AN INVESTIGATION OF THE EFFECTS OF HIGH MOLECULAR WEIGHT GLUTENIN SUBUNITS ON WHEAT TORTILLA QUALITY

by

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Abstract

The wheat tortilla is a chemically leavened circular light colored flat bread. Desirable characteristics for good quality tortilla include large diameter, softness, flexibility and long shelf stability. Important components influencing quality are wheat flour properties, which have not been optimized for tortilla industrial production thus far. The studies presented here investigated the effects of high molecular weight glutenin subunits (HMW-GS) on tortilla quality. Two approaches were employed: biotypes derived from Centurk and OK102 cultivars expressing defined HMW-GS compositions and transgenic wheat lines over-expressing HMW-GS 10.

Analysis of protein expression and protein extractability were conducted to characterize wheat flours and suitable assays carried out to determine the respective dough properties. Tortillas were prepared by the hot-press method and quality parameters were measured at days 0, 2, 4, 7 and 14.

Tortillas derived from Centurk biotypes possessing HMW-GS 2*, 7+9, 2+12, 2*, 7+8, 5+10 and 2*, 7+9, 5+10 exhibited superior texture profiles over time, but smaller diameters than the biotype 2*, 7+8, 2+12. Tortillas containing HMW-GS 7+9 and 2+12 revealed a texture profile similar to tortillas containing 5+10. Tortillas from the OK biotype 2*, 7+9, 3+12 exhibited larger diameter and texture profiles equivalent to tortillas containing 5+10. Therefore, this biotype showed the best quality within this cultivar.

Tortillas derived from transgenic flours over-expressing HMW-GS 10 exhibited an undesirable rough appearance with decreased diameter, greater thickness, lower rollability scores, lower stretchability and greater rupture force over time. Over-expression of HMW-GS 10 in a wheat line containing 1RS-translocation did not promote the same deleterious effects in tortilla quality as it did in transgenic lines without 1RS translocation.

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Dedication

I dedicate this work to my dear mom Maria Edméa de Resende. She is a wonderful woman that taught me and my sisters the importance of good education, among all other good values that we hold for our lives. I know that earning this Master's degree will be a reward not only for me but especially for her.

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CHAPTER 1 - Literature Review: Tortilla Manufacturing, Formulation and Wheat Flour Properties for Tortilla Production

1.1 Introduction

The tortilla is the traditional unleavened flat bread consumed by native peoples of pre-Columbian Mesoamerica. Originally made from maize (*Zea mays*), the cultivation of wheat (*Triticum aestivum*) in North America led to the development of wheat flour tortillas, which became common in the northern Mexico and in southern American states, such as Texas. The popularity of Tex-Mex cuisine has introduced tortillas to the mainstream American diet. Currently, tortillas are the second best selling bread-type product in the United States. Rapid growth exhibited by the tortilla industry in the US derives not only from the growing Mexican immigrant population but also from a increasing tortilla consumption among Americans (www.tortilla-info.com 05/05/2008). Today, this market accounts for over $ 6 billion annually.

Technically, the wheat tortilla (WT) is a chemically leavened circular flat bread (Waniska 1999). Desirable characteristics for good quality WT include large diameter, high opacity, softness, flexibility, absence of cracking when folded, puffiness, light color and long shelf stability. Continuous research and development has guided the industry towards optimal quality. However, not all parameters influencing WT quality are understood, such as optimal properties of wheat flour required to obtain good quality tortillas.

It is well established that flour properties such as protein content, protein quality and amount of starch damage, determine WT quality (Serna-Saldivar et al. 1988, Waniska et al. 2004). Wheat flour contains approximately 63.0-72.0% starch, 6.0-16.0% protein, 1.0-2.0% lipids and other secondary constituents (Atwell 2001). Each constituent plays a distinct role in the quality of baked goods. Among the chemical constituents found in flour, protein content is 6-16% (by weight) and despite this modest presence in quantitative terms, is the most important component governing the quality of baked products (Shewry and Miflin 1985, Wrigley and Bietz 1988). Protein functionality in baked goods has been extensively studied for the bread industry.

However, the growing tortilla industry has generated a demand for research and development tailored to its needs. Quantitative and qualitative aspects of wheat flour proteins are major factors determining tortilla quality. High molecular weight glutenin subunits (HMW-GS), in particular, influence bread quality but little is known about their effects on tortilla properties. Original experimental research described herein focus on these aspects.

1.2 Wheat Tortilla Manufacturing

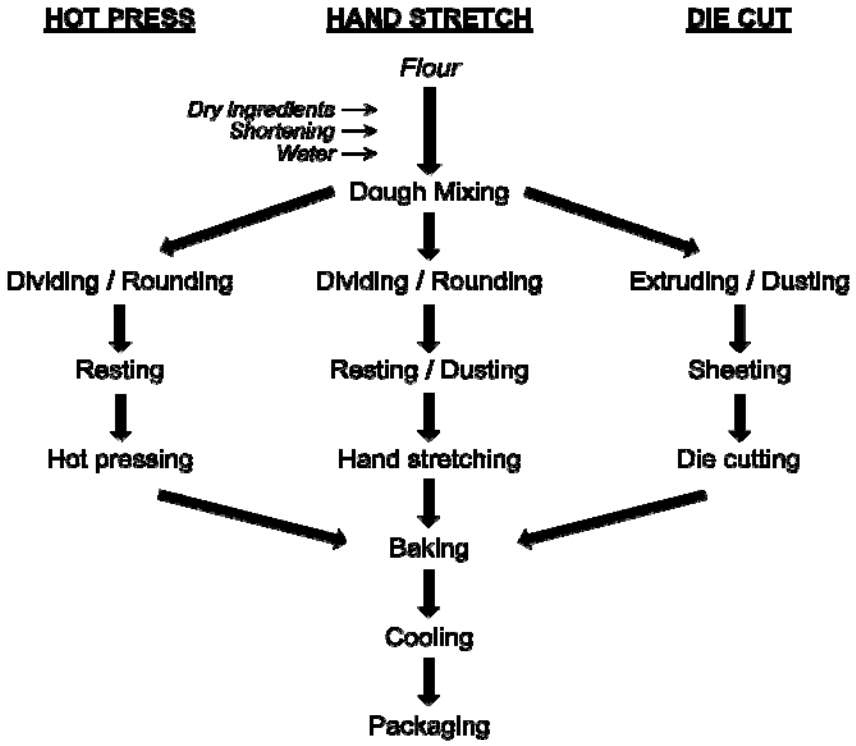
Commercially, wheat flour tortillas can be manufactured through three different methods: hot-press, die-cut and hand-stretch. Major differences between methods reside in how tortilla dough is transformed into tortilla disks. Tortilla disk formation is therefore, method-specific and a major manufacturing step that determines the characteristics of the final product. A summary illustration is depicted in Figure 1.1 (Serna-Saldivar et al. 1988), as this section compares those methods by pointing relevant features in each, as well as differences that affect the characteristics of the final product.

In order to obtain and develop tortilla dough, dry ingredients are thoroughly mixed, shortening is added and further mixing is performed until fine dispersion occurs. The amount of shortening used in the formulation, however, varies according to each method. Die-cut uses less shortening than the other two methods (6-10%), while hand-stretch and hot-press require 8-12% and 10-14%, respectively. Finally, water is added and mixed until a dough is developed. The amount of water used varies in each method and is dependent on the characteristics of the flour. Flours with high protein content, when formulated with reduced shortening, require greater amount of water for the dough development. Hot-press and hand-stretch require approximately the same amount of water (50%, based on flour), while die-cut requires greater amounts of water (54%) in order to produce a stronger dough. Increased dough strength is needed during the sheeting process in this method.

Once a dough is developed, a resting time of 5 min. is necessary before forming appropriately sized balls in order to obtain tortillas with uniform size and shape (Bello et al. 1991). The dough is then subjected to a resting time in all three methods. For hot-press, dough balls are rested for 20-30 min. in a proof chamber at 32ºC and 60-70% relative humidity (RH)

(Bello et al. 1991). Hand-stretch and die-cut doughs require dusting with wheat flour prior tortilla formation.

Figure 1.1: General schemes for the production of wheat tortillas by hot-press, die-cut and hand-stretch methods.



The processing of dough into tortilla is carried out through different maneuver among the methods. In the **hot-press** method, tortillas are formed by pressing the dough balls between two hot platens (around 200ºC) for no more than 1.5 sec. This creates a raw circular tortilla disk. Several conditions in hot-press influence the final tortilla quality, such as the amount of pressure applied to the dough, pressing time, and temperature of the platens (Adams and Waniska 2005, Bello et al. 1991). Longer shelf stability, larger diameter and reduced thickness are obtained using higher pressures of 1,450 psi, instead of 750 or 1,150 psi and using longer press-time of 1.55 sec., instead of 1.15 or 1.35 sec. (Adams and Waniska 2005). Tortilla diameter increases by 5% as platens temperature increases from 191ºC to 218ºC (Bello et al. 1991). The appearance and symmetry of tortillas improves with differential platen temperature, such as 218ºC at the top

and 204ºC at the bottom (Bello et al. 1991). Hot-pressing promotes a sealing effect on the dough and causes steam and carbon dioxide formed in the process to be trapped inside the dough while baking. This improves the characteristics of the final product as it results in greater puffing and opacity (McDonough et al. 1996, Qarooni 1993). In addition, unique features of the hot-press method produce a finished product that is superior to those produced by other methods. As a dough ball is pressed between the platens, the gluten network is forced to stretch in all directions, whereas in die-cut, the gluten is aligned unidirectionally in the sheeting direction only. Thus, hot-pressed tortillas have greater foldability, elasticity and resistance to tearing. These properties make the final product suitable for retail as gourmet table tortillas or for making products such as fajitas and soft tacos.

Tortillas made by the **hand-stretch** method are traditionally used for homes and small restaurants, in which all process is done by hand. This method is not extensively used industrially, due to intense labor demanding and high microbial contamination rates. Industrially, dough balls are flattened into disks by a presser belt. The patties are conveyed into a pair of rolls, both rotating at the same speed and direction to form, at first, ovoid tortilla disks. These are then pressed by another pair of rolls that is positioned at a right angle in relation to the first roll set and a circular tortilla disk is formed. The final shape of the disk is adjusted into a circular shape by hand-stretching on a hot plate (Serna-Saldivar et al. 1988). Characteristics of tortillas produced by hand-stretch include intermediate quality, irregular shape, and a distinctive powdery mouthfeel. A powdery mouthfeel occurs because of increased amounts of flour used in dough dusting. These tortillas are marketed as table tortillas, burritos and used in the composition of fried products.

The formation of tortillas through the **die-cut** method requires an extruder. First, the extruder sheets the dough into a layer that is dusted with flour, rolled and further thinned by a cross roller. The dough is then cut by the tortilla cutter that provides tortillas with a uniform circular size and shape. Excess trimmed from the sheeted dough (~ 5%) are returned to the extruder and sheeted a second time to minimize dough loss (Serna-Saldivar et al. 1988). This method requires stronger dough with higher water absorption than the other methods to enable proper sheeting. The resulting tortillas have low moisture content, low elasticity, increased density, reduced resistance to cracking, and a floury mouthfeel (Serna-Saldivar et al. 1988). The

low moisture content makes die-cut-produced tortillas best suited for fried products such as hard tacos, tortilla chips, salad bowls, chimichangas and frozen products.

Tortillas produced by all three methods are then baked at specific temperature. Since hot-press uses heat prior baking, tortillas are baked at lower temperatures and at longer oven dwell times: 221ºC and 32-38 sec., respectively. Tortillas originated from die-cut and hand-stretch methods are baked at 246ºC for 15-28 sec., and 235ºC for 25-32 sec., respectively (Serna-Saldivar et al. 1988).

Proper preparation for packaging is a major determinant in the final product's quality and market success. Before packaging, tortillas are cooled at 25ºC for approximately 5 to 9 min. Appropriate cooling time is essential to avoid condensation of water inside the package and to prevent tortilla stickiness. Excessive cooling time should be avoided because it increases microbial contamination and moisture loss, which affect tortilla shelf life. Once cooled, tortillas are packed into plastic bags, air is removed and packages are immediately sealed.

Among the three methods described above, hot-press is the most commonly used commercially. Tortillas prepared using this method have the characteristics desired by consumers: increased foldability, resistance to tearing, puffiness and softness. The great majority of recent research and development efforts in tortilla manufacturing employ this method. A small-scale laboratory based hot-press production system was developed in order to study manufacturing variables that affect wheat tortilla quality (Bello et al. 1991). The laboratory based hot-press method, with modifications as described in *Materials and Methods*, was used in experiments described in *Chapters 2 and 3*.

