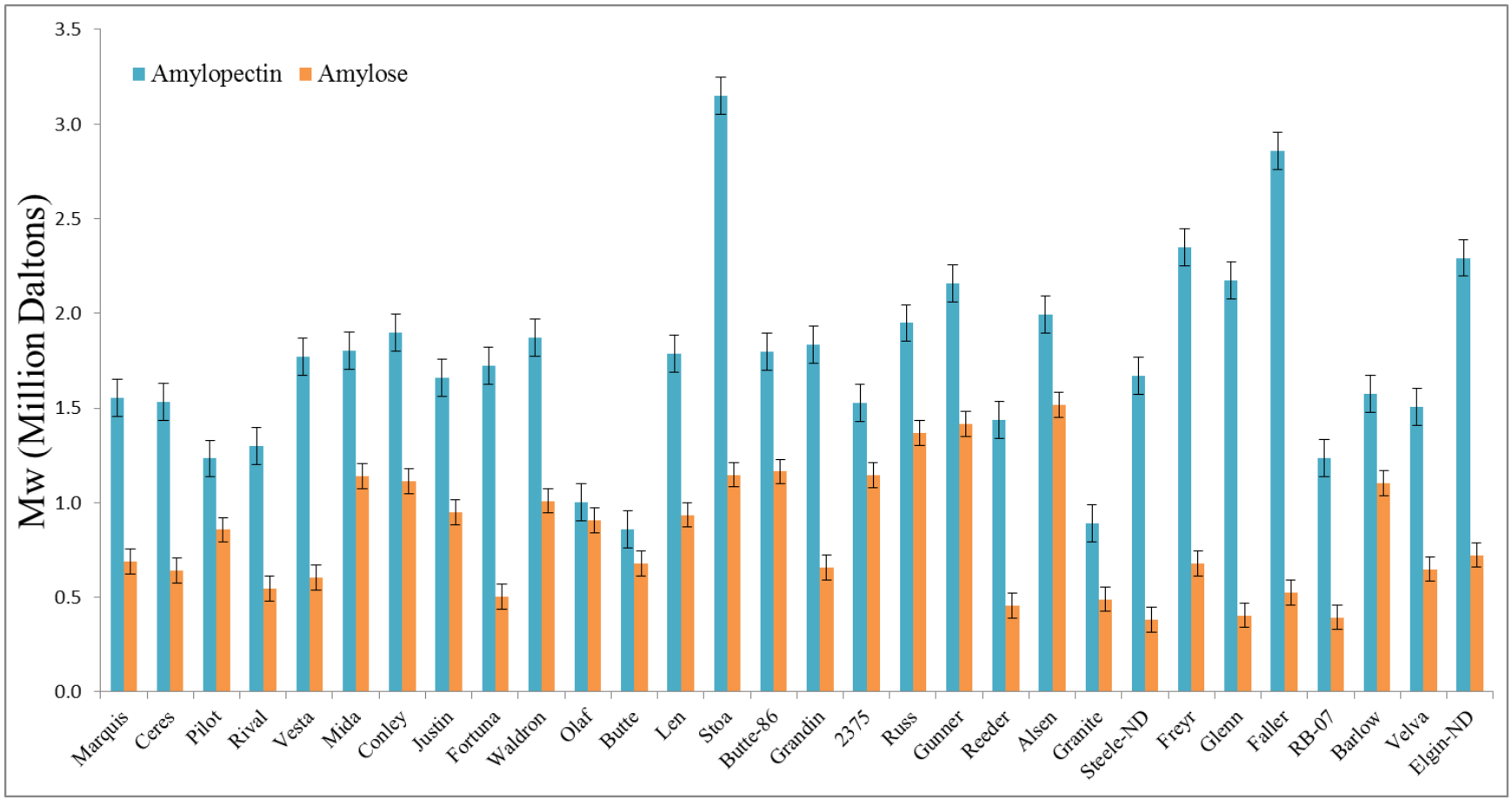


Figure 10. Difference in amylopectin and amylose molecular weights after baking in hard red spring wheat white flour and bread[a,b]

[a] Amylopectin average change is 1.7×10^6 Da and amylose change is 0.8×10^6 Da.

[b] Error bars represent ± Tukey's HSD ($P<0.05$): flour = 9.80×10^4; bread = 6.56×10^4.

Published values for the molecular weights (Mw) of the starch constituents in white flour indicate a typical range of 10–310 million Da for amylopectin and 0.1–2.0 million Da for amylose (Yoo and Jane 2002; Simsek et al. 2013; Simsek et al. 2014b). When comparing the published values to the mean values presented in this paper, it would appear that the amylopectin average ($9.3x10^6$ Da) is low (Figure 8), while the amylose average ($2.1x10^6$ Da) is high (Figure 9); the average difference in amylopectin and amylose Mw after baking is $1.7x10^6$ Da and $0.8x10^6$ Da respectively (Figure 10). However, given that the range for the Mw of amylopectin and amylose is extremely broad, 10^7–10^9 Da and 10^5–10^6 Da, respectively, these averages are within the range reported in literature (Gidley et al. 2010). Furthermore, considerations must be made for differences in experimental procedures. The weights given in this paper were obtained through HPSEC with multi-angle light scattering and refractive index detectors, which allows for the Mw to be measured in absolute terms, instead of relative terms (Grant et al. 2002).

Table 11. Correlation coefficients (r) between starch and year, chemical composition, and enzymatic activities for hard red spring wheat white flour and bread[a,b,c]

		RY	AC	AS	PS	XS	CMA	CAF
Flour	TS	-0.254 NS	0.122 NS	0.209 NS	0.325 NS	0.242 NS	0.106 NS	-0.188 NS
	AM	-0.075 NS	-0.066 NS	0.078 NS	0.150 NS	-0.016 NS	-0.032 NS	-0.081 NS
	APW	0.315 NS	-0.101 NS	-0.376 *	-0.388 *	-0.264 NS	-0.219 NS	-0.059 NS
	AMW	-0.005 NS	0.016 NS	0.062 NS	-0.110 NS	0.064 NS	0.058 NS	-0.048 NS
Bread	TS	0.384 *	-0.387 *	-0.436 *	-0.300 NS	-0.336 NS	-0.312 NS	-0.296 NS
	AM	0.166 NS	-0.086 NS	0.008 NS	-0.227 NS	-0.250 NS	-0.191 NS	0.029 NS
	APW	0.175 NS	0.081 NS	-0.207 NS	-0.287 NS	-0.071 NS	-0.031 NS	0.071 NS
	AMW	0.015 NS	-0.025 NS	0.049 NS	0.032 NS	0.086 NS	0.119 NS	-0.155 NS
	CAP	0.252 NS	-0.218 NS	-0.302 NS	-0.239 NS	-0.287 NS	-0.266 NS	-0.150 NS
	CAM	-0.016 NS	0.031 NS	-0.005 NS	-0.095 NS	-0.036 NS	-0.069 NS	0.107 NS

[a] RY, release year; AC, ash content; AS, α-amylase; PS, endo-protease; XS, xylanase; CMA, *p*-coumaric acid; CAF, caffeic acid; TS, total starch; AM, amylose; APW, amylopectin weight; AMW, amylose weight; CAP, change in amylopectin; CAM, change in amylose.
[b] All column titles, excluding RY, correspond to hard red spring whole wheat parameters.
[c] NS, not significant; * Significant at 0.05 probability level.

