Evaluation of the Chemical and Functional Stability of Intermediate Wheatgrass (*Thinopyrum intermedium*) over Storage and in Response to Steam Treatment

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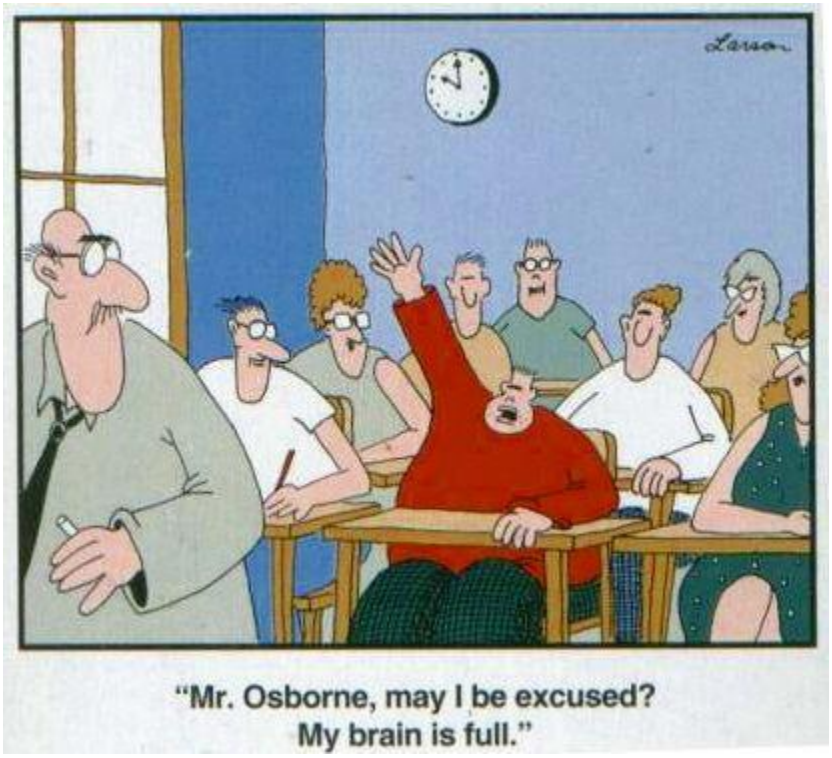
Dedication

To my dad. Thank you for the laughs along the way.



Gary Larson, The Far Side®

Abstract

Annual crop farming is degrading the environment at a faster rate than it can be restored, causing soil erosion and water run-off with subsequent loss of nutrients and biodiversity. Yet, the world population currently relies on annual cereal grains to supply 40% of their calorie needs and 60% of their protein needs. The high dietary demand for cereal grains, together with the high rate of soil degradation from annual farming and growing consumer interest in sustainably-sourced food creates space in the market for a more sustainable grain. Perennial intermediate wheatgrass (IWG), *Thinopryum intermedium*, is a promising grain to fulfill this role, owing to its good flavor, breeding potential, and superior environmental benefits due to its extensive root system and long growing season. Understanding the storage stability of IWG and identifying ways to improve its stability will not only help incentivize farmers to plant IWG, but will also help make IWG competitive against existing grains on the market.

The objectives of this study were: (1) evaluate the effect of steam treatment on antioxidant content and activity, enzyme activity, and progression of hydrolytic and oxidative rancidity in IWG compared to hard red wheat (HRW) over storage at different temperatures; (2) evaluate the effect of steam treatment of IWG grains on the functionality of its whole flour over storage at different temperatures; (3) determine the overall safety of the grains by assessing presence of chemical residues, such as pesticides and mycotoxins, heavy metals, allergens, and anti-nutrient factors.

Pre-storage, compositional analysis of IWG and HRW was carried out following official AOAC and AACCI methods. Steam treatment was carried out by subjecting 30 g aliquots of IWG and HRW groats to 100°C and 95% relative humidity conditions in a proofing oven for 60 minutes. Steamed and non-steamed samples were stored at 45°C, ambient (22 ± 2°C), and 4°C at 0.43 water activity for 6 weeks, 6 months, and 12 months respectively. Samples were analyzed periodically for lipoxygenase and lipase activity, hydroxycinnamic acid content, carotenoid content, antioxidant activity, and indicators of hydrolytic and oxidative rancidity, including free fatty acids and hydroperoxides, respectively. Lipoxygenase activity pre- and post-steam treatment was analyzed using the

ferrous oxidation-xylenol orange (FOX) assay, and lipase activity was determined spectrophotometrically using a copper soap assay. Hydroxycinnamic acids and carotenoids were quantified using high performance liquid chromatography. Antioxidant activity pre- and post-steam treatment was analyzed using 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging and leucomethylene blue (LMB) assays. Free fatty acids and hydroperoxides were quantified according to AOAC titration methods 940.28 and 965.33, respectively. Functionality parameters were also measured at the beginning, middle, and end of storage at each storage temperature. Rheological and mixing properties were assessed using a Farinograph® and a texture analyzer equipped with a Kieffer rig. Starch pasting properties were monitored using a MicroVisco-Amylograph®. Bread baking tests were performed according to AACCI 10-10.03 method.

IWG had significantly higher protein, insoluble fiber, and fat content than HRW, along with a higher lipase activity. HRW had a relatively higher lipoxygenase activity than IWG. IWG also had significantly higher hydroxycinnamic acid and carotenoids concentrations than HRW, along with higher antioxidant activity. The steam treatment employed in the present study did not inactivate lipoxygenase and lipase. Throughout storage, lipase activity significantly decreased in non-steamed grains over ambient (22 ± 2°C) storage but otherwise was retained over accelerated (45°C) and refrigerated (4°C) storage. However, an after-ripening effect was evident in lipoxygenase activity at ambient and 45°C temperatures, with significant increases in activity over storage. Hydroxycinnamic acid content was retained throughout storage, with prolonged storage inducing increases to its content, as well as increases to antioxidant activity at higher storage temperatures. Carotenoid content decreased throughout storage in IWG and HRW, to a greater degree at higher storage temperatures, as was expected due to its high susceptibility to oxidation. Development of oxidative and hydrolytic rancidity in IWG was minimal throughout storage of groats. Although the steam treatment was not able to effective in inactivating enzymes, it demonstrated a minimal, but positive effect on antioxidant activity and content, as well as a slight inhibitory effect on hydroperoxide formation over storage. IWG demonstrated a significantly higher antioxidant content and antioxidant activity and lower hydroperoxide content than HRW throughout storage. In

terms of hydrolytic rancidity, IWG showed higher lipase activity and free fatty acid concentration than HRW throughout storage.

IWG showed increases to dough development time, resistance to extension, and loaf volumes over storage, denoting an increase in dough strength, although no significant increases to dough stability time were evident. IWG also had improvements to starch pasting viscosities over storage, including peak viscosity and hold viscosity, at all temperatures. The effects of steaming on functionality were temperature-dependent, with positive effects on dough development time and resistance to extension during storage at 45°C and improvements to starch pasting viscosities during storage at ambient temperature. Steaming appeared to have a negative impact on functionality at refrigerated storage temperatures. Steaming did not have an effect on bread loaf volumes of IWG over storage. Although overall functionality of IWG remained inferior to HRW throughout storage, HRW had significant decreases in several functionality parameters over storage. While steaming had a positive effect on some of these parameters (e.g. extensibility, loaf volume), it had a negative impact on others (e.g. starch pasting viscosities). Bread firmness was not significantly affected over storage in either grain.

The present study was the first to evaluate the storage stability of IWG and investigate steam treatment as a mode of improving storage stability of IWG. The results of the study provide additional incentives to farmers and food manufacturers alike by highlighting IWG's competitive storage stability. The present study demonstrated not only its high tolerance to storage but in some regards, its positive response to storage. Furthermore, this research formed the foundation for establishing a more effective method of steam treatment in a currently ongoing storage study on IWG flour.

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Chapter 1: Literature Review

1.1 Introduction

The last five decades have seen a twofold increase in world population growth that has been mirrored by a twofold increase in grain production (Pimentel & Wilson, 2004; Brown, 2010). The three foremost produced and consumed grains worldwide are wheat, rice, and maize, all three of which are annual crops (Beta & Isaak, 2016). While farming of these annuals yields the grains that feed the world, it also results in a disconcerting amount of environmental harm. Annual farming has been associated with soil erosion, nitrogen leaching, and water-run off among other problems (Cox, Glover, Van Tassel, Cox, & DeHaan, 2006; Glover et al., 2010). In 1973, the Rodale Research Center (RRC) started investigating perennial crops as a potential solution to this problem.

Perennial crops protect the integrity of the soil, sequester carbon, and require fewer fertilizer, mechanical, and labor inputs (Culman, Snapp, Ollenburger, Basso, & DeHaan, 2013; Lewandowski, 2016). However, in order for a perennial grain to be marketable for food consumption, it must be able to compete with annual grains, agronomically, nutritionally, and functionally. In 1990, the RRC evaluated approximately 100 different species of perennial grasses for their potential cultivation as perennial crops (Wagoner, 1990) based on desirable agronomic and nutritional traits. Perennial intermediate wheatgrass (IWG) was identified to have superior qualities and has since been researched and improved through breeding efforts to enhance its marketability. A significant aspect governing the marketability of IWG is its storage stability, which has not yet been addressed and requires further exploration to aid in its success.

Storage stability of a grain can be assessed with regards to microbial contamination, grain viability (germination ability), or quality attributes – organoleptic, functional, and nutritional (Pomeranz, 1992). These factors are, in turn, influenced by both extrinsic factors such as relative humidity, temperature, and light (Kaleta & Górnicki, 2013) and intrinsic factors, namely the grains' moisture content, fat content, enzymes, and antioxidant content (Galliard, 1994). IWG has a higher fat content and

lipase activity than wheat and lower lipoxygenase activity (Tyl & Ismail, 2018). The action of lipase and lipoxygenase on lipids results in hydrolytic and oxidative rancidity, respectively, thereby reducing the overall quality of the grain during storage (Galliard, 1994). At the same time, IWG contains relatively high amounts of antioxidants that could counter oxidative activity (Tyl & Ismail, 2018).

In an effort to extend shelf life of milled grain, manufacturers often refine the flour, thus removing the bran and germ, which constitute problematic enzymes and lipids (Slavin, Jacobs, & Marquart, 2001). However, the consumption of refined flour is contraindicated by the Dietary Guidelines for Americans that encourage intake of whole grains to curtail the onset or progression of a variety of chronic diseases (US Department of Health and Human Services & Agriculture, 2015). This conundrum posed the question of whether storage stability of whole IWG grains can be enhanced without jeopardizing the grain's nutritional profile. To address this challenge, current industrial practices in grain processing must be investigated.

Heat treatments to extend shelf life by inactivating detrimental enzymes are common practices in oat and legume processing. Steam treatment, in particular, is quite effective in inactivating these enzymes while preserving antioxidant activity (Rose, Ogden, Dunn, & Pike, 2008; Bergonio, Lucatin, Corpuz, Ramos, & Duldulao, 2016; Hu, Wang, & Li, 2018) . Given the relatively high amounts of lipids in IWG, steam treatment may be a viable option to prolong its shelf life. However, the effects of storage and steam treatment on functionality need also be addressed. Prolonging the shelf life through steam treatment while maintaining functionality and nutritional quality will fuel efforts to breed IWG for commercial food applications.

1.2 Hypothesis and Objectives

We hypothesize that inactivation of enzymes, namely lipase and lipoxygenase by steam treatment will enhance the storage stability of IWG in terms of hydrolytic and oxidative rancidity. We further hypothesize that a high antioxidant activity coupled with

a lower lipoxygenase activity as compared to hard red wheat will result in a slower progression of rancidity in IWG than in hard red wheat. Finally, we hypothesize that steam treatment will not affect functionality over storage.

Our objectives are as follows:

1. Evaluate the effect of steam treatment on antioxidant content and activity, enzyme activity, and progression of hydrolytic and oxidative rancidity in IWG compared to wheat over storage at different temperatures.
2. Evaluate the effect of steam treatment of IWG grains on the functionality of whole flour over storage at different temperatures.
3. Determine the overall safety of grains by assessing presence of chemical residues, such as pesticides and mycotoxins, heavy metals, allergens, and anti-nutrient factors.

1.3 The Grain Market

1.3.1 Production and Consumption

1.3.1.1 Cereal Grains

According to the International Grains Council, total global consumption of grains has increased from 1,686 million tons in 2007-08 to 2,076 million tons in 2016-17, nearly perfectly matching the increase in production from 1,698 to 2,113 million tons over the same time period. Recent estimates of production reach as high as 2.5 billion tons per year worldwide (Wrigley, 2016).

The grain market is expected to continue to grow over the next five years, with forecasted increases in overall production, consumption, and trade (International Grains Council, 2017). Total grain production over the next five years is projected to increase by 1.2% annually, accompanied by an equivalent increase in projected consumption. The worldwide grain trading market is expected to grow by 1.3% annually, mostly attributed to growing demand in Asia and Africa. Compared to the 900 million tons used each year

for animal feed, approximately 700 million tons of cereal grain worldwide are currently allocated to food markets (International Grains Council, 2017). Industrial uses of grain, specifically biofuel, takes approximately 350 million tons annually. Wheat, rice, and maize comprise 90% of total global cereal crop production (Beta & Isaak, 2016). Among these crops in the United States, wheat production for food consumption is highest (International Grains Council, 2017).

1.3.1.2 Wheat

Wheat production currently ranges from 745-760 million tons per year globally, of which two thirds goes toward human consumption (Wrigley, 2016; USDA Foreign Agricultural Service, 2018). The United States was responsible for 56 million tons of wheat produced in 2016-2017. Despite an overall upward trend in total grain and wheat consumption globally, consumption of wheat in the United States has trended down since a peak of 225 lbs per capita in 1879. In 2015, consumption was only 133lbs per capita. This trend could be a result of the increasing prevalence of wheat intolerance and allergy, as well as fad diets blaming carbohydrates as the source of obesity, diabetes mellitus, and other chronic diseases (Sheats & Jones, 2016). However, wheat remains the "gold standard" among grains because of its superior functional attributes and versatility in end-use applications (Žilić, 2013).

Over the next five years, wheat production and consumption are both projected to increase by 1%. Production growth in recent years has been surpassing total production area growth, which is explained by improvements in total yields. Through the 2022-23 season, production increases are expected to continue to outweigh increases in production area. Wheat trade is also expected to increase by 0.8% over the next five years. In the past decade, Sub-Saharan Africa and Southeast Asia have surpassed North Africa and the Middle East as the top importers of wheat (USDA Foreign Agricultural Service, 2018). Worldwide, the top exporters of wheat are Russia and the European Union, with most of their exported wheat destined for the Middle East and Africa. Despite the current and projected success of the wheat market, the global stock market for total grains decreased

in the 2017-18 season for the first time in five years and projected to continue to decline over the next five years (International Grains Council, 2017).

The failure of annual cereal crop farming to keep up with global demand coupled with a downward trending consumption of wheat leaves room in the market for new and sustainable grains. With growing consumer interest in sustainable agricultural practices, perennial grains will likely establish a foothold in the global grain market.

1.3.2 Grain and Health Benefits

Worldwide, cereal grains account for 60% of total caloric intake and 40% of total protein intake (Wrigley et al., 2016). Intake of grain crops, and specifically whole grains, has been definitively linked to a decrease in risk of several chronic diseases, including cardiovascular disease (Jacobs & Gallaher, 2004; Mellen et al., 2008), type II diabetes (Cho et al., 2013), metabolic syndrome (Esmaillzadeh et al., 2005; Sahyoun et al., 2006), and several types of cancer, including colorectal cancer (Aune et al., 2011) and pancreatic cancer (Lei et al., 2016). Consistent whole grain intake not only reduces the risk for developing type II diabetes, but also helps manage current diagnoses of diabetes. Whole grains have relatively low glycemic indices (Slavin, Jacobs, Marquart, & Wiemer, 2001). Whole grains, owing to their high soluble fiber content, can also reduce cholesterol indices, particularly total serum cholesterol and low-lipoprotein cholesterol, that increase the risk of atherosclerosis and cardiovascular disease (Davis, 2014). While soluble fiber slows gastric transit to optimize absorption of nutrients, insoluble fiber is helpful in promoting gut motility (Slavin, Jacobs, & Marquart, 2001). Dietary fiber decreases the risk for diverticulosis, as well (Slavin, Jacobs, & Marquart, 2001).

The above health effects of grains are attributed to the dietary fiber, resistant starch, oligosaccharides, and phytochemicals, which are concentrated primarily in the bran and germ of the grain (Slavin, 2017). The bran and germ are also a nutrient dense source of B vitamins (specifically thiamin, riboflavin, niacin, and folate), and minerals (specifically iron, selenium, and magnesium), which each carry their own set of additional benefits (U.S. Department of Agriculture, 2015). The bran and germ are

mostly removed during the refinement process (Slavin, Jacobs, & Marquart, 2001), and as such, the U.S. Department of Health and Human Services and the U.S. Department of Agriculture recommends choosing whole grains for at least half of one's total grain intake (US Department of Health and Human Services & Agriculture, 2015). While refined grains are devoid of a bounty of beneficial nutrients, they are also lower in antinutritional factors than their whole grain counterparts.

1.3.3 Anti-nutrient Factors

Anti-nutritional factors lower the bioavailability of certain nutrients in food, such that the amount of nutrients absorbed in the body is lower than that measured in the food. Anti-nutrient factors common in cereal grains include phytate, condensed tannins, and proteinaceous inhibitors of α-amylase and trypsin (Madsen & Brinch-Pederson, 2016). While undesirable to consumers, these compounds have a physiological benefit to plants, preventing predatory species from accessing their essential nutrients.

Phytic acid, or *myo*-inositol-(1,2,3,4,5,6)hexakisphosphate, is the primary source of stored phosphorous in the cereal grain (Lott, 1984; Brinch-pedersen, Madsen, Holme, & Dionisio, 2014). Found in the aleurone layer, it forms salts with a variety of cations, including potassium, zinc, magnesium, manganese, sodium, copper, calcium, and iron (ferric ions) (Lott, 1984; Brinch-Pedersen, Sorensen, & Holm, 2002). Once grain phytate is consumed, the phytate continues to chelate these minerals, along with positively charged proteins and amino acids, which lowers their bioavailability (Brinch-Pederson & Hatzack, 2006). Grains possess endogenous phytase, which hydrolyzes phytate in order to release these minerals and render them more bioavailable, but the amount of phytase in a grain can vary across, and even within, a species (Brinch-pedersen et al., 2014). Breeding techniques, genetic modification, and processing techniques can help to either increase the phytase content in plants or limit the amount of phytate in plants (Kornegay, 2001; Brinch-Pedersen et al., 2002; Egli, Davidsson, Juillerat, Barclay, & Hurrell, 2002; Brinch-pedersen et al., 2014) .

Condensed tannins are water-soluble polymers of flavonols, which are phenolic compounds with antioxidant capabilities (Dorf & Miller, 2007). Condensed tannins can bind to minerals, reducing their bioavailability. They can also bind to proteins and carbohydrates, inhibiting their *in vitro* digestibility. Condensed tannins have also been shown to inhibit digestive enzymes (Jansman, 1993). However, tannins also have health-promoting effects. Polymerized phenolics exhibit higher antioxidant activity than the monomeric constituents alone (Hagerman et al., 1998). Additionally, tannins may have hypocholesteremic, antiulcerogenic, anticarcinogenic, gastroprotective, and cardioprotective effects (Prior & Gu, 2005).

Protein inhibitors of α-amylase and trypsin make up a third class of antinutritional factors in grain. α-amylase inhibitors are found in the endosperm of wheat, barley, rye, and finger millet (Madsen & Brinch-Pederson, 2016). α-amylase inhibitors bind irreversibly to α-amylase, therefore preventing the enzyme from breaking down starch in ingested food. In certain populations, such as diabetics, this may be considered desirable as fewer monosaccharides are released into the bloodstream (Buonocore, Petrucci, & Silano, 1977). α-amylase inhibitors are generally undesirable, as they may prevent consumers from accessing the full nutritional value of the grain, yet Bora (2014) asserts the amounts in cereal grains is nutritionally irrelevant. Trypsin is a proteolytic enzyme responsible for the digestion of protein (Madsen & Brinch-Pederson, 2016). Trypsin inhibitors irreversibly bind to the enzyme and prevent complete breakdown of a protein into its amino acid constituents for absorption. Trypsin inhibitors levels are far more pronounced in legumes such as soybean, but do exist in cereal grains such as wheat, barley, triticale, and rye with the most significant amounts in barley (Sosulski, Minja, & Christensen, 1988; Bora, 2014; Madsen & Brinch-Pederson, 2016). Outside of impairing complete digestion of ingested carbohydrate and protein, α-amylase and trypsin inhibitors can pose additional health problems.

Dual α-amylase and trypsin inhibitors (ATIs) have been characterized in cereal grains (Islamov & Fursov, 2007). Recent evidence strongly suggests that ATIs can cause intestinal inflammation and exacerbate existing inflammatory disorders of the intestine

(Cuccioloni et al., 2016; Zevallos et al., 2017). Researchers have also shown in animal models that the pancreas overcompensates in production of α-amylase and trypsin when their inhibitors are present (Buonocore et al., 1977; Gallaher & Schneeman, 1986). This overcompensation can lead to hypertrophy and hyperplasia of and potential tumor growth in the pancreas. α-amylase and trypsin inhibitors are particularly heat-resistant and protease-resistant (Sosulski et al., 1988; Madsen & Brinch-Pederson, 2016; Zevallos et al., 2017). Reducing agents can be added during processing to help cleave the disulfide bonds of the inhibitors and promote their inactivation.

1.3.4 Disease, Allergy, and Intolerance

Celiac Disease (CD) is a permanent inflammatory disease characterized by the destruction of jejunal and duodenal villi upon gluten protein ingestion (Bao, Green, & Bhagat, 2012). Consequences of such inflammation are malabsorption of nutrients, gastrointestinal distress, and potential psychological reactions, among other symptoms (Koehler, Wieser, & Konitzer, 2014a). The prevalence of CD in the developed world is about 1% (Mustalahti et al., 2010). Cereal grains implicated in the onset of symptoms include wheat, barley, rye, and oats, all of which share a certain degree of homology in protein composition (Koehler, Wieser, & Konitzer, 2014b). Gluten is the protein matrix formed upon mixing flour and water. Gluten is less readily digested *in vitro* because of its high proline content, which results in rigidity of the protein structure, making it less accessible to proteolytic enzymes (Koehler et al., 2014b). In individuals with CD, intact peptides can bind to the intestinal mucosal lining before passing through the epithelial layer to incite a proinflammatory cytokine response and proliferation of intraepithelial lymphocytes. The conventional treatment for CD is a gluten-free diet; adherence to this diet enables regeneration of villi (Koehler, Wieser, & Konitzer, 2014c). CD has been associated with a number of autoimmune, neurological, and malignant diseases, as well as interstitial lung disease and ulcerative colitis (Koehler et al., 2016).

Wheat allergy is one of the eight most common food allergies worldwide (Tatham, 2016). Wheat allergy is an IgE mediated response to one or more of its protein

fractions: albumins, globulins, gliadins, glutenins, or to the less prevalent proteins such as α-amylase inhibitors (Inomata, 2009). It can manifest as baker's asthma, exercise-induced anaphylaxis, contact dermatitis, or classic allergy with gastrointestinal symptoms. Because of the homology in prolamins – or aqueous alcohol-soluble seed storage proteins – from wheat (gliadin), barley (hordein), rye (secalin), and oats (avenin), individuals may have cross-reactivity to these grains, as well. Allergies to maize, sorghum, rice, quinoa, buckwheat, and amaranth are less common but do exist.

Non-celiac/non-allergy wheat sensitivity is a disorder with gastrointestinal symptoms that occur in the absence of an IgE response (Koehler et al., 2016). Researchers hypothesize the role of α-amylase and trypsin inhibitors, oligo-, di-, and monosaccharides, or polyols in its genesis. Other gluten-mediated intolerances include gluten ataxia -- difficulty walking associated with gluten intake, and dermatitis herpetiformis – a skin reaction manifesting in papules, blisters, and pigmentation changes.

Anti-nutritional factors in grain, together with wheat allergy, intolerance, and disease pose challenges to millions of consumers. At the other end of the production chain, current agricultural practices in grain production, specifically of annual grains, pose challenges to grain farmers.

1.3.5 Current Challenges of Grain Production

The population of the world has doubled over the last 45 years and is expected to double again over the next 50 (an annual rate of 1.3%) (Pimentel & Wilson, 2004). The global population relies heavily on cereal grains for sustenance, yet current agricultural practices utilized to grow these cereal grains cause damage to croplands. Accordingly, farmers are constantly seeking out arable cropland. The annual rate of loss of available cropland is just as great as the annual rate of population growth, 1.3%. The combination of a burgeoning world population with a high rate of depletion of land resources results in a continuously decreasing amount of grains per capita in an already malnourished world that gleans 80% of their food supply from cereal grains production.

These alarming facts are met with efforts to find ways to continue to feed the world (Pimentel & Wilson, 2004). One solution has been deforestation to create available farmland, however this is only a temporary solution, as new farmland is subject to the same damage if current agricultural practices continue. Additionally, this approach is environmentally harmful. A second solution has been increasing yields per hectare through breeding and advanced technology. While grain yields per hectare continue to increase from year to year, the rate of increase is slowing down and cannot keep up with the rate of population growth. A third potential solution is the farming of perennial grains, which addresses the fundamental issue of land degradation. Perennial grains mitigate soil erosion, conserve water, reduce greenhouse gas emissions, and require fewer chemical and mechanical inputs (Dinnes, Karlen, Jaynes, Kaspar, & Hatfield, 2002; Culman et al., 2013; Lewandowski, 2016). Perennial plants do present their own challenges. For example, perennials are more susceptible than annuals to seed shattering, as well as lodging (Lawrence, 1983; Lubofsky, 2016). Lodging is a phenomenon in which tall and weak stems cause plants to fall over, making harvesting more challenging and preventing proper seed growth (Lubofsky, 2016). Perennial plants also tend to have smaller seed size and yields than annuals (L. DeHaan, 2015). However, breeding efforts are addressing these problems and have thus far shown significant improvement (Zhang et al., 2016). With continued breeding efforts, perennial agriculture is a promising alternative to annual farming.

1.4 Perennial Grains

1.4.1 Description and Environmental Benefits

The planet's lands were once comprised of almost entirely perennial plants, but since the start of agriculture 10,000 years ago, almost entirely annual species were domesticated for grain production (Cox et al., 2002; Chiras & Reganold, 2010). At present, annual crops, including cereal grains along with legumes and oilseeds, are farmed on approximately 70% of the world's cropland (Cox et al., 2006; Glover, Cox, &

Reganold, 2007), while perennials only account for 13% of harvested cropland according to an estimate by Monfreda, Ramankutty, and Foley (2008). While currently necessary to meet the demands of the world population, prolonged annual farming can exacerbate environmental problems such as soil erosion and water run-off. Furthermore, annual crop farming can perpetuate soil degradation; in fact, 33.5% of cropland worldwide is at high risk of soil degradation, in terms of loss of natural nutrients and biodiversity (Eswaran, Beinroth, & Reich, 1999; Zhang et al., 2011). One estimate suggests that approximately 90% of U.S. soil is being degraded faster than it can be repaired (David, Pimentel, Harvey, Resosudarmo, & Sinclair, 1995). Jackson (2002) argues that a natural systems agriculture approach that takes advantage of the perennial plants naturally present across the world's landscapes could be the solution.

Perennial crops boast several benefits to the environment, many of which are a direct result of their long growing seasons and extensive root systems (DeHaan, Van Tassel, & Cox, 2004). Perennial plants are differentiated from annual plants by their ability to produce annual shoots for harvest over several years without re-planting, while annual crops require re-planting after each harvest season (Monfreda et al., 2008). This difference partly manifests itself in the way annual and perennial plants allocate their carbohydrate stores. Annual plants allocate a greater proportion of carbohydrate to their surface biomass, while perennial crops allocate more carbohydrates to their root system, leading to an overall deeper root system as compared to annuals (DeHaan et al., 2004). The greater allocation of resources to roots prioritizes longevity over yield in many perennial species. These extensive root systems improve soil carbon sequestration, which in turn results in increased soil fertility and overall reduction of greenhouse gas emissions (Boody et al., 2005; Lewandowski, 2016). Additionally, deeper root systems are better able to trap and utilize rainwater, reducing the risk of flooding and soil erosion which can lead to pesticide leaching and loss of essential plant nutrients, including nitrogen (Troeh, Hobbs, & Donahue, 1981; Randall et al., 1997; Culman et al., 2013) . Culman et al. (2013) found that perennial IWG in its second year of production had greater than 85% less nitrogen leaching than annual wheat. Increased carbon sequestration and decreased

nitrogen leaching together can lower nutrient input requirements from fertilizers, benefiting farmers from a cost perspective and preventing pollution of water systems. (Troeh et al., 1981; Lewandowski, 2016). This retention of nutrients allows for development of substantial vegetative biomass, which renders perennial crops an excellent candidate for biofuels, grain and animal feed, and forage (Culman et al., 2013).

Perennial plants offer additional benefits aside from those secondary to extensive root systems. When compared to annuals, perennials tend to be more resistant to drought and frost, as well as disease, therefore lowering the need for herbicide and pesticide application. As perennial plants do not require annual tillage and planting, mechanical and labor costs are lower for perennial croplands as compared to annual croplands, as well (Lewandowski, 2016). Finally, the long growing season along with the ability to persist for several years enables wildlife to develop habitats across perennial croplands (Pimentel et al., 2012). Many agronomists agree that the benefits of perennial crops are most evident with polycultures of perennials and diversification of landscapes, where different perennial species can fulfill various roles (Jackson, 2002).

Given the array of environmental benefits perennial crops offer and growing consumer interest in sustainably sourced food products (IFIC, 2017), perennial crops have a likely niche in the commercial food market. However, continued breeding efforts to optimize their agronomic characteristics and ongoing research to develop end use applications are necessary to incentivize farmers to plant perennials.

1.4.2 Breeding Efforts of Perennial Crops and Intermediate Wheatgrass

Perennial breeding efforts were started in the 1960s in the then Soviet Union under the looming threat of soil degradation due to annual crop farming and the knowledge of environmental benefits from perennial agriculture (Cox et al., 2002; Jackson, 2002). The concept of natural systems agriculture, essentially exploiting the earth's naturally produced perennial crops as a means of addressing environmental distress from conventional agriculture, was developed and refined at the Land Institute in Salina, Kansas in 1977.

The foremost question breeders must address is whether perennial crops can compete with annual crops in terms of being able to feed the ever-growing world population, a factor which is predominately impacted by yield and seed size (Cox et al., 2006). In general, perennial crops tend to produce lower yields and have smaller seeds, characteristics which are a reflection of the allocation of carbohydrate resources to the vegetative root system to remain competitive (L. DeHaan et al., 2005). Murphy et al. (2009) found that 31 perennial wheat breeding lines produced lower yields than two of their annual wheat counterparts (Murphy, Hoagland, Reeves, Baik, & Jones, 2009). Small seed size of perennials poses an additional challenge for breeders. Smaller seed size can negatively impact flour yield, ease of use, and functionality in end products (Cox et al., 2006; Murphy et al., 2009). Yet, a smaller seed size does have its nutritional advantages, namely higher mineral and fiber contents as a result of higher bran to endosperm ratio (Becker, Wagoner, Hanners, & Saunders, 1991; Murphy et al., 2009).

While lower yields and small seed size are of primary concern to breeders, perennials also pose challenges in terms of ease of harvesting, plant hardiness, and quality of end-use products. Perennials are prone to asynchronous ripening, shattering of seeds, and poor threshability, all of which make harvesting difficult (Cox et al., 2006; Murphy et al., 2009). Consequently, yields are further reduced. Additionally, long-living root systems may provide stable environments for pathogens to thrive in, leading to disease and pest infestation (Ploetz, 2007). Moreover, if perennial species are to replace annual crops, they need to meet the nutritional needs of a growing population (Cox et al., 2006). All these challenges can be potentially addressed through breeding efforts.

Breeding initiatives occur via two primary approaches: domestication of perennial species with desirable traits or wide hybridization, referred to by some as "perennialization" of annual crops (Jackson, 2002; Dehaan et al., 2004; Y. Zhang et al., 2011). Domestication entails artificially selecting for desired phenotypic traits, often seed size and yield, across a single species' gene pool. Hybridization entails cross-breeding a perennial species with an annual species with genes that code for advantageous domestication properties, including high yields, large and non-shattering seeds, lodging

resistance, increased threshability, decreased branching, low dormancy rates, uniform maturation, disease resistance and abiotic stress resistance, quality of seed for end-use products, and low toxicity (Harlan, de Wet, & Price, 1973; Kantar et al., 2016). Perennialization accelerates the breeding process, where domestication of a perennial line with selected traits can take decades. However recent advances in genomic selection have expedited the domestication process in a number of perennials, such as intermediate wheatgrass (Zhang et al., 2014; Kantar et al., 2016). On the other hand, perennialization poses the risk of reproductive sterility (Cox et al., 2006).

Regardless of which approach is taken, the breeding process starts with evaluating wild perennial species for potential to be domesticated based on inherent agronomic qualities. Wagoner (1990) conducted an analysis of 100 wild perennial species, and recognized intermediate wheatgrass (IWG), or *Thinopyrum intermedium*, as being superior for its agronomic qualities, nutritional qualities, and breeding potential (Wagoner, 1990). Efforts to domesticate IWG began at the Rodale Research Center in Pennsylvania in 1983 and continued at the Land Institute in Kansas in 2002. Mass selection, bulk breeding, and more recently genomic selection, have been the primary approaches in developing an ideotype of this promising perennial (Cox et al., 2006; Culman et al., 2013; Zhang et al., 2014). From a breeding perspective, IWG is a favorable target for a couple of reasons. First, high intraspecies genetic variation offers ample opportunity for improvement in selected traits. Second, IWG has shown high heritability of domestication traits (Cox et al., 2006).

Thus far, improvements in domestication traits in IWG have included an enlarged seed size, less seed shattering, and a shorter plant height, which prevents lodging (Zhang et al., 2016). DeHaan (2015) demonstrated a seed size increase of 23% over two selection cycles. DeHaan (2015) also showed progress in amplifying seed yields, with an increase of 77% over the same two selection cycles. While yields remain inferior to those of annual wheat, comparable yields are attainable. IWG is particularly known for its deep root system and as such, concern exists around whether its high vegetative biomass will be compromised in breeding efforts that favor seed size and yield. Yet in an earlier study,

breeding efforts to increase seed yield of IWG did not significantly decrease forage yields (Knowles, 1977). DeHaan (2005) asserts that the trade-off between having deep root systems associated with perenniality or high yields associated with farming of annual crops is rarely static or bivariate, meaning the optimization of one characteristic does not necessarily proportionally affect the other (Dehaan et al., 2005). Furthermore, perennial plants overall are more productive than annuals, better utilizing their resources and requiring fewer inputs beyond the first year of planting, allowing the plants to produce larger yields over time and boasting competitive input to yield ratios. In a study by Glover et al. (2010), perennial grass has maintained yields for the past 75 years, over which time period, it has cumulatively produced greater yields than annual wheat. With controlled artificial selection, DeHaan (2005) argues that high yields do not have to come at the entire expense of deep root systems and subsequent longevity, albeit a negative correlation is realistically expected. The realistic probability of maintaining IWG's unique root system while still improving yields through breeding is a positive incentive for continued research into IWG.

1.4.3 Intermediate Wheatgrass: Thinopyrum intermedium

IWG is genetically similar to common wheat, *Triticum aestivum L.*, belonging to the same taxonomic family of Poaceae (grass family) (Ogle, Loren, & Jensen, 2003). IWG originated in Europe and Asia and now can be found in many parts of the United States' midwestern and western regions. IWG has long been used for hay, pasture, and animal feed. Both seeding and harvest occur in late August to mid-September. IWG stands 3 to 4 feet tall with 4 to 8 inch seed spikes and 4 to 8 mm-wide leaves in a green/blue hue. Around 2 to 6 florets per spikelet florets are usually found in this crop. The preferred growing conditions of IWG are 12-13 in. of rainfall or greater, elevations of 3,500-9,000 feet, loamy or clayey textured soils.

From both an environmental and evolutionary perspective, IWG has the edge over its perennial competitors owing to its particularly dense root system. IWG can produce as much as 7,000 pounds (dry weight) of roots per acre in solely the top 8 inches of soil

(Ogle et al., 2003). This extensive root system has been shown to reduce nitrogen leaching by 86% and increase carbon sequestration by 13% (Culman et al., 2013). The impressive amount of biomass derived from its root system has prospective applications as biofuel along with its existing applications for feed, forage, and hay. In its first year of production, the vegetative biomass of IWG is on par with that of annual wheat, but it significantly outweighs wheat by the second year, with 71% more kilograms per hectare produced.

IWG is considered to be a high yielding perennial (Culman et al., 2013) with relatively large seed size (Wagoner, 1990). Under irrigated conditions, IWG has typical yields of 504 to 616 kilograms per hectare, but it is still able to produce yields of 168 to 280 kilograms per hectare in dry conditions (Weik, Kaul, Kubler, & Aufhammer, 2002; Ogle et al., 2003). Culman et al. (2013) measured yields as high as 1390-1662 kilograms per hectare in the second year of production. Yields peak in the second to third year of production and wane thereafter (Wagoner, 1990; Culman et al., 2013). Part of what enables higher yields of IWG is its ease of harvest. Factors that facilitate harvest in IWG are a relatively high threshability index, an erect culm, synchronous maturation and flowering, and resistance to shattering (Wagoner, 1990). These factors are not yet on par with those of annuals, but are superior in comparison to other eligible perennial species.

Another means by which IWG outcompetes its fellow perennials is via its high adaptability (Culman et al., 2013). Wagoner (1990) noted IWG to be hardy and stress-resistant. IWG is tolerable to slightly acidic to mildly saline conditions, cold weather, drought, flooding, and fire (Ogle et al., 2003). As noted previously, it also performs well over a wide range of elevations. IWG has also been recognized for its good flavor and nutrient density, which is of relevance to its end use applications in animal feed and potential commercial food applications (Wagoner, 1990; Becker et al., 1991; Murphy et al., 2009; Tyl & Ismail, 2018) . Lastly, IWG seeds dry quickly post-harvest, which is a promising characteristic with regards to its storage stability (Wagoner, 1990).

1.5 Overview of Chemical Composition of Intermediate Wheatgrass

In the grain industry, wheat is recognized as the "gold standard" due to its exceptional gluten proteins profile and the array of baking applications it can be used in (Zilic, 2013). Therefore, grains are often compared to wheat as a measure of potential success from an agronomic, functional, and nutritional lens. From a nutritional lens, IWG possesses a competitive nutritional profile. Due to its small seed size, IWG has a large ratio of bran to endosperm (Rahardjo et al., 2018). Therefore, many of the nutrients housed in the bran and the aleurone, namely phytochemicals, fiber, and micronutrients comprise a greater portion of the seed as a whole ((Rahardjo et al., 2018; Tyl & Ismail, 2018). The total fiber content of IWG on a dry basis is approximately 16-17%, of which 13% is insoluble and 3% is soluble (Marti, Qiu, Schoenfuss, & Seetharaman, 2015; Rahardjo et al., 2018; Tyl & Ismail, 2018). Wheat, by comparison, has approximately 13% total dietary fiber (Dhingra, Michael, & Rajput, 2012).

The higher fiber content of IWG does come at a cost, as the arabinoxylans comprising a large portion of the fiber in IWG can compete with gluten for water which ultimately affects bread volume (Kantar et al., 2016). Higher fiber contents also come at the cost of total starch, because of the smaller proportion of endosperm (Becker et al., 1991). Marti et al. (2015) measured IWG to have a significantly lower total starch content (46.7% dry basis) than its hard red wheat counterpart (74.9%). A lower starch content imparts a unique texture in baked products formulated with IWG flour, and may potentially interfere with flavor development (Kantar et al., 2016). Differences in starch structure of IWG are evident in unique starch pasting profiles as seen by Marti et al. (2015) and Rahardjo et al. (2018). When compared to the pasting profile of hard red wheat, IWG exhibited a significantly higher pasting temperature but significantly lower peak viscosity and setback. The lower setback value may indicate lower tendency to retrograde (Marti et al., 2015), which is promising from a shelf life standpoint.

With regards to protein, IWG is a double-edged sword, offering both a superior protein content when compared to hard red wheat and an inferior protein profile. IWG boasts a total protein content of 20-21% dry basis according to results by Marti et al.

(2015) and Tyl & Ismail (2018), while wheat has 13.2-15% total protein. IWG also has higher quantities of all essential amino acids, with the exception of the limiting amino acid lysine (Becker et al., 1991). While higher protein content may help market IWG, the protein profile is far less marketable. IWG is deficient in high molecular weight glutenins (Rahardjo et al., 2018; Tyl & Ismail, 2018), which are an essential component of the gas-holding gluten matrix in leavened baked goods, as they contribute to the elasticity of bread dough. In evaluating the dough strength by Farinograph, Rahardjo et al. (2018) found shorter dough development times and lower stabilities and further measured significantly lower loaf heights compared to wheat. Fortunately, IWG has some redeeming characteristics that could enhance baking quality. For one, IWG proteins have an appreciable amount of thiols that could participate in disulfide exchange contributing to gluten strength, albeit they are less readily accessible than in hard red wheat (Marti et al., 2015). Secondly, IWG has shown promising progress in terms of protein aggregation ability, which is central to forming a gluten matrix. These characteristics are aided by breeding efforts to increase glutenin content (Kantar et al., 2016).