1.3 Ingredient Formulation for Wheat Tortilla Manufacturing

In the cultural birthplace of tortillas, Mexican families prepared tortillas for immediate consumption. Therefore only the fundamental ingredients flour, oil, salt and water were necessary. Such traditionally made tortillas have shelf-life of 2-4 days and are, therefore, unsuitable for commercial sale. Industrial production required formulation to achieve long shelf life and to maintain properties desired by consumers. The four fundamental ingredients are currently present in all industrial formulations, but additional ingredients such as antimicrobial and leavening agents, emulsifiers, gums and acidulants are used to obtain a final product with

commercially desirable characteristics. In addition, ingredients and their respective concentrations vary according to the adopted manufacturing method, intended shelf life and properties of available flour.

Flour is the major ingredient in tortilla formulation as it accounts for 80.0-95.0% of the dry matter and provides structure to the product. The gluten proteins (glutenin and gliadin) are responsible for the formation of a protein network with visco-elastic properties. For industrial tortilla production, flour is milled from hard wheat, bleached and enriched with thiamin, niacin, riboflavin, iron, folic acid and low levels of malted barley flour (Waniska 1999). All-purpose and soft wheat flours are used to some extent (Serna-Saldivar et al. 1988). Protein content requirements vary depending on the manufacturing process in place. Hot-press systems use flours with a protein content raging from 9.5 to 11.5%, while hand-stretch and die-cut use 10.0-11.5% and 11.5-14%, respectively. The die-cut method requires greater protein content in order to produce a stronger dough. Greater protein content also increases mixing tolerance, which is desirable in die-cut in order to reprocess dough trimmings. When flour does not contain the desired characteristics, oxidizing agents can be used to strengthen the dough and improve mixing tolerance for the die-cut method. Conversely, hand-stretched and hot-pressed tortillas use reducing agents to weaken the gluten network, decreasing mixing tolerance and subsequently obtain a dough with decreased elasticity that will allow for tortillas with increased diameter. The influence of different wheat proteins on final tortilla quality is subject of experimental research described in *Chapters 2 and 3*. Therefore further flour aspects of requirements for tortilla production are discussed later in this *review of literature*.

The role of **water** in tortilla manufacturing is to disperse all other ingredients, hydrate flour and activate the leaving system. Water is the second most abundant ingredient and accounts for 45.0 to 55.0% (baker's percentage [BP]). Variations in the amount of water used is a function of the flour's protein content, protein quality, starch properties, especially damaged starch, presence of other ingredients in the formulation such as gums, hydrocolloids, shortening and the method of tortilla formation. Water's physico-chemical characteristics that should be considered in tortilla production are temperature, pH and hardness. Water temperature should be adjusted before addition to the dry ingredients in order to obtain a dough temperature of 28ºC, which is optimal for dough resting (Serna-Saldivar et al. 1988). Water with basic pH produces undesirable colored tortillas and may affect the amount of acidulants needed in the formulation. Acidic water

causes premature CO_2 release and delays fermentation in yeast-leavened tortillas by retarding or even killing yeast cells (Serna-Saldivar et al. 1988). Water hardness is another factor that should be observed in tortilla production. Hardness is defined as the content of calcium and magnesium ions in parts per million (ppm). Water is classified as soft, medium-hard or hard as it exhibits less than 50, 100-150 or over 200 ppm, respectively. The ideal water hardness for tortilla production is medium-hard (AIB 2006).

Shortening or oil has important roles in dough processability and tortilla quality. The primary function of shortening is lubrication, as it facilitates dough expansion and improves dough handling by decreasing stickiness. It also improves shelf life by interacting with proteins and starch during mixing, baking and cooling (Serna-Saldivar et al. 1988). Fats, to enhance tortilla's flavor and tenderness, are used at concentrations varying from 6.0-15.0% (BP). The liquid form (oil) is used in die-cut and hand-stretch methods, while the solid form (shortening) is used in hot-press method. Liquid oil facilitates manipulation and incorporation into the dry ingredients, however it does not prevent oxidation of the lipids as shortening. Therefore, products made with shortening generally have a longer shelf life than those made with oil.

Salt (sodium chloride) is used for flavor at concentrations ranging from 1.3-2.0% (BP). Salt enhances the desirable flavors of a food product by increasing the perception of sweetness and masking off-tastes (Gillette 1985), contributing to the final product's taste. In addition, salt causes a gluten toughening effect that influences dough properties (Serna-Saldivar et al. 1988). Salt-containing dough exhibits decreased stickiness that favors machinability, and increased elasticity that contributes to tortilla puffing by improving gas retention (AIB 2006). Salt decreases water activity in tortillas, improving shelf life. Excess salt exerts an inhibitory effect on yeast, therefore tortilla dough containing yeast should receive salt after fermentation has been carried out.

Chemical leavening agents are composed of bases and acidic salts. In the presence of moisture and heat, they react to each other to form a neutral salt, carbon dioxide (CO_2) and water (Dubois 1981). CO_2 derived from that reaction causes tortillas to puff during baking, filling the pre-existing air cells incorporated during mixing. The resulting tortillas exhibit increased tenderness, decreased density and are white-colored, provided changes in the surface structure promoted by CO_2 (Serna-Saldivar et al. 1988). Chemical leavening agents are used at 1.0-2.0% (BP) in tortilla formulation. Several bases and acids are available for use as chemical leavening

agents. The standard alkali used in leavening systems is sodium bicarbonate ($NaHCO_3$) that produces tortillas with increased height, volume and opacity (Bejosano and Waniska 2004). Other limited used alkalis include potassium bicarbonate, ammonium bicarbonate, sodium carbonate and potassium carbonate. The acidic salts available for baking are: organic acids (fumaric, sorbic, citric, ascorbic, propionic, malic acids), tartarates, monocalcium phosphate (MCP), sodium acid pyrophosphate (SAPP), glucono delta lactone (GDL), sodium aluminum phosphate acidic (SALP), sodium aluminum sulfate (SAS), dimagnesium phosphate (DMP) and dicalcium phosphate dihydrate (DCPD or DCP). The choice of acid depends on the desired reaction rate and it can be determined by measuring the amount of CO_2 released during mixing. If the acid has a fast reaction rate, most CO_2 is released during mixing, consequently reducing the final tortilla volume, provided gas production is absent during baking. In this case, opacity will also be decreased because formation and maintenance of gas in the tortilla during baking contributes opacity in the product. Slow reacting acids allow for CO_2 release during baking as heat increase the reaction rate. Table 1.1 depicts the reaction rate of acidic salts.

Table 1.1: Rate of reaction of acid salts.

Leavening Acid	Reaction rate
Sodium Aluminum Phosphate (SALP)	Very slow
Dicalcium Phosphate Dihydrate (DCPD)	Very slow
Sodium Aluminum Sulfate (SAS)	Very slow
Sodium Acid Pyrophosphate (SAPP)	Slow
Glucono Delta Lactone (GDL)	Slow
Monocalcium Phosphate, Anhydrous, Coated (MCP)	Slower, intermediate
Monocalcium Phosphate Monohydrate (MCP)	Intermediate
Cream of Tartar	Rapid
Tartaric Acid	Very rapid

The most commonly used acids in chemical leavening of tortillas are sodium aluminum sulfate (SAS), sodium aluminum phosphate (SALP) and fumaric acid. The latter is used in combination with other salts and its major function is to acidify the formula. It was demonstrated that SALP and SAS yield better tortillas than SAPP. Addition of fumaric acid to tortillas with SALP and SAS increased tortilla shelf life (Cepeda et al. 2000).

Biological leavening agents are not extensively used in tortilla formulation. When used, they are no more than 1.0% (BP) and in the form of activated dry yeast *Saccharomyces cerevisiae* (Serna-Saldivar et al. 1988). Yeast requires at least 45 min. to start fermentation once flour has been mixed with water and dough is developed. This makes this leavening process excessively long and therefore economically disadvantageous. However, biological leavening constitutes an attractive strategy to enhance flavor development. Yeast metabolism produces organic acids and ethanol that contribute to the final flavor of baked products. Fermentation improves tortilla texture by increasing moisture retention in the final product. Special care should be taken when yeast fermentation is used because it produces enzymes that disrupt disulfide bonds in the protein network, causing a slackening effect in the dough.

Preservatives aim at inhibiting microbial growth and therefore extend tortilla shelf life. They are widely used in tortilla formulation within the range of 0.2-0.4% (BP). The most common preservatives are sodium and calcium propionates and potassium sorbate and they may be combined in a single formula. Tortilla pH is a factor that should be considered when using mold inhibitors. Such chemicals require an ideal pH range in the formulation in which they exhibit optimal performance. Propionates require pH 5.5, while potassium sorbate needs pH 6.5. It has been shown that the effectiveness of antimicrobial agents increased as the pH of tortilla decreased from 6.8 to 5.5 (Friend et al. 1995). Shelf life can be extended by over 12 days when tortilla formulation includes calcium propionate at pH 5.5 or potassium sorbate at pH 6.0 (Friend et al. 1995). In order to decrease tortilla's pH, acidulants are used. Among the most used preservatives, potassium sorbate is more effective and expensive than propionates. Calcium propionate is the most used in tortilla manufacturing provided its effectiveness, cost and tolerance to yeast activity. Sodium propionate is extensively used in chemically leavened products, since calcium propionate interferes with baking powders reactivity.

Acidulants, most commonly fumaric, malic, phosphoric, acetic and citric acids, are mainly used to decrease tortilla pH and consequently provide an appropriate environment for the

effectiveness of preservatives. Most acidulants used in tortilla formulation are fast acting acids, therefore have an initial effect in which CO_2 production occurs during mixing. Resulting tortillas may present poor volume and opacity if the amount of alkaline compounds is not adjusted to compensate initial loss. Fumaric acid is the most used acid in tortilla formulation because it is less soluble and consequently presents less interference on the leavening system. In addition, fumaric acid provides better tortilla properties when compared with other acidic compounds. It has been shown that tortillas made at pH 5.5 with citric acid had lesser diameter than tortillas made with fumaric acid at the same pH. Although all doughs made at pH 5.8 exhibited good machinability and baking performances, tortillas made with citric or malic acid presented inferior puffing when compared to counterparts made with fumaric acid (Friend et al. 1995). However, fumaric acid has a potential to react with sodium bicarbonate during mixing, therefore, interfering in the leavening system. In order to avoid such interference, fumaric acid is widely used in the encapsulated form. In this preparation, it becomes soluble and available to acidify the formulation only when tortillas are baked, as high temperature breaks the encapsulating film. Tortillas made with encapsulated fumaric acid have greater opacity when compared to appropriate controls (AIB 2006).

Emulsifiers are amply used in tortilla formulation for their dual function as dough strengtheners and crumb softeners. They promote beneficial effects such as improvement of dough machinability and gas retention, resulting in final products with increased puffing. Emulsifiers reduce dough proofing time, confer greater resistance to over-mixing, increase water absorption, decrease crumb firming and staling rate, improve texture and symmetry of the product and decrease the amount of shortening required in the formulation (Kamel 1993). Several emulsifiers are currently in use such as mono- and diglycerides, sodium stearoyl-2-lactylate (SSL), calcium stearoyl-2-lactylate (CSL), lecithin, diacetyl tartaric acid esters of monoglycerides (DATEM) and polysorbates. The most used in tortilla production are SSL and mono- and diglycerides, at a maximum amount of 0.5% and 1.0% (BP), respectively (Serna-Saldivar et al, 1988). Doughs prepared with 0.5% monoglyceride or SSL have better surface texture and machinability than dough without emulsifiers. In addition, tortillas made with this level of monoglycerides or SSL have superior quality than tortillas made with higher levels. (Friend et al. 1995). SSL is largely used in commercial tortillas at levels of 0.25 and 0.5% of flour. However, it has been shown that the ideal level in formulations using whole-wheat flour is

0.125%, which provides larger tortillas with improved rollability and stretchability (Akdogan et al. 2006).

Reducing agents affect dough properties and final product by breaking or blocking disulfide bonds in the gluten network. This allows for shorter mixing time and improved machinability (Serna-Saldivar et al. 1988). Such agents are most often used in tortilla formulation when hot-press or hand-stretch methods are used. Compounds in this ingredient class include L-cystein, glutathione, bisulfites and sodium metabisulfite. L-cystein in concentrations up to 10 ppm, improves dough and tortilla properties, whereas greater concentrations produce large diameter tortillas with poor symmetry and decreased rollability. The use of L-cystein requires strict monitoring of processes such as mixing and resting time (Friend et al. 1995).