Similar to before, a dendogram was prepared to determine if there were any significant groupings between genotypes (Figure 11). Cluster 1, which constitutes the cultivars Freyr to

Conley, had release years of 1955, 1972, and 2004–2007; cluster 2, with cultivars Vesta through Ceres, had the largest range of release years 1910–1966, 1979–1989, and 2004–2013; cluster 3, which contained cultivars RB-07 through Butte, had release years of 1939, 1977–1999, and 2002–2007; and cluster 4, with cultivars Stoa to Pioneer-2375, were released in years 1944–1969, 1984–2009. After noticing the wide spread of release years, it should be apparent that none of the clusters were significantly (P>0.05) different from each other, which means there were no differences that corresponded to changes in the historic or modern cultivars.

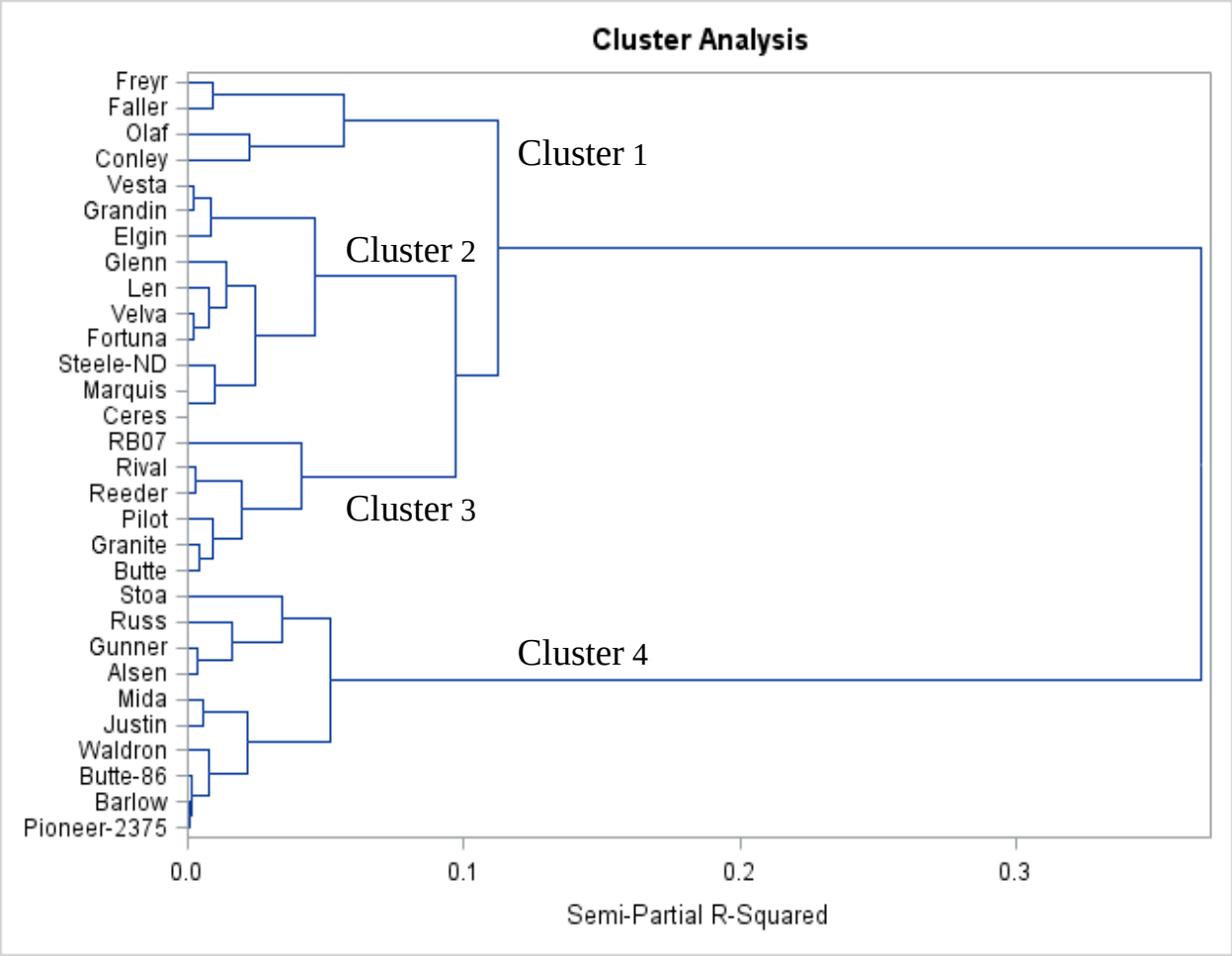


Figure 11. Cluster analysis for amylose and amylopectin amounts and molecular weights in bread made from hard red spring wheat; Cluster 1 (Freyr–Conley) 1955, 1972, and 2004–2007, Cluster 2 (Vesta–Ceres) 1910–1966, 1979–1989, and 2004–2013, Cluster 3 (RB-07–Butte) 1939, 1977–1999, and 2002–2007, Cluster 4 (Stoa–2375) 1944–1969, 1984–2009.

5.5. Digestibility of starch in bread

Starch constitutes 60–75% of the wheat grain weight and is a very important dietary component, since it consists of nearly 70–80% of calories consumed by humans worldwide (Delcour and Hoseney 2010a). Furthermore, starch is the only polysaccharide humans can digest; however, not all starch fractions are digested or digestion occurs at different rates. These nutritionally important starch fractions include rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) (Englyst et al. 1992). RDS is made primarily from amylopectin, and is readily hydrolyzed by gut enzymes and absorbed. SDS, on the other hand, contains mostly amylose, and is digested and absorbed more slowly in the gut. The remaining starch consists of the indigestible or resistant portions of starch; instead of being broken down and absorbed in the stomach and small intestine, this fraction passes to the large intestine where it undergoes fermentation by microflora present in the colon (Patil 2008; Stone and Morell 2009). Besides the starch fractions, two other important parameters are the hydrolysis index (HI) and the glycemic index (GI). HI is a value given to indicate the amount of starch that is broken down by enzymes in the gut, while GI is a measure of the blood glucose response after consumption of a particular food, where the rate and extent of the digestion of starch is reflected in the magnitude and the duration of the glycemic response (Englyst et al. 1992; Brouns et al. 2013). For this research study, an estimated GI (eGI) was used, which involved an *in vitro* experimental procedure that was designed to mimic the digestion of starch in the human gut, due to the infeasibility of using human participants to study the actual GI of each HRS wheat cultivar (Englyst et al. 1992).

Starch digestibility research initially began with human studies, where a food sample of known starch amount was consumed, and the resulting fecal matter was then analyzed for any

remaining starch to estimate the starch digestibility (Langworthy and Deuel 1920). However, while this approach worked well for basic digestion analysis, it failed to consider the different fractions and digestion rates of starch, which wouldn't be recognized until much later (Englyst et al. 1992; Birkett and Brown 2008). Due to the newness of starch digestion analysis via *in vitro* methods, there are no historical data available for HRS wheat, or any other type of wheat.

For all starch fractions measured in bread, significant differences ($P<0.001$) were observed between genotypes. However, except for TS in bread (as mentioned in the previous section), none of the values were significantly ($P>0.05$) correlated to release year (Table 13). Englyst and Hudson (1996) determined the following means for white and whole wheat bread parameters, respectively: TS = 41.7, 35.0; RDS = 37.4, 32.1; SDS = 3.7, 1.4; and RS = 0.6, 1.5 g starch/100 g of food as eaten (Englyst and Hudson 1996). The current means for TS (68.1%), SDS (31.5%), and RS (7.9%) were higher than the literature, while the RDS (28.7%) mean was lower (Table 12). These results are encouraging as they indicate that starch in HRS wheat products is less rapidly digested than typical bread products, which are likely made from other classes of wheat, especially winter wheat. However, in consideration that no details were provided by Englyst and Hudson (1996) on the bread samples used, such as how they were made or where they were obtained, it cannot be concluded that starch from HRS wheat is less digestible than other wheat classes, since the samples may have contained some HRS wheat.