When looking at total fat content, IWG has a greater amount (3.2% dry basis) than whole wheat flour (2.8%) (Tyl & Ismail, 2018). The fat content is of most interest with regards to storage stability. The higher fat content of IWG could render IWG more susceptible to rancidity over storage. This is because lipids can serve as substrates for certain enzymes, namely lipase and lipoxygenase, that catalyze hydrolysis and oxidation of the lipids, respectively (Galliard, 1994). Lipids are also susceptible to autooxidation, a non-enzymatic spontaneous oxidation in the presence of oxygen. The products of these reactions impart off flavors that decrease overall sensory acceptability, thereby limiting shelf life (Galliard, 1994). Therefore, higher lipase and lipoxygenase activity is undesirable for overall storage. Tyl and Ismail (2018) measured significantly lower lipoxygenase activity in 13 IWG breeding populations and forage varieties than in HRW. However, lipase activity was variable across different IWG samples, with some exhibiting significantly greater lipase activity than HRW and others exhibiting significantly lower activity.

Though the total fat content and enzyme activity could predispose IWG to rancidity over storage, the high bran to endosperm ratio of IWG lends relatively higher quantities of antioxidants than wheat (Tyl & Ismail, 2018) that could help counter oxidative rancidity over storage. The primary classes of antioxidants within wheat grain are carotenoids, phenolic acids (namely hydroxycinnamic acids), tocopherols, and lignins (Yu, 2008; Luthria, Lu, & Maria John, 2015). Of those mentioned, Tyl and Ismail (2018), measured the carotenoids lutein and zeaxanthin and the hydroxycinnamic acids *trans*-ferulic acid, *trans*-*p*-coumaric acid, and *trans*-sinapic acid, and found that most IWG populations contained higher levels than wheat. Factors impacting rancidity in IWG over storage, including fat content, lipid enzymes, and antioxidant content, will be central to the further discussion of its storage stability.

1.6 Storage and Storage Stability of Grains

1.6.1 Common Storage Conditions

Directly after harvest, grains are either stored in silos or grain elevators, or directly transported to a flour or feed mill or malt house. Prior to storage, grains must be dried in order to prevent microbial growth and insect infestation. A moisture level between 13 and 14% is considered maximal for most grains to prevent these issues (Pomeranz, 1992; Wilcke & Hellevang, 1992; Grundas & Wrigley, 2016). Fumigation or carbon dioxide treatment of silos can further hinder microbial growth and pest infestation. IWG, as mentioned earlier, dries quickly, which is advantageous to farmers (Wagoner, 1990). When drying grains, temperatures should not exceed 35°C, as changes in functionality can occur at higher temperatures (Grundas & Wrigley, 2016). Wilcke and Hellevang (1992) assert that an ideal storage temperature is below 15.5°C to prevent insect and mold growth. They further assert that a temperature difference between the storage bin and the outside environment should not exceed approximately 10°C to avoid moisture transfer into the bin. In order to avoid a large differential, silos and elevators may be aerated to cool the grains and prevent moisture retention. The same principle

holds true for relative humidity, as a large differential between storage environment and external environment can lead to transfer of moisture to the grains (Pomeranz, 1992).

Given the number of factors that influence shelf life of grains, there is no single answer as to how long each cereal grain can be stored before deemed unfit for consumption, whether by measure of safety or quality parameters. However, researchers universally agree that seed moisture content along with relative humidity and temperature of the storage environment are central to storage stability, directly influencing other secondary factors that impact shelf life. These factors include grain respiration, microbiological or pest infestation, germination capacity, and rancidity that are discussed in the following section. In general, stored intact grains can remain fit for consumption with minimal quality deterioration for several years so long as proper humidity and temperature conditions are observed and moisture content of the grain is maintained below the critical level (Pomeranz, 1992; Grundas & Wrigley, 2016). If hermetically sealed and stored free of oxygen, most grains have a shelf life of 8-12 years ("Storage Life Of Dry Foods," n.d.).

1.6.2 Factors Impacting Shelf Life in Grains

According to Kaleta and Górnicki (2013), the primary parameters that impact shelf life of grains are grain respiration, microbial (especially fungal) or pest infestation, and germination capacity. In addition, rancidity, functional changes, and nutrient loss can also influence shelf life (Tait & Galliard, 1988; Pomeranz, 1992; Galliard, 1994).

1.6.2.1 Grain Respiration

Grain respiration is defined as the oxidation of carbohydrate resulting in the production of carbon dioxide, water, and heat. Uncontrolled respiration of stored grain is deleterious, as the heat produced can cause germ-damaged grain, otherwise referred to as "sick grain" (Pomeranz, 1992). Heat damage, or discoloration to grain caused by excessive heat, may also occur. Heat damage can cause protein denaturation and loss of processing quality (Wang, Dowell, & Chung, 2001). Additionally, respiration can

interfere with the ability of the grain to germinate, which is unfavorable in such end-use industries as malting (Sauer, Meronuck, & Christensen, 1992). Uncontrolled respiration also leads to loss of dry matter in grain from the extensive oxidation of carbohydrate and fat (Pomeranz, 1992). The acceptable limit of dry matter loss leading to quality loss is debated; researchers have argued dry matter loss in the range of 0.04 to 1% is the limit for human consumption, and 2% for animal feed (Kaleta & Górnicki, 2013). Grain respiration is impacted by moisture content, temperature of storage, aeration during storage, microbial contamination, grain damage, and grain hardness (Pomeranz, 1992; Kaleta & Górnicki, 2013).

Arguably the most significant influence on respiration is intrinsic moisture content of the grain and corresponding relative humidity of the external environment. The "critical moisture value" is defined as the limit above which both respiration and spoilage will substantially increase. Researchers assert that the critical level of moisture content (13-14%) for a grain often corresponds with a relative humidity of approximately 70-75% or less, the level below which mold spore germination is inhibited (S. Pixton & Warburton, 1971; Pomeranz, 1992). The effect of temperature on the relationship between relative humidity and interstitial moisture content of the grain is negligible, according to Pomeranz (1992), as his review revealed minimal changes in measured relative humidity (and corresponding moisture content) per change in temperature. The effect is only slightly more pronounced as relative humidity values decrease.

While temperature does not exert a significant effect on the relative humidity of the storage environment, it can be damaging, wherein it accelerates respiration of the grain. High temperatures will continue to promote respiration until the chemical reaction underlying respiration is precluded by low amount of substrate (i.e. oxygen, carbohydrate, or fat), inactivation of catalyzing enzymes, or high amounts of product (i.e. carbon dioxide) that inhibit the forward direction of the reaction.

Aeration of grain storage facilities supplies the grain with oxygen. This is a disadvantage in the respect, as it promotes grain respiration and the affiliated

consequences, yet it is also an advantage as it prevents moisture accumulation, which is deemed more influential on shelf life (Pomeranz, 1992).

A large proportion of the carbon dioxide produced in a grain storage compartment is due to microbial respiration. Heat produced during microbial respiration can, in turn, facilitate growth of other, thermophilic fungi, promote caking (at 50-55°C), and lead to "heat damage." "Heat damage" refers to the phenomenon in which respiration occurs at an advanced rate whether within high moisture grains, storage microbes, or both collectively, leading to the rapid production of heat and a dark discoloration of the grain. In a sort of a positive feedback loop, the heat produced from the respiratory reaction increases the temperature of the storage environment which further accelerates grain respiration (Coleman & Rothgeb, 1927).

Other factors impacting grain respiration include grain damage and hardness. Grain with a large percentage of damaged kernels heats more readily and therefore, has a higher rate of respiration. Grain damage is a reflection of the growing environment and harvesting practices. Finally, grain hardness also impacts respiration rate. The hardness of grain refers to its physical resistance to compression and friability of the endosperm (Hrušková & Švec, 2009; U.S. Wheat Associates, 2011). Soft grains tend to have higher respiration rates and therefore more oxidation over a shorter period of time compared to hard grains (Pomeranz, 1992; Kaleta & Górnicki, 2013).

1.6.2.2 Microbes, Insects, and Pests

Microbial and pest contamination primarily influence the shelf life of grain from a safety standpoint. The most common bacterial threats to grain belong to the following families: *Pseudomonadaceae, Micrococcaceae, Lactobacillaceae,* and *Bacillaceae* (Kaleta & Górnicki, 2013). However, bacterial growth in stored intact grain is uncommon, as the minimal relative humidity required for most bacterial species is 90%, which far exceeds the limit of 70-75% grain storage facilities observe (Pomeranz, 1992).

There are more than 150 species of molds, fungi, and yeasts that have been discovered in cereal grains. A moisture content of grain beyond the critical level, and a

significant microbiological count at storage onset can all exacerbate fungal growth. Physically damaged grain is also more susceptible to fungal growth. The molds and fungi most relevant to grain deterioration can be categorized into “field fungi” and “storage fungi,” and are assigned to either category based on where they are most commonly found on the grains, as well as their criteria for growth (Sauer et al., 1992). “Field fungi” survive at high moisture contents (above 20%) and high relative humidity (90-100%) and therefore favor grains prior to harvest or before drying post-harvest. The most common “field fungi” belong to the following families: *Alternaria, Fusarium, Helminthosorium, Claviceps,* and *Cladosporium* (Sauer et al., 1992; Fletcher & Blaney, 2016).

“Storage fungi” on the other hand, are adapted to low moisture environments and outcompete hydrophilic fungi during storage (Sauer et al., 1992). Some "storage fungi” are even halophilic. Thorough research has confirmed that while these fungi may be present in the field, they do not invade the seed or cause damage until favorable environmental conditions are reached during storage. The most common “storage fungi” in cereal grains are *Aspergillus spp*. and *Penicillium spp.* To a lesser extent, other storage fungi may be present in wet grains or prior to drying, namely *Nigrospora spp., Rhizopus spp., Mucor spp., Wallemia sebi*, and *Chrysosporium fastidium*.

Microbiological, and specifically fungal, infections can render stored grain undesirable or unsafe for a number of reasons, including visible growth, unpleasant odor, discoloration, changes in chemical and nutritional composition, reduced germination ability, caking in late stages of damage, dry matter loss and heat damage in the grain from fungal respiration, and mycotoxin contamination. Additionally, fungi and molds can produce secondary toxic metabolites, referred to as myctoxins, which can be poisonous to human and animal life (Sauer et al., 1992; Fletcher & Blaney, 2016). The following group of mycotoxins are most concerning for human consumption: ergot alkaloids (*Claviceps spp.),* aflatoxins (*Aspergillus spp.*), and trichothecenes (*Fusarium spp.*). Ochratoxins (*Aspergillus ochraceus* and *Penicillium verrucosum*), fumonisms (*Fusarium spp.*), zearalenone (*Fusarium spp.*), and phomopsins (*Diaporthe toxica*) are dangerous for

animal consumption but do not significantly affect humans. Of those mentioned, the most notable mycotoxins in wheat species are the ergot alkaloid toxins and the trichothecenes.

Mites and pests in grains can carry the aforementioned bacterial and toxigenic fungal species, and it is therefore pivotal to prevent their presence during grain storage (Kaleta & Górnicki, 2013). Additionally, these pests can feed on grain, reducing the overall nutritional quality of the grain, specifically iron and vitamin B content.

1.6.2.3 Germination Capacity

Germination capacity has been extensively studied as a determinant of overall shelf life in grains. Seed viability is an important characteristic among food manufacturers and farmers alike. Germination is desired in industries such as malting, as previously mentioned, as well as for production of seeds for future planting. Loss of germination ability can also indicate other damage to the grain, including dry matter loss and microbial contamination (Kaleta & Górnicki, 2013). Karunakaran et al. (2001) argues that once germination capacity decreases to 90%, damage to the grain is pronounced enough such that the safe storage limit has been reached. Wallace et al. (1983) defined the minimum limit of germination capacity indicating overall deterioration of grain to be slightly lower, at 85%.

1.6.2.4 Hydrolytic and Oxidative Rancidity

Over storage, sensory acceptability of the grain can be significantly impacted by rancidity of the lipid component, which primarily is a chemical consequence of enzymatic hydrolysis by lipase (hydrolytic rancidity) and oxidation via non-enzymatic or enzymatic pathways (oxidative rancidity) (Pomeranz, 1992; Galliard, 1994). Lipase hydrolyzes triacylglycerols into free fatty acids. Free fatty acids, especially polyunsaturated fatty acids, compared to triglycerides, are particularly prone to subsequent oxidation. In a study measuring the storage stability of whole grain wheat flour, the increase in free fatty acids at 20°C was accompanied by a proportional increase in uptake of oxygen with a calculated correlation coefficient of 0.96 (Galliard, 1986a).

Lipoxygenase can enzymatically catalyze the oxidation of fatty acids to produce hydroperoxides. Lipoxygenase, however, is highly preferential to unesterified fatty acids released by lipase. The enzymatic oxidation by lipoxygenase occurs at a much slower rate than the lipolytic action by lipase in intact grains. The lipoxygenase in wheat grain has a particular affinity for linoleic acid and linolenic acid (Galliard, 1994). Fatty acids may also undergo non-enzymatic oxidation. Autooxidation refers to the spontaneous oxidation of fatty acids in the presence of oxygen, catalysts, and light. Autooxidation can also produce hydroperoxides. However, autooxidation is more readily inhibited by antioxidant activity as compared to enzymatic oxidation. Autooxidation can continue to produce lipid oxidation products in the absence of lipoxygenase.

The hydroperoxides generated from both enzymatic and nonenzymatic oxidation are unstable, and they breakdown into alkoxy free radicals which can be substituted with aldehyde, alcohol, or hydrocarbon groups. These secondary oxidation products are often volatile and contribute to off-flavors. Secondary oxidation product hexanal, an aldehyde, had correlation coefficients of 0.92, 0.92, and 0.95 with bitter flavor, rancid flavor, and musty flavor, respectively (Heiniö, Lehtinen, Oksman-Caldentey, & Poutanen, 2002).

Free fatty acids also contribute to off-flavors that lead to decreased sensory acceptability and, therefore, decreased shelf life of grains and grain products (Galliard & Gallagher, 1988; Khan et al., 2011; Lampi et al., 2015). Strong correlation coefficients were derived from measurements of free fatty acids and sensory attributes of stored oat grains in a 2002 study, including a coefficient of 0.90 between a bitter flavor and free fatty acids and a coefficient of 0.85 between a rancid flavor and free fatty acids. (Heiniö et al., 2002). Hansen and Rose (1996) discovered a significant positive correlation between rancidity as measured by a sensory panel and free fatty acid content in bread made from stored wheat flour.

In general, free fatty acids are deemed a greater contributor than oxidized products to the loss of sensory quality (De Almeida, Pareyt, Gerits, & Delcour, 2014; Pomeranz, 1992). Wheat bran lipase can act exceptionally well in low moisture environments and is particularly heat tolerant. (Saunders & Heltved, 1985).

Lipoxygenase, on the other hand, is most active in a hydrated system, though can still act at a slower rate over dry storage. Upon hydration, however, lipoxygenase will rapidly act on polyunsaturated free fatty acids (Tait & Galliard, 1988).

Degradative enzyme activity is far more pronounced in milled grain than in groats for a couple of reasons. First, enzymes within the grain have greater access to the lipids in the germ upon milling. Lipase, which is primarily concentrated in the bran of the grain (Galliard, 1986b; De Almeida et al., 2014), is better able to access the triacylglycerols, which are predominately in the germ, upon milling. In fact, about 50% of triacylgylcerides in wheat grain are located in the germ and remain inaccessible to lipase until milling. (Morrison & Hargin, 1981). To that end, lipoxygenase, which is concentrated primarily in the germ of the kernel (Galliard, 1986b), is better able to access lipid substrate, including fatty acids liberated by lipase, when components of the grain are decompartmentalized during milling. Along with enhanced substrate accessibility, a second factor that lowers storage stability of milled whole grains is the exposure of lipids in the germ to environmental oxygen upon milling, enabling autooxidation over storage (Pomeranz, 1992). While whole grain flour holds a significant nutritional advantage over refined grain flour, it is inferior to refined flour in regards to shelf life. According to Doblado-Maldonado, Pike, Sweley, & Rose (2012), most manufacturers set use-by dates of 3-9 months for whole grain wheat flour and 9-15 months for refined wheat flour, however the authors were careful to point out that the shelf life could be shorter depending on storage conditions.

Though there are many criteria used to assess the shelf life of grains, hydrolytic and oxidative rancidity are most appropriate for assessing the shelf life of IWG for a few reasons. First, as previous research has shown, IWG has a relatively high concentration of fat and relatively high lipase and lipoxygenase activity (Tyl & Ismail, 2018). Furthermore, cereal grains in general have a substantial amount of the polyunsaturated linoleic and linolenic acids (Galliard, 1994). As a genetic relative to wheat, IWG may also contain a high percentage of these constituents that may intensify its predisposition to oxidation, a factor that will be further explored in the present research. It is also of

interest to consider how the high antioxidant content of IWG may slow oxidative progression over storage. Further validating the choice to measure rancidity over other criteria is that manufacturers and grain processers have long relied heavily on free fatty acid content as an indicator of shelf life, often setting limits in material and product specifications (Galliard, 1983; Zeleny & Coleman, 1938). Additionally, taste is the most influential trait on consumer selection of products (International Food Information Council Foundation, 2017), and the relationship of rancidity to odor and flavor is well demonstrated. While hydrolytic and oxidative rancidity along with functionality changes are used in the present study to measure shelf life, shelf life is the cumulative result of changes to several quality parameters over storage.

1.6.2.5 Estimating Shelf Life

The criteria by which end of shelf life in grains is determined varies and often depends on the buyer, as well as the criteria established in the Official United States Standards for Grain. Some criteria, including discoloration (either from fungi or "heat damage"), visible mold, and a musty or rancid odor, are obvious during sampling and does not require further inspection to deem the grain unacceptable. Oftentimes, these conspicuous changes are used as indicators of less readily detectable damage to the grain. This is based on the principle that the same fundamental parameters, i.e. moisture content, storage temperature, and storage time, are central to all deterioration mechanisms and that once a critical value has been measured in a single parameter, it is likely that other criteria have reached critical levels, as well . In fact, Karunakaran et al. (2001) found that moisture content influenced respiration rate, microbial growth, and germination capacity so much so that a decrease in moisture content from 17% to 15% increased shelf life by a factor of 10.

Several researchers have developed algorithms to predict storage stability using specific criteria and maximum or minimum limits of said criteria. Some of these parameter limits have been discussed previously, including percentage of dry matter loss

and percentage of germination capacity. Kaleta and Gornícki (2013) discuss the algorithms developed for predicting shelf life using these parameters in detail.

Mold count may also be used to predict lipid degradation, as increasing mold counts have been associated with reduction in total lipids, breakdown of both free and bound lipids, and decrease in polar lipids, glycolipids, and phospholipids (Daftary & Yeshajahu, 1961; Daftary, Sauer, & Pomeranz, 1970; Pomeranz, Halton, & Peers, 1956). In a study by Pomeranz et al. (1956), a 1,000 fold increase in mold count was accompanied by a 20% decrease in free lipids. A subsequent study revealed a 40% reduction in total lipids in mold count alongside a 2,000 fold increase in mold count (Daftary & Yeshajahu, 1961). Both mold growth and lipid degradation proceed in high moisture, oxygenated environments and are influenced by temperature.

Understanding that moisture content, temperature, and storage stability are fundamental to the varying causes of failure of grain, a study evaluating the shelf life of IWG grains must control for these environmental parameters. In order to discern an effect of time and temperature on rancidity as a potential mode of failure in IWG, the present study employed different time and temperature combinations. Moisture content must also be maintained well below critical levels, as is common practice in the grain industry, to prevent heightened respiration with corresponding dry matter loss, heat damage, and microbial growth that might otherwise interfere with rancidity and functionality assessments.

1.6.3 Antioxidant Stability over Storage

As previously discussed, antioxidants can be key elements in preventing oxidative rancidity over storage of cereal grains (Galliard, 1994). However, antioxidants, themselves, are susceptible to degradation over storage, whether due to environmental factors, such as light, oxygen, and temperature or by sacrificially inhibiting auto- or enzymatic oxidation (Lin, Guo, & Mennel, 2008; Boon, McClements, Weiss, & Decker, 2010). Loss of phytochemicals over storage in grains is directly proportional to the

length of storage and increase in intensity of temperature, moisture content, and other degradative processes (Mellado-Ortega & Hornero-Méndez, 2017).

Cereal grains are a rich source of antioxidants. In wheat, antioxidants are concentrated in the bran, aleurone, and germ fractions (Smith & Hartley, 1983; Lin, Guo, & Mennel, 2008). Furthermore, the antioxidants in these fractions contribute significantly more to antioxidant activity than antioxidants found in the endosperm (Zieliński & Kozłowska, 2000). Of the plethora of antioxidants in wheat, Tyl & Ismail (2018) compared carotenoid and hydroxycinnamic acid content to that found in wheat. IWG was found to have significantly higher contents of the carotenoids lutein and zeaxanthin and the hydroxycinnamic acids *trans*-ferulic acid, *trans*-sinapic acid, and *trans*-*p*-coumaric acid. These antioxidants are particularly relevant over storage as a result of their high concentration and previously demonstrated relationship to antioxidant activity in several wheat species (Haila, Lievonen, & Heinonen, 1996; Zieliński & Kozłowska, 2000; Adom & Liu, 2002). The relationship to activity and stability over storage of hydroxycinnamic acids and carotenoids will be discussed in more detail.

Hydroxycinnamic acids are cinnamic acid derivatives, distinguished by the degree of hydroxyl groups on the phenol ring (Yu & Cheng, 2008). Hydroxycinnamic acids include ferulic, sinapic, *p*-coumaric, and caffeic acids. Phenolic acids, including hydroxycinnamic acids, are predominately found in the bran fraction of grain (Gallardo, Jiménez, & García-Conesa, 2006). These antioxidants are frequently associated with high antioxidant activity in grains. They participate in radical scavenging by hydrogen transfer (Onyeneho & Hettiarachchy, 1992; Toda, Kumura, & Ohnishi, 1991), reduction by electron transfer (Sroka & Cisowski, 2003), and metal chelation (K Zhou, Yin, & Yu, 2006).

Adom, Sorrells, & Liu (2003), Zieliński & Kozłowska (2000), Adom & Liu (2002), and Choi, Jeong, & Lee (2007) reported a significant positive correlation between total phenolic content and total antioxidant activity. Phenolic compounds exist in free, soluble-conjugated, and insoluble bound forms (Adom et al., 2003). Bound phenolics are esterified to cell wall polysaccharides, often pentosans like arabinoxylans (Smith &

Hartley, 1983). Bound phenolic compounds are in greater abundance than free forms and, therefore, contribute significantly more to antioxidant activity than free phenolics Accurate quantification of these phytochemicals requires an alkaline hydrolysis step (Smith & Hartley, 1983; Adom et al., 2003; Moore et al., 2005; Kim, Tsao, Yang, & Cui, 2006). The primary esterified phenolic acid in cereal grains is ferulic acid (Smith & Hartley, 1983). Ferulic acid is also the primary phenolic compound in wheat gluten (Labat, Morel, & Rouau, 2000). *p*-coumaric acid and sinapic acid are also found in noteworthy amounts in wheat and wheat relatives and IWG, contributing to overall antioxidant activity (Gallardo, Jiménez, & García-Conesa, 2006; Tyl & Ismail, 2018).

Literature is sparse regarding stability of phenolic acids over storage in grains. The little research that does exist quantifies phenolic compounds as a whole, which includes not only phenolic acids but also flavonoids and tannins. However, considering ferulic acid is the primary contributer to antioxidant activity of wheat (K Zhou, Su, & Yu, 2004), it can be deduced that changes to overall phenolic quantities and respective antioxidant activity over storage reflects, to an extent, changes to hydroxycinnamic acids. An accelerated storage study of intact wheat grains showed that antioxidant activity over 25, 60, and 100°C storage remained unchanged in some antioxidant activity assays and increased in others (Cheng et al., 2006). These changes were temperature-dependent, with the higher temperatures producing greater increases in antioxidant activity and total phenolic content.

Increases in antioxidant activity and total phenolic content over storage have been observed in a variety of foods and explained by a number of mechanisms (Duodu, 2011). Longer storage times and higher storage temperatures can induce aglycosylation, or the release of bound phenolics from the cell wall (Hopia & Heinonen, 1999). These released phenolics can subsequently contribute to antioxidant activity. They can also form polymers with additional antioxidant capacity (Randhir, Kwon, Lin, & Shetty, 2009). Additionally, the breakdown of polymerized antioxidants such as condensed tannins can release simple phenolic acids (Fares, Platani, Baiano, & Menga, 2010). In storage conditions favoring the Maillard reaction or caramelization, products exhibiting

antioxidant activity may also be produced (Michalska, Amigo-Benavent, Zielinski, & del Castillo, 2008; Chiremba, Taylor, & Duodu, 2009; Žilić et al., 2013).

While increases to antioxidant activity and phenolic content of stored grains are possible, oxidation of phenolic acids can still occur over storage (Krygier, Sosulski, & Hogge, 1982). Albeit, oxidation of antioxidants is far more pronounced in milled grains owing to the decompartmentalization of cell components and exposure to oxygen. Compared to intact cereal grains, Narwal, Jaswal, Sehgal, Sheoran, & Gupta (2012) showed a 25-45% reduction in antioxidant activity of whole wheat flour across temperatures of -20°C to 60°C over 60 days of storage.

Carotenoids have also been shown to protect oxidative stability of various food oils (Kiritsakis & Dugan, 1985; Fakourelis, Lee, & Min, 1987; Jung & Min, 1991). In cereal grains as a whole, lutein is the most predominant carotenoid (Adom et al., 2003). Lutein and zeaxanthin are the predominant carotenoids in wheat, followed by β-carotene (Abdel-Aal et al., 2002; Fratianni, Irano, Panfili, & Acquistucci, 2005). Tyl and Ismail (2018) also found lutein and zeaxanthin to be the predominant carotenoids in IWG. However, oxidized carotenoids have been shown to be more effective antioxidants than β-carotene (Haila et al., 1996). Lutein and zeaxanthin are stereoisomers and belong to the xanthophyll group of carotenoids, which are oxygenated derivatives of carotenes (Alves-Rodriguez & Shao, 2004; Cuttriss, Cazzonelli, Wurtzel, & Pogson, 2011).
Lutein and zeaxanthin act by single oxygen quenching and reduction of free radical species (Conn, Schalch, & Truscott, 1991; Sies, Stahl, & Sundquist, 1992; Haila et al., 1996; Baltschun et al., 1997; Edge, McGarvey, & Truscott, 1997) . Antioxidant activity of carotenoids related to concentration of oxygen, structure of carotenoids, and presence of other antioxidants (Krinsky, 1993). In fact, Haila, Lievonen, & Heinonen (1996) found that lutein was most efficient at inhibiting formation of hydroperoxides of triacylglycerols in the presence of γ-tocopherol, a phenomenon formerly observed by Terao, Yamauchi, Murakami, & Matsushita (1980). which they discussed to be a result of γ-tocopherol inhibiting degradation of lutein. On the other hand, contradictory research has shown the lutein and β-carotene may act as prooxidants (Terao, Yamauchi,

Murakami, & Matsushita, 1980; Haila et al., 1996) . Choi et al. (2007) was further unable to demonstrate a significant correlation between total carotenoid content and antioxidant activity as measured by a variety of assays. Despite these opposing conclusions regarding the role of carotenoids in oxidative stability over storage, their positive implications for health (Zaheer, 2017) and high concentration in IWG warrants further investigation over storage of IWG.

Carotenoids are prone to degradation over storage by a number of deteriorative mechanisms. Total carotenoid content decreases over storage of grains and flour in a first-order degradative reaction (Galleschi et al., 2002; Hidalgo & Brandolini, 2008; Mellado-Ortega, Atienza, & Hornero-Méndez, 2015; Mellado-Ortega & Hornero-Méndez, 2017). Degradation is especially influenced by temperature. Mellado-Ortega & Hornero-Méndez (2017) measured ~72% carotenoid retention after 12 months storage at -32°C, and only 10% retention at 50°C. Autooxidation of carotenoids proceeds quite rapidly, especially in isolated organic extracts (Boon et al., 2010). Photooxidation and an acidic environment are also responsible for loss of carotenoid content over time (Boon et al., 2010). Carotenoids can further be consumed by participating in inhibitory behavior towards prooxidant species, losing antioxidative capacity in the process. Moreover, carotenoids can be oxidized by lipoxygenase over storage (McDonald, 1979), which endorses the hypothesis that steam treatment to inactivate lipoxygenase will enhance storage stability of IWG.

1.6.4 Functionality Changes Over Storage

Functionality has been proven to improve in the beginning stages of storage before waning over prolonged periods (Pomeranz, 1971, 1992; Kibar, 2015). A review of literature by Tipples (1979) showed that functionality improves up to a maximum of two to four months of storage of intact grains, after which time it decreases for the remainder of storage. Grain flour is also subjected to a short period of storage prior to baking to optimize baking quality (Yoneyama, Suzuki, & Murohashi, 1970; Wang & Flores, 1999; Kibar, 2015). Researchers have cited the following improvements to baking quality upon

short-term storage of grains and flour: higher water absorption, as well as higher hydration rate, better mixing tolerance, higher dough viscosity and stability, enhanced gas retention, and heightened loaf volumes (Wang & Flores, 1999; Kibar, 2015).

This boost to functionality conferred by short term storage has been attributed primarily to changes in the protein profile of the grain. Oxidation of thiols over storage results in disulfide bridges that help stabilize the gluten network (Tipples, 1995; Wieser, 2007). The disulfide bridges form between cysteine residues of gliadin and glutenin proteins, leading to polymerization (Yoneyama et al., 1970; Ariyama & Khan, 1990; Wrigley & Bekes, 1999). Furthermore, small molecular mass gliadins, albumins, and globulins can also aggregate over storage (Rao, Vakil, & Sreenivasana, 1978; Ariyama & Khan, 1990). Extended storage periods may induce conformational changes, manifesting in an increase in aggregation and a decrease in hydrophilicity and solubility (Kozlova & Nekrasov, 1956). Further studies have elaborated on these changes to gluten with prolonged storage, noting loss of wet gluten content, decreasing glutenin content with accompanying loss of elasticity, reduced hydration capacity, and decreased Farinograph dough stability (Cenkowski, Dexter, & Scanlon, 2000; Kibar, 2015; Baik & Donelson, 2018).

Changes to the structure and content of lipids and starch can likewise alter dough functionality of grains and, therefore, end use applications. Alterations to the lipid profile over storage, including loss of polar lipids and enzymatic lipolysis, can have significant ramifications to end use functionality of the grain. Daftary & Yeshajahu (1961) showed that not only did the content of polar lipids (comprising glycolipids and phospholipids) rapidly decline over storage, but that rate of decline was faster than both the rate of decline of triacylglycerides and the rate of increase of free fatty acids. Polar lipids help to stabilize the gluten network via hydrophilic interactions with gliadins and hydrophobic interactions with glutenins (Shogren, Finney, & Hoseney, 1969). Therefore, the loss of polar lipids over storage leads to loss of stability of the gluten network (Morrison, 1979). Daftary & Yeshajahu, (1961) and Daftary et al. (1970) have suggested that the polar

lipids are metabolized by fungi and molds that multiply over storage in high moisture grains at high storage temperatures.

Free fatty acids released through the action of endogenous lipase in stored groats or flour are responsible for not only unfavorable organoleptic changes in grain, but also potential functionality changes (Tait & Galliard, 1988; G. Zhang & Hamaker, 2005). Tait and Galliard (1988) discovered an inverse relationship between lipase activity and loaf volume, suggesting that higher free fatty acid concentrations are associated with lower bread volumes. They further elucidated the effect of fatty acid unsaturation on loaf volume, showing that *cis*-unsaturated fatty acids had a greater negative effect on bread loaf volume than saturated fatty acids. These results were in agreement with those of Bell, Daniels, and Fisher (1979) who reported low loaf volumes in breads baked with added oleic and linoleic acids. Bell et al. (1979) hypothesized that unsaturated fatty acids in a dough system, whether from lipolytic action or added during processing, bind to the shortening lipids, thereby reducing the capacity of the added shortening to interact with gluten and starch in a dough system. This hypothesized mechanism is further supported by Bell, Daniels, and Fisher (1979), who showed a significant reduction in loaf volume with the addition of unsaturated fatty acids in the presence of shortening but no such difference in the absence of shortening Bookwalter, Lyle, and Nelsen (1991) proposed an alternative mechanism, suggesting that free fatty acids can react with sodium bicarbonate, a leavening agent, in a dough system, and therefore prevent rising.

These results do not go undisputed. Gerits, Pareyt, and Delcour (2014) experimented with addition of exogenous lipases as a means of studying the role of free fatty acids in breadmaking, and they incidentally found a significant positive correlation between free fatty acids and bread loaf volume. However, the researchers were careful to point out that their results indicated a correlation, not a causation. Rose et al. (2011) did not find a strong correlation coefficient (0.65) between storage length and bread loaf volume. Regardless, the relatively high concentration of lipase in IWG compared to wheat together with the predominance of unsaturated fatty acids linoleic and oleic acids, warrant baking quality and rheological measurements over storage of IWG.

Lipid oxidation can also influence baking quality. Sullivan, Near, and Foley (1936) asserted based on his research that unsaturated fatty acids have a minimal influence on rheological and baking quality unless they are oxidized. Upon oxidation, a loss in dough elasticity, vulnerability to dough fracture, and decrease in bread volume were observed, although no mechanism was proposed. Schaich and Karel (1976) explained that free radicals generated by lipid peroxidation can cause denaturation of proteins, along with destruction of individual amino acids, polymerization, and cross-linking.

Aside from the impacts on baking qualities like loaf volume and crumb structure, changes to lipids can also impact starch pasting profiles over storage. Starch pasting profiles influence the suitability of a grain to different types of products. A common starch pasting profile is shown in **Figure 1**. Peak viscosity refers to the maximum viscosity reached in starch-water or flour-water slurry as starch granules gelatinize upon heating (CWB Brabender). A high peak viscosity is indicative of higher water holding capacity and granule swelling ability, both directly impacting ease of cooking and baking. Breakdown refers to the extent of decrease in viscosity as granules rupture and disintegrate during continued heating and stirring. A low breakdown value is reflective of a high paste stability during heating and is measured as the difference between peak viscosity and minimum viscosity or holding strength. Final viscosity, also referred to as "cold paste viscosity" reflects the stabilized viscosity of the paste upon cooling. Setback is calculated as the difference between final viscosity and minimum viscosity and represents the extent of increase in viscosity upon cooling. Setback and final viscosity values are both indicative of the tendency of amylose and amylopectin to reassociate into a gel or crystalline structure by forming hydrogen bonds, a phenomenon known as retrogradation. Amylose molecules reassociate more readily owing to their small molecule size and unbranched structure (Singh & Anderson, 2004). Higher setback and final viscosities correlate with a higher degree of reassociation of starch components. Retrogradation can be undesirable as it causes staling and loss of crispness of baked goods, as well as changes to flavor and aroma (Morris, 1990). Retrogradation also

reduces starch digestibility. However, it can be desirable in products such as pasta as it increases hardness and reduces stickiness (Farhat, 2004).

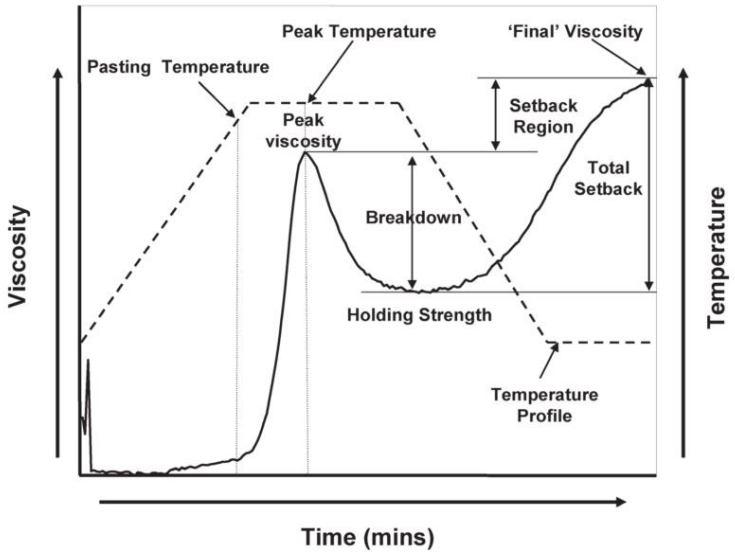

Figure 1: A common starch pasting profile obtained using a Micro-Visco-Amylograph® (MVAG) (Saunders, Izydorczyk, & Levin, 2011). Used with permission.

Free fatty acids formed over storage in grain can increase gelatinization peak viscosity, holding viscosity, and final viscosity, along with setback and breakdown values (Zhang & Hamaker, 2005; Fierens, Helsmoortel, Joye, Courtin, & Delcour, 2015; Gerits, Pareyt, & Delcour, 2015; Frauenlob, Hetebrij, Amico, & Schoenlechner, 2018)The increase in pasting viscosities of stored groats or whole flour is attributed to presence of amylose-lipid (AM-L) complexes (Hayfa Salman & Copeland, 2007; Fierens et al., 2015), which form when starch and lipids are heated together (Kugimiya, Donovan, & Wong, 1980). The insoluble complexes AM-L complexes aggregate in an aqueous system and form an intergranular network that results in increased viscosity values (Conde-Petit & Escher, 1992; Putseys, Lamberts, & Delcour, 2010). AM-Ls form when amylose takes on a helix conformation in the presence of aliphatic and ampiphilic ligands such as monodiacylglycerols; the nonpolar chain of the ligand complexes to the hydrophobic interior of the helix, while the polar head resides outside of the helix

(Conde-Petit & Escher, 1992; Micus, Hixon, & Rundle, 1946; Putseys et al., 2010). AM-L inclusion complexes may form in unmilled groats or develop after milling in conditions favoring gelatinization of starch. Free fatty acids and monoacylglycerols form AM-L complexes to a greater extent than diacylglycerols; AM-L complexes cannot form with triacylglycerols (Eliasson, 1994; Fierens et al., 2015). Degree of AM-L complex formation is influenced by degree of unsaturation and length of the fatty acid chain (Fierens et al., 2015; Tufvesson, Wahlgren, & Eliasson, 2003), with a lower degree of unsaturation and higher chain length favoring complex formation owing to the hydrophobic nature of the interior of amylase helices. Free fatty acid formation through lipolysis over storage increases the propensity of AM-L complexes to develop. Salman & Copeland (2015) substantiated this conclusion by measuring a positive correlation of ~0.81 between final viscosity and fat acidity. As the concentration of fatty acids increased over storage, the final viscosity also increased. Amylopectin can also complex with lipids over storage and increase viscosity in pasting profiles by increasing rigidity of the starch granules (Conde-Petit & Escher, 1992). While higher final viscosity and setback values are commonly associated with increased retrogradation, AM-L complexes may in fact inhibit reassociation of starch compounds through steric hindrance (Eliasson & Ljunger, 1988). Though peak, holding, and final viscosities are frequently shown to increase upon storage of grain due to AM-L inclusion complexes, theoretically AM-L complexes could also decrease viscosity values by reducing granule swelling capacity (Putseys et al., 2010).

AM-L complexes are also responsible for delayed gelatinization. According to Gerits, Pareyt, & Delcour (2015), mono-diacylglycerols and free fatty acids can form an insoluble surface around starch granules, preventing water absorption and thus, swelling and gelatinization of the starch. Higher lipid concentrations and longer fatty acid chain lengths are cause for greater delay in swelling and gelatinization (Eliasson, Carlson, Larsson, & Miezis, 1981).

The release of free fatty acids over storage of IWG may therefore increase the formation of AM-L complexes that impact pasting. IWG not only possesses a high

protein content, but is also susceptible to high free fatty acid content over storage as a result of its high lipase content. Therefore, it is necessary to observe how the starch pasting profiles of IWG may change over storage as a means of discussing its shelf life in terms of functional performance.

Protein may also interact with starch over storage and impact pasting profiles. In rice grains over storage, a decrease in swelling capacity of starch with corresponding decreased solubility of starch, was noted (Jood, Kapoor, & Singh, 1993). The researchers concluded that protein-starch interactions restricted entry of water into the granule, delaying gelatinization and ability of amylose to leach from the granule, resulting in reduced solubility. Although this phenomenon was explained for changes to starch pasting profiles of rice grain, IWG's high protein could render it susceptible to similar issues.

While storage studies in wheat and a variety of other commonly consumed grains examining development of rancidity and functionality changes as indicators of shelf life are well-documented, no such studies exist for IWG. The chemical composition of IWG, previously elucidated, predisposes it to certain changes over storage that have not formerly been explored. As discussed, the relatively high fat and enzyme content of IWG predispose IWG to rancidity over storage, while its high antioxidant content and activity may counteract rancidity. Hydrolytic and oxidative changes to lipids over storage may also induce functionality changes. Understanding the mechanisms of deterioration in IWG helps inform the processing strategies employed to elongate shelf life, such as heat treatment of grains.

1.7 Heat Treatment to Enhance Storage Stability of Grains

Lipid degradation is a substantial mode of failure in cereal grains, impacting its end use quality through sensory and functionality changes. Minimizing lipid degradation over storage starts with controlling moisture content of the grain, as well as the external storage environment, especially relative humidity, temperature, and light. Beyond control

of physical parameters, certain processing measures may be undertaken to enhance the grain's stability. Heat treatment of grains has been shown to be an effective and efficient means of elongating storage life of grains by inactivating the aforementioned enzymes responsible for hydrolytic and oxidative rancidity. Researchers often monitor inactivation of peroxidase, a relatively thermally stable enzyme, as an index of lipase and lipoxygenase inactivation (Bookwalter et al., 1991). Peroxidase can also participate in oxidative reactions, often favoring phenolic substrates (Žilić et al., 2010).