Oxidizing agents are used in tortilla formulation tailored to the die-cut method, provided these agents increase dough mixing tolerance. Oxidizing agents used in tortilla production include potassium bromate, ascorbic acid, azadicarbonamide (ADA).

Hydrocolloids are rarely used in tortilla formulation due to their high cost and the requirement for increased process control. However, such additives exert beneficial effects to the final product. Their function is to improve shelf life by decreasing moisture loss and retarding staling. It does so by binding water at a proportion of as much as 100-fold their weight (AIB 2006). As hydrocolloids limit moisture loss in tortillas, they also prevent tortillas from sticking to each other when stacked and packed. Hydrocolloids are normally used at concentrations of 0.25-0.50% (BP). The most commonly used in tortilla formulations are guar gum, xanthan gum, and carboxymethyl cellulose (CMC). Natural gums (arabic, guar and xanthan) and CMC used in low concentrations (0.2% and 0.3%, respectively), promote better dough machinability and produce tortillas with fewer translucent areas, when compared to higher concentrations. CMC is more effective in improving tortilla rollability than natural gums (Friend et al. 1993).

Enzymes are used to modify rheological and physical properties of dough and final product. The choice of an enzyme is based on the intended effect and a list of those used in tortilla formulation would include amylases, proteases, xylanases, lipase, lipooxygenase, glucose oxidase, peroxidase, and transglutaminase. Potential problems in using enzymes are extended processing and reduction of process tolerance, provided enzymes function in strict pH and temperature ranges. For these reasons, enzymes are seldom used in tortilla formulation.

1.4 Effects of Wheat Flour Properties and its Proteins on Tortilla Quality

Flour properties are the major determinant of the quality of industrially manufactured wheat tortillas. To date, optimal flour characteristics to maximize quality are well established for bread making, but not for wheat tortilla production. Tortilla processing requires dough with high viscosity and low elasticity in order to produce tortillas with large diameters (Waniska 1999). Doughs for bread making, however, require high viscosity and elasticity to produce high volume breads. Although dough property requirements are different for these two products, the tortilla industry uses flours that were developed for bread making. Consequently, the properties of the final product are not ideal and adjustments in formulation and processing are needed to achieve desired characteristics. Therefore, a better understanding of cereal and flour chemistry influencing the final quality of tortillas is necessary in order to develop flours more suitable for tortilla production.

Wheat flour contains approximately 75% starch and 6-16% protein. Secondary components of wheat flour include lipids and non-starch carbohydrates. Endosperm proteins are classified into four classes, based on their solubility (Osborne 1907): albumins are soluble in water; globulins are soluble in dilute salt solution, but insoluble in water; gliadins are soluble in alcohol solutions and glutenins are soluble in dilute acid or base solutions. The albumins and globulins correspond to the physiologically active proteins (enzymes) and are present mainly in the aleurone cells, bran and germ and low levels in the endosperm. Gliadins and glutenins are storage proteins located in the endosperm and function as a source of amino acids for germination. Gliadins and glutenins form a complex protein network, which has a fundamental role in the making of baked products. This protein network, called gluten, is formed when wheat flour is hydrated and mechanical force is applied to the mixture in the form of mixing. Gluten has a visco-elastic structure capable to hold gas, which allows for gas trapping, rising and increased volume in the final product. Gliadins are responsible for viscosity, while glutenins confer elasticity to dough (MacRitchie 1987). Such properties of wheat gluten proteins are unique as no other cereal presents these physico-chemical abilities.

Gliadins are monomeric proteins with apparent molecular weight of 15-60 kDa (Wrigley et al. 2006), encoded at the loci *Gli-1* and *Gli-2* on the short arms of group-1 and group-6 chromosomes, respectively. All three homeologous wheat genomes (A, B and D) have the *Gli-1* and *Gli-2* loci. Based on electrophoretic mobility, gliadins are further subdivided in α, β, γ and ω

(Brown and Flavell 1981, Wrigley and Shepherd 1973). Among these proteins, ω-gliadin have the highest molecular weight, followed by γ, β and α, respectively.

Glutenins are polymeric proteins represented by two sub-groups: high molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS). *Glu-1* loci located on the long arms of group-1 chromosomes of all three wheat genomes encode HMW-GS. Each locus is composed of two tightly-linked genes, designated x- and y-type. This genetic configuration generated the theoretical expectation of six HMW-GS on hexaploid wheat. However, a variable number of subunits, from three to five, have been observed, due the occurrence of gene silencing. The y-type gene present at the *Glu-A1* locus is always silent, whereas the x-type gene at the *Glu-A1* and the y-type gene at the *Glu-B1* are expressed only in some cultivars (Shewry et al. 2006). Table 1.2 depicts common HMW-GS coded by genes in the *Glu1* loci (Payne and Lawrence 1983). The nomenclature of the HMW-GS was originally based on their apparent molecular sizes in SDS gel electrophoresis, after reduction of disulfide bonds. Subunits were named in a numerical order, in which higher molecular size received smaller numbers (Payne and Lawrence 1983). Improvement in the nomenclature system was introduced in order to name HMW-GS with intermediate molecular weight that were discovered after numerical designations of the subunits was devised. For example, HMW-GS were named with decimal numbers or a number accompanied by a symbol, such as in HMW-GS 2.2 and 2*. LMW-GS are encoded by genes on the short arm of group-1 chromosomes designated *Glu-3* (Galova et al. 2002, MacRitchie and Lafiandra 2001).

The key fundamental step in dough development is hydration of proteins (Wrigley et al. 2006). In the development of dough, gliadins and glutenins interact through chemical bonds such as hydrogen, hydrophobic, disulfide and dityrosine bonds (Bushuk 1998, Tilley et al. 2001), resulting in a complex protein matrix which determines the final structure of the dough. Since gluten proteins have an important contribution to dough formation that ultimately determines the quality of the final product, total protein content is a parameter that is widely used to estimate the quality of wheat. Protein quality found in a given wheat cultivar is a second factor used to predict grain quality. Wheat cultivars exhibit allelic variation of genes coding for gluten proteins that ultimately influence baking performance (Moonen et al. 1982, 1983, Payne and Corfield 1979, Payne et al. 1981).

Table 1.2: Common HMW-GS coded by *Glu1* loci.

HMW-GS coded by *Glu-1* loci		
Glu-A1	***Glu-B1***	***Glu-D1***
1	17 + 18	5 + 10
2*	13 + 16	2 + 12
	7 + 9	3 + 12
	7 + 8	
	6 + 8	
	20	

Hot-press tortilla processing requires flour with intermediate to high protein content (9.5-11.5%), which were shown to provide good dough processability and superior quality tortillas (Waniska et al. 2004). It is well established that protein content has a direct correlation with shelf stability, stretchability and foldability of tortillas (Qarooni et al. 1994, Wang and Flores 2000, 1999, Waniska et al. 2004). Flours with high protein content produce tortillas that maintain longer rollability, ultimately increasing shelf life (Friend et al. 1995). In studies using flours fractionated based on different milling streams or different particles sizes, fractions containing the highest protein content produced tortillas with greater rupture distances and foldability than fractions with low protein content (Wang and Flores 2000, 1999). Flours containing low amount of protein (<9.5%) produce dough that is easy to manipulate and tortillas with increased diameter. However, such flours produced tortillas with very low shelf stability, poor rollability and consequently have limited applicability in tortilla processing (Wang and Flores 2000, 1999, Waniska et al. 2004). On the other hand, when flour exhibits excessively high protein content (>12.5%), processing becomes difficult due to excessive dough strength and tortillas have decreased diameter due to shrinkage after hot press (Waniska et al. 2004).

Protein quality is determined by both gliadins and glutenins, which define the final quality of baked products (Mondal et al. 2008, Moonen et al. 1982, Payne and Corfield 1979). Investigation on how protein quality affects tortilla properties began approximately 10-15 years ago and several experimental approaches have been undertaken. Methodologies included correlation studies between dough strength and tortilla properties, supplementation of flour with

gluten or individual classes of gliadins or glutenins and the use of near-isogenic lines differing in protein composition.

Dough strength is a measurement of protein quantity as well as protein quality. Optimal level of protein strength to obtain tortillas of good quality was determined using 61 commercial tortilla flours (Sullins 1997). It was demonstrated that flours which produced doughs with intermediate protein strength had the best smoothness and softness. Flours exhibiting weak protein strength produced tortillas with significantly larger diameter, but shorter shelf life than intermediate and strong flours. Very strong flours produced tortillas with long shelf life, but were difficult to process provided the toughness of the dough (Sullins 1997).

Gluten proteins influence tortilla stability (Pascut et al. 2004, Suhendro et al. 1993, Uthayakumaran et al. 2003). It has been demonstrated that glutenin is important for tortilla stability (Uthayakumaran et al. 2003). A wheat cultivar null for all three *Glu-1* loci produced dough with very low resistance to extension and extensibility. Tortillas had increased diameter, poor rollability and lower puncture force (Uthayakumaran et al. 2003). Improvements in gluten functionality, and consequently in tortilla properties, can be achieved by supplementation of flour with gluten, gliadins or glutenins. Supplementation of control tortilla flour with 2-3% vital wheat gluten improved tortilla stability, increased shelf life, did not change dough properties and reduced tortilla diameter (Suhendro et al. 1993). Pascut (2004) examined the effect of flour supplementation with gliadin or glutenin on tortilla properties using tortilla, pastry and bread flours. Addition of glutenin to pastry and tortilla flours increased shelf stability but decreased tortilla diameter. Reduced diameter was also observed when glutenin was added to bread flour without an increase in shelf stability. Gliadin supplementation in all three flours promoted increased shelf stability without decreasing tortilla diameter.

Several correlations exist between specific HMW-GS and good quality bread, but little information is available regarding their contribution to tortilla quality. Early studies revealed that glutenin, in specific HMW-GS 1 and the HMW-GS 5+10 improved bread quality (Payne and Corfield 1979, Payne et al. 1981). Those studies were based on the indirect SDS-sedimentation test, in which the size of the gluten sediment after suspension in SDS solution is correlated with gluten strength. Baking tests confirmed that HMW-GS 5+10 have a beneficial effect in bread quality. In addition, HMW-GS 2* was correlated to good bread properties (Moonen et al. 1982, 1983). The beneficial effects promoted by HMW-GS 5+10 are superior to HMW-GS 3+12 and

2+12 on bread making (Campbell et al. 1987, Cressey et al. 1987, Lawrence et al. 1988, Ng and Bushuk 1988). In addition, HMW-GS 5+10 confers higher resistance to dough extension than its counterpart HMW-GS 2+12 (Lawrence et al. 1987). HMW-GS 5, 10, 9, 1 and 2* are also directly correlated to dough strength, while HMW-GS 2+12 are inversely correlated (Branlard and Dardevet 1985). Based on differences in bread making performance promoted by allelic variation of HMW-GS, a quality score system was proposed and is shown in Table 1.3 (Payne et al. 1987). *Glu-A1*, *Glu-B1* and *Glu-D1* scores are added to determine potential quality of a flour.

Table 1.3: Quality scores of individual or pair of HMW-GS.

Score	*Glu-A1*	*Glu-B1*	*Glu-D1*
4	-	-	5+10
3	1	17+18	-
3	2*	7+8	-
2	-	7+9	2+12
2	-	-	3+12
1	null	7	4+12
1	-	6+8	-

It has been demonstrated that variation in the HMW-GS composition also alters tortilla properties (Mondal et al. 2008). Near-isogenic lines have been useful to study the effect of specific glutenin or gliadin classes on tortilla properties. HMW-GS 1, 5+10 and 2+12 are important for tortilla stability and they provide elasticity to the dough. Tortillas without those proteins have large diameter, but shelf stability is compromised. HMW-GS 5, in particular, has been shown to play a role on tortilla shelf life when combined with HMW-GS 10. In the absence of HMW-GS 5, tortillas are larger, but with short shelf stability. Absence of HMW-GS 17+18 does not appear to decrease tortilla properties. Tortillas made with flour that does not contain these proteins have larger diameter and good shelf stability (Mondal et al. 2008). This study is, to date, the one single publication describing the influences of HMW-GS on tortilla properties and was performed using near-isogenic lines null for one or more genes that occupy the *Glu1* loci. More studies are necessary to evaluate the effects of other combinations of HMW-GS and their

effect on tortilla properties. Determination of protein functionality influencing tortilla quality is essential to overcome problems deriving from the lack of flours tailored to tortilla production.