Table 12. Digestibility of starch in bread made from hard red spring wheat[a]

Genotype	RY	TS (%)	RDS (%)	SDS (%)	RS (%)	HI	eGI
Marquis	1910	62.58	26.68	32.81	3.09	103.04	97.02
Ceres	1926	66.14	28.52	31.50	6.13	96.13	91.06
Pilot	1939	66.23	27.81	31.26	7.17	101.20	95.43
Rival	1939	70.76	27.60	32.55	10.62	98.82	93.38
Vesta	1942	69.78	29.48	31.94	8.36	98.71	93.28
Mida	1944	62.57	28.48	26.41	7.68	104.41	98.20
Conley	1955	66.05	28.87	32.79	4.40	94.01	89.23
Justin	1963	66.43	26.47	34.39	5.57	95.97	90.93
Fortuna	1966	71.55	32.64	29.37	9.55	98.24	92.88
Waldron	1969	73.23	31.44	32.04	9.75	97.06	91.87
Olaf	1972	65.19	27.30	29.09	8.79	101.74	95.89
Butte	1977	65.34	26.93	28.95	9.47	94.26	89.45
Len	1979	64.76	28.16	32.50	4.11	99.76	94.19
Stoa	1984	63.22	28.73	30.83	3.65	105.73	99.34
Butte-86	1986	66.50	26.49	32.37	7.64	96.04	90.98
Grandin	1989	69.48	26.22	35.03	8.23	94.77	89.89
2375	1990	66.34	27.92	29.07	9.35	95.62	90.63
Russ	1995	71.96	29.96	32.88	9.12	97.72	92.43
Gunner	1995	70.49	25.91	33.89	10.69	95.58	90.58
Reeder	1999	67.61	25.72	32.40	9.49	101.41	95.61
Alsen	2000	66.71	28.09	33.52	5.10	96.79	91.63
Granite	2002	70.78	25.93	34.57	10.27	93.39	88.70
Steele-ND	2004	70.54	30.24	31.72	8.58	99.00	93.53
Freyr	2004	70.52	33.18	29.65	7.68	97.26	92.04
Glenn	2005	72.88	32.32	30.78	9.78	99.69	94.13
Faller	2007	73.22	31.62	31.42	10.18	99.21	93.72
RB-07	2007	65.58	27.34	31.36	6.88	101.75	95.90
Barlow	2009	65.56	30.53	26.59	8.45	96.24	91.16
Velva	2012	73.85	29.21	34.37	10.27	95.95	90.91
Elgin-ND	2013	66.57	31.24	28.60	6.73	96.75	91.60
HSD[b]		5.50	1.65	2.29	1.66	3.33	2.87

[a] RY, release year; TS, total starch; RDS, rapidly digestible starch; SDS, slowly digestible starch; RS, resistant starch; HI, hydrolysis index; eGI, estimated glycemic index.
[b] Tukey's honest significant difference ($P<0.05$).

For the digestibility parameters in HRS wheat bread, HI and eGI, significant differences ($P<0.001$) were observed between genotypes and none of the values were significantly ($P>0.05$) correlated to release year (Table 13). Commercial white bread, which is used as a control in *in*

vitro starch digestibility studies, typically has a HI of 100, and different wheat breads have HI values that range from 73.0 to 112.3 (Åkerberg et al. 1998). The average HI value obtained (98.2) falls within the acceptable range of published values, as does the eGI average (92.9), since the eGI is calculated using the HI in *in vitro* starch digestion assays (Table 12). Usually, the GI value for whole wheat bread (66) is slightly lower than the GI for white bread (70) and sucrose (67) (Foster-Powell et al. 2002). In an earlier study, the GI was reported at 72 for whole wheat bread, 69 for white bread, and 59 for sucrose (Jenkins et al. 1981); these earlier values are commonly used by anti-wheat advocates in an attempt to convince consumers that both whole wheat and white breads generate a more significant glycemic response than that of sugar (Davis 2014). However, these anti-wheat advocates fail to consider the differences in available carbohydrates, which can impact the glycemic response (Brouns et al. 2013).

GI, as mentioned previously, is a measure of the blood glucose response after consumption of a food, which is compared to the blood glucose response after intake of 50 g of glucose (Englyst et al. 1992; Brouns et al. 2013). Glycemic load (GL), on the other hand, is determined by multiplying the GI by the amount of available carbohydrates in the food, e.g. both low-GI/high-carbohydrate and high-GI/low-carbohydrate foods have the same GL (Patil 2008). If the amount of available carbohydrates in each bread loaf made from HRS wheat is considered to be analogous, given the lack of significant differences in carbohydrate and starch composition, and seeing that the GL is determined from the GI, the GL values for each HRS wheat cultivar should display a similar or identical trend to the eGI values.

Even though breeders and researchers are trying to reduce the GI in wheat products, by increasing amylose and decreasing amylopectin in wheat starch to make it digest more slowly (higher SDS) or much less digestible (higher RS), it is not a straightforward or simple process

(Delcour and Hoseney 2010a; Jones 2012; Brouns et al. 2013). Not only is starch digestibility affected by the amylose to amylopectin ratio, but several other factors, including particle size, starch–protein interactions, physical form, and method and time of cooking, can further complicate an already difficult process (Patil 2008). Furthermore, researchers would need to also investigate the digestibility of nonstarch polysaccharides, such as cellulose and arabinoxylan, in their efforts to reduce the GI in wheat products (Stone and Morell 2009).

Table 13. Correlation coefficients (r) between starch digestibility and year, chemical composition, and enzymatic activities for bread made from hard red spring wheat[a,b,c]

	RY	AC	AS	PS	XS	CMA	CAF
TS	0.384 *	-0.387 *	-0.436 *	-0.300 NS	-0.336 NS	-0.312 NS	-0.296 NS
RDS	0.232 NS	-0.089 NS	-0.202 NS	-0.447 *	-0.251 NS	-0.280 NS	-0.254 NS
SDS	-0.004 NS	-0.187 NS	-0.369 *	0.084 NS	-0.020 NS	0.195 NS	0.217 NS
RS	0.360 NS	-0.311 NS	-0.089 NS	-0.102 NS	-0.243 NS	-0.397 *	-0.420 *
HI	-0.258 NS	0.269 NS	0.225 NS	0.155 NS	0.299 NS	0.161 NS	0.019 NS
eGI	-0.258 NS	0.269 NS	0.225 NS	0.155 NS	0.299 NS	0.161 NS	0.019 NS

[a] RY, release year; AC, ash content; AS, α-amylase; PS, endo-protease; XS, xylanase; CMA, *p*-coumaric acid; CAF, caffeic acid; TS, total starch; RDS, rapidly digestible starch; SDS, slowly digestible starch; RS, resistant starch; HI, hydrolysis index; eGI, estimated glycemic index.
[b] All column titles, excluding RY, correspond to hard red spring whole wheat parameters.
[c] NS, not significant; * Significant at 0.05 probability level.

A dendogram was made to further confirm the non-significance between results (Figure 12). Cluster 1, which constitutes the cultivars Rival to Len, had release years of 1910–1944, and 1972–2007; cluster 2, with cultivars Velva through Alsen, were from release years 1926–1963, and 1986–2012; and cluster 3, which contained cultivars Waldron through Pioneer-2375, had release years of 1942–1977, and 1990–2013. Just like the cluster results for starch characterization, it should be clear that none of the clusters were significantly (P>0.05) different from each other, and that the starch digestibility of the modern wheat cultivars does not differ from the starch digestibility of the historic cultivars.