Several methods of heat treatment exist, including micronization, wet heat treatments, and dry heat treatments. Alternative methods of enzyme inhibition not requiring thermal processing have also been explored, such as addition of salt solutions during grain conditioning (Doblado-Maldonado, Arndt, & Rose, 2013). It is important to consider not only the extent to which each method inactivates enzymes, but also the extent to which each method affects antioxidant activity and content, as antioxidants largely contribute to shelf life and ultimate nutrient density of a grain. The effectiveness of a given method is also a function of the substrate type and condition, as well as the parameters of the designated heat treatment. In order to accurately compare each of the methods, one must account for their impact on enzymatic activity, antioxidant content and activity, along with nutritive consequences (if any), functionality changes, organoleptic repercussions, and industrial cost and feasibility in a large-scale system.

1.7.1 Types of Heat Treatment

1.7.1.1 Micronization

Micronization is a high temperature, short time processing technique that utilizes near infrared technology to pass heat through food and extend shelf life by inactivation of problematic enzymes. Micronization rapidly increases internal temperature of the grain and internal water vapor pressure (Fasina, Tyler, Pickard, & Zheng, 1999). Micronization is currently used in the food industry to shorten cooking times of legumes by decreasing hardness, lower moisture content of foods susceptible to spoilage, destroy microbial species and pests, and inactive antinutritional factors such as trypsin (Sun, Watts, Lukow,

& Arntfield, 2006). Micronization has been shown to significantly reduce the activity of problematic enzymes such as lipase and peroxidase and extend shelf life as a result (Deepa & Umesh Hebbar, 2017), however its effect on antioxidant content and activity is variable across studies (Žilić et al., 2010, 2013).

1.7.1.1 Dry Heat Treatment

Dry heat can be administered to foods via conventional oven, blast oven, kiln, and microwave, among other avenues, and can occur at a wide variety of controlled temperatures, often 100 to 230°C. Lipase reductions of 35-50% have been observed in wheat bran subjected to dry heat treatment in a conventional oven. Significant (74 to 93%) reductions were achieved in whole wheat kernels subjected to conventional heating and microwave heating, respectively, (De Almeida, Pareyt, Gerits, & Delcour, 2014). Other studies were not initially successful in lowering free fatty acid content in dry-heated grains, however observed significantly lower free fatty acids and significantly greater sensory scores in treated samples following prolonged storage (Yadav, Anand, Kaur, & Singh, 2012; Bergonio et al., 2016).

Results are mixed when it comes to the effect of dry heat on antioxidant activity and content and strongly depend on the type of dry heat treatment, the heat treatment parameters, and the substrate. Following dry oven heating of sorghum at 121°C for 25 minutes, Cardoso et al. (2014) measured significant retention of phenolic compounds and significant increases in total vitamin E compounds and antioxidant activity. Rose, Ogden, Dunn, and Pike (2008) did not observe significant changes to total phenolics or antioxidant activity of wheat after dry heat at 175°C for up to 30 minutes or microwave treatment for up to 120 seconds on high power. On the contrary, a separate study reported a decrease in total phenolics of wheat bran after dry oven heating at 170°C for 16 minutes (Y. Hu et al., 2018). Both groups observed a significant decrease in carotenoids, which are known to be particularly heat sensitive (Mellado-Ortega & Hornero-Méndez, 2017). The differences in results across these studies can be explained by the variability in

heating parameters, with higher temperature-time combinations leading to greater losses in content and activity of antioxidants.

Conclusions regarding storage stability of dry heated grains are similarly mixed. In addition to partial destruction of carotenoids, Hu et al. (2018) measured a significant increase in peroxides after heat treatment, which coincided with a decrease in the ratio of unsaturated to saturated fatty acids, together suggesting heat treatment helped facilitate autooxidation under those conditions. While shelf life-limiting oxidative rancidity may be promoted by dry heat treatment according to the result of Hu et al. (2018), dry heat treatment may also stymie hydrolytic rancidity. For example, Ekstrand et al., (1993) showed that preemptive dry heat treatment was beneficial in long-term storage, as evidenced by a significantly lower amount of free fatty acids in heated oat samples compared to untreated oat samples after 16 months of storage despite incomplete lipase inactivation with dry heat.

1.7.1.3 Wet Heat Treatment

Several methods of wet heat treatment have been researched as a means of prolonging shelf life of grains or bran fractions through enzyme inactivation, including autoclaving (De Kock, Taylor, & Taylor, 1999; P. Jacobs, Hemdane, Delcour, & Courtin, 2016), cooking in water (Cardoso et al., 2014), steam treatment (Bookwalter, Lyle, & Warner, 1987; De Almeida et al., 2014; Ekstrand et al., 1993; Meera, Bhashyam, & Ali, 2011; Molteberg, Magnus, Bjørge, & Nilsson, 1996), and superheated stream treatment (Head, Cenkowski, Arntfield, & Henderson, 2011; Y. Hu et al., 2018). Wet heat treatments operate on the principle that water has higher thermal conductivity and specific heat capacity than water (Electrical Research Association, 1967).

De Almeida et al. (2014), who looked at the activity of various enzymes in milled flour from steam-treated wheat grains, showed a decrease in lipase activity by 84.4%, peroxidase activity by 98.9%, endoxylanase activity by 99.1%, and α-amylase activity by 85.4%. Their findings are corroborated by those of Meera et al. (2011) and Ekstrand et al. (1993a). Ekstrand et. al (1993) who looked at oats as opposed to wheat or sorghum

grains, not only reported that lipase was not detected after steam treatment, but also reported that free fatty acid content (which also negatively influences flavor quality), was significantly higher in oat grains that had not been heat-treated. The authors were not able to achieve complete enzyme inactivation with dry heat treatment as they were with steam treatment.

Steam treatment outperformed other heat treatments in the results shown by Rose, Ogden, Dunn, and Pike (2008). They studied three methods of heat treatment, namely dry heat, microwave, and steam, on lipase activity in whole wheat flour. While all methods achieved a significant reduction in lipase activity, steam treatment achieved a maximum lipase reduction of 96%. However, all three methods were successful over storage with minimal differences in phenolic content and free fatty acid content across the methods at 6 months' time. The superiority of wet heat treatment, more specifically steam, was further demonstrated in brown rice by Bergonio, Lucatin, Corpuz, Ramos, and Duldulao (2016). Steam treated rice had the least amount of free fatty acids, as well as the greatest increase in antioxidant activity. Neither Bergonio et al. (2016), Hu et al. (2018), nor (Rose et al., 2008) observed significant change to total phenolic content following steam treatment.

Researchers determined that steam treatment is more effective at inactivating enzymes than dry heat as a cumulative result of 1) higher enthalpy of steam compared to dry air that allows heat to better penetrate the kernel (Chungcharoen, Prachayawarakorn, Tungtrakul, & Soponronnarit, 2015; Y. Hu et al., 2018) and 2) the higher susceptibility of enzymes to denaturation in a wet than in a dry environment (Bergonio et al., 2016). The benefits of steam treatment, however, were not seen in all methods of wet heat treatment. Sorghum grains cooked in water retained only 74% of total phenolic content, 86.3% of total vitamin E content and expressed a significant decrease in antioxidant activity (Cardoso et al., 2014). As concluded by the researchers, cooking in water can cause antioxidants to leach out of the grain.

A notable disadvantage to hydrothermal processing is the potential to accelerate microbial growth post-treatment if additional drying precautions are not taken to keep the

moisture content below critical levels. Secondly, with thermal treatments, there is the potential to induce autooxidation in the presence of circulating oxygen, as heat serves as a catalyst (Galliard, 1994).

Heat treatment could enhance the overall storage stability of IWG, however it is important to choose a heat treatment that maintains its exceptional antioxidant status while significantly reducing the high quantities of lipase and lipoxygenase. Steam treatments are advantageous for their superior ability to inactivate enzymes without disrupting antioxidant activity, as results consistently show. Furthermore, steam treatment can exert positive effects on dough functionality, as will be further discussed.

1.7.2. Effects of Thermal Treatment on Dough Functionality

The principle goal of thermal processing treatments is to inactivate enzymes like lipase that create free fatty acids, and thus, it should follow that these treatments may improve baking quality. In autoclaved wheat bran samples where lipase activity was reduced by 90%, De Kock et al. (1999), measured significant increases in loaf volume and height of breads made with flour consisting of treated bran compared to breads made with untreated wheat bran. The researchers determined that the increase in loaf volume and size was a response to the cumulative effects of reduced enzymatic activity and a decrease in glutathione upon hydrothermal treatment. Glutathione is a tripeptide that reduces disulfide linkages within the gluten matrix, thus weakening interactions (Chen & Schofield, 1996; De Kock et al., 1999). The results of de Kock are in agreement with those of Bookwalter, Lyle, and Nelsen (1991) who also found a significant increase in corn bread loaf volume in response to steam treatment of maize kernels. While research consistently supports the claim that heat treatment enhances loaf dimensions, heat treatment is not always beneficial for end use applications, as some thermal treatments may disrupt the native structure of protein and starch within cereal grains, ultimately altering rheological properties in a dough system.

While crude protein content was either minimally decreased (De Kock et al., 1999; Žilić et al., 2010) or not decreased at all (Bookwalter et al., 1987; Sun et al., 2006)

in various thermally treated grains, the protein profile was altered. Sun et al. (2006) reported significant decreases in monomeric proteins and soluble glutenins, and significant increases in insoluble glutenins and residue insoluble proteins in spring wheat subjected to micronization at 100°C. They further studied the effect of protein profile changes on rheology of micronized spring wheat and discovered significant decreases in dough stability and strength, as well as dough extensibility. Deepa and Hebbar (2016) observed similar results in maize subjected to micronization at 200°C for 4 minutes, with a significant decrease in albumins and globulins due to polymerization and a significant increase in high molecular G3-glutelins. Both research groups concluded that the changes in protein profile are related to denaturation of the more heat sensitive monomeric proteins with subsequent exposure of hydrophobic groups, leading to protein aggregation, formation of disulfide bonds, and protein insolubility. Researchers have demonstrated a decrease in protein solubility of isolated proteins fractions from soybeans, maize, barley, lentils, and wheat grains subjected to micronization (Arntfield et al., 1997; Fasina et al., 1999; Sun et al., 2006; Žilić et al., 2010). Runyon, Sunilkumar, Nilsson, Rascon, and Bergenståhl (2015) measured an approximately 50% decrease in solubility of protein fractions from steam-treated oats, along with a decrease in albumins and prolamins and the formation of globulin (a hexamer) aggregates. Such undesirable changes have been found across the spectrum of heat treatments. A study on the rheological effects of dough from wheat bran exposed to a variety of heat treatments found that steaming, autoclaving, and toasting all prolonged dough development time (Caprez, Arrigoni, Amadò, & Neukom, 1986).

The gluten matrix in wheat is highly important in the baking quality and end use application (Schofield & Booth, 1983). LeGrys, Booth, and Al-Baghdadi, (1981) found positive correlations between loaf volume and elasticity (R=0.95) and loaf volume and viscosity (R = 0.97). The disruption of the proteins that comprise the gluten matrix, namely glutenins and gliadins, therefore has irreparable repercussions on quality. The relationship between baking quality of wheat and conformational changes of gluten proteins due to heat has been demonstrated by multiple research groups (LeGrys et al.,

1981; Schofield, Bottomley, Timms, & Booth, 1983). Schofield et al. (1983) represented overall gluten functionality with loaf volume and crumb texture measurements, and summarized a progressive loss of functionality with increasing temperature with a near complete loss of functionality over 75°C. LeGrys et al. (1981) also found a near complete loss of baking quality (loaf volume and crumb structure) in heated gluten at 80°C. Schofield et al. (1983) found these changes to be due to unfolding of the tertiary structure of the protein followed by disulfide cross-linking and sulphydryl bond formation, thus reducing the extensibility of the dough and increasing elasticity beyond a desirable level.

Changes in starch functionality could also contribute to changes in rheology of dough made from thermally treated grain. Thermal treatments can lead to pregelatinization (Arntfield et al., 1997; Fasina et al., 1999; Žilić et al., 2010). Pregelatinized starches do not require cooking to form a gel in later applications and offer certain advantages such as good dispersability in wet or dry formulations and good freeze-thaw stability (BeMiller, 2003). Pregelatinized starch is common in the oat industry (Runyon et al., 2015). Thermally treated grain can further lead to decreases in peak, breakdown, setback, and final viscosities. Yadav et al. (2012) found significant decreases in these parameters in pearl millet flour after 100 seconds of microwave heating. The same researchers observed similar trends in steam-treated pearl millet, with a noted decrease in each parameter as length of steam treatment increased (Yadav, Anand, et al., 2012; Yadav, Kaur, Anand, & Singh, 2012). Yadav et al. (2012) also pointed out the advantages of reduced setback values, particularly a reduced risk of retrogradation and syneresis. While previous research has characterized the functionality of IWG, changes in functionality over storage and as impacted by various treatments are not yet addressed. Understanding how the functionality of IWG changes in response to hydrothermal treatment and prolonged storage will be essential in choosing processing conditions and ultimate end-product applications.

1.8 Conclusions

Breeding efforts and research to characterize, develop, and improve IWG have proceeded since 1988 (L. DeHaan et al., 2013). Agronomists, geneticists, and food scientists have since achieved successful improvements to yield, seed size, and a number of other agronomic traits (DeHaan, 2015; Zhang et al., 2016), as well as completed an extensive chemical and functional characterization of the perennial grain (Rahardjo et al., 2018; Tyl & Ismail, 2018). One critical variable surrounding its readiness for market that remains unaddressed is its storage stability. Manufacturers and food processers rely heavily on indicators of storage stability to determine a grain's quality (Zeleny & Coleman, 1938; Galliard, 1983). Consumers also refer to shelf life as a factor in their food selections. Therefore, studying the storage stability of IWG is of utmost importance to its overall commercial success. Thus, it is important to identify the factors that contribute most significantly to deterioration of a given grain in order to best devise strategies to improve storage stability of that grain. Due to its previously determined high fat content and enzyme activity, which are linked to development of hydrolytic and oxidative rancidity, along with its high antioxidant content linked to retardation of oxidative rancidity (Galliard, 1994; Tyl & Ismail, 2018), rancidity progression is the most appropriate means by which to measure storage stability of IWG. Efforts to enhance storage stability of IWG should therefore address how best to curtail hydrolytic and oxidative rancidity. Steam treatment is among the most promising strategies, as it retains antioxidant content and activity while significantly reducing content of problematic enzymes (Rose et al., 2008; Bergonio et al., 2016; Hu et al., 2018). While the benefits of steam treatment on storage stability of grains are well documented, it is also important to consider how steam treatment can impact functionality of IWG over storage.

IWG's success depends, in part, on its end use applications and therefore functionality changes over storage can influence its ultimate marketability. Thus, measuring rheological, baking, and pasting properties in relation to storage length and steam treatment will help determine storage and treatment conditions that can lead to optimal functional performance.

The present work will provide a foundational understanding of the key determinants in the storage stability of IWG and a potential strategy to prolong its shelf life. This information will help guide future breeding initiatives to select for traits favorable to shelf life. Manufacturers and grain processers can capitalize on the work to choose the conditions and treatments most favorable to storage of IWG and its end products. And finally, answering the question of its storage stability will provide additional information to farmers to help inform their decision to plant IWG.

Chapter 2: Using Rancidity Parameters, Enzyme Activity, and Antioxidant Status to Assess Storage Stability of Intermediate Wheatgrass Groats Subjected to Steam Treatment

2.1 Overview

Intermediate wheatgrass (IWG), *Thinopryum intermedium*, is a perennial grain with superior environmental benefits that has the potential to be commercialized as a food ingredient. Understanding the storage stability of IWG and identifying ways to improve its stability will help incentivize farmers to plant IWG for commercial use. The aim of this work was to evaluate the effect of steam treatment on antioxidant content and activity, enzyme activity, and progression of hydrolytic and oxidative rancidity in IWG compared to hard red wheat (HRW) over storage at different temperatures. IWG had significantly higher protein, insoluble fiber, and fat content than HRW, along with a higher lipase activity. HRW had a relatively higher lipoxygenase activity than IWG. IWG also had significantly higher hydroxycinnamic acid and carotenoids concentrations than HRW, along with higher antioxidant activity. The steam treatment employed in the present study did not inactivate lipoxygenase and lipase, however development of oxidative and hydrolytic rancidity in IWG was minimal throughout storage of the groats. Hydroxycinnamic acid content was retained throughout storage, and prolonged storage also promoted increases to content, as well as increases to antioxidant activity at higher storage temperatures (45°C). Carotenoid content decreased throughout storage in IWG and HRW, to a greater degree at 45°C than ambient and refrigerated storage temperatures, as was expected. Although the steam treatment did not inactivate enzymes, it demonstrated a minimal, but positive effect on antioxidant activity and content, as well as a slight inhibitory effect on hydroperoxide formation over storage. IWG demonstrated a significantly higher antioxidant content and antioxidant activity and lower hydroperoxide content than HRW throughout storage. In terms of hydrolytic rancidity, IWG showed higher lipase activity and free fatty acid concentration than HRW throughout storage. The present study was the first to evaluate the storage stability of IWG and investigate steam treatment as a mode of improving storage stability of IWG.

The results of the study demonstrated that IWG has an acceptable storage stability, which is an important criterion for the grain's marketability.

2.2 Introduction

Cereal grains account for 60% of the world's caloric intake and 40% of the world's protein intake (Wrigley, 2016). Worldwide, the three most cultivated cereal grains are wheat, rice, and maize (Beta & Isaak, 2016). However, these annual crops are degrading the environment at a faster rate than it can be restored (Pimentel et al., 1995), causing soil erosion and water run-off with subsequent loss of nutrients and biodiversity (Eswaran et al., 1999). The high dietary demand for cereal grains, together with the high rate of soil degradation from annual farming and the growing consumer interest in sustainably-sourced food (International Food Research Council Foundation, 2017) creates space in the market for a more sustainable grain.

Perennial intermediate wheatgrass (IWG), *Thinopryum intermedium*, was identified at the Rodale Research Institute in 1990 as a promising sustainable grain to owing to its superior environmental benefits. Due to its extensive root system, IWG reduces soil erosion and nitrogen leaching, sequesters relatively high amounts of carbon, and requires fewer pesticide inputs than annual grains (Wagoner, 1990; DeHaan et al., 2005). IWG has since undergone several breeding cycles, which have improved its yield, seed size, and ease of harvest (DeHaan, 2015; Zhang et al., 2016). IWG has also been characterized for its chemical and functional properties (Rahardjo et al., 2018; Tyl & Ismail, 2018). However, IWG's success in the market further relies on its storage stability. In fact, consumers recognize shelf life as a key determinant in food selection. Determining the storage stability and identifying ways to improve its stability will help make IWG competitive against existing grains on the market.

In a storage environment with properly controlled relative humidity and temperature, cereal groats can remain acceptable for consumption, from a safety and quality standpoint, for several years (Grundas & Wrigley, 2016; Pomeranz, 1992). The ultimate mode of failure in stored groats may stem from microbial contamination,

heightened grain respiration leading to loss of dry matter, discoloration, and/or decreased germination ability, or hydrolytic and oxidative rancidity (Kaleta & Górnicki, 2013; Pomeranz, 1992). Microbial contamination and grain respiration can be controlled by maintaining appropriate environmental conditions, namely low relative humidity, and temperature, and reduced exposure to light and oxygen. Though affected to a certain extent by environmental conditions, hydrolytic and oxidative rancidity are largely dependent on the composition of the grain, specifically the lipid content and the presence of enzymes and antioxidants and their respective activity (Galliard, 1994). Thus, efforts to extend the shelf life of IWG must focus on its composition in relation to rancidity.

Rancidity is the off-flavor that develops over storage because of hydrolytic or oxidative changes to the lipid component of the grain (Galliard, 1994). Lipase and lipoxygenase enzymes are responsible for hydrolytic and oxidative rancidity, respectively, but autooxidation can also occur in the presence of oxygen and/or light. The products of these enzymatic reactions, namely free fatty acids from lipolytic action and secondary oxidation products, such as hexanal, from lipoxygenase action or autooxidation, contribute to bitter, rancid, and musty flavors in the grain (Heiniö et al., 2002). Grains with higher amounts of lipid, specifically polyunsaturated fatty acids, as well as lipase and lipoxygenase are particularly susceptible to rancidity (Galliard, 1994). Free fatty acids, released by the action of lipase, are more susceptible to oxidation than triglycerides. Former research has shown IWG to have a similar amount of lipoxygenase as HRW but higher amounts of fat and lipase than hard red wheat (HRW) (Tyl & Ismail, 2018), which is concerning for shelf life.

Antioxidants in grains, however, may hinder the progression of enzymatic and non-enzymatic oxidative rancidity. Significantly higher quantities of antioxidants, including the hydroxycinnamic acids *trans*-ferulic, *trans-sinapic*, and *trans-p*-coumaric and the carotenoids lutein and zeaxanthin were found in IWG compared to HRW (Tyl & Ismail, 2018). It has been widely demonstrated that hydroxycinnamic acids, particularly ferulic acid, contribute significantly to antioxidant activity in wheat and other cereal grains (Zieliński & Kozłowska, 2000; Adom & Liu, 2002; Zhou et al., 2004; Choi et al.,

2007). The antioxidative properties of carotenoids, especially in the presence of other antioxidants such as γ-tocopherol, are also well-documented (Fakourelis et al., 1987; Jung & Min, 1991; Haila et al., 1996). Due not only to their health benefits *in vivo* (Slavin, 2017), but also to their high potential to inhibit oxidation over storage of grains, protecting the integrity of these antioxidants should be a key consideration when contemplating strategies to improve shelf life of IWG.

One potential strategy to address shelf life concerns of IWG is breeding efforts to decrease the concentration of problematic enzymes (Kantar et al., 2016). A second potential strategy is heat treatment to inactivate these enzymes, a strategy that is currently employed with several grains, including oats, sorghum, barley, maize, millet and rice (Bookwalter et al., 1991; Ekstrand et al., 1993; Molteberg, Vogt, Nilsson, & Frolich, 1995; Fasina et al., 1999; Meera et al., 2011; Yadav, Kaur, et al., 2012; Bergonio et al., 2016). Heat treatment takes several forms, among which are micronization, dry heat, extrusion, and wet heat treatments. Steam treatment, a wet heat treatment, is often favored for its ability to retain antioxidant activity while still achieving a high degree of enzyme – most commonly lipase – inactivation (Rose et al., 2011; Bergonio et al., 2016; Hu et al., 2018). Steam treatment was thus considered the best option for IWG, as the relatively high levels of lipase and lipoxygenase could be targeted without compromising the high antioxidant activity and content.

Therefore, the overall objective of this study was to evaluate the effect of steam treatment, over storage at different temperatures, on antioxidant status, enzyme activity, and progression of rancidity in IWG. We hypothesized that due to inactivation of enzymes, namely lipase and lipoxygenase, steam treated IWG will have less hydrolytic and oxidative rancidity over storage than untreated IWG. We further hypothesized that a high antioxidant activity coupled with a lower lipoxygenase activity as compared to hard red wheat would result in a slower progression of oxidative rancidity in IWG than in hard red wheat.

2.3 Materials

Intermediate wheatgrass (IWG) was harvested from Rosemount, MN in August 2015 and represented an improved breeding population originating from TLI Cycle 2. Hard red wheat (HRW) was a bulk sample consisting of harvests from St. Paul and Lamberton, MN in July 2015. Both IWG and HRW were kindly supplied by the Agronomy and Plant Genomics Department at the University of Minnesota. Both IWG and HRW grains were dehulled and cleaned. Samples were sent to Covance Laboratories (Madison, WI, USA) to be analyzed for fatty acid composition, amino acid composition, mycotoxins, phytic acid, trypsin inhibitor, and gluten protein allergens (**Figures 8,9; Tables 9-12, Appendix A**). Glycine standard (99%, 502-211) was purchased from Leco, Corp. (St. Joseph, MI, USA). Enzymes and assay kits for total dietary fiber (K-TDFR-100A/K-TDFR-200A 04/17), total starch (K-TSTA-50A/K-TSTA-100A 06/17), and amylose/amylopectin ratio (K-AMYL 12/16) were purchased from Megazyme International Co. (Wicklow, Ireland). Celatom® (diatomaceous earth), tris(hydroxymethyl)aminomethane (TRIS), and 2(*N*-morpholino) ethanesulfonic acid (MES) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydroxycinnamic acid standards ferulic acid (99.4%), trans-*p*-coumaric acid (98%), and sinapic acid (>99%) were purchased from MP Biomedicals, LLC (Santa Ana, CA, USA), Acros Organics (Morris, NJ, USA), and Sigma-Aldrich, respectively. Carotenoid standards, zeaxanthin (>98%) and lutein (>97%) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and Sigma-Aldrich, respectively. 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich, while (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) was purchased from Acros Organics, and benzoyl leucomethylene blue from TCI America (Portland, OR, USA). Analytical oleic acid and linoleic acid (>98.5%) standards were purchased from Sigma-Aldrich. Standardized 0.1 M sodium thiosulfate aqueous solution was purchased from Ward's Science (Rochester, NY, USA). Xylenol orange tetrasodium salt was purchased from Sigma-Aldrich. Olive oil was purchased fresh locally. All other chemicals were of

reagent grade or higher and were purchased from Fisher Scientific (Waltham, MA, USA), Sigma-Aldrich, or VWR International (Radnor, PA, USA).

2.4 Methods

2.4.1 Experimental Design

The experiment was laid out as a factorial design with 2 grains (IWG and HRW), 2 treatments (steam-treated and untreated) and 3 storage temperatures, 4°C, 22 (± 2°C), and 45°C. Samples under each storage temperature were pulled out at various time points. "Time 0" samples from each treatment condition were analyzed to establish a baseline for storage measurements. Samples stored at 4°C were pulled from storage and analyzed every 3 months up to 12 months. Samples stored at 22 (± 2°C) were pulled every two weeks up to 16 weeks, then every 4 weeks thereafter up to 24 weeks. Samples stored at 45°C were pulled at day 2, day 4, week 1 and every week thereafter up to 6 weeks. At each time point, analyses included peroxide value, free fatty acids, hydroxycinnamic acid content, and carotenoid content. Antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method and leucomethylene blue (LMB) method, as well as enzymatic (lipase and lipoxygenase) activity, were analyzed at beginning, middle, and end points for each storage temperature. All samples were stored and analyzed in triplicate. Prior to storage or steam treatment, proximate analysis and carbohydrate composition assays were performed on IWG and HRW.

2.4.2 Steam Treatment and Storage

A portion of each of the IWG and HRW samples was subjected to steam treatment. Samples were steam-treated in an even layer (~15 mm) in a proofing oven (Baxter PW2E, Orting, WA, USA) at 100°C and 95% relative humidity for 60 minutes. Steam treated groats were subsequently equilibrated at ambient conditions overnight prior to storage alongside non-steam treated groats. Relative humidity was controlled by storing samples in sealed desiccators with saturated potassium carbonate solution. Saturated potassium carbonate has a water activity of ~0.43 (or relative humidity of

~43%) across a wide range of temperatures (Fontana, 2007), which is well below the minimum level required for bacterial and mold growth (Pixton & Warburton, 1971; Pomeranz, 1992). Furthermore, equilibrium moisture content charts (Agnew, n.d.) devised from moisture isotherms show that at all storage temperatures at a relative humidity of 45%, moisture contents of wheat grain equilibrate below the critical moisture level of 13-14% that has been implicated in quality and microbiological degradation. All desiccators were protected from light to prevent the initiation of photo-oxidation. Each grain sample (30 g) was stored in uncovered polystyrene 100 mm x 15 mm petri dishes with a level surface. Samples for functionality testing were stored in ~300 g aliquots in uncovered vessels with a depth of 15 mm and a level surface. Ambient (22 ± 2°C) and refrigerated (4°C) storage temperatures were chosen to mimic common storage conditions, while 45°C was intended for accelerated storage conditions.

2.4.3 Milling

Following each designated storage period, grain samples were milled using an Udy Cyclone Sample Mill (Udy Corporation, Fort Collins, CO, USA) and passed through a 0.5 mm screen. The mill operated between 10,000 and 16,000 rpm. Each stored sample (~30 g) was milled individually, and the mill was thoroughly cleaned and allowed to cool between samples. The resulting flour samples were immediately analyzed for moisture content using an infrared instrument (OHaus MB45, Parsippany, NJ, USA) and subsequently stored in sealed containers with nitrogen-flushed headspace at -20°C until further analyses were conducted. Milled flour samples were used for all analyses.

2.4.4 Proximate Analysis

Proximate analysis was performed in triplicate following standard methods of analyses. Protein content was determined following the AOAC 990.03 Dumas nitrogen combustion method (AOAC International, 2016) using a Nitrogen Analyzer (LECO® TruSpecNTM, St. Joseph, MI, USA). A nitrogen conversion factor of 5.70 was used. Fat content was determined following the AOAC 922.06 Mojonnier method (AOAC

International, 2016). Moisture content was determined following the AACCI method 44-40.01 vacuum oven method (AACCI International, 2010). Ash content was measured following the AOAC method 923.03 dry ashing method (AOAC International, 2016). Finally, total carbohydrate content was determined by difference.

2.4.5 Carbohydrate Composition

2.4.5.1 Dietary Fiber

Total dietary fiber, including total soluble and total insoluble, were analyzed following AOAC method 991.43 (AOAC International, 2016) with the Megazyme Total Dietary Fiber Assay Kit. Samples (1 g each), in duplicate, were dissolved in 40 mL 0.05 M MES-Tris buffer (pH 8.2) and sequentially digested with heat stable α-amylase (50 µL, 95-100°C, 30 min), protease (100 µL, 60°C, 30 min), and amyloglucosidase (200 µL, 60°C, 30 min). Each digested sample was filtered through Celatom® and washed with 78% ethanol, 95% ethanol, and acetone, sequentially. The residue, constituting insoluble dietary fiber (IDF), was dried and weighed. Soluble dietary fiber (SDF) contained in the filtrate was precipitated with 95% ethanol and subsequently filtered through Celatom®, and washed with 78% ethanol, 95% ethanol, and acetone, sequentially. SDF in Celatom® was dried and weighed. Dietary fiber results were corrected for protein and ash by the Kjeldahl (AOAC 981.10) and dry ashing methods (AOAC 942.05), respectively (AOAC International, 2016).

2.4.5.2 Total Starch

Total starch was analyzed in triplicate following AOAC method 996.11 (AOAC International, 2016) with the Megazyme Total Starch Assay Kit. Total starch was quantified spectrophotometrically as a function of glucose concentration, following reaction with glucose oxidase-peroxidase (GOPOD) reagent, using standard concentrations of D-glucose. The specific method was as written in section “e” of the Megazyme procedure for “Determination of starch in samples which also contain D-glucose and/or maltodextrins.”

2.4.5.3 Amylose/Amylopectin Ratio

Amylose/amylopectin ratio was analyzed following the conconavalin A (ConA) precipitation procedure using a Megazyme kit. After isolation of starch from other flour components following kit instructions, amylopectin was precipitated out with ConA solution. The precipitate containing amylopectin and the supernatant containing amylose were digested separately by α-amylase and amyloglucosidase to produce D-glucose. Following reaction with GOPOD reagent, the concentration of D-glucose in samples was determined spectrophotometrically at 510 nm using a similarly treated starch control with known amylose/amylopectin ratio. The ratio of D-glucose in the precipitate and supernatant was used to calculate amylose/amylopectin ratio.

2.4.6 In Vitro *Enzyme Activity Assays*

2.4.6.1 Ferrous Oxidation-Xylenol Orange (FOX) Lipoxygenase Activity Assay

Lipoxygenase activity was measured utilizing the method established by Li and Schwarz (2012). Flour (1g), in triplicate, was extracted in 50 mL polypropylene centrifuge tubes using 20 mL of 0.1 M potassium phosphate buffer (pH 6.0) for 30 minutes on ice while stirring on a magnetic stir plate at approximately 800 rpm in the dark, after which extracts were filtered through 9 mm filter paper. An aliquot (10µL) of the filtered extract was subsequently added to 120 µL of the same potassium phosphate buffer and 30 µL of linoleic acid substrate emulsion. The linoleic acid emulsion was prepared by adding 250 µL linoleic acid to 0.5 M borate buffer with 250 µL of Tween, clarified with 650 µL 1 M sodium hydroxide, with a final concentration of 8 mM linoleic acid. The reaction was allowed to proceed for 5 minutes, during which time lipoxygenase from the sample oxidized linoleic acid to hydroperoxides. Following the reaction period, 2.85 mL of ferrous oxidation-xylenol orange (FOX) reagent was added. The FOX reagent was comprised of 250 µM ammonium ferrous sulfate, 25 mM sulfuric acid, 100 µM xylenol orange, 4mM BHT, and 90% (v/v) methanol. Reaction mixtures sat for 10 minutes in the dark at room temperature, during which time ferric ions (formed by

oxidative action of hydroperoxides on ferrous ions) oxidized xylenol orange to produce a colorimetric compound, the absorbance of which was measured at 560 nm. A blank containing 10 µL of deionized distilled water (DDW) in lieu of sample extract was analyzed to account for autooxidation products in the linoleic acid solution. Absorbance values were used to calculate lipoxygenase activity **(Equation 1**), and lipoxygenase activity was defined as µmoles (units *"U"*) of lipid hydroperoxides formed per minute at room temperature per gram (g) of sample on a dry basis. A sample calculation can be found in **Appendix B**.

Equation 1

Lipoxygenase activity (U/g)

$$= \frac{(A_s - A_b)}{\varepsilon_{mM}} \times Vol_{reaction}\ (L) \times \frac{Vol_{Extraction}\ (mL)}{Vol_{Aliquot}\ (mL)} \times \frac{1}{t \times l\ \times Sample\ Wt\ d.b.\ (g)} \times \frac{1000\ \mu moles}{1\ mmol}$$

Where:

A_s = Absorbance of sample at 560 nm

A_b = Absorbance of blank at 560 nm

ε_{mM} = 47 $mM^{-1} \cdot cm^{-1}$ (Molar extinction coefficient for lipid hydroperoxides in a methanol-based reagent) (Vega, Karboune, Husson, & Kermasha, 2005)

$Vol_{Reaction}$ = Total reaction volume in L

$Vol_{Extraction}$ = Total extraction volume in mL

$Vol_{Aliquot}$ = Total aliquot assayed in mL

t = incubation time in minutes

l = path length = 1 cm for standard cuvette

2.4.6.2 Lipase Activity Assay

Using an adaptation of the method established by Rose and Pike (2006), lipase activity was measured indirectly in defatted IWG and HRW flour via spectrophotometric

analysis of liberated oleic acid in samples incubated with olive oil. Flour samples were defatted three times with a 50/50 mixture of diethyl ether and petroleum ether in a 5:1 flour to solvent ratio, mixing for 30 minutes on a shaker, with subsequent centrifugation at rpm for 5 minutes. The extracted fat in ether was discarded, and the defatted flour was retained. The flour samples were subsequently dried under nitrogen. The defatted flour (1 g), in triplicate, was vortexed with 500 µL of olive oil and 200 µL of DDW in 15 mL glass round bottom centrifuge tubes and placed in a 45°C incubator for 4 hours in the dark. A control was prepared for each sample by mixing defatted flour from the respective sample (1 g) with 500 µL of olive oil and 200 µL of DDW in 15 mL glass round bottom centrifuge tubes, however controls were not incubated in order to account for oleic acid present in the olive oil prior to the incubation. The olive oil was extracted from each sample (following incubation) and control and retained in order to measure oleic acids. Extraction was carried out in duplicate by adding 5 mL hexane to the centrifuge tube, vortexing, and centrifuging for 5 min at 523 × g. Olive oil extracts in hexane were dried down under nitrogen and reconstituted in 4 mL of isooctane. Reconstituted extracts were subsequently vortexed with 1 mL of 275 mM cupric acetate reagent (adjusted to a pH of 6.1 with pyridine). Copper salts of oleic acids solubilized into the isooctane layer were measured spectrophotometrically at 715 nm. Oleic acid external standards (1-10 mM) were prepared in 4 mL of isooctane and similarly vortexed with 1 mL cupric acetate reagent before measuring the absorbance. **Equation 2** was used to calculate lipase activity. Lipase activity was expressed as micromoles (units "*U*") of oleic acid liberated per hour per gram (*g*) of sample on a dry basis. Standard curve and sample calculation can be found in **Appendix C**.

Equation 2.

$$Lipase\ Activity\ = \frac{[(4.5\ mL) \times (A_s - A_b)]}{[(\varepsilon_M) \times t\ \times l\ \times Sample\ Weight\ d.b.\ (g)]} \times \frac{1000\ \mu Eq/mL}{1\ mol/L}$$

Where:

4.5 mL = Total reaction volume

A_s = Absorbance of sample at 715 nm

A_b = Absorbance of blank at 715 nm

ε_M = Molar extinction coefficient of oleic acid ($M^{-1} \cdot cm^{-1}$) at 715 nm determined through slope of oleic acid standard curve

t = Incubation time in hours

l = Path length = 1 cm for standard cuvette

2.4.7 Antioxidant Quantification

2.4.7.1 Hydroxycinnamic Acid (HCA) Quantification

HCA quantification was carried out as outlined by Vaidyanathan and Bunzel (2012). Sample flour aliquots (200 mg each), in triplicate, were subjected to alkaline hydrolysis to de-esterify arabinoxylan-bound phenolic compounds in order to allow quantification of the sum of both free and bound hydroxycinnamic acids. De-oxygenated sodium hydroxide (5 mL of 2 M) was added to each flour sample in 15 mL glass round bottom centrifuge tubes with 5 glass beads to aid in mixing and prevent clumping. Subsequently, samples were thoroughly vortexed then left for 18 hours in the dark. Following hydrolysis, samples were acidified by adding 1 mL of 12.2 N concentrated hydrochloric acid to each centrifuge tube and vortexed. Hydroxycinnamic acids were then extracted from each sample matrix three times with 5 mL of diethyl ether. Following each extraction, samples were centrifuged at 2,090 × g for 5 minutes. The supernatants from three extractions were combined into a separate vial, dried down under nitrogen, then reconstituted in 2 mL of 75% methanol. Hydroxycinnamic extracts were analyzed using a Shimadzu Scientific Instruments high-performance liquid chromatography (HPLC) System (Colombia, MD, USA) equipped with SIL-10AF auto injector, LC-20AT pump system, CTO-20A column oven, SPD-M20A photo diode array detector, and a CBM-20A communication module. A Phenomenex (Torrence, CA, USA) Luna phenylhexyl column (250 x 4.6 mm, 3 µm particle size) and guard column (30 x 4.6 mm, 5 µm particle size) of the same material were used. Column temperature and flow rate

were maintained at 45°C and 0.75 mL/min, respectively. Solvent A was 1 mM trifluoroacetic acid in water, and solvent B was 0.1 mM trifluoroacetic acid in 90/10 acetonitrile/water. Following the injection of 20 µL of sample extract, elution was performed with a gradient profile modified from Dobberstein and Bunzel (2010): 13% phase B from 0-10 minutes, 13-20% from 10-20 minutes, 20% held for 3 minutes, 20-25% from 23-28 minutes, 25-50% from 28-32 minutes, 50-70% from 32-35 minutes, 70-13% from 35-37 minutes, and reequilibration at 13% for 8 minutes. External standard curves were prepared using known concentrations of ferulic acid (0.1-0.4 mg/mL), *p*-coumaric acid (0.01-0.06 mg/mL), and sinapic acid (0.005-0.07 mg/mL) in 75% methanol. Detection and quantification of hydroxycinnamic acids was performed at 280 nm. Results were expressed as µg hydroxycinnamic acid (ferulic, *p*-coumaric, or sinapic) per gram of sample. Standard curves (**Figure 11**) and a sample calculation can be found in **Appendix D**.

2.4.7.2 Carotenoid Quantification

Lutein and zeaxanthin quantification was carried out as outlined by Abdel-Aal, Young, Rabalski, Hucl, and Fregeau-Reid (2007). Sample aliquots (500 mg), in triplicate, were extracted in 15 mL glass round bottom centrifuge tubes with water-saturated butanol three times, with extraction volumes of 2 mL, 1.5 mL, and 1.5 mL. Samples were extracted on a shaker (Labline Scientific Instruments, Mumbai, Maharashtra, India) for 1 hour at 175 rpm with intermittent vortexing every 10 minutes with subsequent centrifugation at 523 × *g* for 5 minutes. Extracts from all three extractions were collected in 5 mL volumetric flask and brought to volume with water-saturated butanol. IWG and HRW extracts, 1 mL and 1.5 mL respectively, were dried down under nitrogen followed by reconstitution in 400 µL and 150 µL of water-saturated butanol, respectively. Samples were analyzed using a Shimadzu Scientific Instruments HPLC system described in Section 2.4.7.1. Separation was performed using a ProntoSil (Bischoff, Leonberg, Germany) C30 column (250 x 4.6 mm, 5 µm particle size) with a ProntoSil guard column (4.0 x 10 mm, 5 µm particle size) of the same material. Column temperature and flow

rate were maintained at 35°C and 1 mL/min, respectively. Mobile phase A was 81/15/4 methanol/methyl *tert*-butyl ether/water, and mobile phase B was 90/10 methyl *tert*-butyl ether/methanol. Following the injection of 50 µL of sample extract, elution was performed with a gradient profile adopted from Tyl, Marti, Hayek, Anderson, and Ismail (2018): 0-40% phase B from 0-9 minutes, 40-90% from 9-12 minutes, 90% held for 3 minutes, 90-0% from 15-20 minutes, and reequilibration at 0% for 5 minutes. External standard curves were prepared using known concentrations of lutein (2.0-10 µg/mL) and zeaxanthin (0.2-7.5 µg/mL) in water-saturated butanol. Detection and quantification of carotenoids was performed at 450 nm. Results were expressed as mg zeaxanthin or lutein per 100 g of sample. Standard curves (**Figure 12**) and a sample calculation can be found in **Appendix E**.