CHAPTER 2 - Effect of High Molecular Weight Glutenin Subunit Composition on Wheat Tortilla Quality

2.1 Introduction

The tortilla is a circular, light colored flat bread that was once considered an ethnic food, but has entered the mainstream American diet. Today, the tortilla industry is a large consumer of the hard wheat flour produced in the US. Better understanding of the flour properties that influence tortilla quality has become essential. Characteristics of good quality tortilla include large diameter, opacity, puffiness and long shelf stability. A strong correlation between flour protein content and tortilla quality parameters has been well established (Qarooni et al. 1994, Wang and Flores 2000, 1999, Waniska et al. 2004). The qualitative aspect of protein composition in wheat flour also plays a role in tortilla quality and a substantial demand exist for investigation on the effect of specific gluten proteins on tortilla quality.

The qualitative aspects of gluten proteins have been the subject of extensive investigation under the focus and needs of the bread industry. Several correlations have been demonstrated between specific gluten proteins and quality characteristics of bread. High molecular weight glutenin subunits (HMW-GS) compose a group of wheat gluten proteins that have a significant effect on bread quality. HMW-GS are coded by genes located on the long arm of the group 1 chromosomes of the three homeologous wheat genomes. The HMW-GS loci in each of the three genomes, A, B and D, are designated *Glu-A1*, *Glu-B1* and *Glu-D1*, respectively. Each locus is occupied by two tightly linked genes, which code for x- and y-type of HMW-GS. Allelic variation in each locus is observed in that there have been over 20 HMW-GS identified to date. Such extensive variability in HMW-GS composition in wheat flours accounts for the wide differences in baking performance among wheat cultivars (Payne 1987a, Shewry et al. 1989, 1992).

Several sources of wheat plants expressing a defined HMW-GS composition have been used to investigate the relationship between specific HMW-GS and bread quality. Among those are wheats of different varieties, biotypes obtained from the same variety, near isogenic lines (NIL) and biotypes derived from recombinant inbred lines (RIL) (Branlard and Dardevet 1985,

Burnouf and Bouriquet 1980, Carrillo et al. 1990, Lawrence et al. 1988, Lawrence et al. 1987, Payne 1987b, Rousset et al. 1992). In a study comparing wheat varieties of known HMW-GS composition, it was demonstrated that HMW-GS 2*, 5+10 and 7+9 are positively correlated to dough strength and tenacity, while subunits 1, 17+18 and 13+16 are correlated to dough extensibility (Branlard and Dardevet 1985). Using biotypes derived from a common variety but expressing a defined subset of HMW-GS, it was shown that dough resistance to extension is strongly influenced by proteins coded by *Glu-D1* and that HMW-GS 5+10 conferred greater resistance to extension then 2+12. Smaller differences were observed for the proteins coded by *Glu-A1* (2*>1>null) and *Glu-B1* (7+9>20 and 7+8>7+9) (Lawrence et al. 1987).

NIL have also served as models to investigate the relationship between HMW-GS composition and functional properties. NIL are developed by repetitive backcrossing that introduces single genes into a common genetic background. Homogeneous genetic backgrounds allow for appropriate investigation on the effect of specific protein on end-use functionality. Investigations conducted with subsets of NIL in which one or more of the *Glu-1* loci were deleted determined that dough mixing strength and bread making quality is dramatically decreased as HMW-GS are subtracted from the original flour composition (Lawrence et al. 1988, Payne 1987b). Among the NIL with deletions in the *Glu-1* locus is a set of 3 lines derived from the cultivar Sicco (Payne 1987b). The original HMW-GS composition in Sicco is 1, 7+9, 5+10. Deletion of HMW-GS 1 alone caused a small decrease in SDS-sedimentation volume and bread loaf volume, but this decrease was accentuated when both HMW-GS 1 and 5+10 (null line at *Glu-A1*+*Glu-D1*) were deleted. Another set of NIL with deletions in one or more *Glu-1* loci were developed through crossing between the mutant cultivars Olympic (null at *Glu-B1*) and Gabo (null at *Glu-A1* and *Glu-D1*). The resulting NIL lines had HMW-GS composition varying from 0 to 5 subunits (Lawrence et al. 1988). From this set, it was demonstrated that loss of subunits 5+10 and/or 17+18 had a greater effect on mixograph time to peak than loss of subunit 1. This indicated that strong doughs are obtained when subunits 5+10 or 17+18 are present.

RIL (or biotypes) also represent a useful tool to investigate the relationship between protein composition and end-use functionality. RIL originate from repetitive crossing with a close relative and, therefore, also exhibit a largely homogeneous genetic background. This makes RIL suitable for investigation of the effects of specific HMW-GS compositions on the quality of baked products. This study made use of two sets of RIL derived from Centurk (CT) and OK102

(OK) cultivars to investigate the effect of specific HMW-GS on tortilla quality. Four biotypes from CT and four biotypes from OK were examined. Each biotype expresses five different HMW-GS. Different HMW-GS composition in the RIL was provided by allelic variation in the *Glu-B1* and *Glu-D1* loci in both cultivars, while the HMW-GS coded by *Glu-A1* was subunit 2* in all biotypes. Among the CT biotypes, the HMW-GS expressed by *Glu-B1* were 7+8 or 7+9, while the HMW-GS expressed by *Glu-D1* were 5+10 or 2+12. Among OK biotypes, the HMW-GS coded by *Glu-B1* were 6+8 or 7+9 and the HMW-GS coded by *Glu-D1* were 5+10 or 3+12.

2.2 Materials and Methods

Plant Materials and Experimental Design

To investigate the effects of HMW-GS on tortilla quality, flours derived from the two winter wheat cultivars Centurk (CK) and OK102 (OK) were used. Seeds were planted as a randomized complete block design during fall of 2004 at the University of Nebraska Agricultural Research and Development Center in Mead, NE. Basic flour characteristics such as protein content, water absorption and mixing time, were determined by the USDA-ARS HWWQL (Manhattan, KS) and are described in Table 2.1. Flour protein content (N x 5.7) was determined using AACC Method 46-40A (Leco Corp., St. Joseph, MI). Optimal water absorption and mixing time were determined using a 10-g mixograph (National Mfg. Co., Lincoln, NE) according to AACC Method 54-40A. Flours with similar protein content were chosen to compose the experimental groups in this study. CT exhibited an average protein content of 10.51% and OK flours had 10.62%. Four biotypes from each cultivar possessing different HMW-GS composition (Table 2.1) were used to produce tortillas, which were subsequently analyzed through an array of physical parameters to assess quality.

Protein Analysis

To confirm HMW-GS composition in each sample, protein electrophoresis on a microfluidic chip (Lab-on-a-chip) was performed. Protein was extracted from flour samples (100 mg) with 1 ml of 50% n-propanol containing 5% β-mercaptohetanol under constant vortexing for 30 min. at room temperature. Following centrifugation at 13,400 x *g* for 5 min., the supernatant

protein extract was retrieved and an aliquot (4 µl) was used for sample preparation to be loaded on the capillary chip (Lab-on-a-chip; Agilent Bioanalyzer 2100, Agilent Technologies, Palo Alto, CA), in accordance with the manufacturer recommendations.

Table 2.1: Protein content, mixing times and flour absorption of biotypes derived from Centurk and OK102 cultivars.

Centurk HMW-GS composition	Protein content (%)	Mixing time (min)	Flour Absorption (ml)
2*, 7+9, 2+12	10.30	3.38	63.10
2*, 7+9, 5+10	10.42	5.00	62.80
2*, 7+8, 2+12	10.66	3.13	63.30
2*, 7+8, 5+10	10.64	5.50	63.60
OK102 HMW-GS composition	**Protein content (%)**	**Mixing time (min)**	**Flour Absorption (ml)**
2*, 7+9, 3+12	10.59	4.00	62.20
2*, 7+9, 5+10	10.60	9.50	63.10
2*, 6+8, 3+12	10.61	3.38	62.10
2*, 6+8, 5+10	10.66	7.38	63.20

Tortilla Formulation and Processing

The hot-press method, adapted to a research laboratory setting (Akdogan et al. 2006), was used to make tortillas (either 700 or 800 g of flour). Wheat flour was mixed with other dry ingredients in a commercial mixer with a paddle (Kitchen-Aid, model KSM-90, St Joseph, MI) at low speed (speed 1) for 2 min. Dry ingredients included: 1.50% salt (Norton International, Chicago, IL), 0.50% sodium propionate (Caravan Ingredients, Lenexa, KS), 0.40% potassium sorbate (Caravan Ingredients, Lenexa, KS), 0.58% sodium aluminum sulfate (Budenheim USA, Inc., Plainview, NY), 0.60% sodium bicarbonate (Baking soda, Arm & Hammer, Princeton, NJ) and 0.24% encapsulated fumaric acid (Balchem Corp., New Hampton, NY). All percentages are

expressed as “baking percentage”, therefore calculated based on wheat flour. Vegetable shortening (Crisco, Orrville, OH) was added to 6.00% and mixing maintained for additional 6 min. Distilled water heated to 35°C was added slowly over 1 min. and the dough developed by mixing at higher speed (speed 2) for additional 4 min. The amount of water used in the formulation was 10 ml less than the water absorption determined by the mixograph analysis for each 100 g of flour.

Dough samples were placed in a closed plastic container, rested for 5 min. at room temperature, divided into 40 g pieces and rolled into balls using an automatic rounder (Round O Matic dough rounder, AM Manufacturing, Dolton, IL). Additional resting in a proof chamber at 35°C with 70% relative humidity was maintained for 30 min.

Dough balls were pressed using a tortilla dough press (TXA-SS Tortilla Press, DoughXpress, Pittsburg, KS) with both top and bottom platens set at 71°C for 10 sec. under the “thin” setting. Immediately after pressing, tortillas were baked on a griddle (DoughPro, model 1520) at 160°C, for 30 sec. on each side, followed by an additional 10 sec. on each side. Tortillas were allowed to cool on a metal baking rack for about 5 min, packaged into plastic bags and stored at room temperature, protected from light.

Tortilla Quality Tests

Tortilla quality was assessed by diameter and texture measurements. Tortilla texture was determined subjectively by the rollability test, and objectively using a texture analyzer. Tests were performed 2 hours after baking and this time point was designated d0. At days 2, 4, 7 and 14, only tortilla texture tests were performed.

Two diagonal diameters were measured on each tortilla using a ruler and the values were averaged. Diameter mean of 32 tortillas was determined in each experimental sample, except in sample CT 2*, 7+8, 5+10, in which diameter measurements were conducted in 28 tortillas.

The subjective rollability test (Friend et al. 1995) was performed by individually wrapping tortillas around a 1.0 cm diameter wooden dowel and visually inspecting the wrapped tortilla. Rollability determinations were assigned according to a scale ranging from 1 (impossible to roll due to breakage) to 5 (no cracking or breaking). At d0, three tortillas from each experimental group were evaluated by this test. At d2, four tortillas were assessed and at d4, d7 and d14, the subjective rollability test was performed on five tortillas from each experimental

group and values were averaged. In group CT 2*, 7+8, 5+10, rollability was performed on three tortillas at d0 and d2 and four tortillas at d4, d7 and d14 due to limited flour and thus fewer tortillas than the other flours were produced.

The objective extensibility test (Akdogan et al. 2006) was performed using a texture analyzer (model TA.XT.Plus, Texture Technology Corp., Scarsdale, NY). Values of rupture force (Fr) and stretchability (distance at Fr) were derived from the force-distance graph. Two tortillas from each experimental sample were evaluated by this test at each of the analysis time points. Each tortilla provided four strips and the mean of eight measurements was determined for each experimental sample, at each time point.

Statistical Analysis

Means, standard error of the mean and plots were derived with Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, WA). One way analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) analysis at level of 0.05 were performed to determine whether there were significant differences among experimental samples (SAS statistical software package, SAS Institute, Cary, NC).