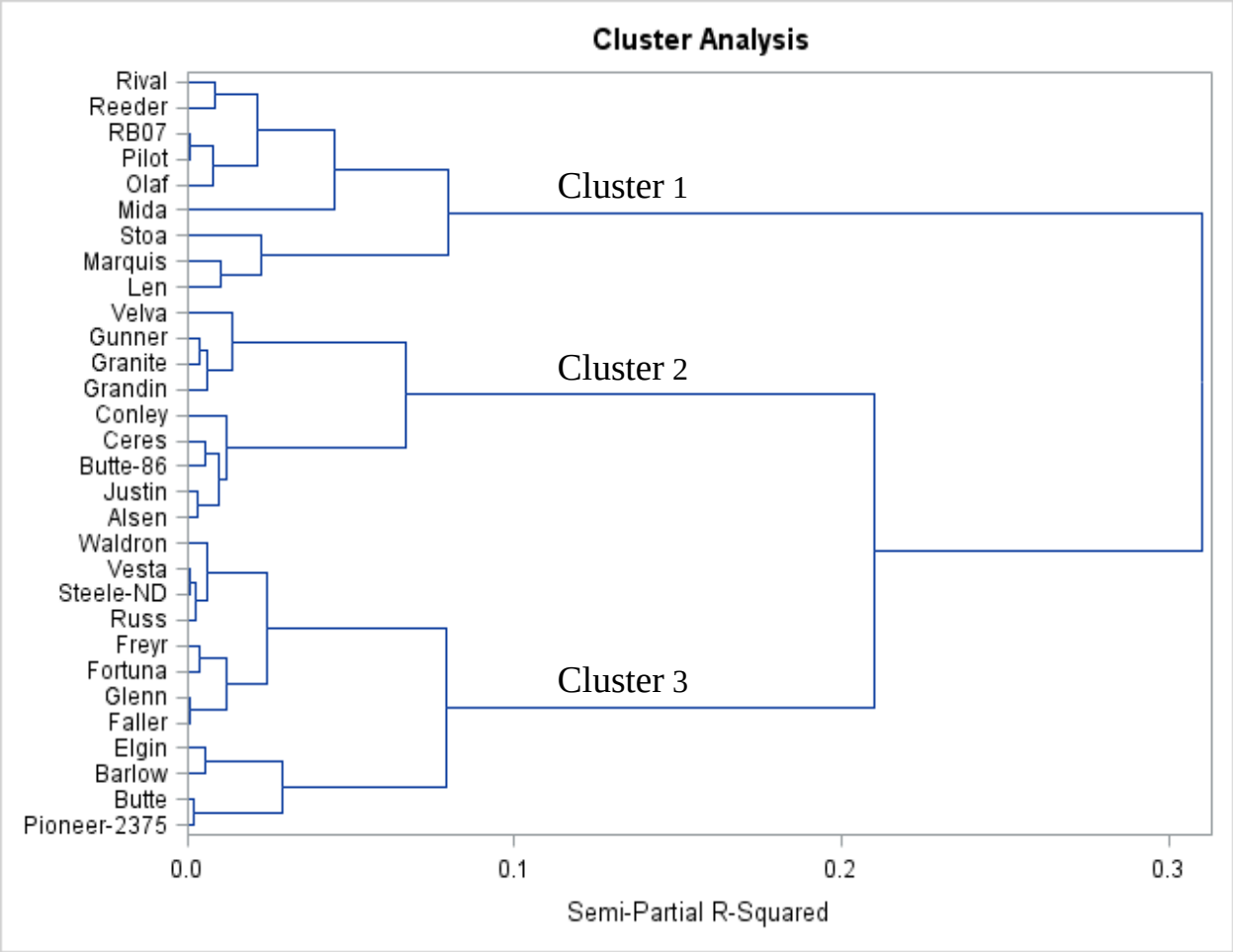


Figure 12. Cluster analysis for starch digestibility in hard red spring wheat bread; Cluster 1 (Rival–Len) 1910–1944, and 1972–2007, Cluster 2 (Velva–Alsen) 1926–1963, and 1986–2012, Cluster 3 (Waldron–2375) 1942–1977, and 1990–2013.

It is important to repeat that the only significant (P<0.05) change observed in the starch profiles for HRS whole wheat, white flour, and bread, in regards to release year, was the TS amount in the bread samples, which demonstrated a positive correlation (r = 0.38) to release year (Table 11). However, equally important observations are that the r value for TS in bread is comparatively low and that the starch digestibility of the bread was not significantly (P>0.05) correlated to release year (Table 13). Therefore, even though the TS in bread made from HRS wheat increased, the amount of bioavailable starch did not increase. This is important because many believe that an increase in TS content in bread would lead to an increase in the amount of

available glucose, which would lead to a higher GI and a more pronounced glycemic response as claimed by anti-wheat diet advocates (Davis 2014).

CHAPTER 6. SUMMARY AND CONCLUSIONS

In light of the substantial increase in concerns over possible negative effects on the end-use and nutritional quality of wheat produced from advanced selection, breeding, or agronomic techniques, it was necessary to compare and evaluate any differences between a selection of historic and modern HRS wheat. Thirty different historic and modern HRS wheat cultivars, grown in the same location and year, were analyzed for variances in proximate, specifically ash, protein, lipid, and fiber, enzyme activity, phenolic acid, and starch properties.

Many of the parameters measured in the whole wheat and white flour samples, including PC, CF, TS, total AX, DF, and total free and bound phenolic acids, were not significantly ($P>0.05$) correlated to release year. Of the remaining parameters, significant ($P<0.05$) differences in relation to release year existed for AC in whole wheat and white flour, P, K, Zn, and A/X in whole wheat, α-amylase and xylanase activities in whole wheat and white flour, endo-protease activity in whole wheat, and vanillic, *p*-coumaric, caffeic and syringic acid in whole wheat. Most of the parameters measured in white flour and bread, including amylose content, amylopectin and amylose Mw, difference in amylopectin and amylose Mw after baking, RDS, SDS, RS, HI, and eGI were not significantly ($P>0.05$) correlated to release year. However, while the TS in white flour was not significantly ($P>0.05$) related to release year, the TS in bread displayed a significantly ($P<0.05$) positive correlation.

The results observed in this study were significantly different ($P<0.001$) between genotypes; however, the majority of the results obtained were not significantly ($P>0.05$) correlated to release year, which initially supports our hypothesis. Moreover, while the AC, and α-amylase and xylanase activities in white flour and TS in bread were significantly ($P<0.05$) correlated to release year, our hypothesis was further supported when cluster analysis could not

clearly distinguish between the historic and modern cultivars. However, cluster analyses of the AC, mineral, and phenolic acid concentrations in whole wheat, which were significantly (P<0.05) related to release year, were largely able distinguish between the historic and modern cultivars and partially disproves our hypothesis.

Even though the results did not completely prove our hypothesis, enough evidence was obtained to contradict claims that selection and breeding efforts have caused extensive changes in grain composition and nutritional properties from historic to modern HRS wheat cultivars. Furthermore, of the whole wheat results that were significantly (P<0.05) correlated to release year, our findings imply that only changes observed in the ash, mineral, and phenolic acid concentrations may have an effect on consumer health. Although significant (P<0.05) differences were observed in the white flour parameters mentioned above, it's possible that these results were the product of contamination from the bran and germ fractions during milling.