2.4.8 In vitro *Antioxidant Activity Assays*

In vitro antioxidant activity was evaluated in triplicate using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and the leucomethylene blue (LMB) assay. The DPPH assay measures antioxidant activity by electron transfer (otherwise referred to as radical scavenging), while LMB measures antioxidant activity by electron-transfer coupled with hydrogen atom transfer (also known as reduction). Using two different methods of analysis enables the assessment of antioxidant activity via different mechanisms of lipid oxidation inhibition (Frankel & Meyer, 2000).

2.4.8.1 DPPH Antioxidant Activity Assay

Antioxidant activity, as measured by radical scavenging activity, was determined according to the DPPH method outlined by Guo and Beta (2013). The following extracts were analyzed in triplicate against the external standard trolox, an analog of vitamin E: 1) Alkaline hydrolysate extracts as prepared for hydroxycinnamic acid quantification (Section 2.4.7.1), further diluted threefold in 75% methanol, and 2.) 50% acetone (10:1 solvent/sample) extracts. The latter was prepared by extracting flour samples (200 mg) twice in 2.5 mL microcentrifuge tubes with 1 mL of 50% aqueous acetone (Lv, Yub, Lu,

et al., 2012). Extraction was performed on a shaker at 200 rpm for 18 hours then for another 1 hour followed by centrifugation at 16,000 × g for 10 minutes. After each extraction, supernatants were collected in 2 mL volumetric flasks and brought to volume with 50% acetone.

An aliquot (100 µL) of alkaline hydrolysate extracts and an aliquot (200 µL) of acetone extracts were each reacted with 3.9 mL and 3.8 mL, respectively, of 60 µM DPPH radical solution, prepared in methanol. Following incubation at room temperature in the dark for 1 hour, absorbance of samples was read at 515 nm against a blank of 60 µM DPPH radical solution. Trolox standards (100-700 µM) were measured following the same preparatory steps. The DPPH assay operates by the hydrogen atom transfer (HAT) mechanism; as antioxidants reduce the DPPH radical, the radical changes from its purple oxidized form to its bleached reduced form and thus, reducing capacity can be measured as a function of change in absorbance as compared to the control. The DPPH radical scavenging activity (%) of both extracts and trolox standards was calculated using **Equation 3.** Using standard trolox concentrations and their respective DPPH radical scavenging activities, an external standard curve was constructed. The equation of the standard curve was then used to express results in µmoles trolox equivalents (TE) per gram of sample based on the previously calculated DPPH radical scavenging activity of each sample (**Equation 4**). Trolox standard curves (**Figure 13**) and sample calculations can be found in **Appendix F**.

Equation 3.

$$DPPH\ Radical\ Scavenging\ Activity\ (\%) = \left(1 - \frac{A_{sample}}{A_{blank}}\right) \times 100\%$$

Where:

A_{sample} = Absorbance of sample at 515 nm

A_{blank} = Absorbance of blank (60 µM DPPH radical solution) at 515 nm

Equation 4.

$$\mu moles\ TE = \left(\frac{\%\ DPPH\ Scavenging\ Activity - b}{m}\right) \times \frac{Vol_{Extract}}{Vol_{Aliquot}} \times d \times \frac{Vol_{Extract}}{1000\ mL} \times \frac{1}{wt\ sample\ (g)}$$

Where:

b = Y-intercept of equation of the standard curve for % DPPH radical scavenging activity of trolox standards

m = Slope of the equation of the standard curve

$Vol_{Extract}$ = Total extraction volume in mL

d = dilution factor

$Vol_{Aliquot}$ = Aliquot volume in mL

2.4.8.2 Leucomethylene Blue (LMB) Antioxidant Activity Assay

LMB antioxidant assay was performed following the method outlined by Bright, Stewart, and Patino (1999) with slight modifications using alkaline hydrolysate extracts of IWG and HRW, which were obtained for hydroxycinnamic acid quantification (**2.4.7.1**). Sample extracts were further diluted by a factor of 2 in 75% methanol. An aliquot (50 µL) of sample extract or trolox standard (0.1-2 mM) was added to 3 mL 0.2 M de-oxygenated phosphate buffer (pH 6.75), 100 µL 15 mM EDTA (ethylenediamenetetraacetic acid) disodium salt/16 mM ferrous sulfate heptahydrate solution, 1 mL 8.05 mM linoleic acid solution in 50 mM borate buffer (pH 9.0) and 100 µL 16 mM hydrogen peroxide solution in that order. After 10 minutes of incubation at room temperature, 1 mL of the reaction mixture was added to 2 mL of the leucomethylene blue reagent, made up of 131 µM leucomethylene blue, 8% (v/v) DMF, 1.4% (w/v) Triton-X, and lyophilized bovine hemoglobin in 0.2 M de-oxygenated potassium phosphate buffer (pH 5). After 30 minutes of reaction time at ambient temperature in the dark, samples and trolox standards were read at 666 nm against a leucomethylene blue reagent blank. A blank was prepared following the aforementioned procedure using 50 µL DDW in lieu of sample extract. Absorbance values of trolox standards (0.1-2 mM) were used to construct an external standard curve against which

sample activity was measured. Results were reported in mmoles of TE per gram of sample (**Equation 5**). Trolox standard curve (**Figure 14**) and sample calculation can be found in **Appendix G**.

Equation 5.

$$\frac{mmol\ TE}{g} = \frac{[(A_{blank} - A_{sample}) - b]}{m} \times \frac{Vol_{Extract}}{Vol_{Aliquot}} \times d \times \frac{Vol_{Extract}}{1000\ mL} \times \frac{1}{Sample\ wt\ (g)}$$

Where:

A_{blank} = Absorbance of blank at 666 nm

A_{sample} = Absorbance of sample at 666 nm

b = Y-intercept of equation of the standard curve plotting Δ Absorbance at 666 nm of trolox concentrations (0.2-2 mM)

m = Slope of the equation of the standard curve plotting Δ Absorbance at 666 nm of trolox concentrations (0.2-2 mM)

$Vol_{Extract}$ = Total extraction volume in mL

$Vol_{Aliquot}$ = Aliquot volume in mL

d = dilution factor

2.4.9 Hydrolytic and Oxidative Rancidity

The fat extracts used for determining both free fatty acids and peroxide value were prepared in the same manner. An appropriate amount of flour to yield approximately 100 mg of fat (6.2 g of HRW and 5.3 g IWG) was extracted three times using 50:50 (v/v) diethyl ether to petroleum ether solvent in a 5:1 solvent/flour ratio for the first two extractions and a 3:1 ratio for the third. Samples were extracted using a Labline shaker in the dark for 30 minutes each time, followed by centrifugation at 2,090 × g for 5 minutes. Supernatants from all three extractions were pooled and filtered through 9 mm filter paper then dried down under nitrogen in pre-weighed 50 mL glass centrifuge vials. The

final weights of fat extracts were used in **Equations 6** and **7** following the analyses. Nitrogen-flushed fat extracts were sealed and stored at -20°C until analysis, which occurred within 1 week of extraction. The resultant defatted sample from fat extraction for free fatty acid analysis was utilized for lipase analysis (**2.4.6.2 *Lipase Activity Assay***. Both IWG and HRW samples were analyzed in triplicate for free fatty acids and peroxide value.

2.4.9.1 Free Fatty Acids

Total free fatty acid content was determined as a measure of hydrolytic rancidity following a scaled-down version of AOAC method 940.28 (AOAC International, 2016). Nitrogen-flushed crude fat extracts were quantitatively transferred into Erlenmeyer flasks with 2 x 5 mL of neutralized ethanol. After the addition of phenolphthalein, samples were titrated with standardized 0.01 M sodium hydroxide until a permanent faint pink color was produced. Titrant volumes were used to calculate the percent concentration of free fatty acids (**Equation 6**) expressed as oleic acid per 100 g of flour on a dry basis. A sample calculation can be found in **Appendix H.**

Equation 6.

$$\%Free\ fatty\ acids\ = \frac{[(V_s - V_b) \times \frac{N\ NaOH}{1000\ mL} \times \frac{282\ g}{mol}]}{Weight\ flour\ d.b.(g)} \times 100$$

Where:

V_s = Volume of sodium hydroxide titrated to neutralize sample

V_b = Volume of sodium hydroxide used to neutralize blank

N NaOH = Normality of NaOH (Eq/L)

282 g/mol = Molecular weight of oleic acid

2.4.9.2 Peroxide Value

Peroxide value was determined as a measure of primary oxidation following a scaled-down and modified version of AOAC method 965.33 (AOAC International,

2016). Nitrogen-flushed crude fat extracts were quantitatively transferred into Erlenmeyer flasks with 2 x 5 mL of 3:2 acetic acid to chloroform solution. Saturated potassium iodide solution (1 mL) was subsequently added to the crude fat extracts and swirled for 1 minute, during which time potassium iodide reacted with fatty acid hydroperoxides to form iodine. After the reaction period, 10 mL of deoxygenated water and 1 mL of 1% soluble starch solution were added; starch forms a blue-purple color complex with iodine. Samples were titrated with 0.001 N sodium thiosulfate ($Na_2S_2O_3$) to end point, or loss of blue-purple color, which indicated all iodine formed upon hydroperoxide-induced oxidation of potassium iodide had been exhausted to form sodium iodide. Titrant volumes were used to calculate peroxide value, expressed as milliequivalents of peroxide per 1000 g of oil using **Equation 7**. A sample calculation can be found in **Appendix I**.

Equation 7.

$$\frac{mEq\ hydroperoxides}{1000\ g\ fat} = \frac{[(Vol_{sample} - Vol_{blank})\ \times N\ Na_2S_2O_3 \times 1000]}{Weight\ fat\ (g)}$$

Where:

Vol_{sample} = Volume of $Na_2S_2O_3$ (mL) used to titrate sample

Vol_{blank} = Volume of $Na_2S_2O_3$ (mL) used to titrate blank

$N\ Na_2S_2O_3$ = Normality of $Na_2S_2O_3$ (Eq/L)

282 g/mol = Molecular weight of oleic acid

2.4.10 Statistical Analyses

One-way analysis of variance (ANOVA) was done using R Version 3.3.1 (The R Foundation, 2016) to determine differences among samples with either storage time, or sample type as factors. Differences among the means were determined using Fisher's Least Significant Difference (LSD) test ($P < 0.05$). Two-way ANOVA was carried out to assess

for interaction effects among treatment variables, including storage time, storage temperature, and sample type on various dependent variables. Welch's two-sample t-tests were performed to analyze differences in proximate and carbohydrate composition of IWG and HRW. Pearson's product-moment correlation coefficients ($P < 0.05$) were calculated using R to test for linear relationships between dependent variables. Correlation coefficients depicting relationships among antioxidant concentrations, antioxidant activity, rancidity markers, and enzyme activity can be found in **Table 14-Table 16 (Appendix L)**. Two-way ANOVA summary tables can be found in **Table 21-32 (Appendix O).**

2.5 Results and Discussion

2.5.1 Differences in Chemical Composition of IWG and HRW

IWG had significantly higher protein, ash, and insoluble fiber compared to HRW **(Table 1**), which is a consequence of its relatively smaller size and hence higher bran to endosperm ratio (DeHaan & Ismail, 2017; Rahardjo et al., 2018). For the same reason, the starch content of IWG was significantly lower than that of HRW. Previous reports have shown that IWG consistently has higher protein content than HRW (Becker et al., 1991; Marti et al., 2015; Rahardjo et al., 2018; Tyl & Ismail, 2018). However, the protein content of IWG is lower than determined by Becker et al. (1991), which is a result of breeding efforts that lead to increased seed size **(Table 13, Appendix J)**. As albumin and globulin are found primarily in the germ and the aleurone layers of the seed (Šramková, Gregová, & Sturdík, 2009) a reduced bran to endosperm ratio would result in lower overall protein content. IWG also had a relatively high fat content, albeit not significantly higher than that of HRW on a dry basis. A high total fat content, coupled with a high content of polyunsaturated linoleic and oleic fatty acids **(Figure 8, Appendix A),** predispose IWG to oxidative and hydrolytic rancidity.

Table 1. Nutrient composition (on dry basis) of intermediate wheatgrass and hard red wheat.

	Protein g/100 g flour	Fat g/100 g flour	Ash g/100 g flour	Starch g/100 g flour	Amylose g/100 g starch	Insoluble fiber g/100 g flour	Soluble fiber g/100 g flour
IWG	**17.2***	**5.24**	**2.40***	**58.0**	**21.7**	**15.7***	**2.42**
HRW	**15.2**	**4.38**	**1.79**	**67.7***	**25.5**	**11.1**	**1.62**

*Indicates significant differences between samples according to the Welch Two Sample t-test ($P \leq 0.05$), n=2 for protein and fiber analyses and n=3 for all other analyses.

Non-steamed IWG had significantly higher lipase activity compared to non-steamed HRW, predisposing it more to hydrolytic rancidity over storage **(Table 2**). Yet, compared to HRW, IWG had significantly lower lipoxygenase activity, which is responsible, in part, for oxidative rancidity. These trends corroborate those demonstrated by Tyl and Ismail (2018). Furthermore, IWG had significantly higher concentrations of carotenoids, namely lutein and zeaxanthin, and hydroxycinnamic acids, namely ferulic and *p*-coumaric acid, than HRW **(Table 3**). The carotenoid and hydroxycinnamic acids content of IWG are within the range reported by Tyl and Ismail (2018) for several IWG lines. The higher antioxidant content compared to HRW contributes to a higher overall antioxidant activity in IWG. Antioxidant activity of acetone extracts of IWG as measured by DPPH was significantly higher than that of HRW extracts **(Table 4).** The relatively high antioxidant content of IWG with corresponding higher activity could help delay oxidative rancidity over storage.

Table 2. Enzymatic activity of steamed and non-steamed IWG and HRW over accelerated (45°C), ambient (22 ± 2°C), and refrigerated (4°) storage.

Assay	Storage Temperature	Storage Time	Sample			
			IWG Steamed	IWG Not Steamed	HRW Steamed	HRW Not Steamed
Lipase Activity (Mean U/g flour (d.b.)[β]	45°C	0 Weeks	3.60^{aA}	3.09^{aA}	1.95^{bA}	2.09^{bA}
		3 Weeks	2.80^{aA}	2.93^{aA}	1.23^{bA}	1.43^{bA}
		6 Weeks	3.34^{aA}	3.34^{aA}	1.69^{bA}	1.69^{bA}
	22 ± 2°C	0 Months	3.60^{aA}	3.09^{aA}	1.95^{bA}	2.09^{bA}
		3 Months	2.05^{aB}	2.14^{aB}	1.65^{abA}	1.41^{bB}
		6 Months	2.99^{aA}	2.68^{aAB}	2.00^{bA}	1.61^{bB}
	4°C	0 Months	3.60^{aA}	3.09^{aA}	1.95^{bA}	2.09^{bAB}
		6 Months	3.52^{aA}	3.91^{aA}	2.01^{bA}	2.44^{bA}
		12 Months	2.89^{aA}	3.30^{aA}	2.05^{bA}	1.90^{bB}
Lipoxygenase Activity Mean U/g flour (d.b.)	45°C	0 Weeks	4.79^{bA}	5.00^{bB}	5.30^{aB}	5.36^{aB}
		3 Weeks	4.75^{bA}	5.11^{abB}	5.20^{aB}	4.94^{abC}
		6 Weeks	5.44^{bA}	5.45^{abA}	5.78^{abA}	5.88^{aA}
	22 ± 2°C	0 Months	4.79^{bB}	5.00^{bA}	5.30^{aB}	5.36^{aB}
		3 Months	5.90^{bA}	5.75^{bA}	6.92^{aA}	6.76^{aA}
		6 Months	5.96^{bA}	6.05^{bA}	6.55^{abA}	7.14^{aA}
	4°C	0 Months	4.79^{bB}	5.00^{bA}	5.30^{aB}	5.36^{aB}
		6 Months	5.58^{abA}	5.02^{bA}	5.85^{aA}	6.20^{aA}
		12 Months	5.39^{aAB}	5.42^{aA}	5.66^{aAB}	5.50^{aB}

[β]d.b.: Units per gram of flour on a dry basis. Lowercase superscripts represent significant differences ($P \leq 0.05$) across IWG and HRW samples within single time point; capital superscripts represent significant differences within each grain sample across time points according to the Fisher's LSD means comparison test.

Table 3. Antioxidant content of steamed and non-steamed IWG and HRW.

	Carotenoids		Hydroxycinnamic Acids		
	Lutein	**Zeaxanthin**	**Ferulic Acid**	**Sinapic Acid**	***p*-Coumaric Acid**
IWG Steamed	**3.31**^{A}	**0.40**^{A}	**794**^{A}	**66.8**^{A}	**19.6**^{A}
IWG Not Steamed	**3.36**^{A}	**0.39**^{A}	**700**^{A}	**40.5**^{A}	**19.1**^{A}
HRW Steamed	**0.29**^{B}	**0.008**^{B}	**463**^{B}	**55.2**^{A}	**11.4**^{B}
HRW Not Steamed	**0.29**^{B}	**0.009**^{B}	**489**^{B}	**39.3**^{A}	**12.4**^{AB}

Capital superscripts represent significant differences ($P < 0.05$) across samples according to the Fisher's LSD means comparison test.

Table 4. Antioxidant activity of steamed and non-steamed IWG and HRW as measured by DPPH and LMB over accelerated (45°C), ambient (22 ± 2°C), and refrigerated (4°) storage.

Assay	Storage Temperature	Storage Time	Sample			
			IWG Steamed	IWG Not Steamed	HRW Steamed	HRW Not Steamed
DPPH[β] – Alkaline Hydrolysates (Mean µmol TE[δ]/g flour)	45°C	0 Weeks	53.8^{aA}	53.1^{aB}	49.1^{aA}	47.2^{aB}
		3 Weeks	56.7^{aA}	57.5^{aAB}	51.1^{aA}	52.2^{aA}
		6 Weeks	62.1^{aA}	62.7^{aA}	55.9^{bA}	52.2^{bA}
	22 ± 2°C	0 Months	53.8^{aA}	53.1^{aA}	49.1^{aA}	47.2^{aA}
		3 Months	60.7^{aA}	56.8^{abA}	44.3^{bA}	48.0^{abA}
		6 Months	58.9^{abA}	65.9^{aA}	52.8^{abA}	47.2^{bA}
	4°C	0 Months	53.8^{aA}	53.1^{aA}	49.1^{aA}	47.2^{aA}
		6 Months	46.3^{aA}	47.9^{aA}	35.9^{aB}	36.0^{aB}
		12 Months	43.7^{aA}	46.1^{aA}	42.5^{aAB}	36.8^{aB}
DPPH – Acetone Extracts (Mean µmol TE/g flour)	45°C	0 Weeks	20.1^{aA}	18.6^{aAB}	10.0^{bB}	10.2^{bAB}
		3 Weeks	18.5^{bA}	21.4^{aA}	9.48^{cB}	9.41^{cB}
		6 Weeks	19.3^{aA}	14.1^{bB}	22.7^{aA}	12.0^{bA}
	22 ± 2°C	0 Months	20.1^{aB}	18.6^{aC}	10.0^{bB}	10.2^{bB}
		3 Months	38.6^{aA}	36.2^{aA}	15.4^{bA}	12.8^{bA}
		6 Months	25.2^{aB}	28.4^{aB}	6.79^{bC}	6.78^{bC}
	4°C	0 Months	20.1^{aA}	18.6^{aA}	10.0^{bA}	10.2^{bA}
		6 Months	17.8^{aA}	15.9^{aAB}	6.46^{bB}	6.31^{bB}
		12 Months	12.9^{aB}	12.1^{aB}	2.91^{bC}	3.54^{bC}
LMB[γ] – Alkaline Hydrolysates (Mean mmol TE/g flour)	45°C	0 Weeks	0.26^{aB}	0.30^{aC}	0.28^{aB}	0.23^{aB}
		3 Weeks	0.11^{bB}	0.53^{aB}	0.45^{aAB}	0.37^{aB}
		6 Weeks	1.25^{abA}	1.40^{aA}	0.52^{cA}	1.10^{bA}
	22 ± 2°C	0 Months	0.26^{aB}	0.30^{aA}	0.28^{aA}	0.23^{aA}
		3 Months	0.20^{aB}	0.30^{aA}	0.11^{aB}	0.14^{aA}
		6 Months	0.69^{aA}	0.47^{aA}	0.34^{aA}	0.46^{aA}
	4°C	0 Months	0.26^{aA}	0.30^{aA}	0.28^{aA}	0.23^{aA}
		6 Months	0.32^{aA}	0.35^{aA}	0.24^{aA}	0.23^{aA}
		12 Months	0.24^{aA}	0.40^{aA}	0.21^{aA}	0.28^{aA}

[β]DPPH: 2,2-diphenyl-1-picryl-hydrazyl; [γ]LMB=leucomethylene blue; [δ]TE: Trolox Equivalents. Lowercase superscripts represent significant differences ($P \leq 0.05$) across grain samples within single time point; capital superscripts represent significant differences within a grain sample across time points according to the Fisher's LSD means comparison test.

2.5.2 Effect of Steaming on Enzyme and Antioxidant Activity

The steam treatment was conducted in a proofing oven set at 100°C and 95% relative humidity for 60 minutes. This steaming method did not result in a significant decrease in lipoxygenase activity **(Table 2)**, however preliminary steam treatment optimization showed a 25% decrease in lipoxygenase activity of steamed IWG over 12 days of storage at 60°C (**Figure 15, Appendix K**). With the preliminary observation where enzyme activity continued to decrease in the steamed grain over accelerated storage, it was assumed that steam treatment would delay oxidation over storage as a function of decreasing lipoxygenase activity. With a gradually decreasing lipoxygenase activity over storage, it was reasoned that fewer substrates would be enzymatically oxidized, lowering the overall amount of primary and secondary oxidation products produced.

Lipase was not significantly inactivated with this steam treatment. Using a more direct method, in which samples were steamed in a single layer on a sieve placed atop a boiling water bath, Rose et al. (2008) achieved a significant reduction (96%) in lipase activity. De Almeida et al. (2014) were able to achieve 84% reduction in lipase activity following the same method. Sakou, Takahashi, and Yoshii (2010) used a superheated steam treatment method that involved raising the temperature as high as 300°C. Using this method, they were able to achieve not only a high degree of lipase inactivation (approximately 95%), but also near-complete inactivation of lipoxygenase. However, at temperatures beyond 150°C, they also observed adverse effects, such as a significant increases in starch damage.

On the other hand, the steam treatment employed in the present study did not impact the antioxidant content **(Table 3**) and activity **(Table 4).** The retention of antioxidant content following steam treatment of intact cereal grains was also observed by Rose et al. (2008), Bergonio et al. (2016), and Hu et al. (2018).

Overall, the steaming technique employed in this study was not sufficient to inactive lipase and lipoxygenase. Future studies should examine more direct methods of steam treatment to achieve greater reduction in enzyme activity. However, steaming did,

in fact, preserve antioxidant activity and content, which will be discussed in **2.5.4** and **2.5.5**.

2.5.3 Changes to Enzyme Activity over Storage

Over refrigerated storage at 4°C and accelerated storage at 45°C, there was no significant difference in lipase activity of pre-storage samples and samples at the end of storage (12 months and 6 weeks, respectively) **(Table 2).** Clayton and Morrison (1972) stored spring and winter wheat whole flour for 4 months at 37 °C and also found no significant change to lipase activity. At ambient storage (22 ± 2°C), lipase activity decreased over storage in non-steamed samples, while steamed samples retained enzyme activity throughout storage, with a significant decrease mid-storage. This decrease in steamed samples mid-storage followed by an increase to original levels could potentially be explained by a phenomenon known as after-ripening. In low moisture dry storage, seeds may leave a state of dormancy and resume metabolic activity (Bewley, Bradford, Hilhorst, & Nonogaki, 2013). IWG maintained higher lipase activity than HRW throughout storage at all three temperatures, which would ultimately translate to higher free fatty acid values in IWG over storage as compared to HRW. The steaming method employed in the present study did not result in significant changes in lipase activity between steamed and non-steamed grains over storage at any temperatures. These observations further justify the need for a more direct steam treatment to inactivate lipase in IWG.

Similarly, no significant change in lipoxygenase activity occurred over storage at 4°C for any samples. However, at higher storage temperatures, significant increases in lipoxygenase activity occurred. At ambient storage, all samples but the non-steamed IWG showed a significant increase in lipoxygenase activity. Over accelerated storage at 45°C, all samples but steamed IWG had a significant increase in lipoxygenase activity. However, the length of storage had an effect, with no significant increases observed until the end of accelerated storage. These results showing an effect of temperature on lipoxygenase activity reflected those published by Malekian et al. (2000), who showed

significant increases in lipoxygenase activity in stored rice bran at ambient temperature and a significant decrease over refrigerated storage.

In addition to rice bran, an increase in lipoxygenase activity over storage has been described in milled rice (Dhaliwal, Sekhon, & Nagi, 1991), wheat flour (Nielsen & Hansen, 2008), and wheat seeds (Liu, Li, Guo, Liu, & Zhao, 2016). According to Barna & Pogány (2001), lipoxygenase activity is known to increase during ageing of plants. After-ripening may explain the increase in lipoxygenase activity of stored IWG and HRW over ambient and accelerated storage.

No significant differences in lipoxygenase activity between steamed and non-steamed IWG were noted throughout storage at any temperature. Differences between steamed and non-steamed HRW were also negligible. These results were in opposition to preliminary steam results that showed a 25% decrease in lipoxygenase activity of steamed IWG over 12 days of storage at 60°C. It was expected based on these preliminary results that steaming would not only lead to a decrease in lipoxygenase over storage at 4°C, ambient, and 45°C temperatures, but also significantly distinguish lipoxygenase activity of steamed IWG from that of non-steamed IWG throughout storage. However, a more extensive investigation revealed a substantial decrease in lipoxygenase activity between 45°C and 60°C (Xu et al., 2016). Xu et al. (2016) found that while 4 minutes at 65°C was sufficient to inactivate lipoxygenase, virtually all enzyme activity was retained after the same time period at 45°C. If enzyme activity is not completely eliminated, as was the case in the present study, the effects of after-ripening can otherwise cause increases to lipoxygenase activity with prolonged storage. These results suggest that while steaming was partially effective in inhibiting lipoxygenase in a preliminary storage study, it did not inactivate lipoxygenase sufficiently to elicit long-term inhibition of the enzyme over storage in the present study.

2.5.4 Changes to Antioxidant Content over Storage

By the end of storage at 45°C, lutein and zeaxanthin significantly decreased in steamed IWG by 46% and 56%, respectively, and in non-steamed IWG by 58% and 64%, respectively (**Figure 2)**. At the same storage temperature, lutein and zeaxanthin also significant decreased in steamed HRW by 58% and 50%, respectively, and in non-steamed HRW by 56% and 44%, respectively. By the end of ambient (22 ± 2°C) storage, lutein decreased by 55% in both steamed and non-steamed IWG, which was comparable to the decrease over 45°C storage, however zeaxanthin decreased in steamed IWG and non-steamed IWG by 79 and 80%, respectively. Both steamed and non-steamed HRW retained lutein content over ambient storage, however the very minimal starting amount of lutein in HRW, as well as the variability in data at low concentrations may account for lack of significant difference following 6 months of storage. Zeaxanthin content was lost entirely in steamed and non-steamed HRW at ambient storage. At refrigerated (4°C) storage, lutein also significantly decreased in steamed by 9% and non-steamed IWG by18%, which was to a much lesser extent than over 45°C or ambient storage. In steamed and non-steamed IWG, zeaxanthin decreased by 5 and 18%, respectively, over 4°C storage. Steamed and non-steamed HRW had significant decreases in lutein of 20% and 10%, respectively over 4°C storage, as well. Both steamed and non-steamed HRW retained zeaxanthin content over 4°C storage, however the lack of significance in retention values may again be due a very minimal amount of zeaxanthin in HRW and variability in data at low concentrations. ANOVA results confirmed a significant effect ($P < 0.01$) of temperature on carotenoid concentration.

Carotenoids are particularly prone to oxidation over storage (Boon et al., 2010). Temperature is known to have a significant impact on degradation of carotenoids. Mellado-Ortega & Hornero-Méndez (2017) measured ~72% carotenoid retention after 12 months storage at -32°C, and only 10% retention at 50°C. Several other storage studies on intact cereal grains show significant decreases in carotenoid concentration, as well (Galleschi et al., 2002; Mellado-Ortega et al., 2015; Mellado-Ortega & Hornero-Méndez, 2017). Autooxidation of carotenoids proceeds quite rapidly, especially in isolated organic

extracts (Boon et al., 2010). Carotenoids can further be consumed by participating in inhibitory behavior towards prooxidant species, losing antioxidative capacity in the process. Moreover, carotenoids can be oxidized by lipoxygenase over storage (McDonald, 1979) In the present study, a significant, negative correlation coefficient between each carotenoid concentration and lipoxygenase was measured at each temperature **(Table 14-16, Appendix L)**, suggesting the carotenoids were degraded by lipoxygenase activity over storage in addition to sacrificially reducing prooxidants and scavenging free radicals.

Throughout the entirety of storage at all temperatures, IWG samples maintained significantly higher concentrations of lutein and zeaxanthin than HRW samples. Steaming had a minimal effect on carotenoid retention over 45°C and ambient storage. While there were no significant differences in retention values at the end of 45°C and ambient storage between steamed and non-steamed samples, steamed IWG did have significantly higher lutein and zeaxanthin values than non-steamed IWG at multiple time points throughout storage at these temperatures. Over 4°C storage, steaming of IWG had a statistically significant effect, with significantly greater retention of lutein and zeaxanthin in steamed IWG compared to non-steamed IWG after 12 months of storage. The differences may be explained by the pro-antioxidative effects of steaming (**2.5.5**), which could delay enzymatic and auto-oxidation, which carotenoids are particularly susceptible to (Boon et al., 2010). Differences between steamed and non-steamed HRW were insignificant throughout storage at all temperatures, though carotenoid concentrations were negligible.

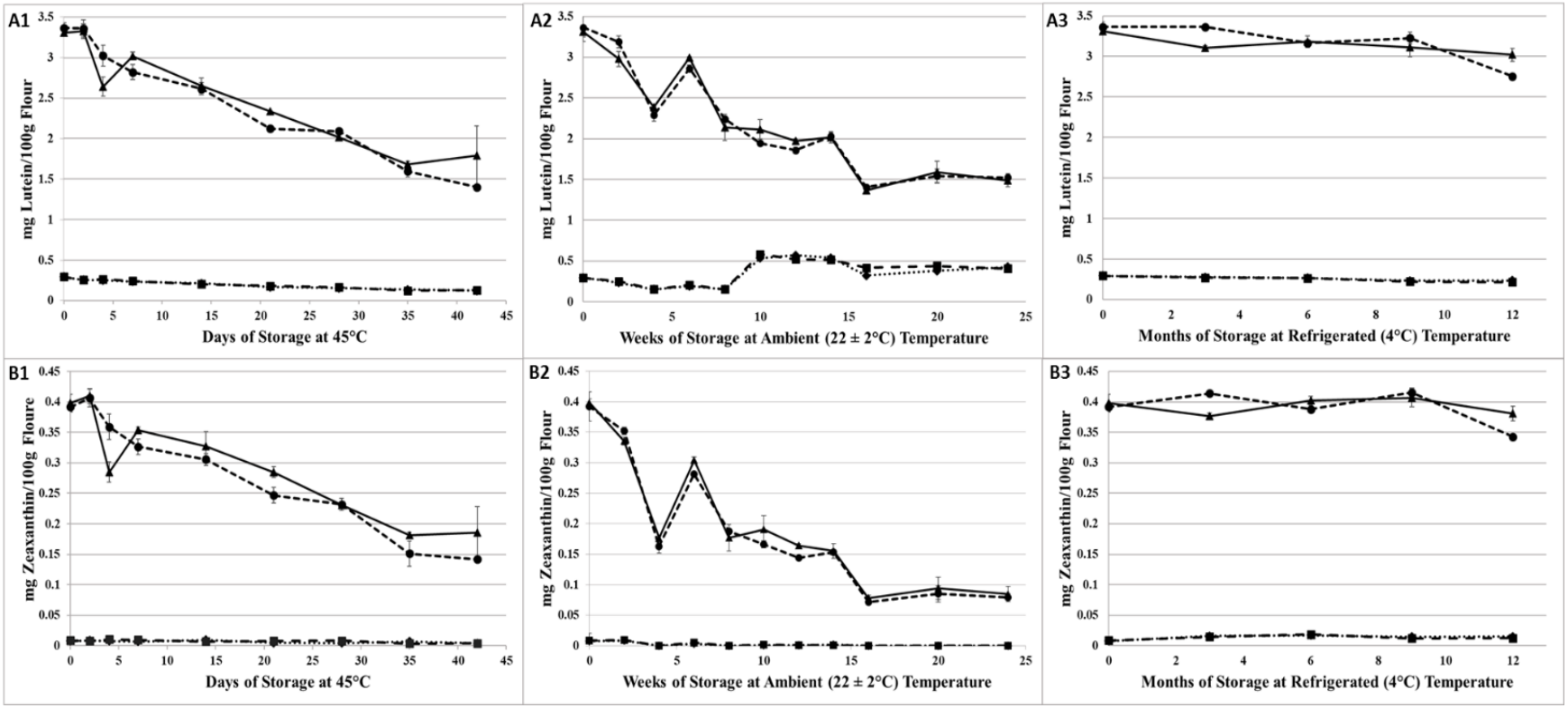

Figure 2. Carotenoids lutein (A) and zeaxanthin (B) over accelerated storage at 45°C (1), ambient storage at 22 ± 2°C (2) and refrigerated storage at 4°C (3). Samples are represented as follows: IWG steamed (—▲—), IWG non-steamed (- -●- -), HRW steamed (···◆···), and HRW non-steamed (—■—). Error bars represent standard error (n=3).

At all storage temperatures, ferulic acid and sinapic acid concentration of steamed and non-steamed IWG and HRW samples remained steady or significantly increased over storage by up to 13% in steamed IWG, 37% in non-steamed IWG, 50% in steamed HRW, and 44% in non-steamed HRW **(Figure 3**). *p*-Coumaric acid concentration significantly decreased by the end of storage in steamed and non-steamed HRW at 4°C (by 75% and 58%, respectively) and in non-steamed HRW at ambient storage (by 80%), although the concentration of *p*-coumaric acid was relatively low in HRW prior to storage. The concentration of *p*-coumaric acid otherwise remained unchanged or increased in all other samples at 45°C and ambient temperatures. These changes in antioxidant content will impact changes in antioxidant activity, which will be discussed in **2.5.5.** The increase in concentration of phenolic compounds has been observed in previous reports on cereal grain storage (Cheng et al., 2006; Liu et al., 2010) and can be explained by a number of mechanisms (Duodu, 2011). Longer storage times and higher storage temperatures can induce aglycosylation, or the release of bound phenolics from the cell wall (Hopia & Heinonen, 1999). Harbaum, Maria, Zhu, and Schwarz, (2008), who measured an increase to hydroxycinnamic acid content over storage in cabbage, explained the increase as the plants' defense mechanism to environmental stress. Post-harvest stressors, in this case dry storage at 4°C and 22°C, stimulate biosynthesis of protective polyphenols. Per ANOVAs, storage temperature had a significant impact on hydroxycinnamic concentration over storage. Higher temperatures induced greater increases in sinapic and *p*-coumaric acid concentration over storage than lower temperatures, whereas ferulic acid saw greater increases over 4°C. Aglycosylation, which proceeds to a greater extent at higher temperatures, may better explain the increase in *p*-coumaric acid and sinapic acid. While post-harvest stress at lower storage temperatures may explain the significant increases to ferulic acid.

Difference in ferulic, sinapic, and *p*-coumaric acid concentration between IWG and HRW were apparent throughout storage at all three storage temperatures, with significantly higher concentrations in both steamed and non-steamed IWG than steamed and non-steamed HRW at a majority of time points. At the end of storage at each

temperature, either steamed IWG, non-steamed IWG, or both, had significantly greater ferulic acid, *p*-coumaric acid, and sinapic acid content than HRW, with the one exception being no difference in sinapic acid content at 12 months of 4°C storage, which reflects the absence of a significant difference at the beginning of storage.

Steaming had a minimal but positive effect on hydroxycinnamic acid concentration of IWG, with significant differences between steamed and non-steamed IWG occurring at multiple time points throughout accelerated (2 days, 3 weeks, and 4 weeks) and ambient storage (2 weeks, 10 weeks, and 20 weeks). Differences in ferulic, sinapic, and *p*-coumaric acid concentration between steamed and non-steamed HRW were negligible. Heat and pressure can oxidize phenolic compounds naturally bound to sugars, fatty acids, and proteins (Randhir et al., 2009). The increases in hydroxycinnamic content due to the effects of both steaming and storage length correlated with increases to antioxidant activity, which will be discussed in the following section.

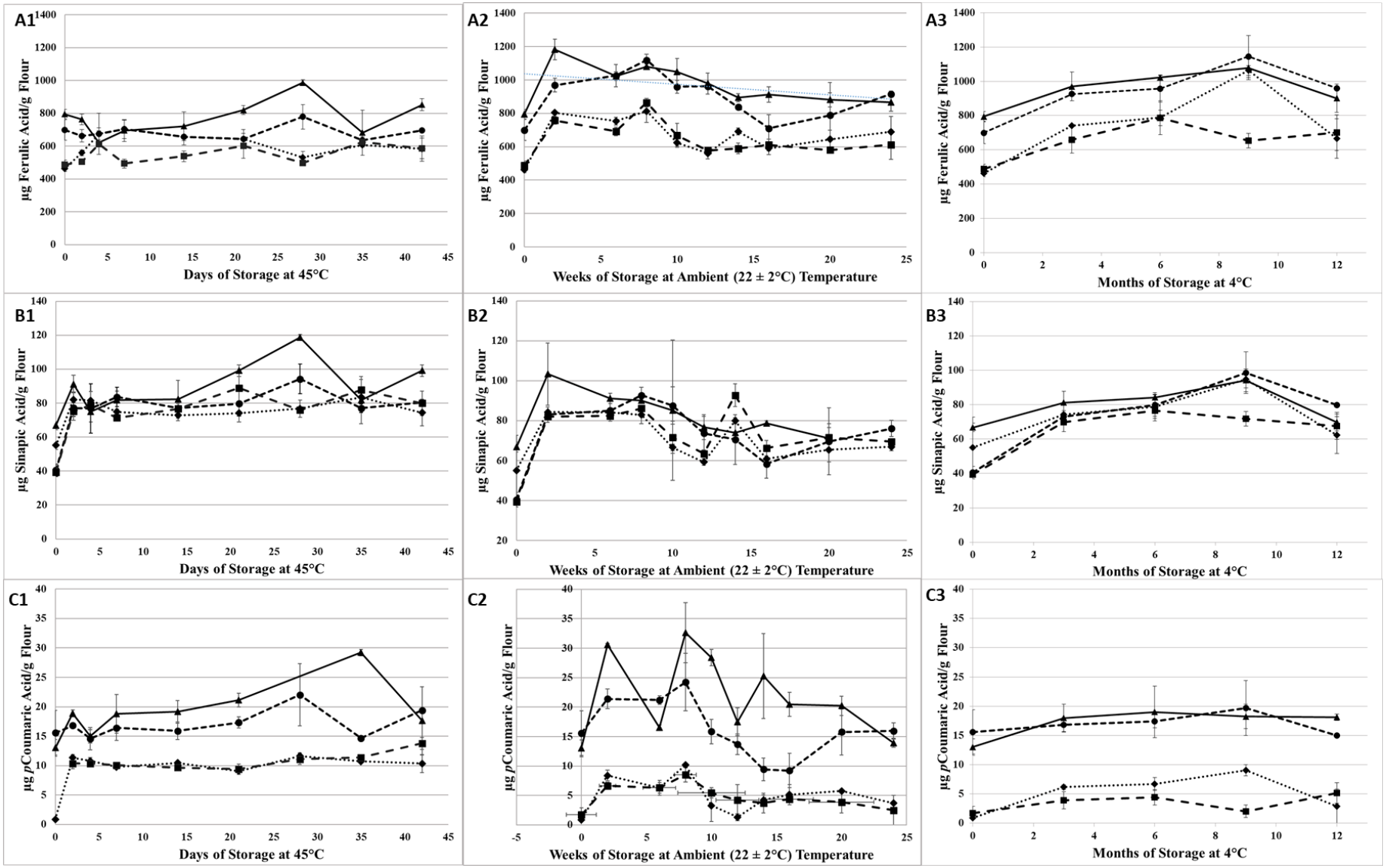

Figure 3. Hydroxycinnamic acids, ferulic acid (A), sinapic acid (B), and *p*Coumaric acid (C) over accelerated storage at 45°C (1), ambient storage at 22 ± 2°C (2) and refrigerated storage at 4°C (3). Samples are represented as follows: IWG steamed (—▲—), IWG non-steamed (- -●- -), HRW steamed (··◆··), and HRW non-steamed (—■—). Error bars represent standard error (n=3).