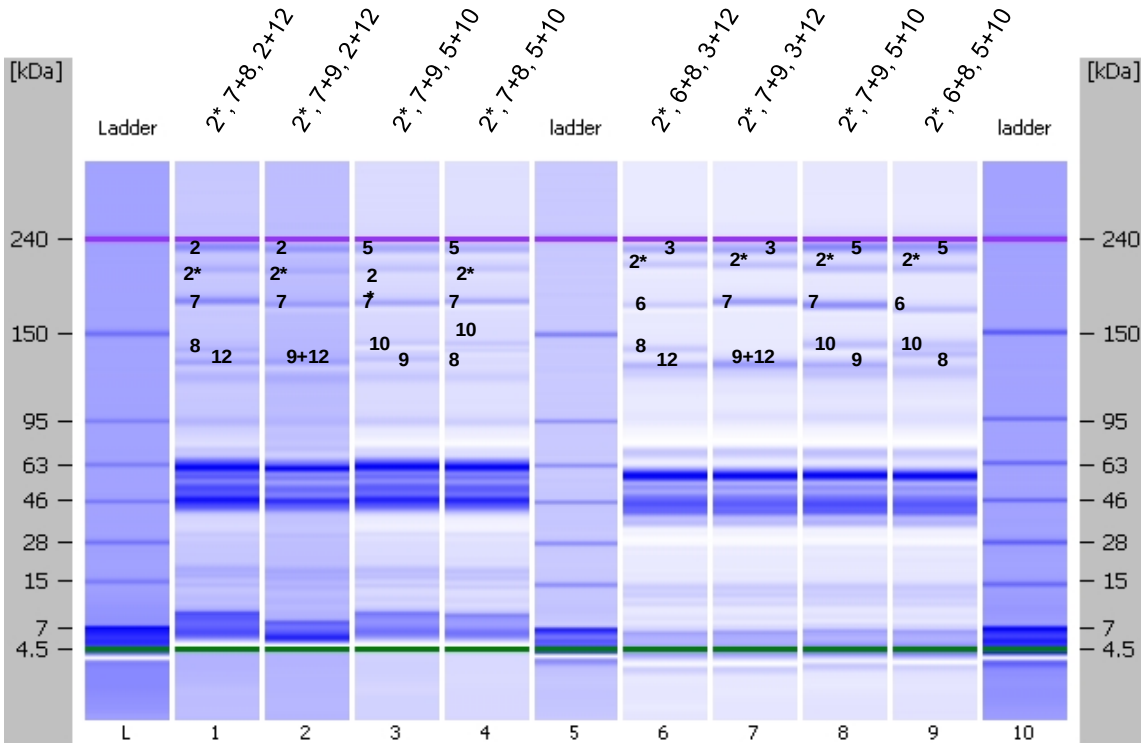
2.3 Results

HMW-GS Composition of CT and OK102 Biotypes

Differences in HMW-GS composition in each biotype of Centurk and OK were characterized by protein electrophoresis on a capillary chip. The results are shown in Figure 2.1. Data from these assays are depicted as digital simulations of protein gel electrophoresis.

Differences in HMW-GS composition are indicated in the gel by the number of each subunit. Protein bands in each lane exhibit mobilities that were expected and in accordance to the HMW-GS of the respective flour. The numbers on the protein bands indicate the HMW-GS identity. HMW-GS 9 and 12 have similar molecular weights and both these proteins are present in the same band in this system.

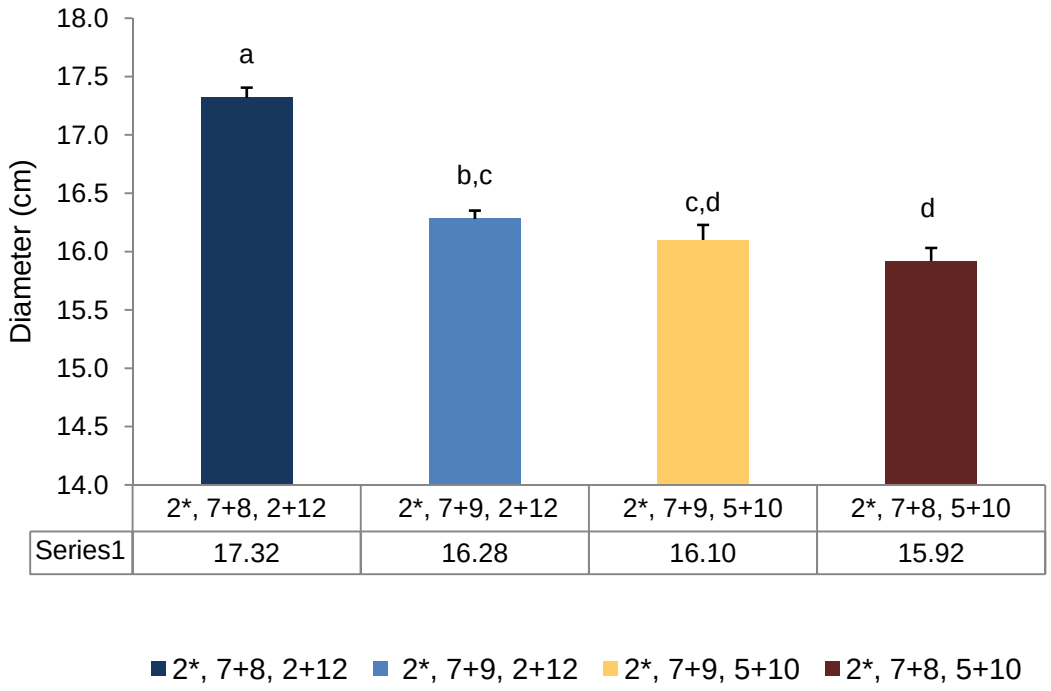
Figure 2.1: Protein gel electrophoresis conducted on a capillary chip. Lanes exhibit bands resolved from protein extracted from CT biotypes (lanes numbered 1 to 4) and OK102 biotypes (lanes numbered 6 to 9). Numbers on bands indicate the expected HMW-GS identity.



Tortilla Quality Parameters from Centurk Biotypes

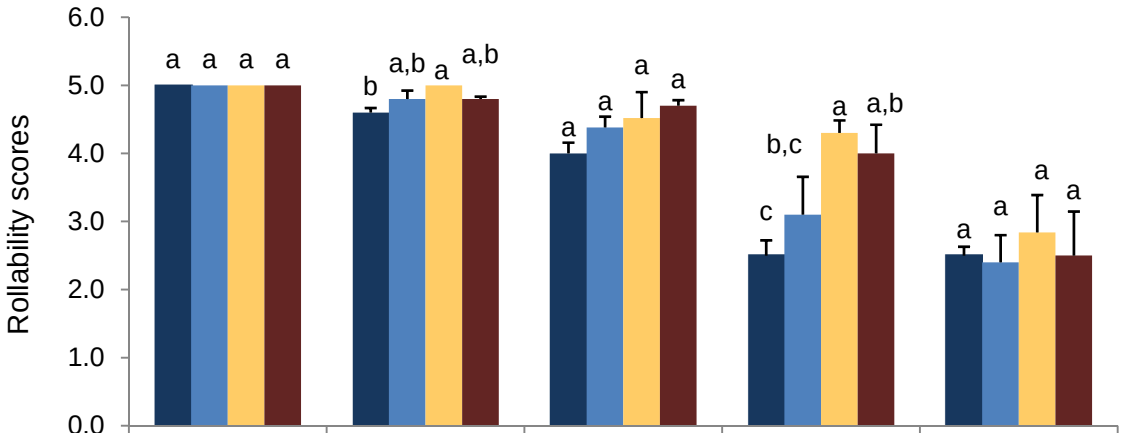
The effects of differing HMW-GS compositions on tortilla quality were investigated by comparing quality parameters of tortillas made with four different biotypes of the CT cultivar over the course of 14 days. Parameters measured included tortilla diameter, rollability, stretchability and rupture force at specific time points. The average tortilla diameter varied from 15.92 cm in biotype 2*, 7+8, 5+10 to 17.32 cm in biotype 2*, 7+8, 2+12 (Figure 2.2). ANOVA analysis indicated that tortillas containing subunits 2*, 7+8, 2+12 had significantly greater diameters than all other biotypes ($P<0.05$).

Figure 2.2: Diameter of tortillas made from four biotypes of the Centurk cultivar. Mean values that exhibit the same letter are not significantly different (P<0.05).



Tortilla texture was determined subjectively by the rollability test at 5 different time points (Figure 2.3). Tortilla rollability was highest for all biotypes at d0. At d2, rollability scores ranged from 4.6 to 5.0 and significant differences in rollability were observed between tortillas made with flour containing subunits 2*, 7+8, 2+12 (score 4.6) and 2*, 7+9, 5+10 (score 5) (P<0.05). At d4, rollability scores were lower than at d2 in all groups, ranging from 4.0 to 4.70. No significant differences in rollability were observed among samples at this time point (P<0.05). At d7, tortilla rollabilities ranged from 2.5 to 4.3. At this time point, tortillas containing HMW-GS 5+10 revealed greater rollabilities than those made with flours containing HMW-GS 2+12 (P<0.05). At d14, no significant differences in rollability were observed among samples.

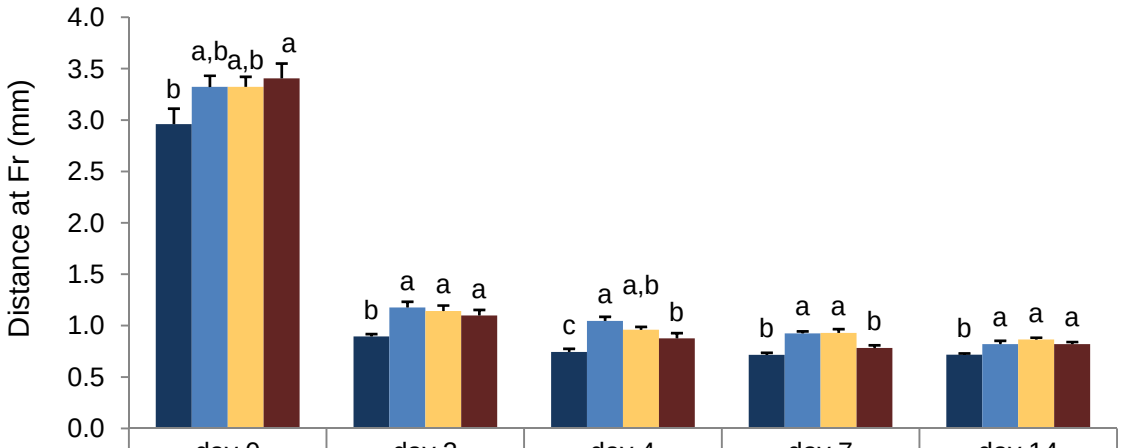
Figure 2.3: Rollability of tortillas made from four biotypes of the Centurk cultivar. Mean values that exhibit the same letter are not significantly different (P<0.05).



	day 0	day 2	day 4	day 7	day 14
2*, 7+8, 2+12	5.0	4.6	4.0	2.5	2.5
2*, 7+9, 2+12	5.0	4.8	4.4	3.1	2.4
2*, 7+9, 5+10	5.0	5.0	4.5	4.3	2.8
2*, 7+8, 5+10	5.0	4.8	4.7	4.0	2.5

Tortilla texture was determined objectively by the extensibility test, performed with a texture analyzer at 5 different time points. Results of stretchability are shown in Figure 2.4. Tortillas derived from all biotypes exhibited greatest stretchability at d0, as values ranged between 2.96 and 3.41 mm. Considerable decreases in stretchability were detected at d2, as values ranged from 0.90 to 1.18 mm. Past this time point, stretchability declined at a modest rate. At d14, tortillas from all biotypes exhibited stretchabilities ranging from 0.72 to 0.86 mm. ANOVA analysis demonstrated that at d0, a significant difference in stretchability was detected between biotypes 2*, 7+8, 2+12 (2.96 mm) and 2*, 7+8, 5+10 (3.41 mm) (P<0.05). At d2, d4 and d14, tortillas containing HMW-GS 2*, 7+8, 2+12 exhibited significantly lesser stretchability than the other three biotypes (P<0.05). At d7, tortillas from the biotype 2*, 7+8, 2+12 exhibited significantly lower stretchability than tortillas with subunits 2*, 7+9, 2+12 and 2*, 7+9, 5+10 (P<0.05).