Considering the whole wheat samples had the greatest number of significant differences, any minor changes in grain composition would solely affect those who regularly consume whole grain bread products, which are only a small part of the population since nearly all consumers prefer the taste, smell, and texture of white bread products. Moreover, the bioavailability of these compounds, especially the phenolic acids, are influenced by the types and quantities of other foods consumed with the whole wheat products, which may negate any concentration differences between the modern and historic HRS cultivars and is determined wholly by the dietary choices of each consumer (Fardet 2010). Therefore, our interpretations of these results indicate that claims citing modern wheat as detrimental to consumer health and the cause of several health problems are unfounded, especially since the digestibility of the nutritionally important starch fractions in historic and modern wheat were not significantly (P>0.05) different.

CHAPTER 7. FUTURE RESEARCH DIRECTIONS

While this research study contains brief examinations of several primary wheat grain components, future studies could be focused on investigating each parameter in greater detail, especially those that would have the greatest impact on human health and nutrition. Moreover, further research is needed to determine if there are significant differences between other nutritionally important micronutrients and phytochemicals in HRS wheat, which would include secondary metabolites, such as carotenoids, flavonoids, and lignans, vitamins (especially B vitamins), phytosterols, choline, and betaine (Piironen et al. 2009). Also, due to the age and storage conditions of the samples used in this study, it would be advantageous to re-examine the same selection of HRS wheat cultivars, but either analyze the samples quickly after harvest or store them at colder temperatures in packaging that is less permeable to oxygen.

Likewise, additional research is needed to examine the bioavailability differences between historic and modern HRS wheat cultivars. For instance, if the lower mineral amounts in modern HRS wheat are more bioavailable than the higher mineral amounts in historic HRS wheat, then the modern cultivars will have the greater nutritional impact. However, research of this nature, which would require human participants, is beyond the capabilities of this laboratory and would need to be outsourced to an appropriate research facility.

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APPENDIX

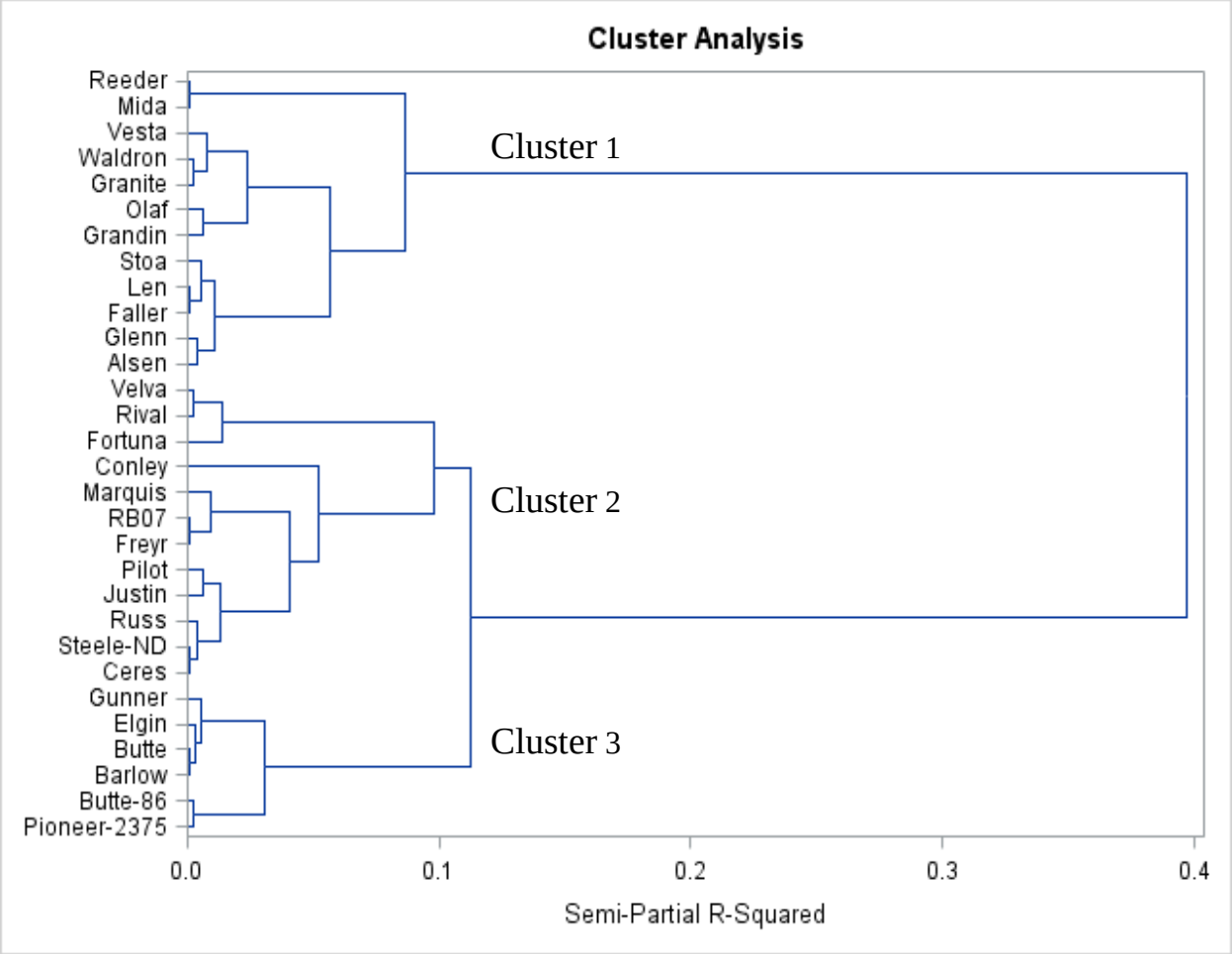

Figure A1. Cluster analysis for dietary fiber in hard red spring whole wheat; Cluster 1 (Reeder–Alsen) 1942–2007, Cluster 2 (Velva–Ceres) 1910–1966, and 1995–2012, Cluster 3 (Gunner–2375) 1977–1995, and 2009–2013.

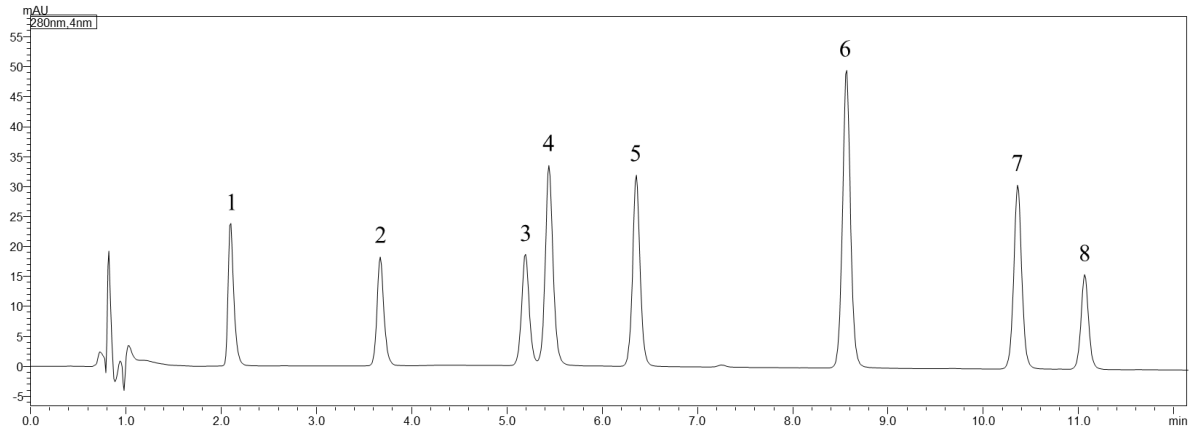

Figure A2. Phenolic acid standard peaks at 280 nm for high performance liquid chromatography analysis of phenolic acids in hard red spring whole wheat; 1, Protocatechuic acid; 2, *p*-Hydroxybenzoic acid; 3, Vanillic acid; 4, Caffeic acid; 5, Syringic acid; 6, *p*-Coumaric acid; 7, Ferulic acid; 8, Sinapic acid.