2.5.5 Changes to Antioxidant Activity over Storage

Changes to antioxidant activity over storage were significantly impacted by storage temperature and length of storage (**Table 4**). At refrigerated and ambient incubation temperatures, antioxidant activity of steamed and non-steamed IWG alkaline hydrolysate extracts as measured by DPPH did not significantly change. However, at 45°C, activity of non-steamed IWG significantly increased over 6 weeks of storage. This same trend was observed in non-steamed HRW over accelerated storage at 45°C, with significantly greater antioxidant activity measured at the end of storage. Similar trends were observed for the activity measured by the LMB assay, with significantly higher antioxidant activity in all samples at 6 weeks of storage at 45°C, and in steamed IWG and HRW at 6 months of ambient storage compared to pre-storage samples. There were no significant changes to antioxidant activity by LMB in any of the samples stored at 4°C. Although grain is not typically stored at temperatures as high as 45°C in order to prevent microbial contamination and heightened grain respiration leading to heat damage, these results do suggest that higher storage temperatures and longer storage times do promote enhanced antioxidant activity over time in IWG and HRW. Cheng (2006) and Liu, Li, and Lei (2010) also found increased antioxidant activity in intact wheat and rice, respectively, over storage. As antioxidant content and activity have been significantly correlated in previous reports (Zieliński & Kozłowska, 2000; Adom & Liu, 2002; Adom et al., 2003), it can be concluded that the significant increases in hydroxycinnamic content over storage in the present study led to significant increase in antioxidant activity over storage. Cheng (2006) noted the effect of temperature, with higher temperatures promoting greater increases in antioxidant activity over storage, which was also the trend for sinapic and *p*-coumaric content. In addition to the significant increase in hydroxycinnamic acids, these results may be explained by progression of the Maillard reaction at higher temperatures (Sikorski, Pokorny, & Damodaran, 2008). In storage conditions favoring the Maillard reaction or caramelization, products exhibiting antioxidant activity may also be produced (Michalska, Amigo-Benavent, Zielinski, & del Castillo, 2008; Chiremba, Taylor, & Duodu, 2009; Žilić et al., 2013). Additionally, bound

phenolics released over longer storage times and higher storage temperatures can form polymers with additional antioxidant activity (Randhir et al., 2009).

The positive impact of temperature on antioxidant activity was further supported by the trends observed in DPPH radical scavenging activity of acetone extracts. While over accelerated storage at 45°C, there were no significant differences between pre-storage and end of storage, antioxidant activity of acetone extracts significantly decreased at 4°C in all samples over 12 months of storage. However, at ambient storage, all samples, steamed and non-steamed, showed a significant increase in activity after 3 months of storage followed by a decrease to original levels by 6 months of storage. One potential explanation for the decrease following 6 months at ambient temperature, as well as 12 months at refrigerated storage, is the oxidation of carotenoids. Lutein and zeaxanthin contents had significant correlation coefficients **(Table 14-16, Appendix L)** with antioxidant activity of the acetone extracts as measured by DPPH. As lutein and zeaxanthin degraded with prolonged storage, they contributed less to antioxidant activity. At 45°C, steamed IWG and HRW samples showed no change and a significant increase to activity, respectively. Non-steamed samples, on the other hand, experienced a dip in antioxidant activity during mid-storage, though beginning and ending antioxidant activity was not significantly different. The greater increase in hydroxycinnamic acids at 45°C as compared to lower temperatures likely balanced the loss of carotenoids, resulting in no significant net loss in antioxidant activity, as was seen at 4°C.

Overall, antioxidant activity of alkaline hydrolysates was significantly greater ($P < 0.05$) than that of acetone extracts. These results were as expected, given the difference in preparation of the two extracts. Alkaline hydrolysates account for phenolic compounds bound to cell wall components that are de-esterified during preparation of the extracts. Acetone extracts account only for free antioxidants in the grain. Not only are bound phenolic compounds in greater abundance than free forms (Smith & Hartley, 1983; Adom et al., 2003; Moore et al., 2005; Kim, Tsao, Yang, & Cui, 2006), they also contribute significantly more to antioxidant activity than free phenolics once freed from cell wall components (Adom & Liu, 2002; Adom et al., 2003).

Differences in antioxidant activity across storage temperatures could be related to changes to the relative concentration of antioxidants, specifically hydroxycinnamic acids and carotenoids, in the stored grains. Over accelerated and ambient storage, total hydroxycinnamic acid content significantly correlated with DPPH radical scavenging activity of both alkaline hydrolysates ($r = 0.693$ and $r = 0.824$, respectively, $P < 0.01$) and acetone extracts ($r = 0.521$ and $r = 0.786$, respectively, $P < 0.01$) (**Table 14-15, Appendix L**). At 4°C, total hydroxycinnamic acids did not significantly correlate with DPPH radical scavenging activity of either acetone or alkaline hydrolysate extracts. These results make sense in light of the aforementioned influence of storage temperature on hydroxycinnamic acid content and activity, with higher storage temperatures inducing increases in both, a trend that was not mirrored over refrigerated storage. While ferulic and sinapic acid both significantly increased over 4°C storage, this was not accompanied by an increase in antioxidant activity. The Maillard reaction and caramelization, both of which produce products with antioxidative capabilities, proceed more readily at higher temperatures (Sikorski et al., 2008), which could explain why increases to antioxidant activity were seen only over accelerated and ambient storage in the present study.

Total hydroxycinnamic acid content did not correlate with antioxidant activity by LMB at either accelerated or ambient storage. At 4°C, ferulic acid exhibited a low, but significant, correlation with antioxidant activity measured by LMB ($r = 0.475$, $P < 0.05$) **(Table 16, Appendix L**). The absence of a significant correlation between hydroxycinnamic acid content and antioxidant activity by LMB at accelerated and ambient storage is as expected considering the antioxidative mechanisms of hydroxycinnamic acids. Hydroxycinnamic acids have been demonstrated in literature to participate in radical scavenging by hydrogen transfer (Toda, Kumura, & Ohnishi, 1991; Onyeneho & Hettiarachchy, 1992), reduction by electron transfer (Sroka & Cisowski, 2003), and metal chelation (Zhou et al., 2006). LMB measures the ability of antioxidants to first inhibit hydroperoxide radical formation and subsequently quench free radicals. Though hydroxycinnamic acids have the ability to quench free radicals, their ability to inhibit hydroperoxide radical formation has not been demonstrated. Overall, observed

results did confirm the significance of hydroxycinnamic acids on antioxidant activity, similar to previous reports that showed significant correlations (r = 0.81-0.99) between total phenolics and antioxidant activity (Zieliński & Kozłowska, 2000; Adom & Liu, 2002; Adom et al., 2003; Choi et al., 2007).

The activity of lutein and zeaxanthin over storage was also temperature-dependent, however given the thermal instability of carotenoids, the trend was opposite that of the hydroxycinnamic acids, with higher storage temperatures causing greater losses of carotenoids and lower temperatures causing greater retention. At 4°C, total carotenoid content significantly correlated with DPPH radical scavenging activity of acetone extracts (r = 0.875, $P < 0.01$), as well as antioxidant activity by LMB (r = 0.417, $P < 0.05$) (**Table 16, Appendix L**). As storage temperature increased, contribution to DPPH radical scavenging antioxidant activity of acetone extracts decreased but remained significant. Over accelerated storage, carotenoids were no longer correlated with DPPH radical scavenging antioxidant activity or LMB antioxidant activity. These results again confirm that carotenoids were degraded with storage and higher storage temperatures, and are best able to contribute to antioxidative activity at refrigerated temperatures. Futhermore, these results support the conclusions of Haila et al. (1996) and Baltschun et al. (1997) respectively, regarding the ability of carotenoids to both inhibit hydroperoxide radical formation (as measured by LMB) and quench free radicals (as measured by DPPH and LMB). Contrary to the results of Choi et al., (2007) who did not detect a significant correlation between carotenoids and antioxidant activity in a variety of cereal grains, these results are promising for IWG, as its high carotenoid content could help prevent enzymatic and auto-oxidation over storage at refrigeration temperatures.

As seen with hydroxycinnamic acid content, steam treatment had a minimal but positive effect on antioxidant activity retention throughout storage. Steaming had a significant effect on DPPH radical scavenging activity of acetone extracts over storage at 45°C. The effect of steaming on antioxidant activity of acetone extracts as measured by DPPH was observed at 6 weeks of 45°C storage, with significantly higher antioxidant activity in acetone extracts of steamed IWG and HRW compared to non-steamed grains.

Although LMB antioxidant activity results showed no significant differences between steamed and non-steamed IWG across temperatures and time points, it was noted that steamed IWG experienced a significant increase in antioxidant activity over storage at room temperature where non-steamed IWG did not.

There were no significant differences in DPPH radical scavenging activity of alkaline hydrolysates between steamed and non-steamed IWG and steamed and non-steamed HRW across all storage times and temperatures. One explanation for the difference in significance of steaming on antioxidant activity between alkaline hydrolysates and acetone extracts is that the 50% acetone solvent could be extracting a variety of antioxidative compounds other than hydroxycinnamic acids, which are extracted by alkaline treatment. These additional extracted compounds, namely carotenoids, flavonoids, tocopherols, and tocotrienols could be more susceptible to steam treatment. 50% acetone has previously been found to be superior to other solvent systems in extracting phenolic antioxidant compounds (Zhou & Yu, 2004).

As with antioxidant content, differences between antioxidant activity of IWG and HRW were apparent throughout storage. Differences in activity were most pronounced in DPPH radical scavenging activity of acetone extracts, where IWG, both steamed and non-steamed, exhibited significantly higher activity than HRW at the beginning, middle, and end of storage at 4°C and ambient storage, as well as the beginning and middle of 45°C storage. Between the middle and end of storage at 45°C, both the hydroxycinnamic acid content and DPPH activity of HRW significantly increased, while antioxidant concentration of IWG remained relatively stable, leading to an insignificant difference in DPPH radical scavenging activity of acetone extracts between the grains at the end of accelerated storage. The difference between IWG and HRW was further evident in DPPH and LMB antioxidant activity of alkaline hydrolysates of IWG and HRW at 6 weeks at 45°C, with higher activity measured in IWG than in HRW. The relatively higher antioxidant activity of IWG throughout storage makes IWG highly competitive against wheat. IWG's high antioxidant activity may also prove beneficial in delaying enzymatic and auto-oxidation over storage.

2.5.6 Progression of Hydrolytic and Oxidative Rancidity over Storage

At ambient (22 ± 2°C) and accelerated (45°C) storage temperatures, steamed and non-steamed IWG experienced significant increases in free fatty acid content over 6 months and 6 weeks of storage, respectively **(Figure 4, A1-A3)**. This trend was also observed at ambient storage in steamed and non-steamed HRW, with significantly greater free fatty acid content at 6 months compared to the beginning of storage. Free fatty acid content of HRW stored at 45°C did not significantly differ between beginning and ending storage times. Over both accelerated and ambient storage, there was a significant negative correlation between free fatty acids and peroxide value (**Table 14,15, Appendix L**). This suggests that free fatty acids are being oxidized by lipoxygenase into fatty acid hydroperoxides, which explains the dip in free fatty acid concentration at certain storage time points that corresponded with an increase in peroxide value (**Figure 4, B1-B3**). At refrigerated storage temperature 4°C, changes to free fatty acid content were insignificant in all but steamed IWG, which showed a significant increase in content after 12 months of storage.

Free fatty acid values in IWG prior to storage (7.9-8.5 g in 100 g fat) were similar to those seen in oats (Ekstrand et al., 1993), and initial free fatty acid values of wheat were similar to those found in wheat (4.3-4.6 g in 100 g fat) (Pixton, Warburton, & Hill, 1975). The researchers measured final concentrations of 13-15% free fatty acids (per total lipid) in heat-treated oats and 30% in not treated oats following 16 weeks of storage at 30°C. While in the present study, free fatty acid concentration following accelerated storage of steamed IWG was similar to that of stored, steamed oats (12.8 g in 100 g fat), non-steamed IWG was also within this range (12.4 g in 100 g fat). This illustrates the storage stability of IWG as compared to other grains of similar fat content.

Kaneko, Nagamine, & Yamada (1995) measured free fatty acid concentration over storage of wheat seeds at 30°C and 8% relative humidity and reported factor increases in palmitic, stearic, oleic, linoleic, and linolenic free fatty acids of 1.3, 1.2, 1.4, 1.3, and 1.6, respectively, over 120 days. A separate study analyzing hydrolytic rancidity over storage of wheat seeds at low and ambient temperatures saw factor increases in free

fatty acid concentration of approximately 1.5-1.7% at low temperatures and 1.8-1.95% at ambient temperatures. Wheat stored in the present study at high temperatures (45°C) showed only a 1-1.1 increase in free fatty acid concentration over 6 weeks. IWG, on the other hand, showed a higher factor increase in concentration of 1.6-1.7 at 45°C, which was slightly lower at ambient storage (1.14-1.2). This substantiates the conclusion that storing IWG at lower temperatures elongates its storage stability. Statistical analysis confirmed that storage temperature did have a significant effect on free fatty acid development. Doblado-Maldonado et al. (2012) and Tsuzuki et al. (2014) also demonstrated the influence of storage temperature, with higher storage temperatures inducing higher rates of increase in free fatty acid concentration.

Throughout the entirety of storage at all three temperatures, IWG maintained significantly higher free fatty acid levels than HRW. These results align with the consistently higher lipase levels measured in IWG as compared to HRW throughout storage **(2.5.3)**. In fact, lipase significantly correlated with free fatty acid concentration at 4°C ($r = 0.646$, $P < 0.01$), ambient temperature ($r = 0.737$, $P < 0.01$), and 45°C ($r = 0.647$, $P < 0.01$) (**Table 14-16, Appendix L**), which corresponds with the high correlation seen by Rose & Pike (2006). While free fatty acid concentration was not significantly different between steamed and non-steamed IWG at the end of accelerated and ambient storage, steamed samples did show significantly higher values at multiple time points throughout storage at these temperatures. After 12 months of refrigerated storage, steamed IWG showed significantly higher levels of free fatty acids. These trends were not observed in HRW, which showed negligible changes to free fatty acid concentration over storage. This is likely a reflection of the relatively low lipase activity in wheat. Overall, these results might suggest that steaming promoted progression of hydrolytic rancidity in IWG over storage. While the increase in free fatty acid content over storage is common in stored cereal grains (Clayton & Morrison, 1972; Molteberg et al., 1996; Rose et al., 2008; Bergonio et al., 2016), the results of this study showing a negative effect of steaming on free fatty acid development in stored grain refute those of

former studies on stored cereal grain and grain flours (Molteberg et al., 1996; Rose et al., 2008; Bergonio et al., 2016).

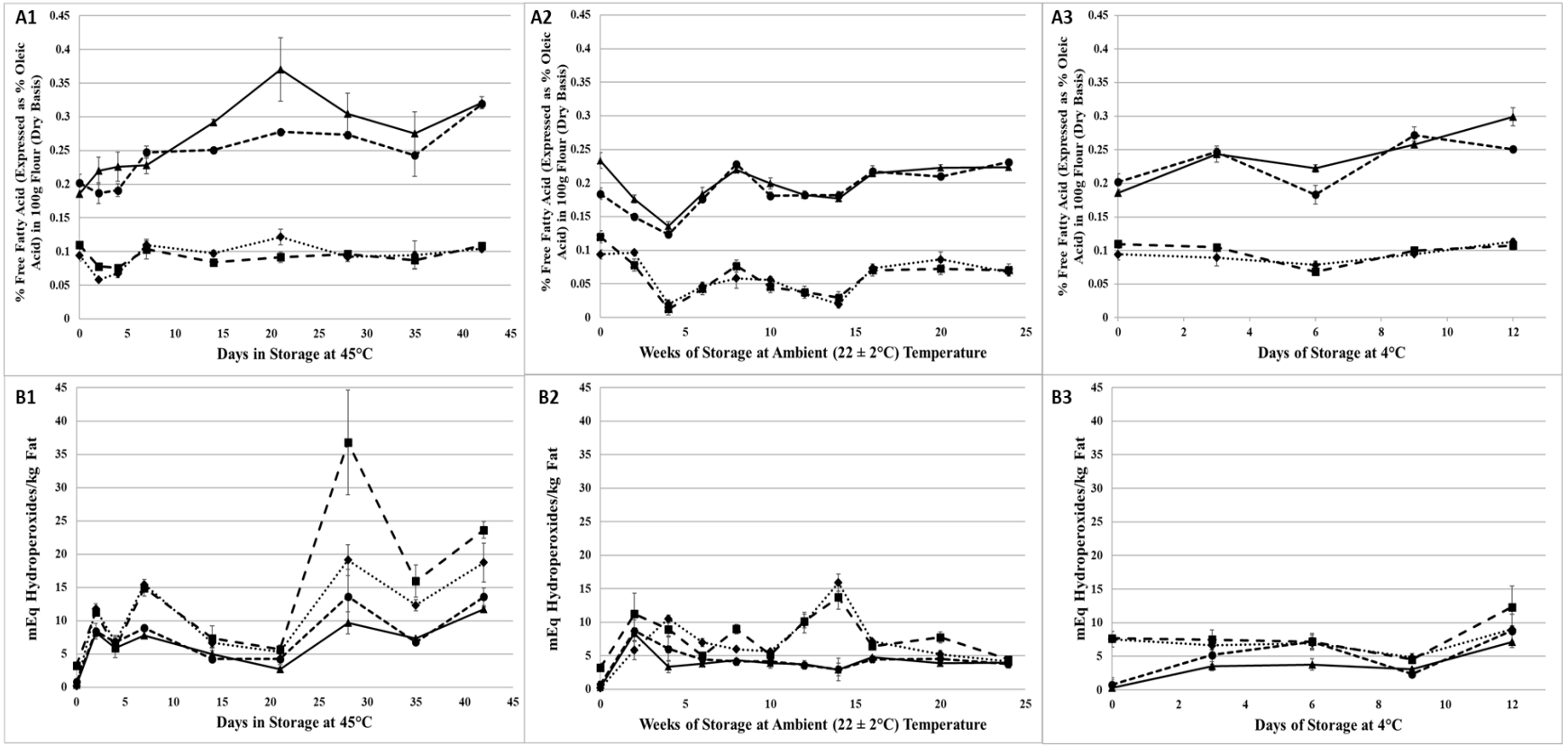

Figure 4. Free fatty acids (A) and hydroperoxides (B) over accelerated storage at 45°C (1), ambient storage at 22 ± 2°C (2) and refrigerated storage at 4°C (3). Samples are represented as follows: IWG steamed (—▲—), IWG non-steamed (--●--), HRW steamed (··◆··), and HRW non-steamed (—■—).

It is important to note that former research demonstrating a significant inhibitory effect of steam on fatty acid development also measured significant inactivation of lipase prior to storage, whereas the present study did not show a significant decrease in lipase activity. Futhermore, it is important to consider changes to fatty acid composition, as well as oxidation progression when analyzing free fatty acid development, as higher free fatty acid accumulation could potentially indicate inhibition of oxidation. For example, Tsuzuki et al. (2014) measured higher free fatty acid concentrations in wheat seeds stored in the presence of an oxygen absorber as compared to a controlled stored sample. The researchers further measured a significant decrease in oxidation-prone polyunsaturated fatty acids in the control sample, ultimately concluding that a storage environment that prevents oxidation alone may increase free fatty acid retention. It is possible this phenomenon occurred in the present research; at storage time points where steamed IWG had higher free fatty acid values than non-steamed IWG, it also had lower hydroperoxide concentration. Therefore, it is appropriate to measure free fatty acid concentration in tandem with fatty acid hydroperoxide concentration in order to understand rancidity trends.

Over storage at 45°C, peroxide activity of all samples was significantly greater at 6 weeks than at the beginning of storage **(Figure 4, B1-B3**). Throughout storage, peroxide value peaked multiple times, which corresponded with dips in free fatty acid measurements. In fact, at 45°C and ambient temperatures, there were significant negative correlations between free fatty acid concentration and peroxide value (**Table 14, Appendix L**). This suggests that free fatty acids are being oxidized by lipoxygenase into fatty acid hydroperoxides.

Over storage at ambient temperature, peroxide values at the beginning and end of storage were not significantly different for any samples, however as with 45°C, peroxide value spiked during various storage time points, then decreased. For IWG samples, steamed and non-steamed, this peak in peroxide activity occurred at the beginning of storage around 2 weeks, after which time peroxide value remained steady with no further significant changes. Both steamed and non-steamed HRW experienced multiple peaks in peroxide value throughout ambient storage. Minimal changes to peroxide value occurred during refrigerated storage, however steamed and non-steamed IWG, as well as steamed

HRW showed significantly higher peroxide values at the end of storage as compared to the beginning of storage. Peroxide values were highest, yet still considerably low, for all samples at the end of 45°C.

Throughout storage at all three temperatures, HRW samples demonstrated higher peroxide values than IWG. These differences in peroxide value between IWG and HRW can be attributed to the higher relative lipoxygenase activity of HRW. At all storage temperatures, lipoxygenase activity correlated significantly with peroxide value ($r = 0.480\text{-}0.719$, $P < 0.05$) (**Table 14-16, Appendix L)**.

Steam treatment prior to storage of IWG and HRW had a small inhibitory effect on hydroperoxide development over storage. At 6 weeks of refrigerated storage, steamed IWG demonstrated a significantly lower peroxide value than non-steamed IWG, however at the remainder of time points, there were no significant differences. At accelerated and ambient storage, no significant differences in peroxide value were apparent. The effect of steaming on hydroperoxide development was similarly minimal for HRW, with significantly lower hydroperoxide content in steamed HRW occurring at 4 weeks of accelerated storage and 8 and 20 weeks of ambient storage. These significant differences between steamed and non-steamed samples were most apparent when peaks in peroxide value were noted. The differences in hydroperoxide content between steamed and non-steamed grains at these time points could potentially be attributed to higher antioxidant activity in steamed grains rather than a difference in lipoxygenase activity. As noted in **2.5.5**, the effect of steaming on antioxidant activity was minimal, yet positive. These results suggest that steaming was only minimally effective at preventing oxidative rancidity. However, overall oxidative rancidity as expressed by hydroperoxide value was relatively minimal over storage in both IWG and HRW.

2.6 Summary and Conclusions

The steam treatment employed in the present study did not inactivate lipoxygenase and lipase. Throughout storage, lipase activity significantly decreased in non-steamed grains over ambient (22 ± 2°C) storage but otherwise was retained over

accelerated (45°C) and refrigerated (4°C) storage. However, an after-ripening effect was evident in lipoxygenase activity at ambient and 45°C temperatures, with significant increases in activity over storage. Yet, despite retention of lipase activity and increases in lipoxygenase activity with storage, development of oxidative and hydrolytic rancidity in IWG was minimal throughout storage of groats, highlighting the storage stability of IWG. The antioxidant content and activity of IWG throughout storage further endorsed its stability. Not only was hydroxycinnamic acid content retained throughout storage, but prolonged storage also promoted increases to content, as well as increases to antioxidant activity at higher storage temperatures. Carotenoid content decreased throughout storage in IWG and HRW, to a greater degree at higher storage temperatures, as was expected. Yet carotenoids contributed to antioxidant activity, especially at ambient and refrigerated storage temperatures. According to these results, storing IWG at refrigerated temperatures can help to preserve carotenoid content, as well as prevent increases to enzyme activity and slow free fatty acid and hydroperoxide product synthesis.

Although steaming did not result in inactivation of enzymes, it demonstrated a minimal, but positive effect on antioxidant activity and content, as well as a small inhibitory effect on hydroperoxide formation over storage. While steaming only minimally enhanced the storage stability of IWG groats via these effects, the results of the present study formed the foundation for establishing a more effective method of steam treatment in a currently ongoing storage study on IWG flour.

In addition, the results of the present study showed that IWG has a superior storage stability to wheat in terms of oxidative rancidity as a result of its superior antioxidant content, antioxidant activity, and relatively lower hydroperoxide content over storage. Yet, in terms of hydrolytic rancidity, IWG showed higher lipase activity and free fatty acid concentration throughout storage. It will be important to determine in future studies effective measures to significantly inactive lipase through steam treatment to reduce hydrolytic rancidity in IWG over storage. Delaying hydrolytic rancidity is particularly important from a sensory standpoint, due to the role free fatty acids play in off-flavor development. In general, free fatty acids are deemed a greater contributor than oxidized products to the loss of sensory quality (De Almeida et al., 2014; Pomeranz, 1992). Strong correlation coefficients were derived from measurements of free fatty acids

and sensory attributes of stored oat grains in a 2002 study, including a coefficient of 0.90 between a bitter flavor and free fatty acids and a coefficient of 0.85 between a rancid flavor and free fatty acids. (Heiniö et al., 2002). The strong relationship between free fatty acids and off-flavors necessitates future sensory studies on stored IWG grain.

Chapter 3: Functional Changes in Stored Intermediate Wheatgrass Grains Subjected to Steam Treatment

3.1 Overview

Perennial intermediate wheatgrass (IWG), *Thinopryum intermedium*, has long been grown as a sustainable crop for forage and animal feed but has recently been recognized for its potential to be integrated into the food system. Characterizing and understanding IWG, including its functionality in food applications and storage stability, will ultimately help promote its marketability. While previous research has characterized the functionality of IWG, changes in functionality over storage and as impacted by various treatments are not yet addressed. The aim of this study was to evaluate the effect of steam treatment of IWG grains on the functionality of its whole flour over storage at different temperatures. IWG showed increases to dough development time, resistance to extension, and loaf volumes over storage, denoting an increase in dough strength, although no significant increases to dough stability time were evident. IWG also had improvements to starch pasting viscosities over storage, including peak viscosity and hold viscosity, at all temperatures. The effects of steaming on functionality were temperature-dependent, with positive effects on dough development time and resistance to extension during storage at 45°C and improvements to starch pasting viscosities during storage at ambient temperature. Steaming appeared to have a negative impact on functionality at refrigerated storage temperatures. Steaming did not have an effect on bread loaf volumes of IWG over storage. Although overall functionality of IWG remained inferior to HRW throughout storage, HRW had significant decreases in several functionality parameters over storage. While steaming had a positive effect on some of these parameters (e.g. extensibility, loaf volume), it had a negative impact on others (e.g. starch pasting viscosities). Bread firmness was not significantly affected over storage in either grain. Overall, these results highlight a positive effect of storage on functionality of IWG and a temperature-dependent effect of steaming. This study provides additional incentives to farmers and food manufacturers alike by highlighting IWG's acceptable storage stability.

3.2 Introduction

Farming of annual wheat, rice, and maize, the top three produced grains worldwide, degrades topsoil, leading to soil erosion, water-run off, nitrogen leaching, and nutrient loss (Cox et al., 2006). There is a current need in the marketplace for a grain that can be sustainably produced, while still meeting the demands of a growing world population and providing the attributes consumers have come to expect from a whole grain. Among these attributes are nutrient density, palatability, and functionality. Intermediate wheatgrass (IWG), or *Thinopyrum intermedium*, has been identified as promising perennial candidate to fulfill these roles (Becker et al., 1991; Wagoner, 1990). Selection of IWG over other grains is rooted in its agronomic benefits related to its perenniality and deep root system (DeHaan et al., 2005), along with its competitive nutrition profile (Rahardjo et al., 2018; Tyl & Ismail, 2018). Initiatives spanning decades to domesticate and improve desirable qualities of IWG, such as ease of harvest and seed and yield size, have proven successful and accelerated the effort to commercialize IWG (DeHaan, 2015). However, in order to incentivize production of IWG, farmers need to know it is marketable. Functionality and storage stability are key drivers of marketability.

Functionality of cereal grains are often compared to that of wheat, the "gold standard" of grain. Wheat has superior viscoelastic and leavening properties that render it capable of performing in a wide variety of end-use applications (Žilić, 2013). These properties are attributed to wheat's gluten forming proteins, more specifically the balance of gliadins and glutenins, storage proteins that heavily impact rheology. Gliadins are responsible for the viscosity and extensibility of a dough, while glutenins are responsible for elasticity and dough strength (Hoseney, 1998). Together, these proteins form the gluten matrix that is essential to the gas holding capacity of leavened products such as bread. Furthermore, the ratio of high molecular weight glutenins (HMWG) to low molecular weight glutenins (LMWG) has been shown to have a significant impact on rheological and functional properties (Dhaka & Khatkar, 2015). A higher HMWG/LMWG ratio has been shown to result in a longer dough development time and an increase in dough stability and bread specific volume.

Previous research has evaluated the functionality of IWG against hard red wheat (HRW). Rahardjo et al. (2018) found that IWG is deficient in HMWG, and accordingly produces a weak gluten matrix with poor gas holding capacity, overall resulting in reduced loaf volumes. Furthermore, the high dietary fiber content of IWG contributed to reduced loaf volume and height. IWG has a high dietary fiber content compared to wheat as a result of its relatively smaller size and thus, higher bran to endosperm ratio (DeHaan & Ismail, 2017; Rahardjo et al., 2018). Though its performance in leavened products does not compete with that of HRW, IWG is well-suited for other food products such as those requiring extensibility, such as flatbread and pita bread, owing to its sufficient gliadin content, and other food products that do not require leavening, such as cookies, tortillas, and crackers.

The starch pasting characteristics have also been previously evaluated by Marti et al. (2015) and Rahardjo et al. (2018). Rahardjo et al. (2018) found IWG to have a higher pasting temperature than HRW, as well as lower pasting viscosities. The protein content of IWG could partially contribute to these trends, as protein can form a matrix via disulfide bonds around starch granules, inhibiting water absorption and swelling (Juliano, Onate, & del Mundo, 1965). The higher fiber content in IWG also explains its lower viscosity values, as both fiber and protein compete with starch for water (Collar, Santos, & Rosell, 2006). Rahardjo et al. (2018) also noted a lower breakdown value in IWG than in HRW, which indicates a higher relative stability of starch granules.

While the functionality of IWG has been investigated, no prior studies have evaluated how its rheological and functional behavior may change over storage and the effect these changes have on shelf life. Changes to the protein, starch, and lipid components of a grain over storage have been shown to directly impact functionality (Tipples, 1995). Oxidation of sulfhydryl groups of gluten-forming proteins can promote disulfide bond formation and polymerization of glutenins and gliadins with resulting improvements to dough stability, gas retention, and loaf volume (Zeleny & Coleman, 1938; Tipples, 1995). However, prolonged storage can lead to loss of elasticity, decreased dough stability, and reduced hydration capacity (Kibar, 2015; Baik & Donelson, 2018). Extended storage periods beyond three or four years may induce conformational changes, manifesting in an increase in aggregation and a decrease in

hydrophilicity and solubility (Kozlova & Nekrasov, 1956). The components of starch are also subject to storage-induced changes. Amylose can undergo conformational changes to form complexes with free fatty acids, which are released due to action of lipase (Eliasson et al., 1981; Fierens et al., 2015; Gerits et al., 2015). Amylose-lipid (AM-L) complexes can delay gelatinization and depending on the composition of the cereal grain as well as granule characteristics, can either increase or decrease pasting viscosities (Conde-Petit & Escher, 1992; Zhang & Hamaker, 2005; Putseys et al., 2010). Amylopectin can interact with lipids, as well, and the rigidity of the resulting structure can lower swelling capacity (Conde-Petit & Escher, 1992).

In addition to effects on starch pasting profiles, changes to the lipid profile of stored grains have large impacts on rheological properties and baking quality. Researchers have also implicated the role of oxidative rancidity in functionality changes, citing loss of dough elasticity, vulnerability to dough fracture, and decrease in bread volume (Schaich & Karel, 1976; Sullivan et al., 1936). Free radicals generated by lipid peroxidation can cause denaturation of proteins, along with destruction of individual amino acids, polymerization, and cross-linking (Schaich & Karel, 1976). Bread volumes may also be reduced by free fatty acids released upon lipolytic action over storage (Tait & Galliard, 1988), however other researchers have contradicted this conclusion (Rose et al., 2011; Gerits et al., 2014). Free fatty acids released via lipolytic action can compete for interaction with shortening lipids, thereby reducing the capacity of the added shortening to interact with gluten and starch in a dough system (Bell et al. 1979). Free fatty acids can also interfere with leavening agents such as sodium bicarbonate, (Bell, Daniels, & Fisher, 1977; Tait & Galliard, 1988; Bookwalter et al., 1991), thereby indirectly reducing loaf volumes.

Understanding the role that lipids play in functionality changes, processing techniques that target lipids may thwart the progression of these changes. Steam treatment is currently used in the grain industry to prolong shelf life of grains with high lipid and enzyme (lipase and lipoxygenase)(Rose et al., 2008). The purpose of steam treatment is to inactivate the enzymes lipase and lipoxygenase that catalyze hydrolytic and oxidative rancidity, respectively. In particular, IWG is susceptible to rancidity due to its relatively high lipid content and enzyme activity (Tyl & Ismail, 2018). Steam

treatment could, therefore, be advantageous for IWG, however it is important to evaluate how steam treatment may impact the functionality of IWG. In addition to curtailing synthesis of lipid peroxides and free fatty acids that can negatively impact functionality, steam treatment, itself, can have an impact on functionality. Heat treatment can cause denaturation albumins, prolamins, and glutenins with subsequent exposure of hydrophobic groups, leading to protein aggregation, formation of disulfide bonds, and protein insolubility (Sun et al., 2006; Runyon et al., 2015; Deepa & Umesh Hebbar, 2017). Consequently, dough stability and strength, as well as dough extensibility, are negatively impacted (Sun et al., 2006). Steam treatment can also lead to pregelatinization of starch (Arntfield et al., 1997; Fasina et al., 1999; Žilić et al., 2010) and lower starch pasting viscosities (Yadav, Kaur, et al., 2012), which can impact ultimate applications of the grain. When choosing a steam treatment method, it is therefore imperative to consider end use functionality and choose a method gentle enough to preserve sufficient functionality yet strong enough to inactivate enzymes. Furthermore, it is important to investigate how this steam treatment can influence functionality with prolonged storage. The present study is the first to evaluate the storage stability of IWG, with regards to both its rancidity and functionality markers, as well as to investigate the effects of steam on these parameters.

The overall objective was to evaluate effects of steam treatment on enzyme activity, antioxidant activity, rheology, starch pasting profiles, and bread quality parameters over storage. We hypothesized that steam treatment and extended storage under different conditions will not have a negative effect on dough functionality and breadmaking.

3.3 Materials

IWG and HRW grains were obtained as described in section **2.3**. All bread baking materials were purchased locally.

3.4 Methods

3.4.1 Experimental Design

The experiment was laid out as a factorial design with 2 grains (IWG and HRW), 2 treatments (steam-treated and untreated) and 3 storage temperatures, 4°C, 22 (± 2°C), and 45°C. Samples under each storage temperature were pulled out at the middle and end of storage for functionality testing. "Time 0" samples from each treatment condition were analyzed to establish a baseline for storage measurements. At each time point, analyses included rheological and testing by Farinograph® and Kieffer Dough and Gluten Extensibility Rig, starch pasting profiles by MicroVisco-Amylograph® (MVAG), and bread baking quality. All samples were stored and analyzed in triplicate.

3.4.2 Dough Functionality

3.4.2.1 Dough Rheology/Mixing Properties

Dough behavior during mixing was determined in triplicate for samples from the beginning, middle, and end storage at each storage temperature using a Farinograph® – AT (C.W. Brabender, Duisburg, Germany) following AACCI method 54-21.02 (AACCI International, 2010). Water absorption values required to reach an optimum dough consistency/torque of 500 BU (Brabender Units) were experimentally determined by mixing flour from each sample (~10 g corrected to default moisture content of 14%) and variable amounts of water in a 10 g mixing bowl set at 30°C. A dough consistency of 500 BU, which is considered optimal for wheat dough (El-Dash, 1978). The water absorption value required to achieve an optimal dough consistency for a given sample was then used to prepare doughs using the respective sample. Flour (~10 g corrected for moisture) was mixed for 1 minute at 30°C at a speed of 63 min^{-1}. The amount of water calculated to achieve the experimentally determined optimal water absorption value for each sample was then added to the flour while mixing. The dough was mixed for 20 minutes, and

dough consistency was measured and plotted as a function of time to create a farinogram (**Figure 5**). The farinogram of a sample was then used to determine its stability arrival, dough development time, and dough stability. Stability arrival is a measure of the time (in min) for a sample to reach the optimal consistency of 500 BU following the addition of water, and it is reflective of the flour's ability to uptake water (Locken & Shuey, 1972). Dough development time is the time (in min) required for a dough to reach peak consistency starting from the time water is introduced. Dough development time is indicative of ideal mixing time. Dough stability is a measure of the time (in min) a sample maintains optimal consistency at 500 BU and is reflective of a sample's tolerance to mechanical action by mixing.

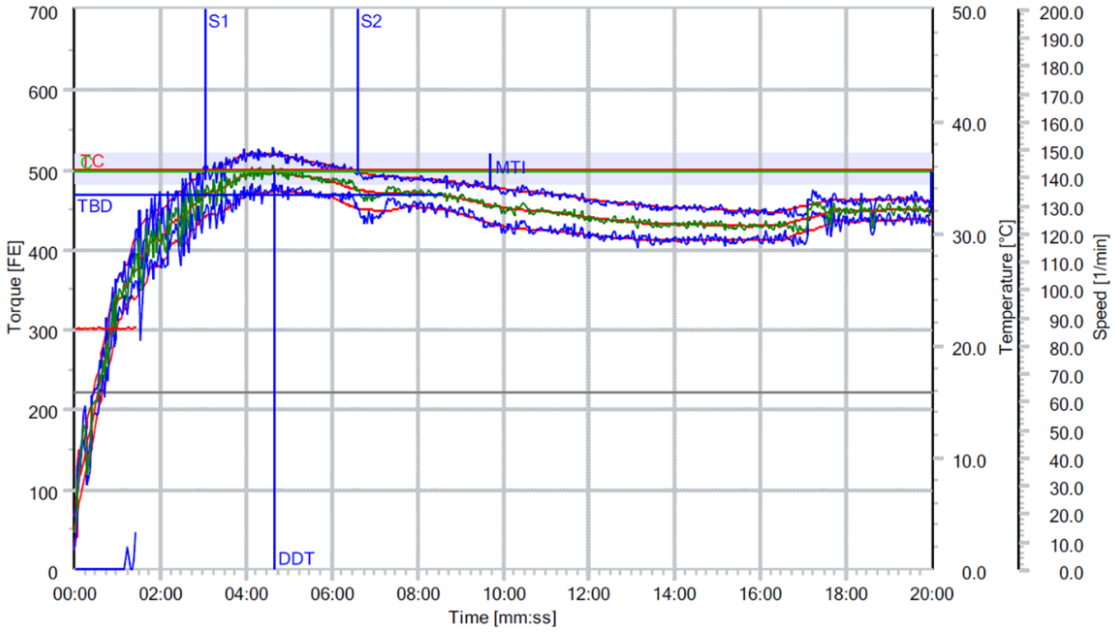

Figure 5: Example of a farinogram, used to plot dough consistency over time as an index of mixing properties of dough as measured using a Farinograph. S1: stability arrival; S2: stability departure; S2-S1: dough stability time; DDT: dough development time.

3.4.2.2 Extensibility Using Kieffer Rig

Dough extensibility and resistance to extension of steamed and non-steamed IWG and HRW dough were tested using a Kieffer Dough and Gluten Extensibility Rig attached to a TA-XT2i texture analyzer (Texture Technologies Corp, Scarsdale, NY, USA). Analysis was conducted in duplicate with three sub-replicates. Dough consistency was first optimized using a Farinograph® – AT, as described in **3.4.2.1** with slight modifications. Salt (0.2 g) was added to flour samples (~10 g corrected to default moisture content of 14%) and the water absorption value necessary to achieve optimal consistency of 500 BU was obtained experimentally for each sample by adding varying amounts of water. Upon reaching optimal consistency at 500 BU, mixing was promptly stopped and a dough ball was formed and allowed to rest in a closed container for 20 minutes. The fresh dough was then pressed and molded into individual strips in the Kieffer mold to approximately 4 mm in width and 50 mm in length. The dough strips were rested for an additional 40 minutes in the mold. Each strip was then placed in the Kieffer microextension rig and stretched vertically as shown in **Figure 6.** The dough strips were pulled at a speed of 3.3 mm/s for a distance of 75 mm. The data was then automatically generated by the Texture Exponent 32 version 6.1.4.0 software (Texture Technologies, Corp. Scarsdale, NY, USA) to provide measurements of dough resistance to extension (mN) and extensibility (mm). The dough resistance to extension (mN) is the measure of the force needed to break the dough, measured by the force generated against the hook attached to the texture analyzer as shown in **Figure 6**. Dough resistance to extension (mN) is an indicator of dough strength. Extensibility (mm) is the distance at which the dough breaks apart, or a measure of dough deformation before it ruptures (M. Wang, Van Vliet, & Hamer, 2004).

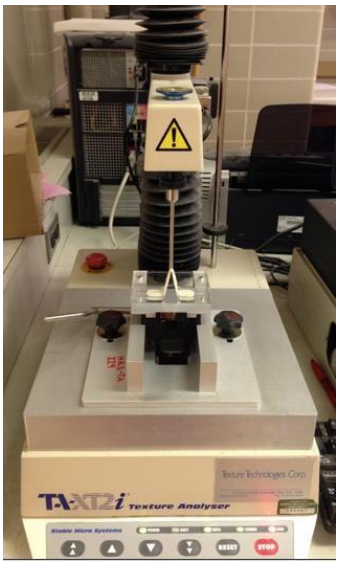

Figure 6: Dough stretched vertically using the Kieffer rig.