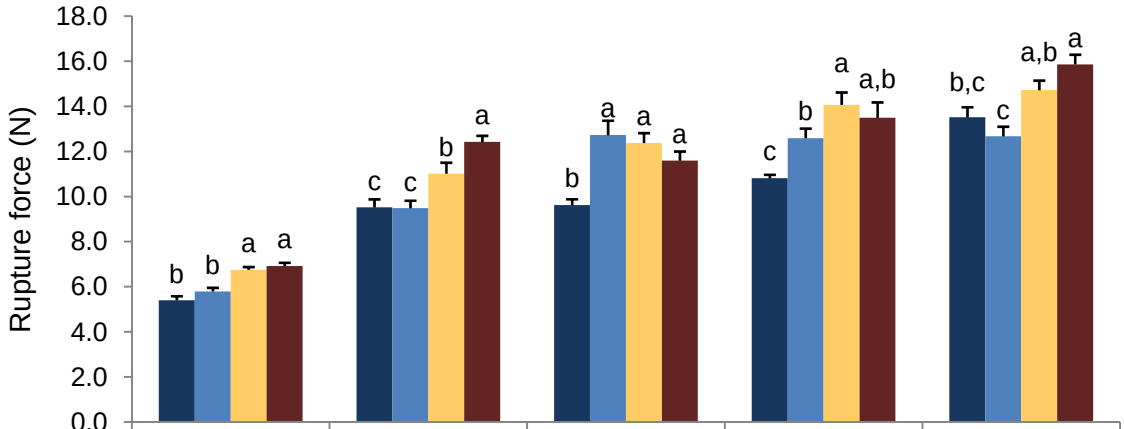
Figure 2.4: Stretchability of tortillas made from four biotypes of the Centurk cultivar. Mean values that exhibit the same letter are not significantly different (P<0.05).



	day 0	day 2	day 4	day 7	day 14
2*, 7+8, 2+12	2.96	0.90	0.74	0.72	0.72
2*, 7+9, 2+12	3.32	1.18	1.05	0.93	0.82
2*, 7+9, 5+10	3.32	1.14	0.96	0.93	0.86
2*, 7+8, 5+10	3.41	1.10	0.88	0.78	0.82

Another parameter measured was rupture force (Fr) and results are depicted in Figure 2.5. Tortillas from all biotypes exhibited lowest Fr at d0, as values ranged from 5.40 to 6.92 N. At d2, a steep increase in Fr was detected as readings of Fr were between 9.48 to 12.42 N. Small Fr increments were recorded at the remaining time points. Tortillas from flours containing HMW-GS 5+10 exhibited greater Fr than tortillas made from flours exhibiting HMW-GS 2+12 at d0 and d2 (P<0.05). At d4, tortillas with HMW-GS 5+10 presented significantly greater Fr than the counterpart flour derived from 2*, 7+8, 2+12 (P<0.05). At d7, tortillas made with flour containing HMW-GS 2*, 7+8, 2+12 exhibited significant lower Fr than all other biotypes (P<0.05). At d14, tortillas from flours containing 2*, 7+9, 5+10 and 2*, 7+8, 5+10 exhibited greater Fr than 2*, 7+9, 2+12 (P<0.05), but tortillas from 2*, 7+9, 5+10 were not different than tortillas from 2*, 7+8, 2+12 (P<0.05).

Figure 2.5: Rupture force of tortillas made from four biotypes of the Centurk cultivar. Mean values that exhibit the same letter are not significantly different (P<0.05).

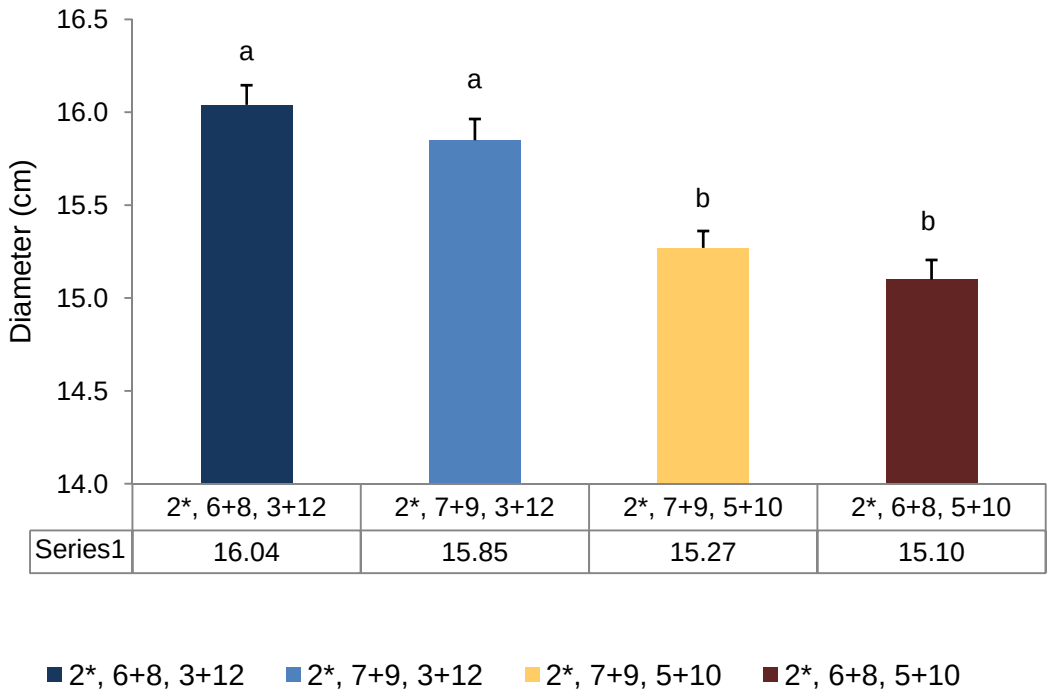


	day 0	day 2	day 4	day 7	day 14
2*, 7+8, 2+12	5.40	9.52	9.62	10.81	13.51
2*, 7+9, 2+12	5.79	9.48	12.73	12.59	12.67
2*, 7+9, 5+10	6.75	11.01	12.38	14.06	14.72
2*, 7+8, 5+10	6.92	12.42	11.59	13.49	15.86

Tortilla Quality Parameters from OK102 Biotypes

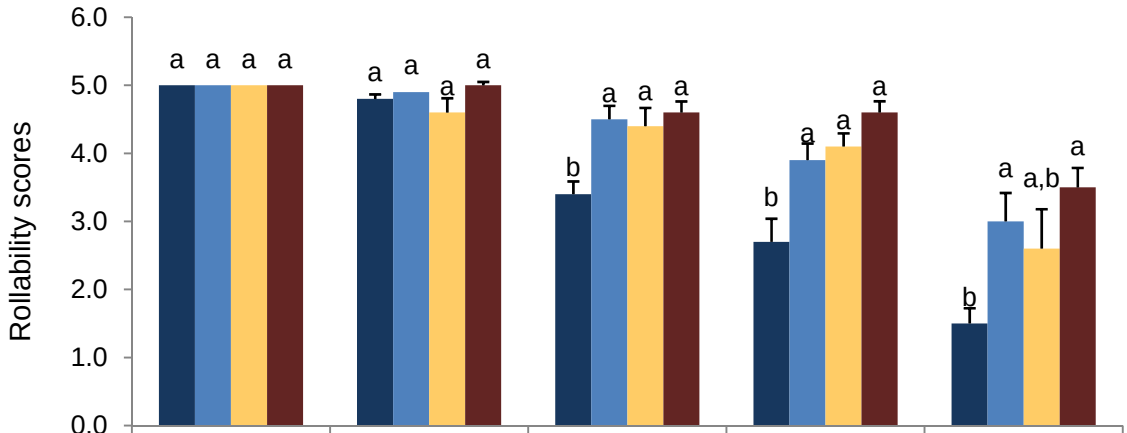
A set of four biotypes originated from the OK cultivar, expressing HMW-GS 2*, 6+8, 3+12; 2*, 7+9, 3+12; 2*, 7+9, 5+10 and 2*, 6+8, 5+10 were employed to investigate the effect of these HMW-GS compositions on tortilla quality. Tortillas made from OK flours had diameters in the range of 15.10 and 16.04 cm (Figure 2.6). ANOVA analysis demonstrated that tortillas made from flours with HMW-GS 3+12 had significantly greater diameter than tortillas made with flours containing HMW-GS 5+10 (P<0.05).

Figure 2.6: Diameter of tortillas made from four biotypes of the OK102 cultivar. Mean values that exhibit the same letter are not significantly different (P<0.05).



Subjective rollability measurements performed at pre-determined time points revealed that, all biotypes produced tortillas with the highest score at d0 (Figure 2.7). At d2, rollability scores ranged from 4.6 to 5.0, with no significant differences among the four biotypes. On the two subsequent days of analysis, d4 and d7, rollability decreased continuously for all samples, reaching values in the range of 3.4-4.6 at d4 and 2.7-4.6 at d7. ANOVA analysis indicated that, at both these time points, tortillas originated from biotype 2*, 6+8, 3+12 exhibited significant lower rollability scores than tortillas from the biotypes 2*, 7+9, 3+12; 2*, 7+9, 5+10 and 2*, 6+8, 5+10 (P<0.05). At d14, rollability scores were between 1.5 and 3.5. Biotype 2*, 6+8, 3+12 exhibited significantly lower score than tortillas made with flours containing 2*, 7+9, 3+12 and 2*, 6+8, 5+10 (P<0.05).

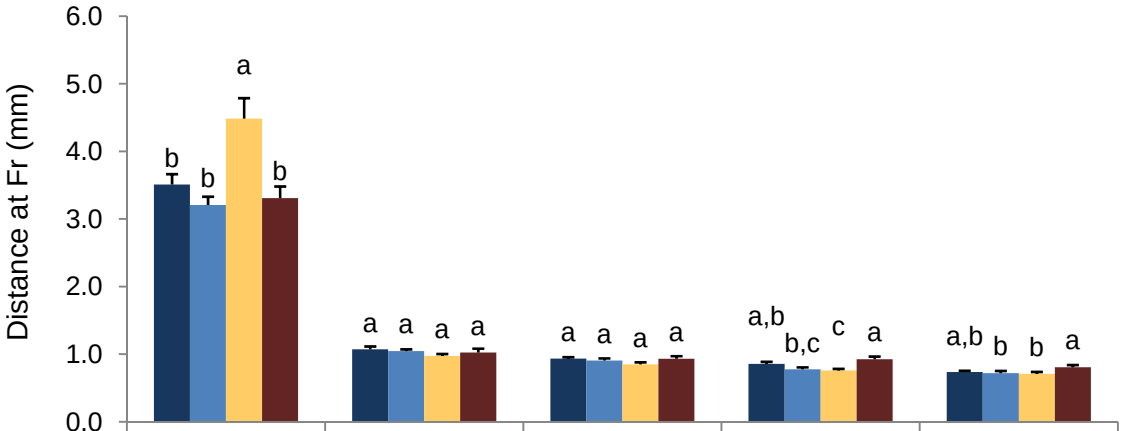
Figure 2.7: Rollability of tortillas made from four biotypes of the OK102 cultivar. Mean values that exhibit the same letter are not significantly different (P<0.05).



	day 0	day 2	day 4	day 7	day 14
2*, 6+8, 3+12	5.0	4.8	3.4	2.7	1.5
2*, 7+9, 3+12	5.0	4.9	4.5	3.9	3.0
2*, 7+9, 5+10	5.0	4.6	4.4	4.1	2.6
2*, 6+8, 5+10	5.0	5.0	4.6	4.6	3.5

Stretchability of tortillas made with OK flours were greatest at d0, decreased substantially at d2 and suffered smaller declines after this time point (Figure 2.8). At d0, tortillas made with flour containing HMW-GS 2*, 7+9, 5+10 exhibited stretchability of 4.48 mm, which was significantly greater than the 3.21-3.51 mm recorded for the other three biotypes (P<0.05). At d2 and d4, stretchabilities ranged from 0.97 to 1.07 mm and 0.85 to 0.93 mm, respectively. At these time points, no significant stretchability differences were detected among all biotypes. At d7 and d14, tortillas with HMW-GS 2*, 6+8, 5+10 presented stretchabilities of 0.93 and 0.81 mm, respectively. These were significantly greater than stretchabilities of tortillas containing 2*, 7+9, 5+10 and 2*, 7+9, 3+12 (P<0.05), but not different than 2*, 6+8, 3+12 (P<0.05).