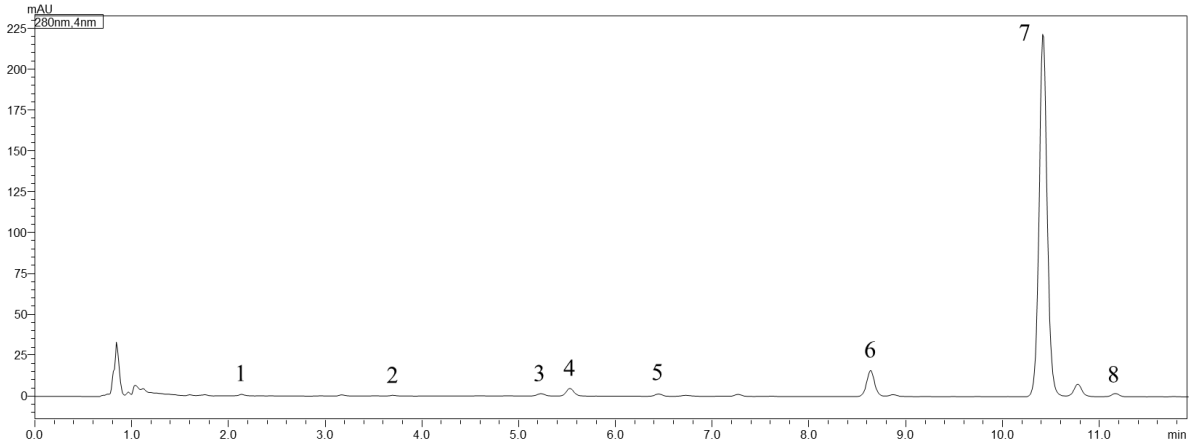

Figure A3. Phenolic acid peaks in hard red spring cultivar ‘Marquis’ from high performance liquid chromatography analysis of whole wheat at 280 nm; 1, Protocatechuic acid; 2, *p*-Hydroxybenzoic acid; 3, Vanillic acid; 4, Caffeic acid; 5, Syringic acid; 6, *p*-Coumaric acid; 7, Ferulic acid; 8, Sinapic acid.

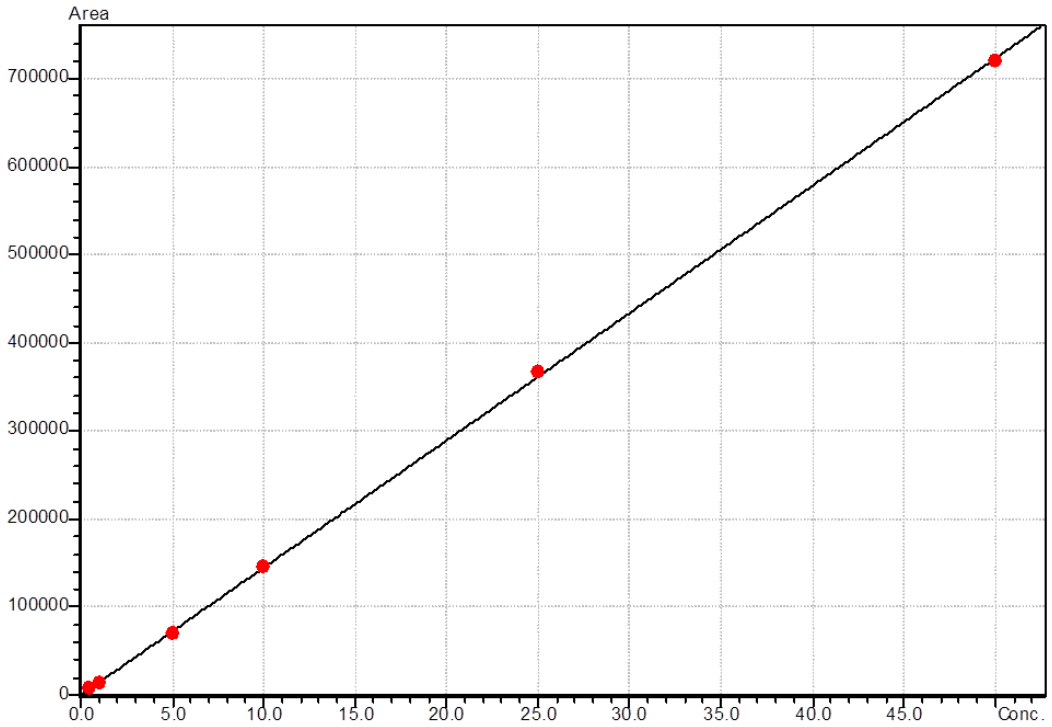

Figure A4. Standard curve generated for high performance liquid chromatography analysis of *p*-hydroxybenzoic acid in hard red spring wheat.

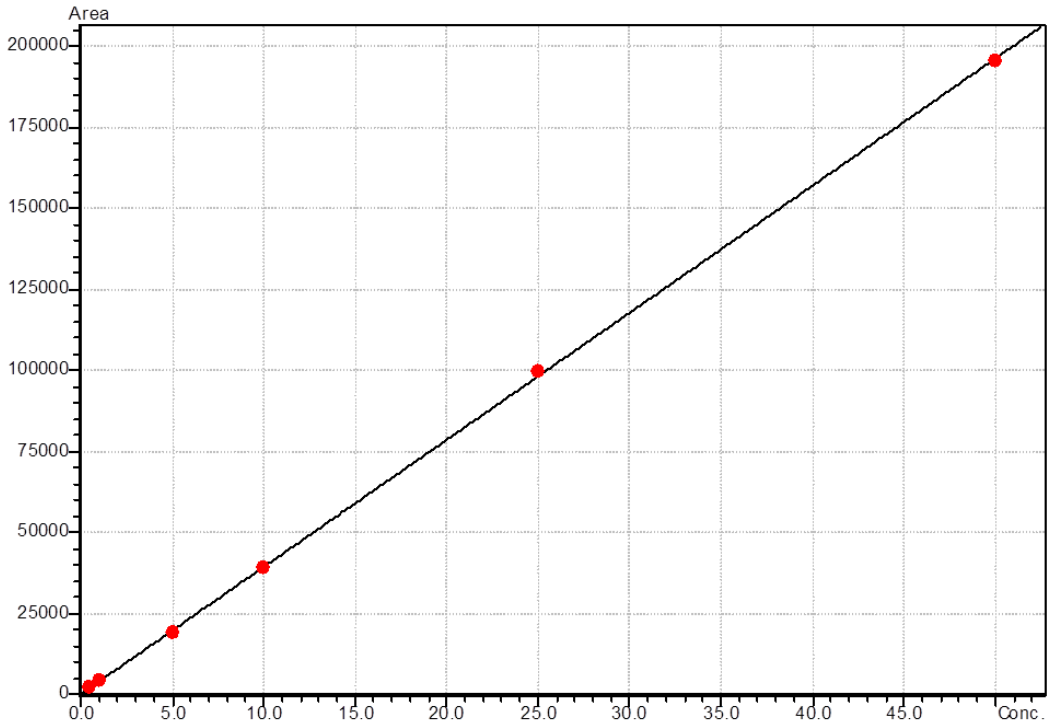

Figure A5. Standard curve generated for high performance liquid chromatography analysis of protocatechuic acid in hard red spring wheat.