3.4.2.3 Pasting Properties

Starch pasting properties were measured in triplicate for IWG and HRW samples from the beginning, middle, and end of storage at each storage temperature using a MicroVisco-Amylograph® (MVAG) (C.W. Brabender®, South Hackensack, NJ, USA). Approximately 15 g of flour (corrected to 14% moisture) was mixed with 100 g of DDW in a rotating, heated bowl with a paddle operating at a speed of 250 min^{-1}. The following temperature profile was applied: 30°C for 1 min, heating from 30°C to 95°C at a rate of 7.5°C/min, holding at 95°C for 5 min, cooling at a rate of -7.5°C/min, and holding at 30°C for 1 min. The following indices were collected: pasting temperature (the temperature at which the initial swelling begins), peak viscosity (the maximum viscosity reached during heating), breakdown (extent of the decrease in viscosity during the holding period), final viscosity, and the setback value (extent of the increase in viscosity during cooling) (Marti et al., 2015). A common pasting profile is shown in **Figure 1.**

3.4.3 Baking Quality

3.4.3.1 Bread Baking Process

Bread baking was conducted in duplicate, with two sub-replicates, following the AACCI method 10-10.03 (AACCI International, 2010), with modifications. First, flour

moisture was determined using an infrared moisture analyzer (OHaus MB45, New Jersey, USA) at 130°C for 4 min. Flour moisture was used to calculate the amount of water necessary to add to the dough to reach optimal dough moisture. For preparation of IWG samples, a final dough moisture of 46.5% was targeted based on the results of a preliminary study that determined optimum moisture content by Farinograph (Marti et al., 2016). For the preparation of wheat controls, 72.4% final moisture was targeted based on optimum moisture level previously determined by Farinograph as in **3.4.2.1.** Flour (50 g), 3 g of sugar, 0.75 g of salt, 2.65 g of yeast, and 1.5 g of shortening were used to make the dough using the Farinograph, along with the calculated amount of water for optimal moisture content. The yeast and sugar solution were kept at 30°C and 85% relative humidity in a proofing oven for 20 min prior to use. The dough components were mixed in a 50 g Farinograph bowl (C.W. Brabender, Duisburg, Germany) until maximum dough consistency was reached. As optimal dough moisture for IWG and HRW was determined using unstored, untreated samples, final dough consistency varied across samples. At maximum consistency, each dough sample was collected, kneaded by hand 10 times, split into two even parts, and then put in the proofer (Baxter PW2E, Orting, WA, USA) at 30°C and 85% relative humidity (RH) for 52 min. Each dough sample was punched by hand 10 times and proofed (30°C and 85% RH) for 25 min. Each dough sample was once more punched by hand 10 times and proofed (30°C and 85% RH) for 13 min. The dough was then sheeted to a 3/16" thickness 5 times, shaped into a ball by hand, re-sheeted to 3/16" thickness 5 times, rolled to a length of 2.1 inches, and placed in a Freshware CB-308RB trapezoidal pan (size 2.8 x 1.5 inches top, 2.1 x 1.2 inches bottom, and 1.1 inches depth) (Los Angeles, CA, USA). The dough was proofed (30°C and 85% RH) for another 33 min before it was subjected to steam for 10 sec followed by baking for 14 min at 425°F in a baking oven (Baxter OV500E1, Orting, WA, USA). The bread was left to cool for one hour before the measurement of bread quality parameters.

3.4.3.2 Bread Baking Quality

The following quality parameters were measured: volume, height, and firmness. Bread volume of each sub-sample was measured in triplicate using the Rapeseed Displacement method, AACCI method 10-05.01 (AACCI International, 2010). Bread's

maximum height was measured using a Neiko 01407A digital caliper (Neiko, Taiwan, China). Bread was cut into slices of 12.5 mm thickness with a bread cutter (Oliver Products Company, Grand Rapids, MI, USA) for analysis of firmness. Three slices from each loaf were analyzed. Bread firmness of each slice was measured following AACCI method 74-09.01 (AACCI International, 2010) using a TA XT-Plus Texture Analyzer (Texture Technologies Corp, NY, USA). The probe used was 1.3 cm (0.5 inch) in diameter and 3.5 cm in length (1 3/8 inches). The force (in g) needed to deform the bread by the probe was recorded. Bread pictures were taken using HP Scanjet (G4050, Palo Alto, CA, USA) at 1200 dpi.

3.4.4 Statistical Analysis

One-way analysis of variance (ANOVA) was done using R Version 3.3.1 (The R Foundation, 2016) to determine differences among samples with either storage time, or sample type as factors. Differences among the means were determined using Fisher's Least Significant Difference (LSD) test ($P < 0.05$). Two-way ANOVA was carried out to assess for interaction effects among treatment variables, including storage time, storage temperature, and sample type, on various dependent variables. Pearson's product-moment correlation coefficients ($P < 0.05$) were calculated using R to test for linear relationships between dependent variables. Correlation coefficients depicting relationships among functionality parameters, rancidity markers, and enzyme activity can be found in **Table 18-20 (Appendix N)**. Two-way ANOVA summary tables can be found in **Table 33-47 (Appendix P).**

3.5 Results and Discussion

3.5.1 Changes in Dough Rheological Properties as Affected by Steam Treatment of IWG and HRW

HRW had a higher water absorption value than IWG **(Table 5)**, similar to the findings of Rahardjo et al. (2018). Steaming significantly affected water absorption values, with dough from steamed grains requiring a greater amount of water to reach optimal dough consistency. An increase in farinograph water absorption has also been

observed by Zhang, Moore, and Doehlert (1998) in dough made from steamed oat grains, as well as by Afshari-jouybari, Peighambardoust, and Azadmard-damirchi (2018) in steam-treated wheat bran. A higher water absorption is more desirable, as it has been shown to correlate with dough stability (a measure of dough strength) and greater bread loaf volumes (Aydoğan, Sahin, Akcacik, Hamzaoglu, & Taner, 2015).

HRW, steamed and non-steamed, had significantly greater dough stability time compared to both steamed and non-steamed IWG **(Table 5**). This is consistent with the results of Marti et al. (2015) and Rahardjo et al. (2018). Dough stability correlates with the strength of the dough (Aydoğan, Şahin, Akcacik, Hamzaoĝlu, & Taner, 2015). The lack of dough stability of IWG can likely be attributed to its deficiency in high molecular weight glutenins (Rahardjo et al., 2018). Dough stability time was not significantly affected by steaming. Heat treatment of grains can lead to denaturation of proteins with subsequent aggregation, loss of solubility, and loss of functionality (Arntfield et al., 1997; Fasina et al., 1999; Sun et al., 2006; Žilić et al., 2010; Runyon et al., 2015; Deepa & Umesh Hebbar, 2017). The steam treatment in the present method was gentle enough so as to not denature proteins and cause loss of dough stability.

Table 5. Corrected water absorption, dough stability, stability arrival, and dough development time of steamed and non-steamed IWG and HRW as measured by farinograph over accelerated (45°C), ambient (22 ± 2°C), and refrigerated (45°C) storage.

Water Absorption for Default Moisture Content (14%)

	Accelerated (45°C) Storage			Ambient Storage (22 ± 2°C)			Refrigerated (4°C) Storage		
	0 Weeks	3 Weeks	6 Weeks	0 Months	3 Months	6 Months	0 Months	6 Months	12 Months
IWG Steamed	64.1^{bA}	64.2^{abA}	62.0^{cB}	64.1^{bAB}	63.6^{bB}	64.8^{bA}	64.1^{bA}	62.7^{bB}	64.2^{bcA}
IWG Not Steamed	62.9^{cAB}	63.5^{bA}	60.4^{dB}	62.9^{cB}	63.5^{bB}	66.0^{aA}	62.9^{cAB}	61.8^{bB}	64.1^{cA}
HRW Steamed	66.8^{aA}	65.1^{aB}	63.4^{bC}	66.8^{aAB}	67.6^{aA}	65.8^{aB}	66.8^{aA}	n.d.$^{\beta}$	65.3^{aB}
HRW Not Steamed	64.6^{bA}	65.0^{aA}	65.1^{aA}	64.6^{bB}	67.1^{aA}	65.7^{aB}	64.6^{bA}	65.2^{aA}	64.6^{bA}

Dough Stability (s)

	Accelerated (45°C) Storage			Ambient Storage (22 ± 2°C)			Refrigerated (4°C) Storage		
	0 Weeks	3 Weeks	6 Weeks	0 Months	3 Months	6 Months	0 Months	6 Months	12 Months
IWG Steamed	38.0^{bA}	44.0^{cA}	62.5^{bA}	38.0^{bA}	37.0^{bA}	34.5^{bA}	38.0^{bB}	51.0^{bA}	36.5^{cB}
IWG Not Steamed	45.0^{bA}	50.5^{cA}	43.0^{bA}	45.0^{bA}	46.0^{bA}	45.5^{bA}	45.0^{bA}	46.0^{bA}	39.5^{cA}
HRW Steamed	200^{aB}	189^{bB}	295^{aA}	200^{aB}	198^{aB}	258^{aA}	200^{aA}	n.d.	176^{bA}
HRW Not Steamed	202^{aA}	299^{aA}	299^{aA}	202^{aB}	194^{aB}	245^{aA}	202^{aA}	210^{aA}	231^{aA}

Stability Arrival (s)

	Accelerated (45°C) Storage			Ambient Storage (22 ± 2°C)			Refrigerated (4°C) Storage		
	0 Weeks	3 Weeks	6 Weeks	0 Months	3 Months	6 Months	0 Months	6 Months	12 Months
IWG Steamed	82.0^{bB}	76.5^{cAB}	89.5^{cA}	82.0^{bA}	63.5^{cA}	79.5^{bA}	82.0^{bA}	73.0^{bA}	65.0^{cA}
IWG Not Steamed	53.5^{cA}	80.0^{cA}	60.0^{dA}	53.5^{cB}	61.5^{cB}	66.5^{bA}	53.5^{cB}	64.5^{bAB}	79.5^{cA}
HRW Steamed	176^{aB}	178^{bB}	246^{aA}	176^{aB}	181^{bB}	201^{aA}	176^{aA}	n.d.	183^{bA}
HRW Not Steamed	186^{aB}	203^{aAB}	221^{bA}	186^{aA}	206^{aA}	194^{aA}	186^{aB}	190^{aB}	225^{aA}

Dough Development Time (s)

	Accelerated (45°C) Storage			Ambient Storage (22 ± 2°C)			Refrigerated (4°C) Storage		
	0 Weeks	3 Weeks	6 Weeks	0 Months	3 Months	6 Months	0 Months	6 Months	12 Months
IWG Steamed	96.0^{bB}	93.0^{cB}	116^{cA}	96.0^{bA}	80.5^{bA}	92.5^{bA}	96.0^{bA}	99.0^{bA}	80.0^{cA}
IWG Not Steamed	78.0^{bB}	102^{cA}	86.0^{dAB}	78.0^{bA}	81.5^{bA}	83.5^{bA}	78.0^{bA}	80.5^{bA}	93.0^{cA}
HRW Steamed	253^{aB}	257^{bB}	353^{aA}	253^{aB}	276^{aB}	291^{aB}	253^{aA}	n.d.	258^{bA}
HRW Not Steamed	265^{aB}	280^{aAB}	329^{bA}	265^{aA}	280^{aA}	278^{aA}	265^{aB}	265^{aB}	331^{aA}

Stability arrival is reflective of the flour's ability to uptake water and form a protein network (Locken & Shuey, 1972). HRW, steamed and non-steamed, had significantly higher stability arrival times than both steamed and non-steamed IWG **(Table 5)**, similar to the findings of Rahardjo et al., (2018). Steaming did not have a significant effect on stability arrival of HRW, however steamed IWG had a significantly higher stability arrival time than non-steamed IWG.

HRW had significantly greater dough development times than IWG. Steaming did not have a significant effect on dough development time of HRW, however steamed IWG had a significantly greater dough development time than non-steamed IWG. These results corroborated those shown by Caprez et al. (1986) and Prakash and Rao (1999). Caprez et al. (1986) found that wheat bran exposed to a variety of heat treatments, including steaming, autoclaving, and toasting, contributed to prolonged dough development time. Dough development time is indicative of ideal mixing time needed to achieve a stable protein network. According to (Cenkowski, 2000), higher dough development time is associated with greater dough strength. Higher arrival and dough development times have a direct impact on processing times of IWG dough. While longer mixing times can ultimately slow industrial processes, they can be advantageous in products that require long fermentation times (Cenkowski, 2000).

HRW had significantly higher resistance to extension, as well as significantly higher extensibility than IWG **(Table 6**). This is consistent with the results of Rahardjo et al. (2018). Wheat has a balance of both glutenins and gliadins, which contribute to elasticity and extensibility, respectively, and an overall stable, high quality dough (Zilic, 2013). High molecular weight glutenins, in particular, contribute to a strong gluten network through the formation of stable stronger protein-protein interactions (Ohm et al., 2010). Rahardjo et al. (2018) found that in comparison to wheat controls, IWG is deficient in high molecular weight glutenins but rich in α-, β-, and γ- gliadins and contains some low molecular weight glutenins. As a result of the deficiency of high molecular weight glutenins, IWG has less elasticity than wheat, thus explaining the lower resistance to extension values. Also, the poor gluten network of IWG limits the extensibility of the dough. Steam treatment did not significantly affect the resistance to

extension or extensibility of IWG, however steamed HRW exhibited significantly higher resistance to extension and extensibility than non-steamed HRW **(Table 6**). Both Prakash and Rao (1999) and Afshari-jouybari et al. (2018) measured significant increases to resistance to extension in steam-treated wheat flour and bran, respectively. However, the researchers saw considerable decreases to extensibility of steam-treated dough, whereas the opposite trend was observed for HRW in the present study. Prakash and Rao (1999) and Afshari-jouybari et al. (2018) related the loss in extensibility to denaturation of gluten forming proteins with heat. Thermal treatments of isolated gluten have confirmed the results seen by these researchers, with temperatures of above 80°C increasing resistance to extension and decreasing extensibility (Cuq & Boutrot, 2000). Cuq and Boutrot (2000) explained that resistance to extension increases upon heating due to "rigidification" of the protein network via covalent cross-linkage formation between proteins. They argued that the same phenomenon is responsible for a decrease in extensibility. Different steaming conditions may have a varying effect on the gluten-forming proteins, explaining differences in observations across studies.

Table 6. Resistance to extension and extensibility of steam treated and not treated IWG and HRW as measured by Kieffer over accelerated (45°C), ambient (22 ± 2°C), and refrigerated (4°C) storage.

	Resistance to Extension (mN)								
	Accelerated (45°C) Storage			**Ambient Storage (22 ± 2°C)**			**Refrigerated (4°C) Storage**		
	0 Weeks	3 Weeks	6 Weeks	0 Months	3 Months	6 Months	0 Months	6 Months	12 Months
IWG Steamed	8.45^{cB}	6.77^{dC}	9.82^{cA}	8.45^{cA}	10.4^{cC}	9.80^{dB}	8.45^{cB}	9.63^{bA}	10.2^{dA}
IWG Not Steamed	6.94^{cB}	8.52^{cA}	6.27^{dC}	6.94^{cC}	9.97^{cB}	18.6^{cA}	6.94^{cC}	9.90^{bB}	16.4^{cA}
HRW Steamed	51.0^{aA}	32.2^{bC}	34.7^{bB}	51.0^{aA}	38.2^{aB}	34.0^{bC}	51.0^{aA}	n.d.$^{\beta}$	27.0^{bB}
HRW Not Steamed	36.8^{bA}	34.7^{aB}	35.8^{aAB}	36.8^{bB}	36.5^{bB}	43.6^{aA}	36.8^{bB}	37.2^{aB}	47.1^{aA}
	Extensibility (mm)								
	Accelerated (45°C) Storage			**Ambient Storage (22 ± 2°C)**			**Refrigerated (4°C) Storage**		
	0 Weeks	3 Weeks	6 Weeks	0 Months	3 Months	6 Months	0 Months	6 Months	12 Months
IWG Steamed	12.0^{cA}	11.4^{cA}	11.2^{cA}	12.0^{cA}	10.9^{dA}	11.0^{cA}	12.0^{cA}	11.5^{cA}	11.5^{cA}
IWG Not Steamed	11.0^{cA}	12.5^{cA}	12.4^{cA}	11.0^{cB}	22.3^{cA}	19.5^{bA}	11.0^{cB}	21.9^{bA}	10.8^{cB}
HRW Steamed	65.0^{aA}	32.7^{bB}	27.9^{bC}	65.0^{aA}	33.5^{bB}	27.9^{aC}	65.0^{aA}	n.d.	58.6^{aB}
HRW Not Steamed	32.7^{bB}	41.7^{aA}	34.1^{aB}	32.7^{bB}	38.9^{aA}	25.5^{aC}	32.7^{bA}	40.1^{aA}	36.4^{bA}

$^{\beta}$No data; sample was improperly stored. Lowercase superscripts represent significant differences ($P \leq 0.05$) across grain samples within single time point; capital superscripts represent significant differences within a grain sample across time points according to the Fisher's LSD means comparison test.

3.5.2 Changes in Starch Pasting Profiles as Affected by Steam Treatment of IWG and HRW

The starch pasting profiles of steamed and non-steamed IWG and HRW prior to storage are shown in **Figure 7**, and the corresponding viscosity data is summarized in **Table 7** and **Table 17 (Appendix M).** Prior to storage, HRW had significantly higher values than IWG for several measured indices. This was expected as, according to Marti et al. (2015), the higher protein and fiber content of IWG interfere with gelatinization and swelling, which ultimately lowers viscosity of the starch slurry. However, in the present study, IWG had a significantly higher pasting temperature than HRW, in line with the results of Rahardjo et al. (2018) but in contrast to the results of Marti et al. (2015). This could be a result of relative changes to the protein and fiber content of IWG over breeding cycles; as the seed size has increased, protein and fiber content have decreased in IWG, yet they remain higher than those of HRW. The pasting temperatures of HRW aligned well with that observed by Rahardjo et al. (2018). However, while the pasting temperatures of steamed and non-steamed IWG were similar to the pasting temperature of whole IWG flour measured by Marti et al. (2015), it was far below the temperature of 79.6°C measured by Rahardjo et al. (2018). The discrepancy can be explained by a difference in instrumentation, as Rahardjo et al. (2018) employed a rapid visco analyzer (RVA) in place of a MVAG, which both the present study and Marti et al. (2015) used.

Pasting temperature indicates the minimum temperature necessary to produce an increase in viscosity of the starch slurry and therefore, can be used to identify processing conditions for various applications. As such, it is important to note whether various treatments of grains, including steam treatments, affect pasting temperature. In the present study, there did not appear to be a significant effect of steaming on pasting temperature for either IWG or HRW.

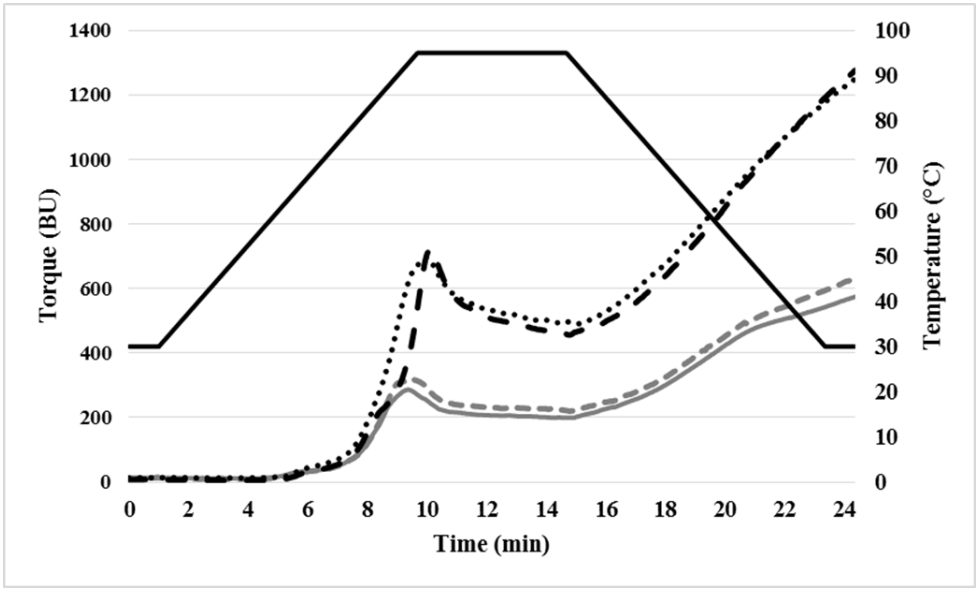

Figure 7. Starch pasting profiles of unstored IWG steamed (—), IWG not ateamed (- - -), HRW steamed (·····), and HRW non-steamed (– –), as measured by MicroVisco-Amylograph® (MVAG).

Table 7. Pasting temperature, and peak, hold, and final viscosities of steamed and non-steamed IWG and HRW as measured by moisture visco-amylograph (MVAG) over accelerated (45°C), ambient (22 ± 2°C), and refrigerated (4°C) storage.

Pasting Temperature (°C)

	Accelerated (45°C) Storage			Ambient Storage (22 ± 2°C)			Refrigerated (4°C) Storage		
	0 Weeks	3 Weeks	6 Weeks	0 Months	3 Months	6 Months	0 Months	6 Months	12 Months
IWG Steamed	62.4^{bA}	61.5^{bA}	62.9^{aA}	62.4^{bA}	60.0^{cAB}	59.3^{abB}	62.4^{bA}	58.8^{bB}	59.9^{abAB}
IWG Not Steamed	62.7^{bA}	62.1^{abA}	61.0^{aA}	62.7^{bA}	60.7^{bcA}	58.2^{bB}	62.7^{bA}	59.8^{bAB}	57.8^{bB}
HRW Steamed	64.2^{abA}	63.7^{abA}	64.6^{aA}	64.2^{abA}	62.8^{abB}	59.9^{aC}	64.2^{abA}	n.d.$^{\beta}$	62.4^{aA}
HRW Not Steamed	65.3^{aA}	64.4^{aA}	58.7^{aA}	65.3^{aA}	62.7^{aB}	59.1^{abC}	65.3^{aA}	61.8^{aB}	60.4^{abB}

Peak Viscosity (BU)

	Accelerated (45°C) Storage			Ambient Storage (22 ± 2°C)			Refrigerated (4°C) Storage		
	0 Weeks	3 Weeks	6 Weeks	0 Months	3 Months	6 Months	0 Months	6 Months	12 Months
IWG Steamed	287^{dB}	286^{cB}	395^{cA}	287^{dB}	265^{bB}	315^{bA}	287^{dAB}	313^{bA}	247^{dB}
IWG Not Steamed	333^{cA}	316^{cA}	386^{cA}	333^{cA}	267^{bB}	219^{cC}	333^{cA}	335^{bA}	316^{cA}
HRW Steamed	769^{aA}	753^{aAB}	734^{bB}	769^{aA}	687^{aB}	562^{aC}	769^{aA}	n.d.	583^{bB}
HRW Not Steamed	729^{bAB}	693^{bB}	757^{aA}	729^{bA}	695^{aB}	558^{aC}	729^{bAB}	760^{aA}	707^{aB}

Hold Viscosity (BU)

	Accelerated (45°C) Storage			Ambient Storage (22 ± 2°C)			Refrigerated (4°C) Storage		
	0 Weeks	3 Weeks	6 Weeks	0 Months	3 Months	6 Months	0 Months	6 Months	12 Months
IWG Steamed	199^{dB}	208^{dB}	274^{cA}	199^{dB}	190^{cB}	228^{bA}	199^{dB}	220^{bA}	163^{dC}
IWG Not Steamed	220^{cB}	230^{cB}	270^{cA}	220^{cA}	192^{cB}	144^{cC}	220^{cA}	224^{bA}	227^{cA}
HRW Steamed	488^{aA}	482^{aA}	431^{bB}	488^{aA}	419^{bB}	364^{aC}	488^{aA}	n.d.	371^{bB}
HRW Not Steamed	457^{bB}	441^{bB}	479^{aA}	457^{bA}	443^{aA}	352^{aB}	457^{bA}	468^{aA}	455^{aA}

Final Viscosity (BU)

	Accelerated (45°C) Storage			Ambient Storage (22 ± 2°C)			Refrigerated (4°C) Storage		
	0 Weeks	3 Weeks	6 Weeks	0 Months	3 Months	6 Months	0 Months	6 Months	12 Months
IWG Steamed	575^{bB}	627^{bB}	757^{bA}	575^{bA}	527^{bA}	594^{bA}	575^{bAB}	603^{bA}	477^{bB}
IWG Not Steamed	633^{bB}	674^{bAB}	799^{bA}	633^{bA}	541^{bB}	468^{cC}	633^{bA}	618^{bA}	613^{bA}
HRW Steamed	1248^{aA}	1372^{aA}	1174^{aA}	1248^{aA}	1180^{aA}	1083^{aA}	1248^{aA}	n.d.	1095^{aA}
HRW Not Steamed	1277^{aAB}	1320^{aA}	1174^{aB}	1277^{aA}	1245^{aA}	1052^{aB}	1277^{aA}	1166^{aA}	1256^{aA}

$^{\beta}$No data; sample was improperly stored. $^{\gamma}$BU = Brabender Units. Lowercase superscripts represent significant differences ($P \leq 0.05$) across grain samples within single time point; capital superscripts represent significant differences within a grain sample across time points according to the Fisher's LSD means comparison test.

Steamed and non-steamed IWG had peak viscosities similar to those observed by Marti et al (2015). Rahardjo et al. (2018) again measured a much higher value, which could again be attributed to differences in instrumentation. Marti et al. (2015) observed a significantly higher peak viscosity in refined HRW compared to IWG, and Rahardjo et al. (2018) observed a significantly higher value in whole HRW flour, as well. Marti et al. (2015) and Collar, Santos, and Rosell (2006) explain that fiber and protein interfere with starch granule swelling, as they compete with starch for interaction with water. The higher fiber and protein content of IWG likely explain its lower peak viscosity. In fact, Lim, Lee, Shin, and Lim (1999) reported a negative correlation coefficient between protein content and peak viscosity. As Juliano, Onate, and del Mundo (1965) explain, polypeptides can form disulfide bonds in and around starch granules, prevent swelling and disruption of the starch granule.

Steaming had a significant effect on peak and hold viscosity of IWG, with significantly lower viscosities in the steamed grain. Yadav, Kaur, et al. (2012) also observed decreased peak viscosity in pearl millet flour that was steam-treated between 10 and 25 minutes and further noted a successive decrease in peak viscosity with increased length of steaming. Ovando-Martínez, Whitney, Reuhs, Doehlert, and Simsek, (2013) further noted this trend in oats that were steamed at 106°C for 20 minutes. The decrease in starch pasting viscosities following hydrothermal treatment of flour could be due to disintegration of starch granules according to Rasper (1980). The opposite effect was observed for HRW, with an increase in peak viscosity due to steaming. The effect seen in steamed HRW has also been seen by Hu, Xing, and Ren (2010) and was explained by changes to the morphology of starch granules due to steaming. A high peak viscosity is indicative of good water holding capacity and granule swelling ability, both directly impacting ease of cooking and baking (CW Brabender). Ma and Baik (2018) found a positive correlation between peak viscosity and specific volume of biscuits. In the present study, positive correlations between peak viscosity and specific loaf volumes were found at both ambient storage temperature ($r = 0.834$, $P < 0.01$) and refrigerated storage temperature ($r = 0.517$, $P < 0.05$) (**Table 19-20, Appendix N**). In part, the water

absorbed by starch during gelatinization helps to maintain the porous structure within the gluten matrix of dough and promote elastic crumb development (Sandstedt, 1961).

There was also a significant difference in hold viscosity between IWG and HRW, similar to the observations of Rahardjo et al. (2018) and Marti (2015). IWG, steamed and non-steamed, had significantly lower breakdown values, a measure of the decrease in viscosity, than steamed and non-steamed HRW. Marti et al. (2015) and Rahardjo et al. (2018) also observed significantly lower breakdown values in IWG as compared to HRW. Marti et al. (2015) further demonstrated this difference to be independent of differences in starch concentration between IWG and HRW and attributed the lower breakdown value of IWG to its high protein and fiber content, which compete with starch for water.

Steaming significantly decreased both the hold viscosity and breakdown value of IWG. Ovando-Martínez et al. (2013) also saw a decrease in breakdown value in steamed oats. As previously noted, this decrease could be due to disintegration of starch granules following steam treatment (Rasper, 1980). The breakdown value is indicative of rigidity of the swollen starch granule and degree of susceptibility to disintegration of the starch granules. (Ma & Baik, 2018) found a significant correlation between breakdown value with specific loaf volume. In the present study, the breakdown value and specific loaf volumes were significantly correlated at ambient temperature ($r = 0.815$, $P < 0.01$) and refrigerated temperature ($r = 0.551$, $P < 0.01$) (**Table 19-20, Appendix N**). In HRW, steaming did not have an impact on breakdown value. Steamed HRW had a higher hold viscosity, as was the trend with peak viscosity, and could be explained by the same rationale. The trends in peak and hold viscosity and breakdown value suggest that steaming, while beneficial to HRW in terms of baking ability, may alter how IWG might be used in baking. However, starch pasting profiles must be evaluated as a whole in order to determine overall processability and applicability.

HRW, steamed and non-steamed, showed significantly higher final viscosities and setback values than steamed and non-steamed IWG. Although the amylose to amylopectin ratio in IWG is similar to that in HRW (**Table 1**), the overall starch content is lower in IWG, which explains its lower final viscosity and setback values. The

measured values for final viscosity and setback value of steamed and non-steamed IWG were slightly higher than the values recorded by Marti et al. (2015). The higher values of final viscosity and setback value, as well as peak viscosity and breakdown value, may be due to an increase in seed size **(Table 13, Appendix J)**, and therefore, an increase in starch content of IWG, with successive harvests due to breeding efforts. In fact, Marti et al. (2015) measured a starch content of only 46.7 g per 100 g flour (dry basis), whereas the present study measured a starch content of IWG of 58.0 g per 100 g flour (dry basis).

Final viscosity, also referred to as "cold paste viscosity", reflects the stabilized viscosity of the paste upon cooling. Setback is calculated as the difference between final viscosity and minimum viscosity and represents the extent of increase in viscosity upon cooling. Final viscosity and setback value are together indicative of susceptibility to retrogradation and syneresis. Retrogradation is the phenomenon in which leached amylose molecules reassociate to form double helices, and leached amylopectin recrystallizes, which together manifest as staling and hardening of the final product (Singh, Singh, & Kaur, 2003; Alcázar-Alay & Meireles, 2015). Retrogradation can be undesirable as it causes staling and loss of crispness of baked goods, as well as changes to flavor and aroma (Morris, 1990). Retrogradation also reduces starch digestibility. However, it can be desirable in products such as pasta as it increases hardness and reduces stickiness (Farhat, 2004). Amylose molecules reassociate much faster than amylopectin molecules and therefore, have a more substantial influence on retrogradation (Singh & Anderson, 2004). Syneresis is a phenomenon in which unbound water separates from the gelled starch (Cui, 2005). This causes watery, inconsistent textures in end products and contributes to staling of baked goods. Lower final viscosity and setback value are related to a lowered tendency to retrogradation and syneresis (Ji, Zhu, Zhou, & Qian, 2010). Therefore, the lower final viscosity and setback values of IWG compared to IWG are promising for the textural stability of products made with IWG over storage.

Steaming did not have a significant effect on either final viscosity or setback value of IWG. Steamed HRW did exhibit a significantly lower setback value than non-steamed HRW, but a comparable final viscosity. These results are contrary to those seen by Yadav, Kaur, et al. (2012), who measured decreases to peak and final viscosity with

hydrothermal treatment. The differences in observations could be due to differences in steam treatment method. In the treatment used by Yadav, Kaur, et al. (2012), the millet grains were soaked to raise the moisture to 30% and subsequently autoclaved, whereas in the present study, wheat and IWG groats were steamed in a proofing oven at their original moisture levels. Too low of a final viscosity indicates a low stability of the cooled starch paste under shear. However, too high of a final viscosity indicates a greater tendency of the starch to undergo retrogradation and syneresis (Singh & Anderson, 2004). Therefore, it is desirable that steam treatment does not markedly affect final viscosity, as either extreme can limit the use of a cereal grain.

3.5.3 Effect of Storage Temperature and Time on Rheological Properties of IWG and HRW Dough

Storage affected various parameters, including water absorption, dough stability time, stability arrival, and dough development time. Water absorption values significantly decreased in steamed grains after 6 weeks of accelerated storage at 45°C, as well as in steamed HRW after 12 months of storage at 4°C **(Table 5**). Storage of grains has been shown to increase water absorption and hydration rate in dough (Wang & Flores, 1999; Kibar, 2015), however this was generally not the case, with the exception of non-steamed IWG over ambient storage. Higher water absorption values have been correlated with greater dough stability and larger bread loaf volumes. The increase in water absorption values of non-steamed IWG over ambient storage was therefore desirable. Furthermore, at the end of ambient storage and refrigerated storage, non-steamed IWG and steamed IWG, respectively, had water absorption values comparable to those of wheat.

Changes in dough stability time were only observed in HRW, with significant increases to dough stability time of steamed HRW at 45°C and both steamed and non-steamed HRW at ambient (22 ± 2°C). Dough stability time is a predictor of overall gluten strength (Aydoğan et al., 2015), which is desirable for baking applications. IWG did not show these same improvements to dough stability time over storage. It is promising, however, that steam treatment did not negatively affect dough stability time of IWG over storage, as it is already significantly lower than that of HRW.

Storage, especially at 45°C, resulted in an increase of stability arrival and dough development time for all samples except for non-steamed IWG. The effect of temperature on dough stability, stability arrival, and development time was significant ($P < 0.01$). Cenkowski (2000) noted a similar effect of storage temperature, with significant increases to stability and mixing times over 40°C storage compared to storage at lower temperatures. Steaming also had an effect on stability arrival and dough development time over storage. At 45°C, stored, steamed grains had both higher stability arrival times and dough development times than non-steamed grains. These trends were not observed over ambient and refrigerated storage. Dough mixing time, including dough arrival time and development time, is a predictor of overall dough strength, with higher mixing times correlating with larger bread loaf volumes, both in the present study ($r = 0.734$-0.961, $P < 0.001$) (Aydoğan et al., 2015). Therefore, the higher dough development times and arrival times induced by storage and steam treatment are positive outcomes in terms of baking applications.

Dough strength, as measured by resistance to extension and extensibility, varied over storage depending on storage temperature and steam treatment. Resistance to extension increased over all temperatures in steamed IWG and over ambient and 4°C storage in non-steamed IWG **(Table 6)**. However, in HRW, resistance to extension decreased over all temperatures in steamed grains, yet increased over ambient and 4°C storage in non-steamed grains. Extensibility of HRW also decreased over ambient storage of non-steamed HRW and over all storage temperatures in steamed HRW, while it did not change for steamed IWG and increased in non-steamed IWG over ambient storage. The differences in trends between IWG and HRW may be explained by differences in dietary fiber content. Differences in resistance to extension and extensibility trends between IWG and HRW over storage could additionally be due to the higher presence of accessible thiols in IWG (Becker et al., 1991; Marti et al., 2015) which could help to stabilize the gluten network via disulfide linkages (Wrigley & Békés, 1999). As thiols are oxidized over storage, they form disulfide bridges between glutenin and gliadin proteins, stabilizing the gluten matrix and thus, enhancing gluten strength (Tipples, 1995).

Length of storage can also impact gluten strength. On one hand, short-term storage has been shown to improve dough strength and gas-holding capacity (Wang & Flores, 1999; Kibar, 2015). On the other hand, prolonged storage can induce conformational changes to the gluten forming proteins that ultimately results in loss of elasticity, reduced hydration capacity, and decreased dough stability (Cenkowski, Dexter, & Scanlon, 2000; Kibar, 2015; Baik & Donelson, 2018). These results may explain why extensibility of non-steamed HRW increased mid-storage at all temperatures and significantly decreased by the end of storage, a trend that was also noted in non-steamed IWG at 4°C.

Steam treatment had a more pronounced effect on the resistance to extension and extensibility of HRW over storage, but did not appear to affect IWG in the same way. Although steaming HRW initially increased its resistance to extension and extensibility prior to storage, steamed HRW showed significant decreases in extensibility across all three storage temperatures over storage, as well as significant decreases in resistance to extension at 45°C and ambient storage. Complex interactions between lipids, starch, and proteins, further altered by steam treatment, throughout storage could partially explain the negative effect of steaming on gluten strength over storage. Zhang & Hamaker (2005) identified a three-component interaction system in sorghum involving starch, lipid, and protein that impacted overall functionality of the grain. Further work exploring how these components interact in IWG and wheat grains over storage and in response to steam treatment may elucidate the mechanisms behind loss of gluten strength in steamed HRW over storage.

Prior to storage, steam treatment did not have a significant effect on resistance to extension or extensibility of IWG. The effect of steam treatment on IWG over storage varied by storage temperature, with increases in resistance to extension of steamed IWG at 45°C and 4°C and decreases at ambient temperature. At the end of storage at 45°C, steamed IWG had significantly higher resistance to extension than non-steamed IWG, however following ambient and refrigerated storage, non-steamed IWG had significantly greater resistance to extension. Steaming negatively impacted extensibility of IWG during storage at ambient temperature, but no significant effects were noted at 45°C or

4°C. Manufacturers and grain processers can capitalize on these results to choose the conditions and treatments most favorable to either elastic or extensible end products.

3.5.4 Effect of Storage Temperature and Time on the Starch Pasting Profiles of IWG and HRW Flour

Changes to starch pasting properties over storage, namely pasting temperature, peak, hold, and final viscosities, breakdown value, and setback value, varied by storage temperature. Over 6 weeks of accelerated storage at 45°C, steamed IWG had a significant increase in peak and hold viscosities **(Table 7)**. Steamed HRW, however, had a significant decrease in peak viscosity over 6 weeks at 45°C and no significant change to hold viscosity. Both non-steamed IWG and HRW showed no significant changes to peak viscosity over accelerated storage, however showed significant increases in hold viscosity. Changes to starch pasting viscosities over storage can be impacted by amylose lipid complexes (AM-Ls). However, there are different theories as to how they impact viscosity.

The first theory suggests that over storage, peak viscosity and hold viscosity can increase due to the formation of AM-Ls. AM-L complexes are inclusion complexes that form when starch and water are heated together (Kugimiya et al., 1980). The amylose takes on a helix conformation within which the hydrophobic end of an aliphatic ligand, such as a free fatty acid, resides (Conde-Petit & Escher, 1992). The insoluble AM-L complexes aggregate in an aqueous system and form an intergranular network that results in increased viscosity values (Conde-Petit & Escher, 1992; Putseys et al., 2010). Amylopectin can also complex with free fatty acids over storage and increase viscosity by increasing rigidity of the starch granules (Conde-Petit & Escher, 1992). This theory could explain the increased peak and hold viscosity values of steamed IWG and the increased hold viscosity values of non-steamed IWG and HRW.

Alternatively, it has been theorized that AM-L complexes could sterically impair swelling of the starch granule, thereby lowering viscosity values (Putseys et al., 2010). Steamed HRW exhibited a different behavior over 45°C storage than steamed IWG, with

a decrease in peak viscosity. The differences in starch pasting viscosity trends of IWG and HRW is likely due to differences in the starch morphology of the two grains. Future research elucidating the grains' starch structures may further the understanding of why these grains react differently to storage.

The breakdown value did not significantly change over accelerated storage for steamed IWG, non-steamed IWG, or non-steamed HRW, while steamed HRW showed a significant increase in breakdown value **(Table 17, Appendix M).**

At ambient (22 ± 2°C) storage, steamed IWG again showed significant increases to peak and hold viscosity **(Table 7)** with no corresponding change to breakdown value **(Table 17, Appendix M)**. Non-steamed IWG, along with steamed and non-steamed HRW, on the other hand, showed significant decreases in both peak and hold viscosity, with corresponding decreases in their respective breakdown values. These results were not consistent with those over accelerated storage, therefore showing an effect of storage temperature on starch pasting behavior. In fact, storage temperature had a significant effect on peak viscosity, hold viscosity, and breakdown value ($P < 0.01$). Prolonged storage at ambient temperature resulted in significant increases in free fatty acid content of steamed and non-steamed IWG and HRW **(Figure 4).** At room temperature, free fatty acid content had a strong, negative correlation with both peak viscosity ($r = -0.873$, $P < 0.01$) and hold viscosity ($r = -0.866$, $P < 0.01$) (**Table 19, Appendix N**). This further supports the theory of Putseys et al. (2010) that inclusion complexes involving free fatty acids and amylose released over storage can sterically inhibit starch granule swelling and lower overall pasting viscosity values, albeit this was not the case for all samples. Additionally, causation statements are limited.

Over refrigerated (4°C) storage, there were minimal changes to peak viscosity, with the exception of a significant decrease to peak viscosity of steamed HRW, as was seen at 45°C and ambient storage temperatures **(Table 7**). Steamed IWG and HRW showed a significant decrease in hold viscosity, consistent with ambient storage results, however non-steamed IWG and HRW did not show significant changes in hold viscosity over refrigerated storage. As discussed in **2.5.6,** free fatty acid content minimally increased over refrigerated storage in non-steamed IWG, as well as steamed and non-

steamed HRW, while it significantly increased in steamed IWG following 12 months of storage at 4°C. Thus, fewer free fatty acids were available to complex with amylose, potentially resulting in a minimally altered peak viscosity. When not considering the effect of AM-L complexes, the effect of steaming on starch pasting viscosities becomes more evident. As discussed in **3.5.2**, steam treatment lowers starch pasting viscosity values by disintegrating starch granules (Rasper, 1980). This may explain the significant decrease in hold viscosity of steamed grains over storage at 4°C, while no change was observed for non-steamed grains. Steamed HRW also showed significant decreases in peak viscosity over 45°C.

The effects of steam on peak and hold viscosities, as well as breakdown value, over storage are important to note due to the implications on the texture of various food products. Although steamed HRW had a higher peak viscosity than non-steamed HRW prior to storage at 45°C and 4°C, it had a significantly lower peak viscosity at the end of storage (6 weeks and 12 months, respectively). Similarly, steaming significantly increased the hold viscosity of HRW prior to storage but ultimately, steamed HRW had significantly lower hold viscosity at the end of storage at all temperatures as compared to non-steamed HRW. These trends in steamed HRW over storage were the same as noted for resistance to extension and extensibility. In fact, peak viscosity and hold viscosity had strong, positive correlations with both resistance to extension and extensibility (**Table 18-20, Appendix N**). Again, interactions among starch, protein, and lipid throughout storage are complex and even more so in reaction to steam treatment. Better understanding these complex interactions could further understanding the trends observed.

The effects of steaming on the peak and hold viscosities of IWG were less straightforward. Prior to storage, steaming significantly decreased both peak and hold viscosities. However, this difference was not maintained over storage at 45°C. At refrigerated storage, steamed IWG did show significantly lower peak and hold viscosity values, yet the opposite was true over ambient storage.