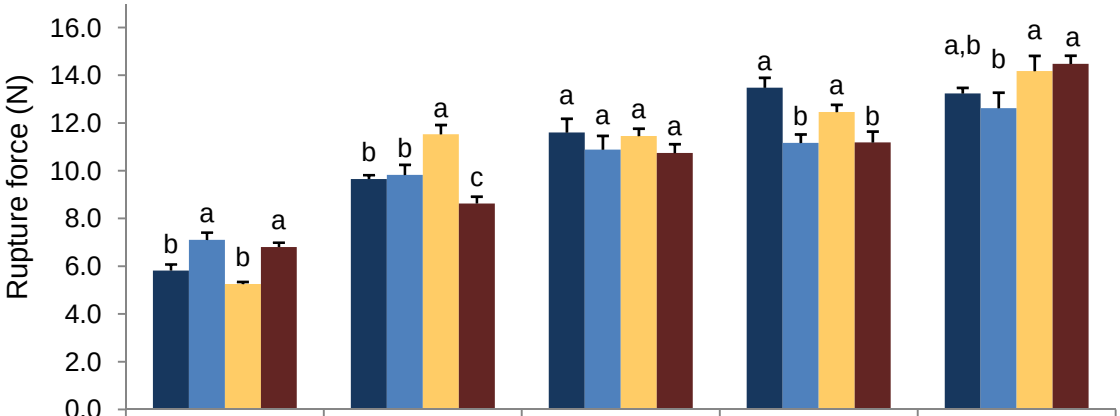
Figure 2.8: Stretchability of tortillas made from four biotypes of the OK102 cultivar. Mean values that exhibit the same letter are not significantly different (P<0.05).



	day 0	day 2	day 4	day 7	day 14
2*, 6+8, 3+12	3.51	1.07	0.93	0.86	0.74
2*, 7+9, 3+12	3.21	1.05	0.91	0.78	0.72
2*, 7+9, 5+10	4.48	0.97	0.85	0.76	0.71
2*, 6+8, 5+10	3.31	1.03	0.93	0.93	0.81

OK biotype tortillas had the lowest Fr values at d0, a substantial increase at d2 and constant smaller increases in the following time points (Figure 2.9). At d0, Fr of tortillas made with flours from all biotypes ranged from 5.25 to 7.10 N. At this time point, tortillas made with flours with HMW-GS 2*, 7+9, 3+12 and 2*, 6+8, 5+10 exhibited significantly greater Fr than flours containing 2*, 6+8, 3+12 and 2*, 7+9, 5+10 (P<0.05). At d2, tortillas containing 2*, 7+9, 5+10 exhibited significantly greater Fr than all other biotypes. Positioned at the other extreme, tortillas containing 2*, 6+8, 5+10 exhibited significantly lower Fr than all other biotypes (P<0.05). At d4, Fr ranged from 10.75 to 11.60 N and no significant differences were observed in Fr of tortillas from all biotypes. At d7, Fr values ranged between 11.17 and 13.48 N. At this time point, tortillas from 2*, 6+8, 3+12 and 2*, 7+9, 5+10 presented significantly greater Fr than tortillas from biotypes 2*, 7+9, 3+12 and 2*, 6+8, 5+10 (P<0.05). At d14, tortillas containing HMW-GS 5+10 had significantly greater Fr than tortillas with 2*, 7+9, 3+12 (P<0.05), but no difference was detected when compared to tortillas with 2*, 6+8, 3+12 (P<0.05).

Figure 2.9: Rupture force of tortillas made from four biotypes of the OK102 cultivar. Mean values that exhibit the same letter are not significantly different (P<0.05).



	day 0	day 2	day 4	day 7	day 14
2*, 6+8, 3+12	5.82	9.65	11.60	13.48	13.24
2*, 7+9, 3+12	7.10	9.82	10.88	11.17	12.62
2*, 7+9, 5+10	5.25	11.53	11.46	12.46	14.18
2*, 6+8, 5+10	6.81	8.63	10.75	11.19	14.48

2.4 Discussion

It is well established that gluten proteins such as HMW-GS, LMW-GS and gliadin affect wheat flour processing (Gupta et al. 1991, Gupta et al. 1989, Lagudah et al. 1988, Payne et al. 1987). Most importantly to this study, expression of different HMW-GS combinations among wheat cultivars account for substantial variability in baking performance observed among flours. It has been established that a decrease in HMW-GS in wheat flour causes a reduction in the flour's performance for bread making (Lawrence et al. 1988, Payne 1987b). This observation has been linked to a decrease in gluten strength. In addition, an array of correlations between specific HMW-GS and bread making potential have been established (Gupta and MacRitchie 1994, Lawrence et al. 1988, Payne et al. 1981, Payne et al. 1987).

In the present study, two sets of RIL derived from CT and OK cultivars were used to determine the effects of specific HMW-GS compositions on wheat tortilla quality. These biotypes express distinct subsets of HMW-GS coded by *Glu-B1* and *Glu-D1* loci as indicated in

Table 2.1. The results indicate that the specific HMW-GS compositions in the flours tested here affect tortilla quality parameters in different manners. However, wheat lines used in this research were grown in one single year and location. It is known that, not only genetics, but also the growth environment plays a relevant role in determining the quality of flour (Johnson et al. 1972, Peterson et al. 1992).

A collection of optimal quality characteristics was not found in any single combination of HMW-GS tested here. Instead, results suggest that a defined HMW-GS may both improve a given quality parameter and negatively affect another. At first, such a pattern was found in CT cultivar. Tortillas made from the biotype 2*, 7+8, 2+12 had significantly greater diameter but exhibited lower rollability scores, stretchabilities and rupture force than the other three biotypes, when tested at different time points. A similar observation was made with the OK cultivars, in which biotype 2*, 6+8, 3+12 had greater diameter than the other biotypes but lower rollability scores over time. It is likely that the biotypes 2*, 7+8, 2+12 and 2*, 6+8, 3+12 from CT and OK cultivars, respectively, exhibited such patterns (larger diameter but lower rollability) because doughs made from these biotypes had lower gluten strength and lower elasticity than the other biotypes. Final tortilla quality is largely influenced by two dough properties. First, tortillas of increased diameter are obtained from doughs with high extensibility and low elasticity (Waniska 1999). Secondly, tortillas with good shelf stability are obtained from doughs with intermediate gluten strength (Sullins 1997). It has been demonstrated that dough with very low gluten strength, derived from flours depleted of HMW-GS, produces tortillas with ~16% increased diameter and very low shelf stability (Uthayakumaran et al. 2003). In addition, it has been shown that an increase in gluten strength through addition of vital wheat gluten or glutenin to flour, promotes a decrease in tortilla diameter and increase in shelf stability (Pascut et al. 2004, Suhendro et al. 1993). This derives from the fact that gluten strength is strictly related to HMW-GS composition and these proteins are also responsible for dough elasticity (MacRitchie 1987). Therefore, doughs with increased strength produce tortillas with good shelf stability (good rollability), but small diameters because of greater dough elasticity. The HMW-GS composition found in biotypes CT 2*, 7+8, 2+12 and OK 2*, 6+8, 3+12 should account for the differences in dough strength and, consequently, in tortilla properties described here. It is well established that certain HMW-GS produce strong gluten, while others confer low gluten strength. HMW-GS 2+12, 3+12 and 5+10 are all coded by *Glu-D1*, however, 2+12 and 3+12 are associated with low

gluten strength, while 5+10 promotes high gluten strength (Payne et al. 1981). The first determinations of such correlations were based on indirect measurements of dough properties such as the SDS-sedimentation test. In this test, a high volume of sediment in a SDS solution correlates to high gluten strength. Flours composed of 2+12 or 3+12 have similar sedimentation volumes, however, they have lower sedimentation volume than that of 5+10 (Payne et al. 1981). Further studies confirmed that 5+10 are superior to 2+12 and 3+12 in bread making performance (Campbell et al. 1987, Cressey et al. 1987, Lawrence et al. 1988, Lawrence et al. 1987, Ng and Bushuk 1988). In this study both biotypes of CT and OK exhibiting decreased rollability scores and the greatest diameters contain subunits that confer poor gluten strength. In addition, HMW-GS 6+8 in the OK are related to very low gluten strength (Payne 1987a). Therefore, the shelf stability of tortillas originated from these biotypes was compromised, while the diameter was favored by low gluten strength. These results suggest that it might be difficult to obtain a single combination of HMW-GS to produce a complete set of desired tortilla quality characteristics.

Tortillas from the CT biotypes 2*, 7+9, 5+10; 2*, 7+8, 5+10 and 2*, 7+9, 2+12 did not reveal significant differences in diameter and texture (rollability, stretchability and Fr) at most time points. However, tortillas from these biotypes exhibited better rollability scores, greater stretchability and Fr over time, but decreased diameter than tortillas from biotype 2*, 7+8, 2+12. These results indicate that the combination of HMW-GS 7+9 and 2+12 provides a gluten structure that is as good as that obtained with 5+10. The combination of HMW-GS 7+9 and 2+12 is associated with low gluten strength measured by the SDS-sedimentation test (Takata et al. 2003), which indicates that flours containing those HMW-GS would not be appropriate for bread making. However, the gluten requirement for bread making (high extensibility and elasticity) differs from that of tortilla making (high extensibility and low elasticity) (Waniska 1999). This difference in dough requirements for bread and tortilla was clearly observed in the results presented here. HMW-GS 7+8 have been shown to exhibit higher gluten strength than 7+9 (Gao and Li 2002, Lawrence et al. 1987). This is substantiated by the HMW-GS score system (Payne et al. 1987), where subunits 7+8 receive a higher quality score (score 3) over 7+9 (score 2), indicating the flours are more suitable for bread making. In this research, tortillas obtained with CT biotypes containing HMW-GS 7+9 were shown to be superior to tortillas with 7+8, especially when combined with 2+12. Additional evidence that ideal dough properties for bread and tortilla differ was obtained from the comparison of tortillas obtained with CT biotypes

containing the pairs 5+10 or 2+12. For bread making, 5+10 is considered better than 2+12 or 3+12 for providing higher gluten strength (Lawrence et al. 1987, Payne 1987a, Payne et al. 1981). A gain in tortilla rollability has been observed with proteins coded by *Glu-D1* (5+10 or 2+12), although the data was not conclusive enough to determine which pair is best for tortilla production (Mondal et al. 2008). It was suggested that HMW-GS 5+10 provides better rollability than 2+12, but the lack of a specific near-isogenic line in that study prevented the authors from determining this. Data presented here supports that HMW-GS 7+9 coded by *Glu-B1* improve the functionality of the pair 2+12 in the tortilla making process. The resulting tortillas have quality characteristics similar to those obtained with the pair 5+10.

Results obtained from the OK cultivar indicated that tortillas from the biotypes 2*, 7+9, 5+10; 2*, 6+8, 5+10 and 2*, 7+9, 3+12 had better rollability scores than tortillas obtained from the biotype 2*, 6+8, 3+12 at most time points. No significant differences were observed for this parameter among tortillas from the former lines. Data from stretchability and Fr tests were not consistent throughout the time points and no conclusive inferences can be made. In addition, tortilla diameters from biotypes containing HMW-GS 5+10 were significantly smaller than tortillas containing 3+12. Therefore, in the OK background, the biotype 2*, 7+9, 3+12 had the best HMW-GS composition to produce good quality tortillas, as observed by larger diameters and good rollability scores over time. The benefits of HMW-GS 7+9 over the 6+8 in tortilla rollability, especially considering 3+12 in the background, is likely to be justified by the higher gluten strength from 7+9, as indicated by its greater sedimentation volume (Payne et al. 1981). Additional evidence is that 7+9 received a higher quality score (score 2) than 6+8 (score 1) in the HMW-GS score system (Payne et al. 1987). When comparing the pairs 5+10 and 3+12, HMW-GS 3+12 provide poorer gluten strength than 5+10, similarly to 2+12 (Payne et al. 1981). However, for tortilla production, the gluten strength provided by the combination of 3+12 and 7+9 has been shown to be superior to that in 5+10. Here again it was shown that variation in the expression of *Glu-B1* (in this case, expression of 7+9) can improve the functionality of 3+12 coded by *Glu-D1*, making this combination suitable for tortilla making. When 3+12 is combined with HMW-GS that confer very poor gluten strength, as 6+8, tortillas have large diameter, but low rollability scores.

2.5 Conclusions

Lines from CT and OK were used to determine the effects of specific combinations of HMW-GS flour compositions on the final quality of wheat tortillas.

Tortillas made with flours derived from CT biotypes 2*, 7+9, 2+12; 2*, 7+8, 5+10 and 2*, 7+9, 5+10 exhibited superior texture profiles over time, but smaller diameters than the biotype 2*, 7+8, 2+12. Optimal tortilla texture is influenced by intermediate to high gluten strength, while diameter is negatively affected by increased gluten strength. HMW-GS 5+10 contribute to higher gluten strength when compared to 2+12 and consequently provide better tortilla texture. However, data presented here indicate that the combination of 7+9 and 2+12 improve tortilla texture.

A similar observation was made with the OK biotype 2*, 7+9, 3+12. HMW-GS 5+10 also confers stronger gluten than 3+12. However, 7+9 combined with subunits 3+12, produced tortilla equal in texture and greater in diameter than those from biotypes containing HMW-GS 5+10. Therefore, within the OK cultivar, the biotype 2*, 7+9, 3+12 was found to produce tortillas with the best quality. Alternatively, the OK biotype 2*, 6+8, 3+12 gave rise to poor quality tortillas.