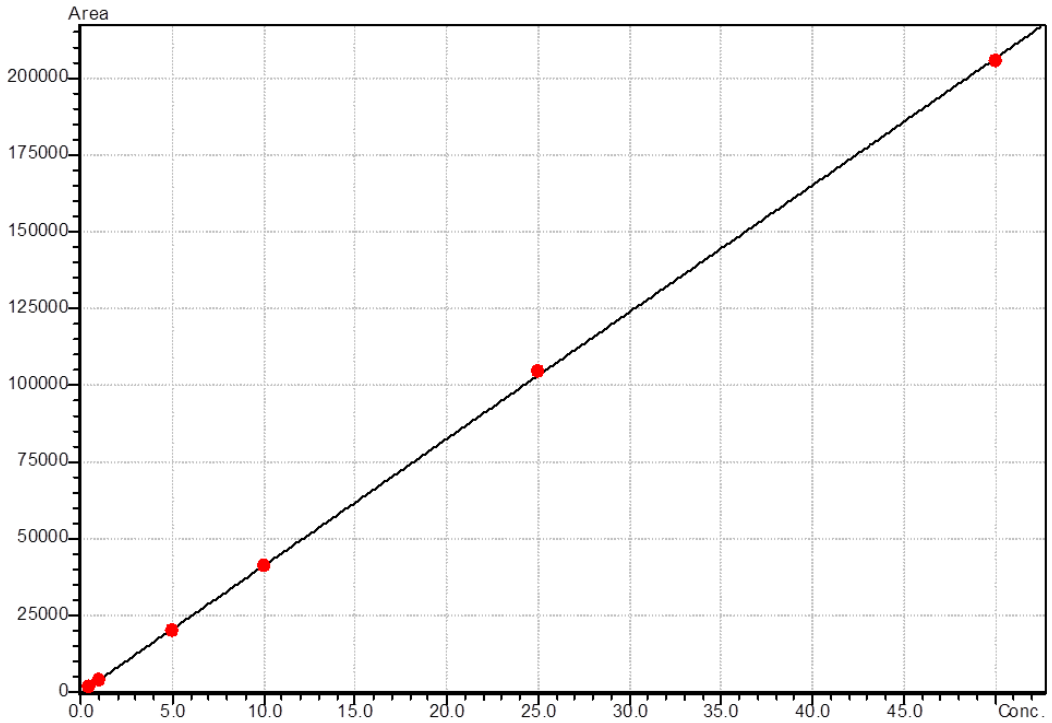

Figure A6. Standard curve generated for high performance liquid chromatography analysis of vanillic acid in hard red spring wheat.

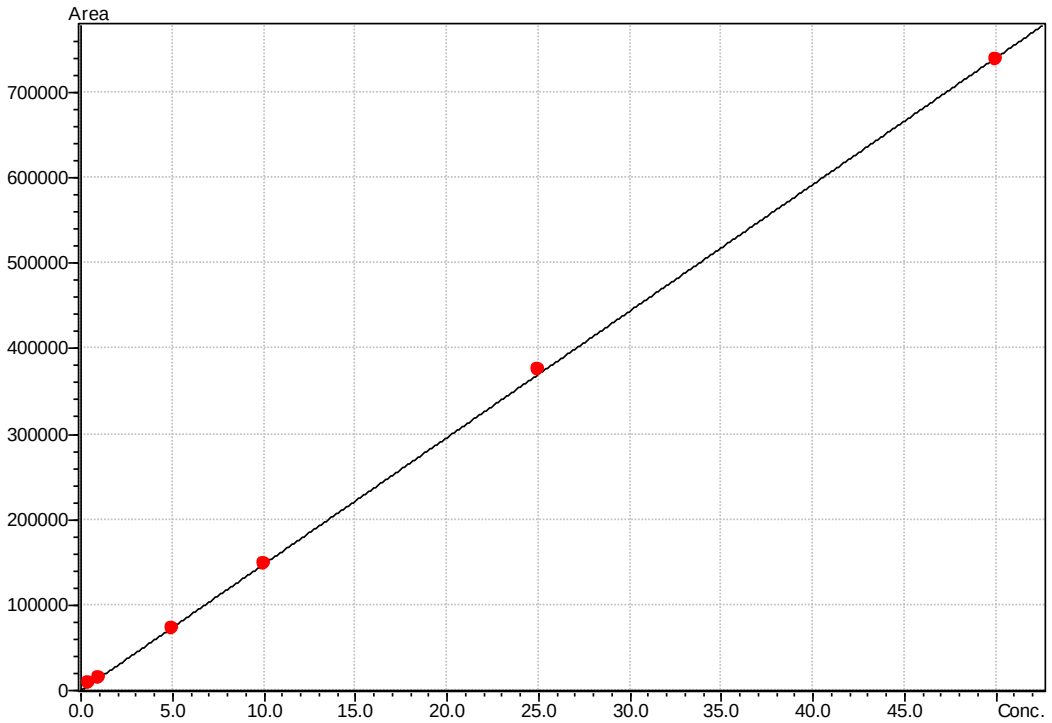

Figure A7. Standard curve generated for high performance liquid chromatography analysis of *p*-coumaric acid in hard red spring wheat.

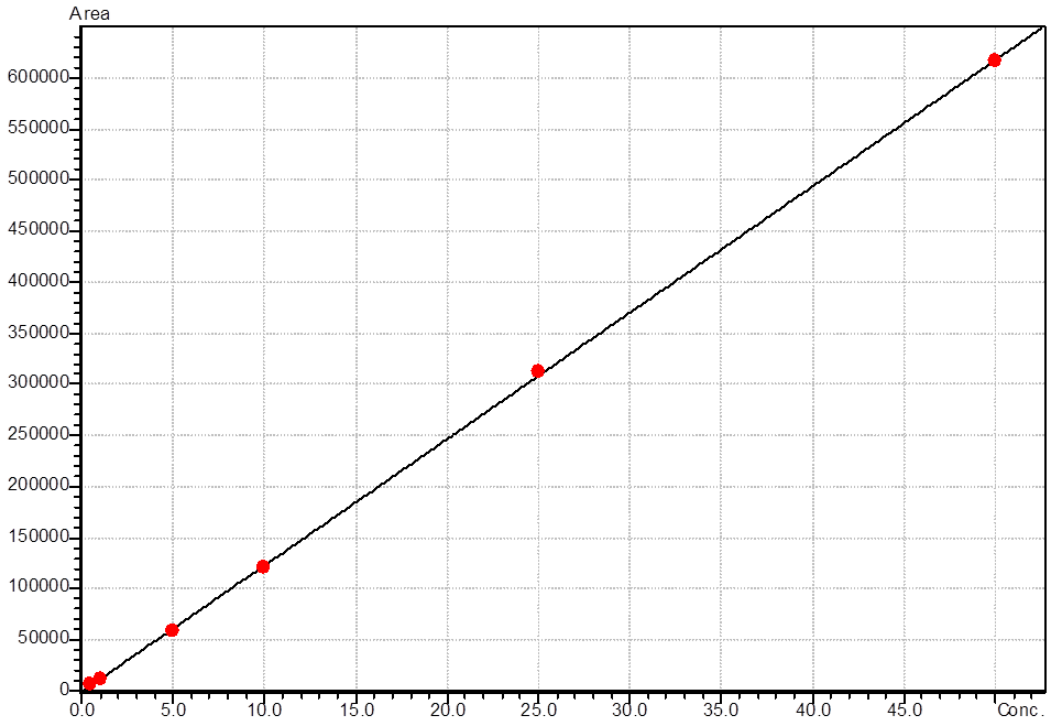

Figure A8. Standard curve generated for high performance liquid chromatography analysis of caffeic acid in hard red spring wheat.