Changes to final viscosity and setback value over storage of grains are important to understanding the susceptibility of starch to retrogradation and syneresis. As with the previously discussed starch pasting properties, storage temperature had a significant

effect on changes to final viscosity and setback value ($P < 0.01$). Over accelerated (45°C) storage, both steamed and non-steamed IWG showed significant increases in final viscosity, however only steamed IWG showed a significant increase in setback value (**Table 7)**. Again, these increases could be explained by Conde-Petit & Escher's (1992) theory of AM-L effects. Neither steamed nor non-steamed HRW showed significant changes to final viscosity or setback value over accelerated storage. As previously discussed, the increases in viscosity of IWG not otherwise seen in HRW may potentially be attributed to differences in starch morphology between the grains, which warrants further study. Although IWG grain showed a significant increase in final viscosity (as well as a significant increase in setback value for steamed IWG), the values were still significantly below those of HRW. Typically, a higher final viscosity and setback value can indicate greater susceptibility to syneresis and retrogradation, both of which can lead to undesirable textural changes. However, AM-L formation over storage may, in fact, sterically hinder retrogradation (Eliasson & Ljunger, 1988). Therefore, the significant increase to final viscosity of IWG at 45°C cannot be confirmed to directly impact retrogradation without further investigation.

Over ambient (22 ± 2°C) storage, non-steamed IWG and HRW showed significant decreases to final viscosity over 6 months of storage, while steamed grains showed no significant change **(Table 7**). Only non-steamed IWG showed a significant decrease to setback value, while setback value remained unaffected in steamed IWG, and HRW (steamed and non-steamed). Decreases to the final viscosity values of non-steamed grains over ambient storage mirrored the results seen in non-steamed grains for peak and hold viscosity. At refrigerated (4°C) storage, there were no significant changes to final viscosity or setback value of steamed or non-steamed IWG or HRW. This makes sense considering the results shown in **2.5.6** demonstrating minimal free fatty acid formation over refrigerated storage. As such, it can be concluded that IWG and HRW grains can be stored at refrigerated temperatures for prolonged periods without implications on final viscosity and setback value. Ultimately, this may reduce retrogradation and syneresis in end products, however, further textural analysis of stored end products is needed to confirm this.

Few changes to final viscosity and setback value were observed as a result of steam treatment. With regards to final viscosity, steaming only had a significant effect at ambient temperature, with a significantly higher final viscosity in steamed IWG as compared to non-steamed IWG following 6 months of storage. While steaming had an effect on the setback value of HRW prior to storage, no effects were observed in either IWG or HRW over storage at all temperatures.

Future research to directly measure textural changes in products, such as baked goods and pasta, made from the flour of stored, steamed IWG grains may be helpful to fully examine the effect of steam on starch pasting properties. Furthermore, changes to α-amylase and amylose content over storage can impact starch pasting properties Amylose molecules reassociate much faster than amylopectin molecules and therefore, have a more substantial influence on retrogradation (Singh & Anderson, 2004). Considering the important role of α-amylase in starch pasting changes and AM-L formation, future research focusing on the measurement of α-amylase activity over storage of steamed grains could help fully understand starch pasting changes, particularly changes to final viscosity and setback value.

3.5.3 Baking Quality over Storage of Treated IWG and HRW

Table 8 shows loaf volume, bread height, and bread firmness measurements in bread made from grain stored at ambient (22 ± 2°C) and refrigerated (4°C) temperatures. Bread loaf volume increased slightly over ambient storage for non-steamed IWG and decreased slightly over storage in steamed HRW, but there were no significant changes to loaf volume of steamed IWG or non-steamed HRW. Over refrigerated (4°C) storage, non-steamed IWG again showed a significant increase to loaf volume, and both steamed and non-steamed HRW showed a significant decrease to loaf volume. Bread height was not significantly affected over storage in any samples over ambient storage, however steamed and non-steamed HRW showed significant decreases to bread height over 4°C storage.

The effect of storage on loaf volumes and heights was dependent on the length of storage and storage temperature. Short term storage has been shown to improve bread

loaf volumes and heights via oxidation of thiols, inducing disulfide bridge formation between glutenin and gliadin proteins, thus enhancing gluten strength (Tipples, 1995). Both oxidation and hydrolysis of lipids over prolonged storage can negatively impact loaf volumes (Sullivan et al., 1936). Schaich and Karel (1976) explained that free radicals generated by lipid peroxidation can cause denaturation of proteins, along with destruction of individual amino acids, polymerization, and cross-linking. This may explain the negative effects of prolonged storage at 4°C, where non-steamed HRW experienced a decrease in loaf volume and both steamed and non-steamed HRW experienced a significant decrease in loaf heights after 12 months of storage. These effects were not noted in steamed and non-steamed HRW over 6 months of ambient storage.

Free fatty acids released via lipolytic action have also been negatively correlated with loaf volumes due to their proposed interference with shortening lipids and reaction with leavening agents such as sodium bicarbonate (Bell, Daniels, & Fisher, 1977; Tait & Galliard, 1988; Bookwalter et al., 1991). In fact, in the present study over ambient and refrigerated storage temperatures, free fatty acid concentration was negatively correlated with bread loaf volume ($r = -0.704$ and -0.449, respectively, $P < 0.05$) and bread height ($r = -0.860$ and -0.761, respectively, $P < 0.05$) (**Table 19-20, Appendix N**). There were not noted differences in loaf volume and height trends between 6 months of ambient and 12 months of 4°C storage of IWG. These results indicate that IWG may withstand longer storage times without detrimental effects on loaf volume and height.

In addition to length of storage, steaming also had a temperature-dependent influence on loaf volumes and height. While steaming was initially effective at improving loaf volumes of IWG and HRW, there were no significant differences in loaf volume between steamed and non-steamed grain after 6 months of ambient storage. However, after 12 months of refrigerated storage, steamed HRW had significantly higher bread loaf volumes and height than non-steamed HRW. Interestingly, refrigerated storage is the only temperature where steamed HRW did not see decreases to peak and hold viscosity, resistance to extension, and extensibility, all of which correlate with dough strength. Perhaps the retention of starch pasting viscosity, elasticity and extensibility in steamed

HRW over refrigerated storage enabled steamed HRW to maintain its higher loaf volumes.

Alternatively, heat treatment of grains can lead to denaturation of the more heat sensitive monomeric proteins with subsequent exposure of hydrophobic groups, leading to protein aggregation, formation of disulfide bonds, and protein insolubility (Arntfield et al., 1997; Fasina et al., 1999; Sun et al., 2006; Žilić et al., 2010; Deepa & Umesh Hebbar, 2017). This may explain why only non-steamed IWG showed increases to loaf volume over ambient and refrigerated storage where steamed IWG did not. This makes sense in light of differences in resistance to extension between steamed and non-steamed IWG over ambient and refrigerated storage. While non-steamed IWG had increases to resistance to extension over ambient and refrigerated storage, which positively correlated with loaf volumes ($r = 0.460\text{-}0.798$, $P < 0.01$), steamed IWG had decreases in these values.

Bread firmness remained largely unaffected over storage, with only a slight significant decrease to firmness occurring in non-steamed HRW after 6 months of ambient storage. Although steaming originally decreased bread firmness in HRW, there were no significant differences between steamed and non-steamed HRW over storage. Steaming did not have an effect on bread firmness in IWG. Although steam treatment had some effects on HRW in terms of loaf volume and bread firmness, it is promising that it did not appear to affect the functionality of IWG. In this respect, the present research supports the hypothesis that steam treatment does not negatively affect the functionality of IWG.

Table 8. Loaf volume, height, and firmness of bread made with steamed and non-steamed IWG and HRW over (ambient 22 ± 2°C), and refrigerated (4°C) storage.

Loaf Volume (mL/g)

	Ambient (22 ± 2°C) Storage			Refrigerated (4°C) Storage		
	0 Months	3 Months	6 Months	0 Months	6 Months	12 Months
IWG Steamed	2.06^{cA}	2.07^{bA}	2.07^{bA}	2.06^{cA}	1.94^{cB}	1.99^{bAB}
IWG Not Steamed	1.72^{dB}	2.03^{bA}	1.98^{bA}	1.72^{dC}	2.05^{bA}	1.89^{bcB}
HRW Steamed	2.64^{aA}	2.31^{aB}	2.35^{aB}	2.64^{aA}	n.d.$^{\beta}$	2.16^{aB}
HRW Not Steamed	2.43^{bA}	2.25^{aA}	2.29^{aA}	2.43^{bA}	2.34^{aA}	1.83^{cB}

Bread Height (cm)

	Ambient (22 ± 2°C) Storage			Refrigerated (4°C) Storage		
	0 Months	3 Months	6 Months	0 Months	6 Months	12 Months
IWG Steamed	3.16^{bA}	3.15^{bA}	3.18^{bA}	3.16^{bA}	3.18^{bA}	3.19^{cA}
IWG Not Steamed	3.07^{bA}	3.08^{bA}	3.06^{bA}	3.07^{bA}	3.16^{bA}	3.18^{cA}
HRW Steamed	4.35^{aA}	3.96^{aB}	4.19^{aA}	4.35^{aA}	n.d.	4.03^{aB}
HRW Not Steamed	4.10^{aA}	3.95^{aA}	4.08^{aA}	4.10^{aA}	3.98^{aA}	3.59^{bB}

Bread Firmness (g)

	Ambient (22 ± 2°C) Storage			Refrigerated (4°C) Storage		
	0 Months	3 Months	6 Months	0 Months	6 Months	12 Months
IWG Steamed	6.15^{aA}	5.99^{aA}	6.14^{aA}	6.15^{aA}	6.02^{aA}	6.04^{abA}
IWG Not Steamed	6.14^{aA}	6.08^{aA}	6.01^{abA}	6.14^{aA}	6.07^{aA}	6.11^{aA}
HRW Steamed	5.81^{cA}	5.82^{bA}	5.86^{bcA}	5.81^{cA}	n.d.	5.84^{cA}
HRW Not Steamed	5.97^{bA}	5.84^{bB}	5.76^{cB}	5.97^{bA}	5.80^{bB}	5.95^{bcA}

$^{\beta}$No data; sample was improperly stored. Lowercase superscripts represent significant differences ($P \leq 0.05$) across groups within single time point; capital superscripts represent significant differences within groups across time points according to the Fisher's LSD means comparison test.

3.6 Conclusions

Over storage, some aspects of IWG functionality were improved. Increases to dough development time, resistance to extension, and loaf volumes denote an improvement to IWG dough strength over storage (Cenkowski, 2000). Effects of steaming on dough strength parameters over storage were temperature dependent. Steaming did not have an effect on bread loaf volumes over storage.

IWG also had improvements to starch pasting viscosities over storage, including peak viscosity and hold viscosity. These improvements could improve specific volume of baked goods as a function of improved starch gelling ability (Ma & Baik, 2018). Storage temperature had a significant effect on final viscosity. Increases to final viscosity may indicate increased susceptibility to retrogradation (Singh et al., 2003), however these conclusions are limited without further textural analysis of end products. Effect of steam treatment was again temperature dependent, with higher viscosity values in steamed IWG after storage at ambient storage.

HRW had improvements to dough stability and dough development time over storage, however loaf volumes and heights were negatively impacted by storage, particularly in steamed HRW, likely due to accompanying decreases to resistance to extension. Extensibility of HRW also decreased with storage, albeit steaming appeared to improve extensibility values.

Storage also negatively impacted starch pasting viscosities of HRW, and steaming further decreased these values over storage. Both grains had decreases in breakdown value over storage, which could potentially negatively impact bread volumes. Bread firmness was not significantly affected over storage in either grain.

Overall, these results highlight a positive effect of storage on functionality of IWG. The effects of steaming on functionality were temperature-dependent, with positive effects on dough strength at 45°C and improvements to starch pasting viscosities at ambient temperature. Steaming appeared to have a negative impact on functionality at refrigerated storage temperatures. Although overall functionality of IWG remained inferior to HRW throughout storage, HRW was inferior to IWG in its ability to maintain functionality over storage, with significant decreases to several functionality parameters over storage.

Chapter 4: Conclusions, Recommendations, and Implications

The steam treatment used in the present study was not effective at inactivating lipase and lipoxygenase. In spite of enzyme retention, IWG groats exhibited acceptable stability over storage. Development of oxidative and hydrolytic rancidity in IWG was minimal throughout storage of groats. Storage also induced increases to antioxidant activity and hydroxycinnamic acid content, particularly over higher (45°C) storage temperatures. Refrigerated storage was most effective at delaying oxidative and hydrolytic rancidity, as well as preserving carotenoid and hydroxycinnamic acid content. Although steaming was not able to inactivate enzymes, it demonstrated a minimal, but positive effect on antioxidant activity and content, as well as a slight inhibitory effect on hydroperoxide formation over storage.

Moreover, storage of IWG groats had positive impacts on functionality, with improvements to loaf volumes, dough development time, resistance to extension, and starch pasting viscosities. Effect of steam treatment on IWG functionality over storage was again temperature dependent, with steam treatment improving dough development time and resistance to extension at 45°C and starch pasting properties over ambient storage. In light of these results, we accept the hypothesis that steam treatment and extended storage under different conditions will not have a negative effect on dough functionality and breadmaking. In fact, prolonged storage improved several functionality parameters, and steaming appeared to have a synergistic effect with storage at certain temperatures (45°C and 22°C) on improving some aspects of dough strength.

In addition, the results of the present study showed that IWG has a competitive storage stability compared to wheat. Despite relatively higher fat content, IWG's lower lipoxygenase activity, superior antioxidant content and activity, and relatively lower hydroperoxide content over storage render it less susceptible to oxidative rancidity than wheat. In terms of hydrolytic rancidity, IWG showed higher lipase activity and free fatty acid concentration than wheat throughout storage. These results make it necessary to optimize steam treatment in order to significantly inactivate lipase and further retard hydrolytic rancidity. Although overall functionality of IWG remained inferior to HRW

throughout storage, HRW was inferior to IWG in its ability to maintain functionality over storage, with significant decreases to several functionality parameters over storage, particularly loaf volumes and starch pasting viscosities.

The present study formed the foundation for establishing a more effective method of steam treatment in a currently ongoing storage study on IWG flour. Understanding the storage stability of flour will provide a more complete picture of IWG's storage stability. The present study also informed additional amendments to the storage study of flour, including the measurement of secondary oxidation products, flavor compounds, and sensory changes. Secondary oxidation products can contribute to off-flavor of stored cereal grains, especially in milled grains, and are a therefore an important determinant of sensory acceptability (Heiniö et al., 2002). Future storage studies on IWG groats and flour may be necessary, as with continued breeding efforts to increase seed size, the composition of IWG may continue to change. Genetic selection of IWG lines with lower lipoxygenase and lipase content is recommended. In addition to the amendments made to the currently ongoing storage study of flour, additional studies should measure textural changes in stored IWG end products in order to better understand the relationship between starch pasting profile changes and end use application. While the present study saw increases in final viscosity of IWG, it is unclear whether and how this will affect retrogradation in final products. Measuring α-amylase activity/falling number of IWG over storage is also recommended, as this will help understand changes to starch pasting profiles and amylose-lipid complex formation.

IWG has long been recognized for its superb environmental benefits (Glover et al., 2010; Culman et al., 2013). In recent decades, IWG was identified as a potential perennial candidate for commercial food use, owing to its relatively superior agronomic properties in relation to other perennials, as well as its excellent nutrient profile (Wagoner, 1990; Becker et al., 1991). Integrating a perennial cereal grain into the food market is a novel idea, one that requires not only extensive breeding efforts to increase yields and improve ease of harvest, but also requires providing meaningful incentives to farmers that prove its marketability. Breeders and agronomists have made excellent headway in enlarging seed size and yield, reducing seed shattering, and a shortening

plant height which prevents lodging (Glover et al., 2010; Zhang et al., 2016). Food scientists have had several strides toward the characterization of IWG evaluation of its end use application as a stand-alone grain and in combination with other cereal grains (Marti et al., 2015; Rahardjo et al., 2018; Catrin Tyl & Ismail, 2018). The present study provides additional incentives to farmers and food manufacturers alike by highlighting its competitive storage stability. The present study demonstrated not only its high tolerance to storage but in some regards, its positive response to storage. Furthermore, this study provides foundational information on how to improve the storage stability of IWG through steam treatment.

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Appendix A: Covance Results

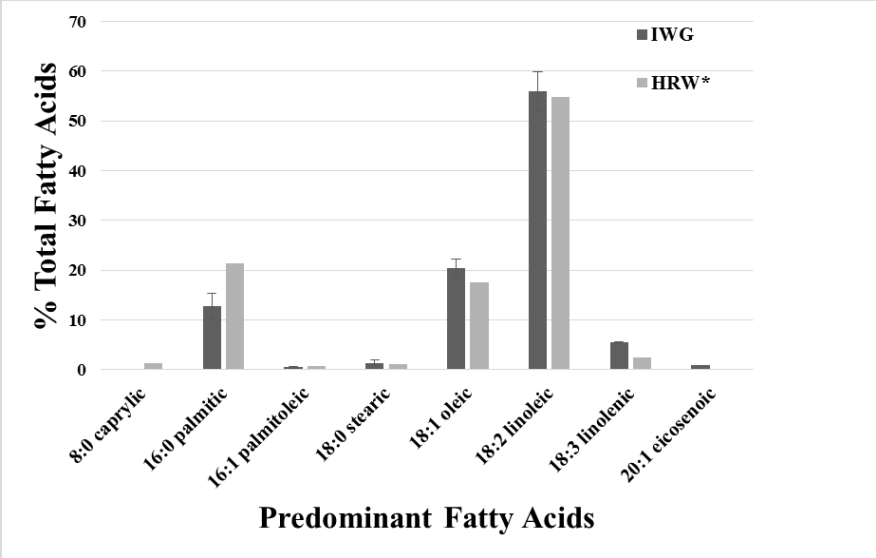

Figure 8. Predominant fatty acids in intermediate wheatgrass (IWG) and hard red wheat (HRW). Error bars represent standard deviation (n = 2). *HRW data from USDA Food Composition Database.

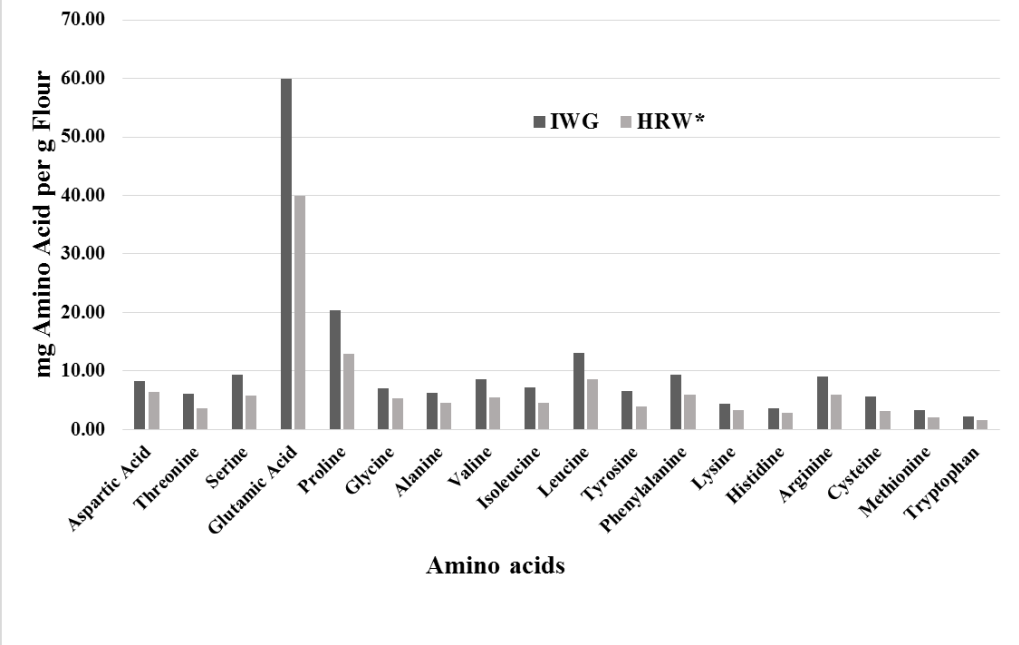

Figure 9. Amino acid profile of intermediate wheatgrass (IWG) and hard red wheat (HRW). *HRW data from USDA Food Composition Database.

Table 9. Mycotoxin levels of IWG and HRW as compared to advisory levels.

Mycotoxin	IWG	HRW	Advisory Levels
	ppb		
15 acetyl deoxynivalenol	<167	<167	
aflatoxin B1	<0.400	<0.400	2 (EU); 20 (sum B1-G2) (FDA)
aflatoxin B2	<0.400	<0.400	20 (sum B1-G2) (FDA)
aflatoxin G1	<0.400	<0.400	20 (sum B1-G2) (FDA)
aflatoxin G2	<0.400	<0.400	20 (sum B1-G2) (FDA)
alpha ergocryptine	271	378	
alpha zearalenol	<28	<28	
beauvericin	0.745	1.43	
citrinin	<200	<200	
deepoxy-deoxynivalenol	<19.0	<19.0	
deoxynivalenol	128	424	1000 (FDA) and 1250 (EU)
deoxynivalenol 3 glucoside	<166	<166	
diacetoxyscirpenol	<15	<15.0	
enniatin A	1.04	0.537	
enniatin A1	1.48	1.59	
enniatin B	5.14	3.62	
enniatin B1	5.31	5.98	
ergocornine	193	162	
ergocristine	633	354	
ergosine	140	31.6	
ergotamine	189	189	
fumagillin	<15.0	<15.0	
fumonisin B1	<19.0	<19.0	
fumonisin B2	<19.0	<19.0	
fumonisin B3	<38.0	<38.0	
fusarenon X	<417	<417	
HT-2 toxin	<19.0	<19.0	
neosolaniol	<200	<200	
nivalenol	<278	<278	
ochratoxin A	<0.700	<0.700	5 (EU)
patulin	<76.0	<76.0	
penicillic acid	<15.0	<15.0	
phomopsim A	<46.0	<46.0	
sterigmatocystin	<0.300	<0.300	
T-2 tetraol	<138	<138	
T-2 toxin	<2.00	<2.00	
T-2 triol	<140	<140	
verruculogen	<76.0	<76.0	
zearalenone	52.2	<6.00	100 (EU)

Table 10. Phytic acid concentration of intermediate wheatgrass (IWG) and hard red wheat (HRW).

Sample	mg/g phytic acid
IWG	14.2
HRW	9.54

Table 11. Trypsin inhibitor concentration of intermediate wheatgrass (IWG) and hard red wheat (HRW).

Sample	TIU*/mg
IWG	<1.00
HRW	<1.00

*TIU = trypsin inhibitor unit

Table 12. Gliadin concentration of intermediate wheatgrass (IWG).

Sample	ppm gliadin
IWG	<5

Appendix B: Sample Calculation for Lipoxygenase Activity

Equation 1.

Lipoxygenase activity (U/g)

$$= \frac{(A_s - A_b)}{\varepsilon_{mM}} \times Vol_{reaction}\ (L) \times \frac{Vol_{Extraction}\ (mL)}{Vol_{Aliquot}\ (mL)} \times \frac{1}{t \times l\ \times Sample\ Weight\ (g)} \times \frac{1000\ \mu moles}{1\ mmol}$$

Where:

A_s = Absorbance of sample at 560 nm = 0.404

A_b = Absorbance of blank at 560 nm = 0.08

ε_{mM} = 47 $mM^{-1} \cdot cm^{-1}$ (Molar extinction coefficient for lipid hydroperoxides in a methanol-based reagent) (Vega et al., 2005)

$Vol_{Reaction}$ = Total reaction volume in L = 0.003 L

$Vol_{Extraction}$ = Total extraction volume in mL = 20 mL

$Vol_{Aliquot}$ = Total aliquot assayed in mL = 0.010 mL

t = incubation time in minutes = 15 min

l = path length = 1 cm for standard cuvette

Sample Weight (d.b.) = 1.0095 g

Lipoxygenase activity (U/g)

$$= \frac{(0.404 - 0.08)}{47\ mM^{-1} \cdot cm^{-1}} \times 0.03\ L \times \frac{20\ mL}{0.010\ mL} \times \frac{1}{15\ min \times 1\ cm \times 1.0095\ g} \times \frac{1000\ \mu moles}{1\ mmol}$$

$$= \frac{\mathbf{2.74\ \mu moles}}{\mathbf{min \cdot g}}$$

Appendix C: Sample Calculation for Lipase Activity

Equation 2.

$$Lipase\ Activity\ = \frac{[(4.5\ mL) \times (A_s - A_b)]}{[(\varepsilon_M) \times t\ \times l\ \times Sample\ Weight\ d.b.\ (g)]} \times \frac{1000\ \mu Eq/mL}{1\ mol/L}$$

Where:

4.5 mL = Total reaction volume

A_s = Absorbance of sample at 715 nm = 0.332

A_b = Absorbance of blank at 715 nm = 0.051

ε_M = Molar extinction coefficient of oleic acid ($M^{-1} \cdot cm^{-1}$) at 715 nm determined through slope of oleic acid standard curve = 87.488

t = Incubation time in hours = 4 hours

l = Path length = 1 cm for standard cuvette

Sample Weight d.b. (g) = 1.0031 g

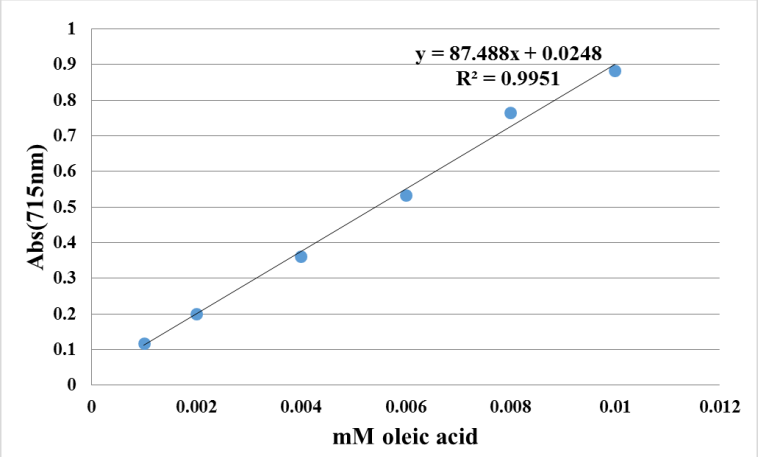

Figure 10: Standard curve of oleic acid plotting mM oleic acid against absorbance at 715 nm. The slope of the line (extinction coefficient of oleic acid) was used to determine lipase activity.

$$Lipase\ Activity = \frac{[(4.5\ mL) \times (0.332 - 0.051)]}{[(87.488\ M^{-1} \cdot cm^{-1}) \times 4\ h\ \times 1\ cm\ \times 1.0031\ g)]} \times \frac{1000\ \mu Eq/mL}{1\ mol/L}$$

$$= \mathbf{3.60}\ \frac{\boldsymbol{mmoles\ oleic\ acid}}{\boldsymbol{h\ \cdot g}}$$

Appendix D. Standard Curves and Sample Calculation for Hydroxycinnamic Acid Quantification

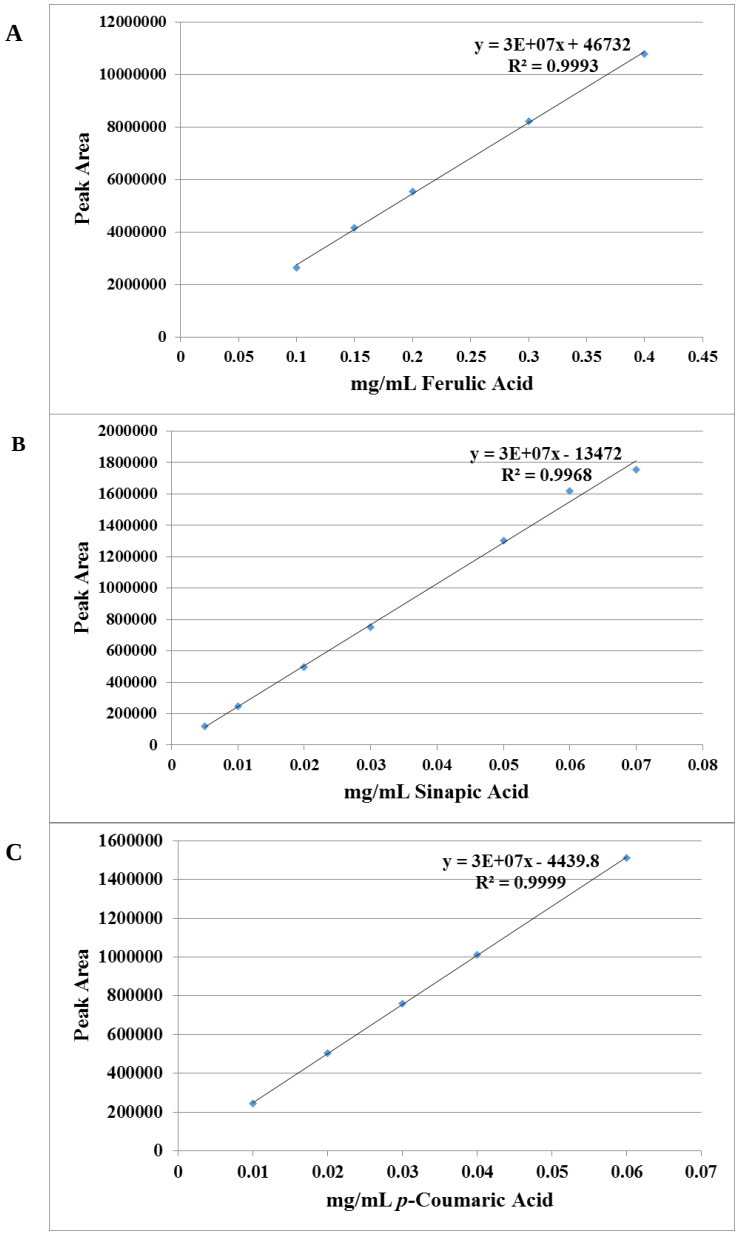

Figure 11: Standard curves of ferulic acid (A), sinapic acid (B) *p*-coumaric acid (C) used to quantify hydroxycinnamic acid content.

Ferulic Acid (µg/g sample) =

$$\frac{(Peak\ Area_{Sample} - b)}{m} \times \frac{1}{Vol_{Extract}} \times \frac{1000\ \mu moles}{1\ mmol} \times \frac{1}{Sample\ wt\ (g)}$$

Where:

b = y-intercept of ferulic acid standard curve = 46732

m = slope of ferulic acid standard curve = 27019519.3

$Vol_{Extract}$ = Volume of reconstituted extracts of hydroxycinnamic acids in 75% methanol = 1 mL

Sample wt (g) = 0.2034

Ferulic Acid (µg/g sample) =

$$\frac{(4598611 - 46732)}{27019519.3} \times \frac{1}{1\ mL} \times \frac{1000\ \mu moles}{1\ mmol} \times \frac{1}{0.2034\ g} = \mathbf{\frac{845.3\ \mu g}{g}}$$

Appendix E. Standard Curves and Sample Calculation for Carotenoid Quantification

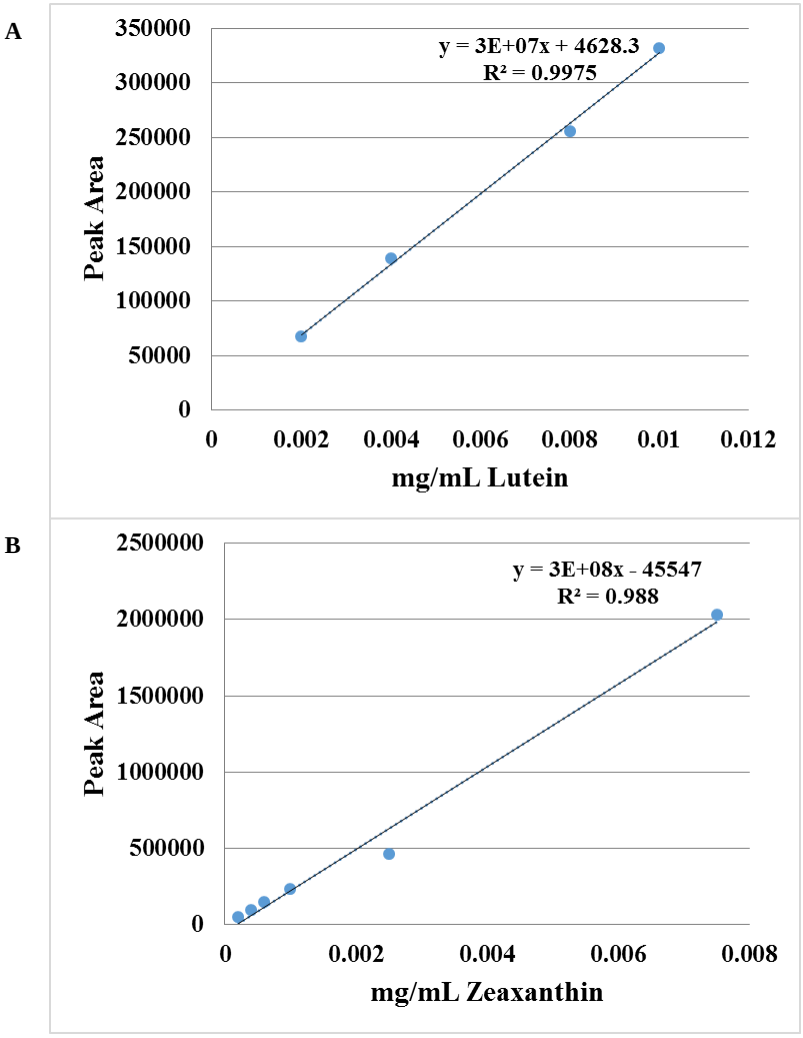

Figure 12: Standard curves of lutein (A) and zeaxanthin (B) used to quantify carotenoids in samples.

Appendix F. Standard Curve and Sample Calculation for DPPH Radical Scavenging Activity

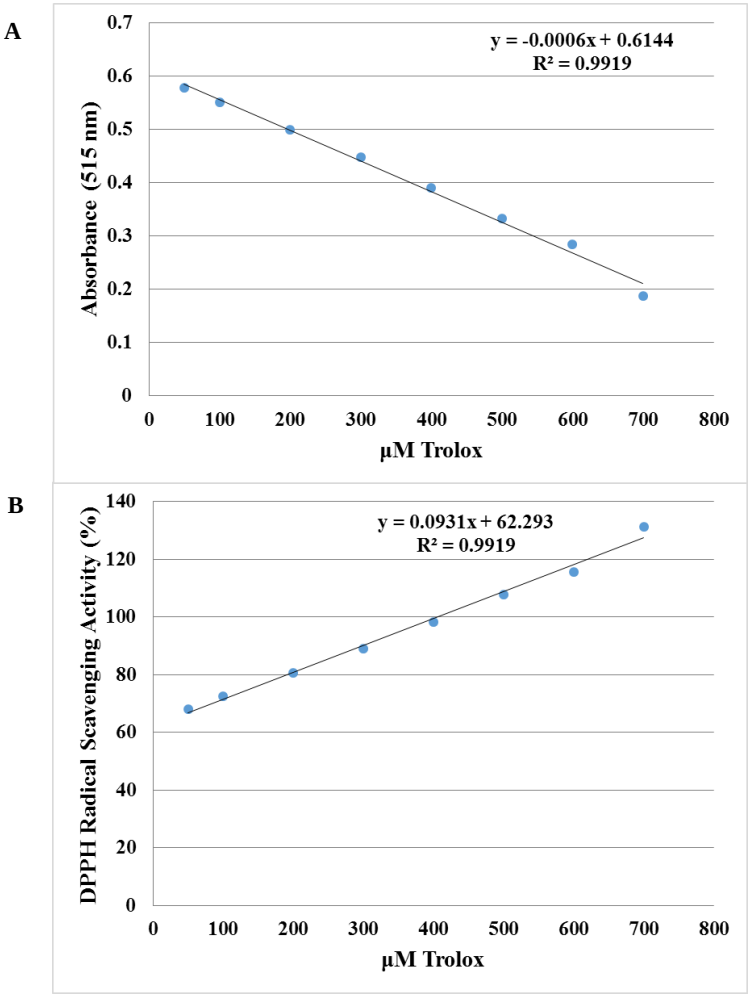

Figure 13: Standard curves plotting trolox concentration against absorbance at 515 nm (A) and trolox concentration against % DPPH radical scavenging activity (B), calculated using Equation 3.

Example 1. DPPH Radical Scavenging Activity of Alkaline Hydrolysates

Equation 3.

$$DPPH\ Radical\ Scavenging\ Activity\ (\%) = \left(\frac{(1 - A_{sample})}{A_{blank}}\right) \times 100\%$$

Where:

A_{sample} = Absorbance of sample or trolox at 515 nm = 0.397

A_{blank} = Absorbance of blank (60 μM DPPH radical solution) at 515 nm = 0.619

Equation 4.

$$\mu moles\ TE = \left(\frac{\%\ DPPH\ Scavenging\ Activity - b}{m}\right) \times \frac{Vol_{Extract}}{Vol_{Aliquot}} \times d \times \frac{Vol_{Extract}}{1000\ mL} \times \frac{1}{wt\ sample\ (g)}$$

Where:

b = Y-intercept of equation of the standard curve for % DPPH radical scavenging activity of trolox standards (**Figure 13**) = 62.293

m = Slope of the equation of the standard curve for % DPPH radical scavenging activity of trolox standards (**Figure 13**) = 0.0931

$Vol_{Extract}$ = Total extraction volume in mL = 1 mL

d = dilution factor = 3

$Vol_{Aliquot}$ = Aliquot volume in mL = 0.1 mL

$$DPPH\ Radical\ Scavenging\ Activity\ (\%) = \left(\frac{(1 - 0.397)}{0.619}\right) \times 100\% = 97.4$$

$$\mu moles\frac{TE}{g} = \left(\frac{97\% - 62.293}{0.0931}\right) \times \frac{1\ mL}{0.1\ mL} \times 3 \times \frac{1\ mL}{1000\ mL} \times \frac{1}{0.200\ g} = 56.6$$

Appendix G. Standard Curve and Sample Calculation for Leucomethylene Blue Antioxidant Assay

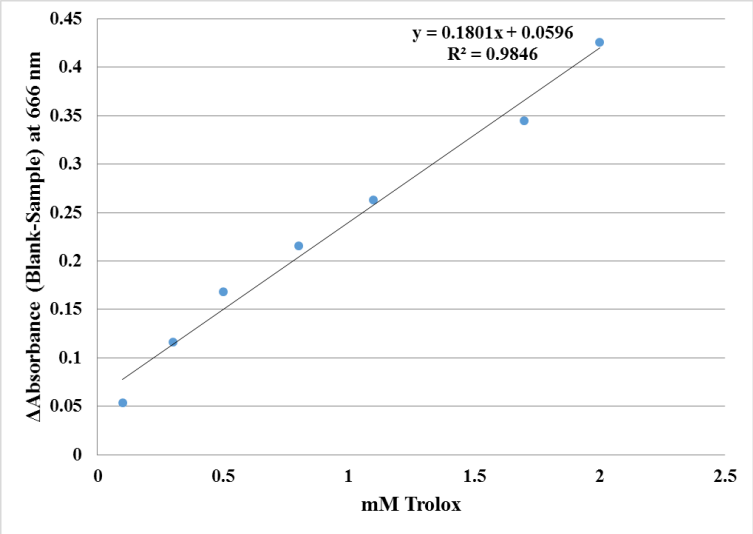

Figure 14: Standard curve plotting concentration of Trolox (0.2-2 mM) against delta absorbance at 666 nm

Equation 5.

$$\frac{mmol\ TE}{g} = \frac{[(A_{blank} - A_{sample}) - b]}{m} \times \frac{Vol_{Extract}}{Vol_{Aliquot}} \times d \times \frac{Vol_{Extract}}{1000\ mL} \times \frac{1}{Sample\ wt\ (g)}$$
$$= \mathbf{0.616}$$

Where:

A_{blank} = Absorbance of blank at 666 nm = 0.707

A_{sample} = Absorbance of sample at 666 nm = 0.467

b = Y-intercept of equation of the standard curve plotting Δ Absorbance at 666 nm of trolox concentrations (0.2-2 mM) = 0.0596

m = Slope of the equation of the standard curve plotting Δ Absorbance at 666 nm of trolox concentrations (0.2-2 mM) = 0.1801

$Vol_{Extract}$ = Total extraction volume in mL = 1 mL

$Vol_{Aliquot}$ = Aliquot volume in mL = 0.05 mL

d = dilution factor = 3

Sample wt (g) = 0.2000 g

Appendix H. Sample Calculation for Free Fatty Acids

Equation 6.

$$\%Free\ fatty\ acids\ = \frac{[(V_s - V_b) \times \frac{N\ NaOH}{1000\ mL} \times \frac{282\ g}{mol}]}{Weight\ flour\ d.b.(g)} \times 100$$

Where:

V_s = Volume of sodium hydroxide titrated to neutralize sample = 4.6 mL

V_b = Volume of sodium hydroxide used to neutralize blank = 0.0 mL

N NaOH = Normality of NaOH in Eq/L = 0.0094 N

282 g/mol = Molecular weight of oleic acid

Weight flour d.b. = 5.41 g

$$\%Free\ fatty\ acids\ = \frac{[(4.6 - 0.0) \times \frac{0.0094\ Eq\ NaOH/L}{1000\ mL} \times \frac{282\ g}{mol}]}{5.41\ g} \times 100 = \mathbf{0.20}\%$$

Appendix I. Sample Calculation for Peroxide Value

Equation 7.

$$\frac{mEq\ hydroperoxides}{1000\ g\ fat} = \frac{[(Vol_{sample} - Vol_{blank})\ \times N\ Na_2S_2O_3 \times 1000]}{Weight\ fat\ (g)}$$

Where:

Vol_{sample} = Volume of $Na_2S_2O_3$ (mL) used to titrate sample = 0.975 mL

Vol_{blank} = Volume of $Na_2S_2O_3$ (mL) used to titrate blank = 0.2 mL

N $Na_2S_2O_3$ = Normality of $Na_2S_2O_3$ (Eq/L) = 0.0094 Eq/L

282 g/mol = Molecular weight of oleic acid

Weight flour d.b. = 5.41 g

$$\frac{mEq\ hydroperoxides}{1000\ g\ fat} = \frac{\left[(0.975\ mL - 0.2\ mL) \times 0.0094 \frac{Eq}{L} \times 1000\right]}{5.41\ g} = \mathbf{8.24}$$

Appendix J. Seed Size Comparison of IWG

Table 13. Seed size comparison of IWG by year.

Samples (Year)	Seed Size (g/1000)
IWG (2004)	3.90
IWG (2015)	5.11
HRW (2015)	32.5

Appendix K. Lipoxygenase Activity of Steamed IWG over Accelerated Storage: Preliminary Trials

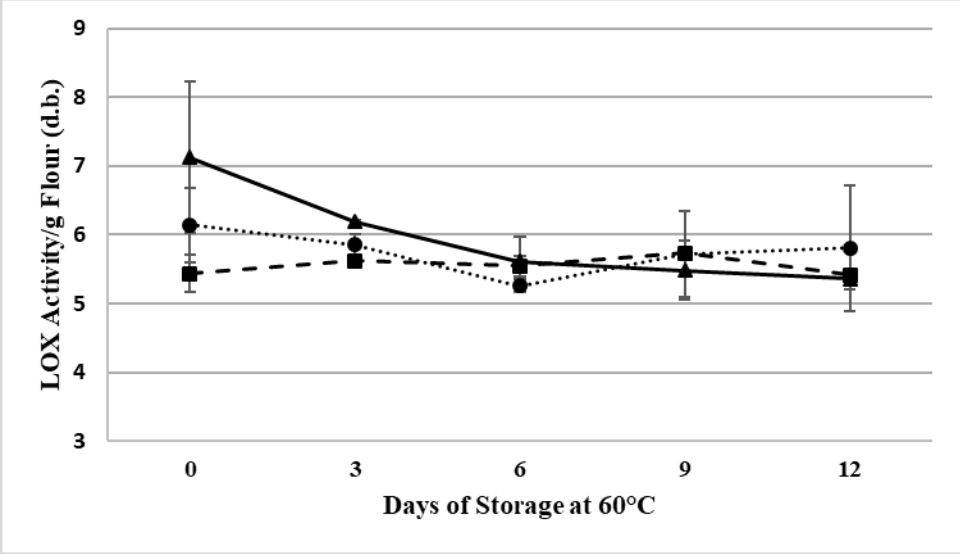

Figure 15. Lipoxygenase activity of steamed (for 0, 30, and 60 minutes) intermediate wheatgrass over accelerated storage at 60°C.

Appendix L. Chapter 2 Correlation Data Tables

Table 14. Accelerated storage (45°C) correlation data depicting relationships among antioxidant concentration, antioxidant activity, rancidity markers, and enzyme activity.

	Lutein Concentration	Zeaxanthin Concentration	Free Fatty Acid Concentration (d.b.)	p-Coumaric Acid Cocentration	Sinapic Acid Concentration	Ferulic Acid Concentration	Peroxide Value	DPPH Radical Scavenging Activity (Acetone Extracts)	DPPH Radical Scavenging Activity (Alkaline Hydrolysates)	LMB Antioxidant Activity	Lipase Activity (d.b.)	Lipoxygenase Activity (d.b.)
Lutein Concentration	1											
Zeaxanthin Concentration	0.997***	1										
Free Fatty Acid Concentration (d.b.)	0.696***	0.680***	1									
p-Coumaric Acid Cocentration	0.304**	0.293**	0.0527	1								
Sinapic Acid Concentration	0.06	0.061	0.212*	−0.482***	1							
Ferulic Acid Concentration	0.556	0.543***	0.637***	0.127	0.689***	1						
Peroxide Value	−0.446***	−0.439***	−0.306**	−0.347***	0.144	−0.212*	1					
DPPH Radical Scavenging Activity (Acetone Extracts)	0.574***	0.593***	0.470**	0.090	0.233	0.559***	0.009	1				
DPPH Radical Scavenging Activity (Alkaline Hydrolysates)	0.282	0.274	0.697***	−0.265	0.571***	0.637***	0.242	0.486**	1			
LMB Antioxidant Activity	−0.185	−0.214	0.213	−0.439*	0.346	0.142	0.636***	−0.152	0.437*	1		
Lipase Activity (d.b.)	0.735***	0.723***	0.647***	0.375*	0.026	0.627***	−0.231	0.539***	0.442**	−0.013	1	
Lipoxygenase Activity (d.b.)	−0.643***	−0.657***	−0.229	−0.334*	−0.001	−0.265	0.719***	−0.066	0.166	0.472*	−0.281	1

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 15. Ambient storage (22 ± 2°C) correlation data depicting relationships among antioxidant concentration, antioxidant activity, rancidity markers, and enzyme activity.

	Lutein Concentration	Zeaxanthin Concentration	Free Fatty Acid Concentration (d.b.)	p-Coumaric Acid Cocentration	Sinapic Acid Concentration	Ferulic Acid Concentration	Peroxide Value	DPPH Radical Scavenging Activity (Acetone Extracts)	DPPH Radical Scavenging Activity (Alkaline Hydrolysates)	LMB Antioxidant Activity	Lipase Activity (d.b.)	Lipoxygenase Activity (d.b.)
Lutein Concentration	1											
Zeaxanthin Concentration	0.968***	1										
Free Fatty Acid Concentration (d.b.)	0.757***	0.663***	1									
p-Coumaric Acid Cocentration	0.687***	0.679***	0.758***	1								
Sinapic Acid Concentration	0.044	0.067	0.101	0.400***	1							
Ferulic Acid Concentration	0.548***	0.530***	0.660***	0.775***	0.715***	1						
Peroxide Value	−0.360***	−0.336***	−0.456***	−0.248**	0.160	−0.122	1					
DPPH Radical Scavenging Activity (Acetone Extracts)	0.535***	0.383*	0.714***	0.615***	0.508**	0.790***	−0.050	1				
DPPH Radical Scavenging Activity (Alkaline Hydrolysates)	0.349*	0.234	0.632***	0.550***	0.541***	0.788***	−0.246	0.620***	1			
LMB Antioxidant Activity	0.067	−0.027	0.474*	0.283	0.123	0.221	−0.403	0.079	0.358	1		
Lipase Activity (d.b.)	0.773*	0.767***	0.737***	0.736***	−0.058	0.428*	−0.481**	0.286	0.371*	0.284	1	
Lipoxygenase Activity (d.b.)	−0.602***	−0.667***	−0.560***	−0.753***	0.270	−0.153	0.480**	−0.225	−0.241	−0.006	−0.642***	1

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 16. Refrigerated storage (4°C) correlation data depicting relationships among antioxidant concentration, antioxidant activity, rancidity markers, and enzyme activity.

	Lutein Concentration	Zeaxanthin Concentration	Free Fatty Acid Concentration (d.b.)	p-Coumaric Acid Cocentration	Sinapic Acid Concentration	Ferulic Acid Concentration	Peroxide Value	DPPH Radical Scavenging Activity (Acetone Extracts)	DPPH Radical Scavenging Activity (Alkaline Hydrolysates)	LMB Antioxidant Activity	Lipase Activity (d.b.)	Lipoxygenase Activity (d.b.)
Lutein Concentration	1											
Zeaxanthin Concentration	0.998***	1										
Free Fatty Acid Concentration (d.b.)	0.905***	0.915***	1									
p-Coumaric Acid Cocentration	0.867***	0.865***	0.798***	1								
Sinapic Acid Concentration	0.108	0.210	0.224	0.122	1							
Ferulic Acid Concentration	0.595***	0.619***	0.623***	0.540***	0.779***	1						
Peroxide Value	−0.383**	−0.371**	−0.247	−0.392**	0.112	−0.035	1					
DPPH Radical Scavenging Activity (Acetone Extracts)	0.877***	0.862***	0.628***	0.874***	−0.122	0.295	−0.593***	1				
DPPH Radical Scavenging Activity (Alkaline Hydrolysates)	0.488**	0.462**	0.343*	0.575***	−0.165	0.055	−0.399*	0.579***	1			
LMB Antioxidant Activity	0.417*	0.411*	0.360	0.258	0.243	0.475*	−0.135	0.260	0.463*	1		
Lipase Activity (d.b.)	0.871***	0.878***	0.646***	0.748***	0.159	0.622***	−0.258	0.780***	0.339*	0.308	1	
Lipoxygenase Activity (d.b.)	−0.561***	−0.536**	−0.390*	−0.674***	0.298	−0.020	0.361*	−0.619***	−0.483**	−0.139	−0.445**	1

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Appendix M. Breakdown and Setback Values of Starch Pasting Profiles over Storage

Table 17. Breakdown and setback values of steamed and non-steamed IWG and HRW as measured by moisture visco-amylograph (MVAG) over accelerated (45°C), ambient (ambient 22 ± 2°C), and refrigerated (4°C) storage.

Breakdown Value (Peak Viscosity – Hold Viscosity) (BU)

	Accelerated (45°C) Storage			Ambient Storage (22 ± 2°C)			Refrigerated (4°C) Storage		
	0 Weeks	3 Weeks	6 Weeks	0 Months	3 Months	6 Months	0 Months	6 Months	12 Months
IWG Steamed	88.5^{cA}	78.0^{bA}	121^{bA}	88.5^{cA}	75.0^{cA}	87.0^{bA}	88.5^{cA}	93.5^{bA}	121^{bA}
IWG Not Steamed	113^{bA}	85.5^{bB}	117^{bA}	113^{bA}	75.5^{cB}	75.0^{bB}	113^{bA}	111^{bA}	89.0^{bB}
HRW Steamed	281^{aB}	271^{aB}	304^{aA}	281^{aA}	269^{aA}	199^{aB}	281^{aA}	n.d.$^{\beta}$	213^{aB}
HRW Not Steamed	272^{aA}	252^{aA}	278^{aA}	272^{aA}	253^{bB}	206^{aC}	272^{aAB}	293^{aA}	253^{aB}

Total Setback (Final Viscosity – Hold Viscosity) (BU)

	Accelerated (45°C) Storage			Ambient Storage (22 ± 2°C)			Refrigerated (4°C) Storage		
	0 Weeks	3 Weeks	6 Weeks	0 Months	3 Months	6 Months	0 Months	6 Months	12 Months
IWG Steamed	376^{cB}	419^{bB}	483^{bA}	376^{cA}	337^{bA}	279^{cA}	376^{cA}	383^{bA}	314^{bA}
IWG Not Steamed	413^{cA}	444^{bA}	529^{bA}	413^{cA}	349^{bB}	324^{cC}	413^{cA}	394^{bA}	386^{bA}
HRW Steamed	715^{bA}	890^{aA}	743^{aA}	715^{bA}	762^{aA}	719^{aA}	715^{bA}	n.d.	725^{aA}
HRW Not Steamed	820^{aA}	879^{aA}	880^{aA}	820^{aA}	803^{aA}	846^{aA}	820^{aA}	698^{aA}	802^{aA}

$^{\beta}$No data; sample was improperly stored. Lowercase superscripts represent significant differences ($P \leq 0.05$) across grain samples within single time point; capital superscripts represent significant differences within a grain sample across time points according to the Fisher's LSD means comparison test.

Appendix N. Chapter 3 Correlation Data Tables

Table 18. Accelerated storage (45°C) correlation data depicting relationships among functionality parameters over storage.

	Water Absorption	Stability Time	Stability Arrival	Dough Development Time	Resistance to Extension	Extensibility	Peak Viscosity	Hold Viscosity	Final Viscosity	Pasting Temperature	Breakdown Value	Setback Value
Water Absorption	1											
Stability Time	0.506*	1										
Stability Arrival	0.544**	0.960***	1									
Dough Development Time	0.530**	0.967***	0.991***	1								
Resistance to Extension	0.737***	0.862***	0.885***	0.892***	1							
Extensibility	0.772***	0.712***	0.704***	0.707***	0.942***	1						
Peak Viscosity	0.707***	0.879***	0.920***	0.923***	0.935***	0.800***	1					
Hold Viscosity	0.621**	0.899***	0.907***	0.920***	0.952***	0.847***	0.944***	1				
Final Viscosity	0.598**	0.906***	0.908***	0.924***	0.911***	0.792***	0.931***	0.982***	1			
Pasting Temperature	0.068	0.153	0.199	0.180	0.312	0.339	0.282	0.246	0.234	1		
Breakdown Value	0.561**	0.910***	0.947***	0.956***	0.946***	0.805***	0.947***	0.969***	0.951***	0.319	1	
Setback Value	0.551**	0.904***	0.897***	0.911***	0.853***	0.715***	0.902***	0.949***	0.989***	0.212	0.920***	1
Peroxide Value	−0.104	0.584**	0.571**	0.570**	0.210	0.051	0.339	0.406*	0.387	−0.219	0.433*	0.395
Free Fatty Acids	−0.601**	−0.733***	−0.782***	−0.791***	−0.835***	−0.744***	−0.826***	−0.800***	−0.781***	−0.340	−0.846***	−0.753***
Lipase Activity	−0.600**	−0.765***	nd	−0.775***	−0.733***	−0.642***	−0.816***	−0.749***	−0.779***	−0.185	−0.746***	−0.794***
Lipoxygenase Activity	0.031	0.619**	nd	0.649***	0.395*	0.241	0.459*	0.562**	0.547**	−0.149	0.551**	0.528**

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 19. Ambient storage (22 ± 2°C) correlation data depicting relationships among functionality parameters over storage.

	Loaf Volume	Loaf Height	Bread Firmness	Water Absorption	Stability Time	Stability Arrival	Dough Development Time	Resistance to Extension	Extensibility	Peak Viscosity	Hold Viscosity	Final Viscosity	Pasting Temperature	Breakdown Value	Setback Value
Loaf Volume	1														
Loaf Height	0.787***	1													
Bread Firmness	−0.391***	−0.648***	1												
Water Absorption	0.592**	0.634***	−0.567**	1											
Stability Time	0.696***	0.941***	−0.764***	0.684***	1										
Stability Arrival	0.741***	0.954***	−0.711***	0.720***	0.960***	1									
Dough Development Time	0.742***	0.943***	−0.753***	0.709***	0.965***	0.985***	1								
Resistance to Extension	0.798***	0.932***	−0.625***	0.771***	0.929***	0.917***	0.922***	1							
Extensibility	0.784***	0.777***	−0.454**	0.659***	0.671***	0.678***	0.678***	0.856***	1						
Peak Viscosity	0.834***	0.934***	−0.673***	0.670***	0.864***	0.914***	0.918***	0.913***	0.819**	1					
Hold Viscosity	0.842***	0.936***	−0.670***	0.654***	0.858***	0.910***	0.909***	0.902***	0.824***	0.997***	1				
Final Viscosity	0.771***	0.908***	−0.674***	0.523**	0.834***	0.900***	0.901***	0.820***	0.697***	0.954***	0.961***	1			
Pasting Temperature	0.528**	0.445*	−0.140	0.101	0.219	0.355	0.363	0.345	0.531**	0.623**	0.627**	0.627**	1		
Breakdown Value	0.815***	0.922***	−0.671***	0.687***	0.866***	0.911***	0.924***	0.920***	0.804***	0.995***	0.985***	0.935***	0.612**	1	
Setback Value	0.715***	0.929***	−0.681***	0.623**	0.927***	0.959***	0.963***	0.911***	0.662***	0.922**	0.909***	0.913***	0.454*	0.932***	1
Peroxide Value	0.356*	0.312	−0.411*	0.353	0.375	0.379	0.347	0.279	0.184	0.354	0.355	0.325	0.16	0.349	0.328
Free Fatty Acids	−0.704***	−0.860***	0.673***	−0.680***	−0.899***	−0.933***	−0.933***	−0.845***	−0.663***	−0.873***	−0.866***	−0.889***	−0.366	−0.875***	−0.918***
Lipase Activity	−0.666***	−0.638***	0.704***	−0.544**	−0.729***	−0.658***	−0.667***	−0.695***	−0.556***	−0.644***	−0.643***	−0.625**	−0.072	−0.641***	−0.678***
Lipoxygenase Activity	0.257	0.392*	−0.556***	0.589**	0.601**	0.546**	0.552**	0.448**	0.161	0.339	0.326	0.34	−0.325	0.353	0.477*

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 20. Refrigerated storage (4°C) correlation data depicting relationships among functionality parameters over storage.

	Loaf Volume	Loaf Height	Bread Firmness	Water Absorption	Stability Time	Stability Arrival	Dough Development Time	Resistance to Extension	Extensibility	Peak Viscosity	Hold Viscosity	Final Viscosity	Pasting Temperature	Breakdown Value	Setback Value
Loaf Volume	1														
Loaf Height	0.680***	1													
Bread Firmness	−0.425***	−0.600***	1												
Water Absorption	0.488*	0.740***	−0.180	1											
Stability Time	0.405	0.844***	−0.426*	0.675***	1										
Stability Arrival	0.319	0.807***	−0.251	0.706***	0.960***	1									
Dough Development Time	0.734***	0.921***	−0.496*	0.708***	0.758***	0.737***	1								
Resistance to Extension	0.460**	0.826***	−0.421*	0.777***	0.941***	0.927***	0.698***	1							
Extensibility	0.456**	0.859***	−0.555***	0.749***	0.831***	0.806***	0.789***	0.811***	1						
Peak Viscosity	0.517*	0.877***	−0.360	0.704***	0.959***	0.939***	0.817***	0.953***	0.842***	1					
Hold Viscosity	0.495*	0.868***	−0.356	0.708***	0.959***	0.942***	0.802***	0.962***	0.841***	0.998***	1				
Final Viscosity	0.440*	0.875***	−0.315	0.682***	0.950***	0.952***	0.793***	0.937***	0.847***	0.976***	0.975***	1			
Pasting Temperature	0.626**	0.715***	−0.158	0.500*	0.421	0.458*	0.656***	0.457*	0.537**	0.575**	0.554**	0.592**	1		
Breakdown Value	0.551**	0.886***	−0.352	0.709***	0.942***	0.913***	0.818***	0.921***	0.824***	0.972***	0.958***	0.946***	0.604**	1	
Setback Value	0.376	0.843***	−0.263	0.627**	0.929***	0.941***	0.759***	0.888***	0.813***	0.937***	0.933***	0.987***	0.587**	0.913***	1
Peroxide Value	−0.214	0.083	−0.039	0.193	0.449*	0.436*	0.109	0.275	0.150	0.311	0.333	0.261	−0.305	0.289	0.246
Free Fatty Acids	−0.449**	−0.761***	−0.439*	−0.561**	−0.828***	−0.842***	−0.757***	−0.794***	−0.804***	−0.899***	−0.899***	−0.885***	−0.593**	−0.840***	−0.858***
Lipase Activity	−0.346*	−0.759***	0.457**	−0.699***	−0.817***	−0.803***	−0.662***	−0.819***	−0.720***	−0.814***	−0.813***	−0.825***	−0.532*	−0.786***	−0.819***
Lipoxygenase Activity	0.216	0.397*	−0.441*	0.411	0.407	0.376	0.381	0.398*	0.350	0.291	0.304	0.305	−0.130	0.286	0.302

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Appendix O. Two-Way Analysis of Variance (ANOVA) Summary Tables for Chapter 2 Statistics

Table 21. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on lipase activity.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	2.824	14.7874	6.976e-06***
Time Point	20	1.212	6.3461	0.0002769***
Steam Treatment	1	0.006	0.0291	0.8652442
Grain Type	1	32.513	170.2438	<2.2e-16***
Temperature X Steam Treatment	2	0.347	1.8144	0.1723773
Temperature X Grain Type	2	0.899	4.7067	0.0128997*
Time Point X Steam Treatment	20	0.102	0.5363	0.7095865
Time Point X Grain Type	20	0.170	0.8878	0.4773203
Grain Type X Steam Treatment	1	0.006	0.0292	0.8650176
Temperature X Grain Type X Steam Treatment	2	0.138	0.7251	0.4887890
Time Point X Grain Type X Steam Treatment	20	0.092	0.4813	0.7492986
Residuals	56	0.191		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 22. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on lipoxygenase activity.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	8.8628	93.8307	<2.2e-16***
Time Point	20	0.6954	7.3620	8.917e-05***
Steam Treatment	1	0.0207	0.2192	0.641644
Grain Type	1	5.9252	62.7305	1.702e-10***
Temperature X Steam Treatment	2	0.0407	0.4304	0.652528
Temperature X Grain Type	2	0.6920	7.3267	0.001573**
Time Point X Steam Treatment	20	0.0834	0.8827	0.480826
Time Point X Grain Type	20	0.1345	1.4234	0.239282
Grain Type X Steam Treatment	1	0.0052	0.0552	0.815145
Temperature X Grain Type X Steam Treatment	2	0.1748	1.8509	0.167297
Time Point X Grain Type X Steam Treatment	20	0.1773	1.8773	0.12836
Residuals	52	0.0945		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 23. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on DPPH radical scavenging activity of alkaline hydrolysates.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	1344.18	34.4127	1.790e-10***
Time Point	20	104.90	2.6855	0.04046*
Steam Treatment	1	1.78	0.0457	0.83156
Grain Type	1	1561.13	39.9669	4.529e-08***
Temperature X Steam Treatment	2	1.63	0.0418	0.95909
Temperature X Grain Type	2	68.89	1.7638	0.1878
Time Point X Steam Treatment	20	5.51	0.1411	0.96616
Time Point X Grain Type	20	17.39	0.4451	0.77546
Grain Type X Steam Treatment	1	42.29	1.0827	0.30258
Temperature X Grain Type X Steam Treatment	2	4.10	0.1049	0.90062
Time Point X Grain Type X Steam Treatment	20	43.95	1.1252	0.35393
Residuals	56	39.06		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 24. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on DPPH radical scavenging activity of acetone extracts.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	798.00	218.3182	<2.2e-16***
Time Point	20	150.36	41.1366	6.348e-16***
Steam Treatment	1	37.40	10.2331	0.00229**
Grain Type	1	2851.81	780.1992	<2.2e-16***
Temperature X Steam Treatment	2	9.76	2.6694	0.07826
Temperature X Grain Type	2	435.49	119.1420	<2.2e-16***
Time Point X Steam Treatment	20	42.10	11.5179	7.314e-07***
Time Point X Grain Type	20	59.97	16.4066	6.658e-09***
Grain Type X Steam Treatment	1	4.82	1.3178	0.25596
Temperature X Grain Type X Steam Treatment	2	7.07	1.9349	0.15414
Time Point X Grain Type X Steam Treatment	20	5.70	1.5604	0.19784
Residuals	55	3.66		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 25. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on LMB antioxidant activity of alkaline hydrolysates.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	0.55618	67,6836	4.893e-12***
Time Point	20	0.84557	102.9009	<2.2e-16***
Steam Treatment	1	0.19446	23.6647	3.165e-05***
Grain Type	1	0.30597	37.2346	9.182e-07***
Temperature X Steam Treatment	2	0.06222	7.5716	0.0021010**
Temperature X Grain Type	2	0.01040	1.2659	0.2961713
Time Point X Steam Treatment	20	0.05428	6.6054	0.0005763***
Time Point X Grain Type	20	0.11382	13.8511	1.376e-06***
Grain Type X Steam Treatment	1	0.00005	0.0061	0.9382433
Temperature X Grain Type X Steam Treatment	2	0.01328	1.6167	0.2148491
Time Point X Grain Type X Steam Treatment	20	0.07091	8.6290	8.412e-05***
Residuals	31	0.00822		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 26. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on ferulic acid concentration.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	951890	95.8261	<2.2e-16***
Time Point	20	88410	8.9002	<2.2e-16***
Steam Treatment	1	106355	10.7066	0.001296**
Grain Type	1	3148458	316.9530	<2.2e-16***
Temperature X Steam Treatment	2	1118	0.1126	0.893591
Temperature X Grain Type	2	50548	5.0886	0.007151**
Time Point X Steam Treatment	20	7340	0.7389	0.781558
Time Point X Grain Type	20	30295	3.0497	4.518e-05***
Grain Type X Steam Treatment	1	11172	1.1247	0.290432
Temperature X Grain Type X Steam Treatment	2	27149	2.7602	0.066148
Time Point X Grain Type X Steam Treatment	20	10791	1.0863	0.368045
Residuals	168	9934		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 27. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on sinapic acid concentration.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	803.08	5.5415	0.004649**
Time Point	20	785.70	5.4215	1.445e-10***
Steam Treatment	1	194.32	1.3409	0.248479
Grain Type	1	1475.43	10.1808	0.001685**
Temperature X Steam Treatment	2	22.57	0.1557	0.855914
Temperature X Grain Type	2	77.34	0.5337	0.587396
Time Point X Steam Treatment	20	132.29	0.9128	0.571462
Time Point X Grain Type	20	536.40	3.7013	1.302e-06***
Grain Type X Steam Treatment	1	704.93	4.8642	0.028737*
Temperature X Grain Type X Steam Treatment	2	396.53	2.7362	0.067625
Time Point X Grain Type X Steam Treatment	20	113.64	0.7841	0.730210
Residuals	173	144.92		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 28. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on *p*-coumaric acid concentration.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	3186.7	400.0200	<2.2e-16***
Time Point	20	66.9	8.3928	<2.2e-16***
Steam Treatment	1	143.2	17.9792	3.721e-05***
Grain Type	1	4090.0	513.4103	<2.2e-16***
Temperature X Steam Treatment	2	53.0	6.6477	0.001675**
Temperature X Grain Type	2	1096.2	137.6013	<2.2e-16***
Time Point X Steam Treatment	20	15.2	1.9085	0.014785*
Time Point X Grain Type	20	32.5	4.0837	1.997e-07***
Grain Type X Steam Treatment	1	59.4	7.4557	0.007015**
Temperature X Grain Type X Steam Treatment	2	55.8	6.9994	0.001211**
Time Point X Grain Type X Steam Treatment	20	11.0	1.3755	0.141123
Residuals	164	8.0		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 29. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on lutein concentration.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	3.77	417.1947	<2.2e-16***
Time Point	20	1.02	112.8108	<2.2e-16***
Steam Treatment	1	0.00	0.3171	0.5740748
Grain Type	1	316.30	34962.3129	<2.2e-16***
Temperature X Steam Treatment	2	0.00	0.3810	0.6837631
Temperature X Grain Type	2	5.84	645.9355	<2.2e-16***
Time Point X Steam Treatment	20	0.02	2.6832	0.0002861***
Time Point X Grain Type	20	1.05	116.0451	<2.2e-16***
Grain Type X Steam Treatment	1	0.01	0.7468	0.3886414
Temperature X Grain Type X Steam Treatment	2	0.00	0.5376	0.5850752
Time Point X Grain Type X Steam Treatment	20	0.02	2.6222	0.0003942***
Residuals	179	0.01		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 30. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on zeaxanthin concentration.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	0.2780	1354.8159	<2.2e-16***
Time Point	20	0.0233	113.4832	<2.2e-16***
Steam Treatment	1	0.0007	3.5221	0.0622121
Grain Type	1	4.1622	20281.3970	<2.2e-16***
Temperature X Steam Treatment	2	0.0001	0.2910	0.7478740
Temperature X Grain Type	2	0.2029	988.4670	<2.2e-16***
Time Point X Steam Treatment	20	0.0006	2.7779	0.0001773***
Time Point X Grain Type	20	0.0190	92.4421	<2.2e-16***
Grain Type X Steam Treatment	1	0.0009	4.5903	0.0335280*
Temperature X Grain Type X Steam Treatment	2	0.0001	0.5855	0.5579358
Time Point X Grain Type X Steam Treatment	20	0.0005	2.5610	0.0005532***
Residuals	176	0.0002		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 31. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on free fatty acid concentration.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	0.08081	266.6879	<2.2e-16***
Time Point	20	0.00905	29.8527	<2.2e-16***
Steam Treatment	1	0.00462	15.2579	<2.2e-16***
Grain Type	1	1.49313	4927.5297	0.0001338***
Temperature X Steam Treatment	2	0.00067	2.2042	0.1133925
Temperature X Grain Type	2	0.00547	18.0680	7.356e-09***
Time Point X Steam Treatment	20	0.00091	2.9902	5.788e-05***
Grain Type X Steam Treatment	1	0.00338	11.1563	0.0010236**
Temperature X Grain Type X Steam Treatment	2	0.00095	3.1448	0.0455214*
Time Point X Grain Type X Steam Treatment	20	0.00019	0.6142	0.8988612
Residuals	175	0.00030		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 32. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on peroxide value.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	382.66	43.2055	7.260e-16***
Time Point	20	179.08	20.2201	<2.2e-16***
Steam Treatment	1	63.82	7.2056	0.007996**
Grain Type	1	938.56	105.9724	<2.2e-16***
Temperature X Steam Treatment	2	42.45	4.7933	0.009452**
Temperature X Grain Type	2	87.93	9.9276	8.411e-05***
Time Point X Steam Treatment	20	20.74	2.3419	0.001777**
Time Point X Grain Type	20	29.37	3.3166	1.095e-05***
Grain Type X Steam Treatment	1	25.63	2.8939	0.090765
Temperature X Grain Type X Steam Treatment	2	9.35	1.0554	0.350362
Time Point X Grain Type X Steam Treatment	20	8.99	1.0147	0.447606
Residuals	168	8.86		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Appendix P. Two-Way Analysis of Variance (ANOVA) Summary Tables for Chapter 3 Statistics

Table 33. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on water absorption values.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	8.789	80.8690	1.193e-13***
Time Point	6	3.798	34.9497	3.706e-13***
Steam Treatment	1	6.892	63.4131	2.809e-09***
Grain Type	1	67.896	624.7494	<2.2e-16***
Temperature X Steam Treatment	2	0.400	3.6830	0.0356629*
Temperature X Grain Type	2	0.032	0.2906	0.7496884
Time Point X Steam Treatment	6	1.785	16.4204	8.878e-09***
Time Point X Grain Type	6	3.252	29.9219	3.356e-09***
Grain Type X Steam Treatment	1	0.259	2.3830	0.1319160
Temperature X Grain Type X Steam Treatment	2	1.184	10.8971	0.0002204***
Time Point X Grain Type X Steam Treatment	5	0.887	8.1660	3.794e-05***
Residuals	34	0.109		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 34. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on dough stability time.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	7577	29.3859	3.882e-08***
Time Point	6	7087	27.4861	1.091e-11***
Steam Treatment	1	1581	6.1317	0.018415*
Grain Type	1	632743	2453.8872	<2.2e-16***
Temperature X Steam Treatment	2	506	1.9629	0.156042
Temperature X Grain Type	2	1695	6.5744	0.003856**
Time Point X Steam Treatment	6	971	3.7653	0.005569**
Time Point X Grain Type	6	1269	4.9220	0.001017**
Grain Type X Steam Treatment	1	1340	5.1973	0.029016*
Temperature X Grain Type X Steam Treatment	2	1199	4.6486	0.016418*
Time Point X Grain Type X Steam Treatment	5	722	2.8010	0.031869*
Residuals	34	258		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 35. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on dough development time.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	11757	53.0160	3.542e-11***
Time Point	6	4429	19.9721	7.674e-10***
Steam Treatment	1	2228	10.0462	0.0032239**
Grain Type	1	520951	2349.1142	<2.2e-16***
Temperature X Steam Treatment	2	1633	7.3651	0.0022008**
Temperature X Grain Type	2	3797	17.1195	7.187e-06***
Time Point X Steam Treatment	6	1179	5.3156	0.0005871***
Time Point X Grain Type	6	2456	11.0737	7.966e-07***
Grain Type X Steam Treatment	1	268	1.2063	0.2797873
Temperature X Grain Type X Steam Treatment	2	2668	12.0305	0.0001120***
Time Point X Grain Type X Steam Treatment	5	2134	9.6232	8.612e-06***
Residuals	34	222		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 36. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on dough stability arrival time.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	1523	31.7366	1.675e-08***
Time Point	6	1829	38.1135	1.058e-13***
Steam Treatment	1	10	0.2078	0.65142
Grain Type	1	258617	5389.5005	<2.2e-16***
Temperature X Steam Treatment	2	202	4.2124	0.02321*
Temperature X Grain Type	2	12	0.2517	0.77889
Time Point X Steam Treatment	6	632	13.1712	1.186e-07***
Time Point X Grain Type	6	459	9.5717	3.583e-06***
Grain Type X Steam Treatment	1	2605	54.2936	1.533e-08***
Temperature X Grain Type X Steam Treatment	2	37	0.7727	0.46972
Time Point X Grain Type X Steam Treatment	5	117	2.4410	0.05403
Residuals	34	48		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 37. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on dough resistance to extension.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	66.3	128.3443	<2.2e-16***
Time Point	6	86.6	167.5194	<2.2e-16***
Steam Treatment	1	1.3	2.5041	0.1181
Grain Type	1	22584.2	43699.3198	<2.2e-16***
Temperature X Steam Treatment	2	41.0	79.2444	<2.2e-16***
Temperature X Grain Type	2	0.5	0.9249	0.4014
Time Point X Steam Treatment	6	210.6	407.5287	<2.2e-16***
Time Point X Grain Type	6	109.4	211.6184	<2.2e-16***
Grain Type X Steam Treatment	1	34.0	65.8771	1.115e-11***
Temperature X Grain Type X Steam Treatment	2	11.3	21.7951	.4380e-08***
Time Point X Grain Type X Steam Treatment	5	93.5	181.0029	<2.2e-16***
Residuals	70	0.5		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 38. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on dough extensibility.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	77.8	18.070	4.706e-07***
Time Point	6	193.7	45.002	<2.2e-16***
Steam Treatment	1	466.1	108.318	7.394e-16***
Grain Type	1	19974.0	4641.577	<2.2e-16***
Temperature X Steam Treatment	2	122.0	28.347	9.593e-10***
Temperature X Grain Type	2	282.3	65.602	<2.2e-16***
Time Point X Steam Treatment	6	446.0	103.647	<2.2e-16***
Time Point X Grain Type	6	293.4	68.186	<2.2e-16***
Grain Type X Steam Treatment	1	1364.1	316.993	<2.2e-16***
Temperature X Grain Type X Steam Treatment	2	199.3	46.298	1.547e-13***
Time Point X Grain Type X Steam Treatment	5	202.7	47.095	<2.2e-16***
Residuals	70	4.3		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 39. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on pasting temperature.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	14.827	7.4239	0.002053**
Time Point	6	26.359	13.1986	9.367e-08***
Steam Treatment	1	2.537	1.2705	0.267341
Grain Type	1	58.708	29.3961	4.469e-06***
Temperature X Steam Treatment	2	1.099	0.5501	0.581813
Temperature X Grain Type	2	1.578	0.7903	0.461647
Time Point X Steam Treatment	6	6.733	3.3714	0.009945**
Time Point X Grain Type	6	1.999	1.0007	0.440399
Grain Type X Steam Treatment	1	0.250	0.1252	0.725607
Temperature X Grain Type X Steam Treatment	2	0.837	0.4191	0.660869
Time Point X Grain Type X Steam Treatment	5	1.423	0.7124	0.618276
Residuals	35	1.997		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 40. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on peak viscosity.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	14415	7.5584	0.001868**
Time Point	6	12644	6.6297	9.532e-05***
Steam Treatment	1	59	0.0310	0.861206
Grain Type	1	2913733	1527.7888	<2.2e-16***
Temperature X Steam Treatment	2	8253	4.3271	0.020928*
Temperature X Grain Type	2	3577	1.8756	0.168339
Time Point X Steam Treatment	6	3507	1.8387	0.119877
Time Point X Grain Type	6	5553	2.9114	0.020743*
Grain Type X Steam Treatment	1	9	0.0047	0.945623
Temperature X Grain Type X Steam Treatment	2	419	0.1098	0.896355
Time Point X Grain Type X Steam Treatment	5	8272	4.3373	0.003552**
Residuals	35	1907		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 41. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on hold viscosity.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	10647	198.0104	<2.2e-16***
Time Point	6	4827	89.7604	<2.2e-16***
Steam Treatment	1	437	8.1222	0.0072809**
Grain Type	1	931635	17325.8393	<2.2e-16***
Temperature X Steam Treatment	2	2393	44.5050	2.430e-10***
Temperature X Grain Type	2	390	7.2584	0.0023068**
Time Point X Steam Treatment	6	2006	37.3010	8.540e-14***
Time Point X Grain Type	6	3021	56.1783	<2.2e-16***
Grain Type X Steam Treatment	1	172	3.2037	0.0821288
Temperature X Grain Type X Steam Treatment	2	514	9.5662	0.0004849***
Time Point X Grain Type X Steam Treatment	5	1845	34.3187	1.509e-12***
Residuals	35	54		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 42. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on final viscosity.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	145472	49.2693	6.654e-11***
Time Point	6	55387	18.7589	1.259e-09***
Steam Treatment	1	4200	1.4225	0.2410180
Grain Type	1	7168164	2427.7582	<2.2e-16***
Temperature X Steam Treatment	2	33062	11.1977	0.0001741***
Temperature X Grain Type	2	10532	3.5672	0.0389093*
Time Point X Steam Treatment	6	27203	9.2133	4.551e-06***
Time Point X Grain Type	6	10816	3.6634	0.0062945**
Grain Type X Steam Treatment	1	17096	5.7900	0.0215331*
Temperature X Grain Type X Steam Treatment	2	13194	4.4688	0.0186881*
Time Point X Grain Type X Steam Treatment	5	18711	6.3371	0.0002754***
Residuals	35	2953		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 43. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on breakdown value.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	2991	17.1705	6.366e-16***
Time Point	6	2576	14.7853	2.454e-08***
Steam Treatment	1	43	0.2456	0.623306
Grain Type	1	477250	2739.4428	<2.2e-16***
Temperature X Steam Treatment	2	227	1.3012	0.285049
Temperature X Grain Type	2	426	2.4444	0.101464
Time Point X Steam Treatment	6	150	0.8592	0.534107
Time Point X Grain Type	6	1419	8.1442	1.510e-05***
Grain Type X Steam Treatment	1	338	1.9381	0.172661
Temperature X Grain Type X Steam Treatment	2	636	3.6504	0.036316*
Time Point X Grain Type X Steam Treatment	5	705	4.0471	0.005287**
Residuals	35	174		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 44. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on setback value.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	48522	22.4991	5.215e-07***
Time Point	6	11054	5.1254	0.0007195***
Steam Treatment	1	52329	24.2643	2.006e-05***
Grain Type	1	2695553	1249.8859	<2.2e-16***
Temperature X Steam Treatment	2	36	0.0167	0.9833979
Temperature X Grain Type	2	7172	3.3257	0.0476062*
Time Point X Steam Treatment	6	2425	1.1246	0.3684033
Time Point X Grain Type	6	7981	3.7007	0.0059408**
Grain Type X Steam Treatment	1	8754	4.0591	0.0516682
Temperature X Grain Type X Steam Treatment	2	204	0.0946	0.9099562
Time Point X Grain Type X Steam Treatment	5	1263	0.5855	0.7109268
Residuals	35	2157		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 45. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on bread loaf volumes.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	0.3958	15.6474	3.624e-07***
Time Point	4	0.3795	15.0035	4.039e-11***
Steam Treatment	1	2.3270	92.0041	<2.2e-16***
Grain Type	1	13.1473	519.8158	<2.2e-16***
Temperature X Steam Treatment	2	0.0780	3.0842	0.04733*
Temperature X Grain Type	2	0.3440	13.5997	2.313e-06***
Time Point X Steam Treatment	4	0.2469	9.7612	2.104e-07***
Time Point X Grain Type	4	0.8247	32.6086	<2.23e-16***
Grain Type X Steam Treatment	1	0.0179	0.7061	0.40147
Temperature X Grain Type X Steam Treatment	2	0.0377	1.4897	0.22723
Time Point X Grain Type X Steam Treatment	3	0.0826	3.2647	0.02186*
Residuals	279	0.0253		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 46. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on loaf heights.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	0.1312	6.3791	0.002764**
Time Point	4	0.1775	8.6309	8.733e-06***
Steam Treatment	1	0.5428	26.3910	2.139e-06***
Grain Type	1	22.6045	1099.0457	<2.2e-16***
Temperature X Steam Treatment	2	0.0098	0.4759	0.623166
Temperature X Grain Type	2	0.0894	4.3454	0.016382*
Time Point X Steam Treatment	4	0.0226	1.0996	0.363127
Time Point X Grain Type	4	0.1546	7.5189	3.784e-05***
Grain Type X Steam Treatment	1	0.1245	6.0549	0.016168*
Temperature X Grain Type X Steam Treatment	2	0.0365	1.7740	0.176704
Time Point X Grain Type X Steam Treatment	3	0.0201	0.9795	0.407073
Residuals	75	0.0206		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.

Table 47. Two-way analysis of variance on the individual effects and interaction effects of storage temperature, storage time, steam treatment, and grain type on bread firmness.

Source of Variation	Degrees of Freedom	Mean Square	F Value	Significance (P Value)
Temperature	2	0.0568	2.3451	0.097723
Time Point	4	0.0702	2.8991	0.022394*
Steam Treatment	1	0.2041	8.4246	0.003997**
Grain Type	1	4.0517	167.2176	<2.2e-16***
Temperature X Steam Treatment	2	0.0230	0.9508	0.387695
Temperature X Grain Type	2	0.0025	0.1037	0.901552
Time Point X Steam Treatment	4	0.0433	1.7872	0.131514
Time Point X Grain Type	4	0.0157	0.6493	0.627817
Grain Type X Steam Treatment	1	0.1244	5.1354	0.024209*
Temperature X Grain Type X Steam Treatment	2	0.0187	0.7710	0.463545
Time Point X Grain Type X Steam Treatment	3	0.0370	1.5261	0.207939
Residuals	279	0.0242		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.01$.