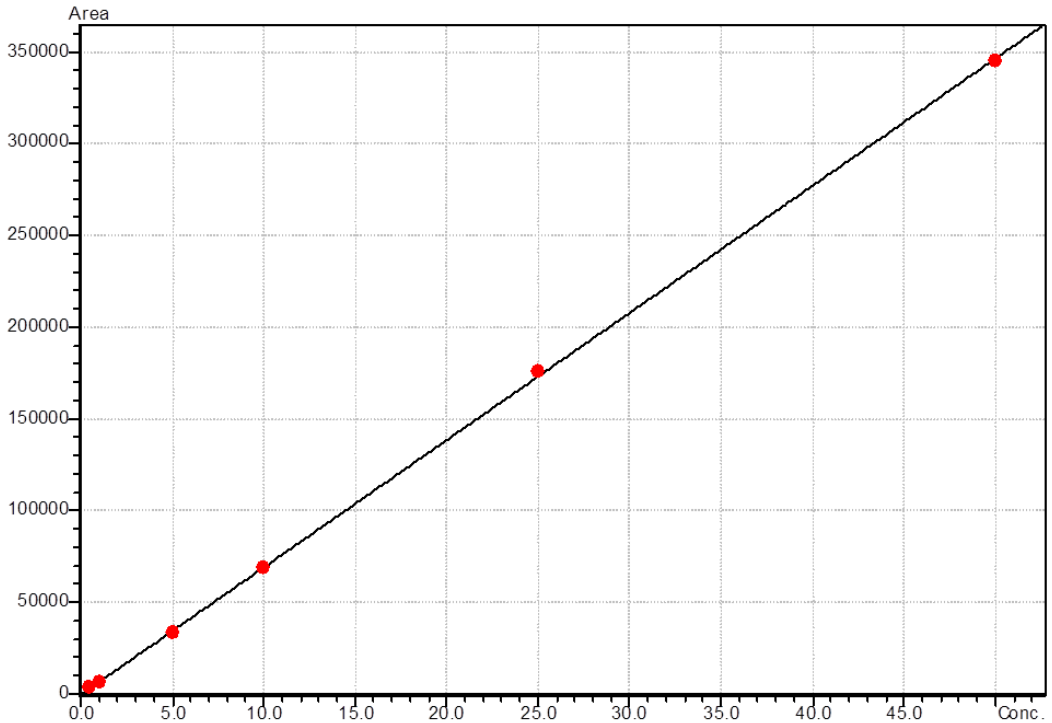

Figure A9. Standard curve generated for high performance liquid chromatography analysis of syringic acid in hard red spring wheat.

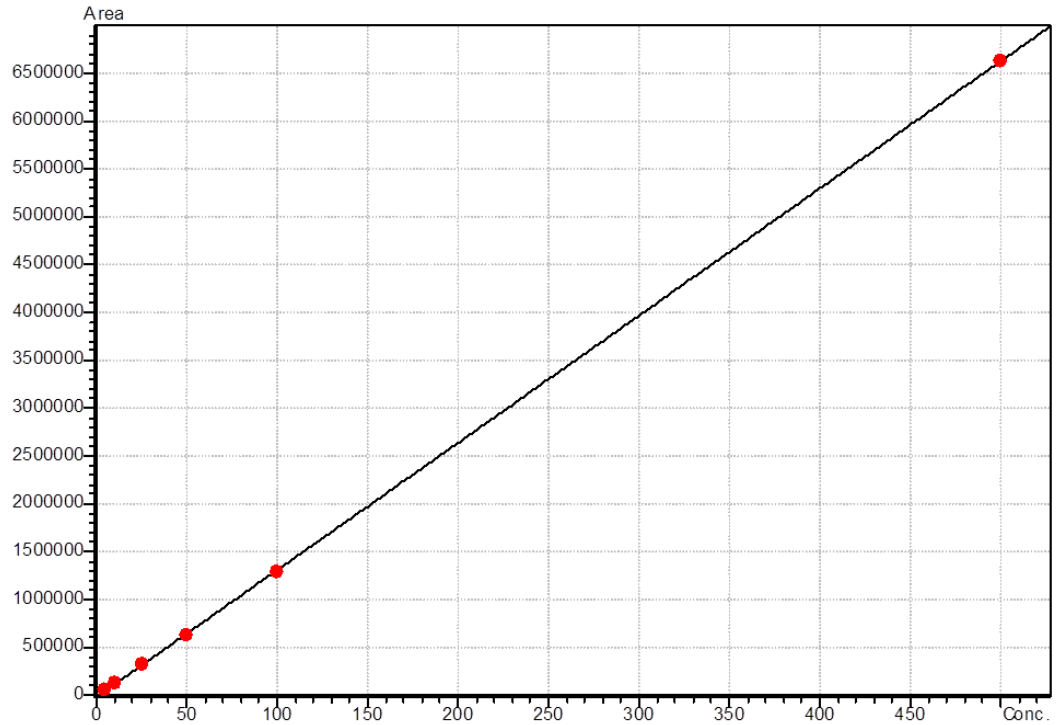

Figure A10. Standard curve generated for high performance liquid chromatography analysis of ferulic acid in hard red spring wheat.

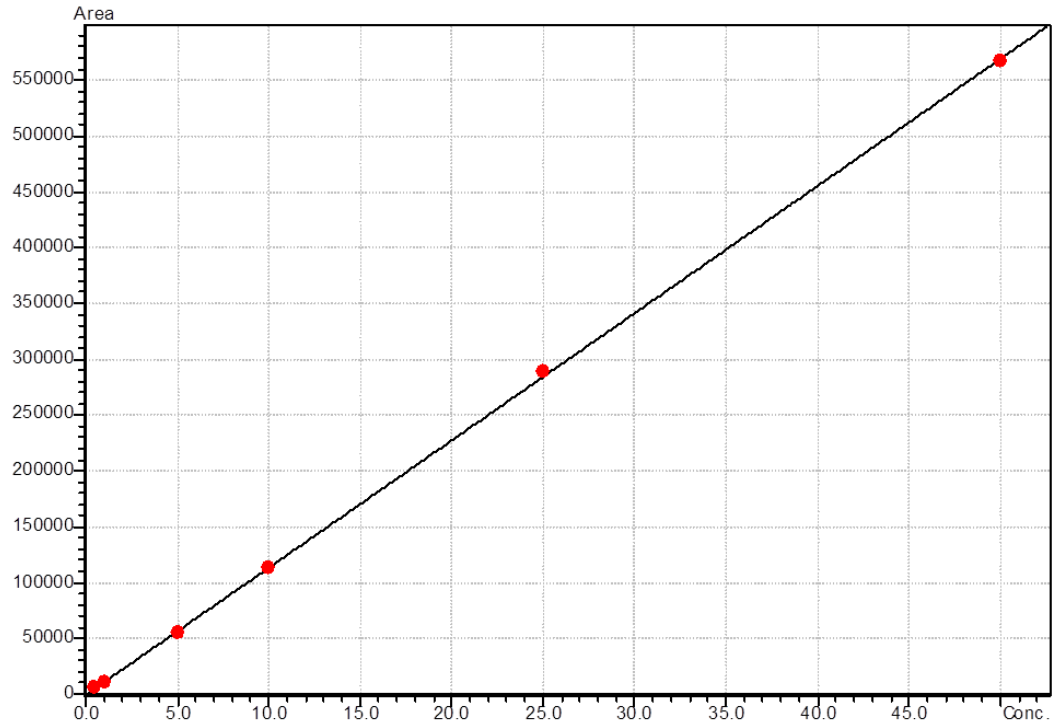

Figure A11. Standard curve generated for high performance liquid chromatography analysis of sinapic acid in hard red spring wheat.