CHAPTER 3 - Effects of High Molecular Weight Glutenin Subunit 10 Over-Expression on Wheat Tortilla Properties

3.1 Introduction

Wheat is a unique cereal in its ability to form dough with viscous and elastic properties. This allows for the production of a variety of food products such as leavened breads, flat breads, pasta, noodles, cookies and cakes. Visco-elastic properties derive from gluten proteins, a group of proteins composed of glutenins (high molecular weight [HMW] and low molecular weight [LMW] glutenin subunits) and gliadins. HMW glutenin composition is highly correlated to dough strength and substantially effects end-use functionality (Shewry et al. 2003). High molecular weight glutenin subunits (HMW-GS) are coded by two tightly linked genes (x- and y-type) located on the long arm of chromosome 1 of the three wheat genomes: A, B and D. Theoretically, six HMW-GS would be expressed in hexaploid bread wheat (3x and 3y), but only three to five subunits are found depending upon the cultivar (Shewry et al. 2006). The HMW-GS coded by the D-genome are known to be responsible for significant variability in dough properties, especially strength and elasticity (Shewry et al. 2003). HMW-GS 5 and 10, coded by x- and y-genes respectively, generally produce stronger dough than the corresponding subunits 2 and 12 (Lawrence et al. 1987). The individual contribution of subunits 5 and 10 on dough properties and final product quality is difficult to assess, since this pair is always expressed together. However, the development of techniques capable to produce transgenic wheats, in which specific subunits are introduced into appropriate genetic backgrounds, have made possible investigation of the contribution of individual HMW-GS to final product quality (Weeks et al. 1993).

Hexaploid tritordeum, derived from the crossing between wild barley and durum wheat, does not possess the D genome and therefore does not express HMW-GS 5 and 10. Introduction of HMW-GS 5 into hexaploid tritordeum caused an increase in dough strength and improved quality of flour to a level suitable for bread making (Barro et al. 2003). Improvement in mixing properties and dough strength was also observed when HMW-GS 5 was genetically introduced in the wheat cultivar L88-31, which expresses HMW-GS 17 and 18 only (Barro et al. 1997).

However, introduction of extra copies of the gene coding for HMW-GS 5 in the wheat cultivar L88-6 that originally had five HMW-GS (1, 17+18, 5+10), produced excessively strong doughs (Rakszegi et al. 2005). It has been suggested that elevated levels of HMW-GS 5 form highly cross-linked polymers, with limited expansion potential (Darlington et al. 2003, Popineau et al. 2001, Rakszegi et al. 2005). Consequently, bread made with transgenic flours over-expressing HMW-GS 5 resulted in low volume loaves with dense and poor crumb structure (Darlington et al. 2003).

Wheat tortillas are extensively consumed in the United States, with annual sales exceeding $6 billion (www.tortilla-info.com 05/05/2008). Characteristics of good quality tortillas include large diameter, high flexibility and opacity, light color and long shelf stability. Tortilla quality parameters such as diameter, flexibility and shelf stability are influenced by wheat gluten proteins (Qarooni et al. 1994, Suhendro et al. 1993, Wang and Flores 1999, Waniska et al. 2004). To date, little is known of how individual HMW-GS affect tortilla quality. HMW-GS coded by *Glu-A1* (HMW-GS 1) and *Glu-D1* (HMW-GS 5+10 or 2+12) contribute to tortilla stability. Flours containing HMW-GS 5+10 produce tortillas with good shelf life. Absence of HMW-GS 5 caused loss of stability but increased tortilla diameter (Mondal et al. 2008).

To determine the effect of HMW-GS 10 over-expression on tortilla quality properties, flours derived from non-transgenic (control) and transgenic sister lines (over-expressing HMW-GS 10) were used to produce tortillas. Subsequently, tortillas were tested by an array of physical and biochemical experiments designed to assess tortilla quality parameters.

3.2 Materials and Methods

Plant Materials and Experimental Design

Transgenic wheat plants over-expressing the gene *Glu-Dy10*, which codes for HMW-GS 10, and their respective controls were made by Dr. Ann Blechl et al. at USDA-ARS Western Regional Research Center, Albany, CA. Two transgenic wheat lines, designated Dy10-E and B52a-6, were produced via particle gun bombardment, using the hard white spring wheat 'Bobwhite' as the recipient. Line Dy10-E was obtained after transformation with a construct containing the endosperm-specific promoter, coding and terminating sequences of the native common gene *Glu-Dy10* (Blechl et al. 2007, Payne and Lawrence 1983). The resulting

transformant over-expressed HMW-GS 10, along with the native HMW-GS 2*, 7, 9, 5 and 10. Line B52a-6 was obtained after transformation with the same promoter, coding and terminating sequences used to develop Dy10-E (Weeks et al. 1993).

The plants of each transgenic line were used as females in controlled matings with hard winter wheat pollen donors. Resultant F_1 plants were again used as females in back-crosses with hard winter wheat male parents to produce BC_1F_1. Seeds from BC_1F_1 were planted to produce BC_1F_2, and this was repeated until BC_1F_4. At this generation, glutenin protein composition was evaluated via SDS-PAGE. Based on HMW-GS abundance, putative transgenic lines were identified in three different groups. Each group was composed of control and transgenic samples with the same genetic backgrounds (Table 3.1), from which samples used in the experiments presented here were derived.

Table 3.1: Pedigrees of wheat populations from which flour samples were derived.

Groups	Pedigree
1	Dy10-E/N97S286//TAM202
2	Dy10-E /W96-495W//N86L177
3	B52a-6/Jagger//Heyne

Bobwhite carries the 1BL.1RS wheat-rye chromosomal translocation derived from Kavkaz (Graybosch 2001). In addition, TAM202, a parent used to develop population 3, carries the Amigo 1AL.1RS wheat-rye chromosomal translocation. Presence of 1RS in control and transgenic lines was determined by detection of rye-derived secalin proteins in SDS-PAGE analysis of the ethanol-soluble protein fraction (Lookhart et al. 1991).

Flour samples employed in the experiments and results described here were tested for their protein content and mixing properties at USDA-ARS HWWQL (Manhattan, KS) and the results of these tests are presented in Table 3.2. Control and transgenic flours from the three groups described in Table 3.1 were paired by similar protein contents. Two sets of samples were chosen from groups 1 and 3, designated “A” and “B”. Samples in each set originated from the same parents, but are different in HMW-GS composition. The 1RS rye-translocation is present in

group 3B only. Transgenic 3B refers to the transgenic line without 1RS translocation, while 3B-1RS refers to the transgenic line with 1RS translocation.

Table 3.2: Protein content, flour absorption and mixing times of control and transgenic flours.

Experimental group, HMW-GS composition	Protein Content (%)	Flour Absorption (ml)	Mixing Time (min)
1A: 2*, 7+8, 5+10			
Control	10.27	60.1	5.00
Transgenic	10.22	60.0	40.00
1B: 2*, 7+9, 5+10			
Control	12.31	63.5	4.63
Transgenic	11.85	58.7	No time*
2: 2*, 7+9, 5+10			
Control	11.89	62.8	4.13
Transgenic	11.99	62.9	No time*
3A: 1, 7+9, 5+10			
Control	11.27	61.7	3.13
Transgenic	11.65	62.4	23.50
3B: 1, 17+18, 5+10			
Control	9.90	59.4	5.13
Transgenic, without 1RS	10.13	57.8	19.50
Transgenic, with 1RS	10.48	60.4	9.50

* Mixing time was not achieved before 40 minutes.

Protein Analysis

To determine the level of HMW-GS 10 over-expression in flour, two types of protein analysis were carried out. First, separation of proteins on a microfluidic chip (Lab-on-a-chip) was employed as means of rapid semi-quantitative analysis and to determine experimental pairs of control and transgenic flours. Subsequently, reverse phase high performance liquid chromatography (RP-HPLC) was used to quantify HMW-GS 10 over-expression in transgenic relatives to control flour samples.

Two other protein analyses were performed. The insoluble polymeric protein analysis was used to evaluate protein extractability from flour and to calculate the percentage of unextractable proteins (%IPP). Finally, size exclusion high performance chromatography (SE-HPLC) in conjunction with multiangle laser light scattering (MALLS) was conducted to determine the size of polymeric proteins in control and transgenic flours.

Lab-on-a-chip Analysis. Protein was extracted from flour samples (100 mg) with 1 ml of 50% n-propanol containing 5% β-mercaptohetanol under constant vortexing for 30 min. at room temperature. Following centrifugation at 13,400 x *g* for 5 min, the supernatant protein extract was retrieved and an aliquot (4 µl) was used for the preparation of the sample to be loaded on the capillary chip (Lab-on-a-chip; Agilent 2100 Bioanalyzer, Agilent Technologies, Palo Alto, CA), in according to the manufacturer recommendations.

Reverse-phase High Performance Liquid Chromatography Analysis. Monomeric proteins were extracted from flour samples (100 mg) twice by solubilization in 1 ml of 7.5% n-propanol containing 0.3M NaI and constant vortexing for 30 min. at room temperature (DuPont et al. 2005). Supernatants were discarded and pellets were washed in 1 ml of deionized water for 5 min. Polymeric proteins were extracted from the pellet twice with 1 ml of 50% n-propanol containing 2% β-mercaptoethanol for 30 min. at 40°C. Aliquots (500 µl) of the two extractions were pooled together and samples (300 µl) were alkylated with 20 µl 4-vinylpyridine for 15 min. at 60°C. The resulting protein sample was analyzed by RP-HPLC (Agilent 1100 Series, Agilent Technologies, Palo Alto, CA). Briefly, protein samples (1 µl) were injected into a reverse phase Poroshell 300SB-C8, 2.1 x 75 mm, 5 µm particle size column (Agilent Technologies, Palo Alto, CA) kept at 65°C. Solvent flow rate was 0.7 ml/min and composed of a non-linear gradient of water (A) and acetonitrile (B), both containing 0.1% trifluoroacetic acid. The gradient was as follow: from 0 to 1 min., 23% B; from 1 to 3 min., the gradient increased from 23 to 30% B; from 3 to 11 min., increased from 30 to 44% B; from 11 to 12 min., the gradient decreased from 44 to 23% B and kept at 23% B until 13 min. Detection of protein peaks was carried out by a UV detector at 206 nm (Naeem and Sapirstein 2007). HMW-GS 10 over-expression was calculated by the ratio between the area under the curve of peaks derived from transgenic and control, normalized to their respective protein contents. This RP-HPLC analysis protocol was performed on protein samples derived from two different extractions for each flour sample and the results were expressed as mean of these two measurements.

Insoluble Polymeric Protein Analysis. Monomeric proteins were extracted from flour samples (100 mg) twice by solubilization in 1 ml of 7.5% n-propanol containing 0.3M NaI and constant vortexing for 30 min. at room temperature (DuPont et al. 2005). Supernatants were discarded and the resulting pellets were lyophilized (Labconco Corporation, Kansas City, MO) and their protein content was determined by LECO FP-428 nitrogen determinator (St. Joseph, MI). Insoluble polymeric protein percentage (%IPP) was calculated by multiplying nitrogen values by a conversion factor of 5.7 (Bean et al. 1998).

Size-exclusion High Performance Liquid Chromatography and Multiangle Laser Light Scattering Analysis. To determine the molecular weight of protein polymers in the insoluble fraction of control and transgenic flour samples, SE-HPLC and MALLS was performed. Soluble polymeric proteins (SPP) were extracted from flours (100 mg) twice by solubilization in 50 mM sodium phosphate (1 ml, pH 7), containing 1% SDS and vortexing for 5 min. at room temperature. After centrifugation, the supernatants were discarded. Pellets were resuspended in 1 ml of the same solvent and IPP were extracted from pellets via sonication (Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA) for 30 sec. at power setting 10 W. Resulting protein extracts were analyzed by SE-HPLC (Agilent 1100 Series, Agilent Technologies, Palo Alto, CA), coupled to a MALLS detector (DAWN EOS, Wyatt Technology Corp. Santa Barbara, CA). SE-HPLC was conducted using a 300.0 x 7.8 mm BioSep S4000 column (Phenomenex, Torrance, CA), kept at 50°C, with a constant gradient composed of 50% acetonitrile/0.1% TFA, flow rate of 0.5 ml/min and during 30 min. per analysis. To determine the molecular weight average molar mass (M_w) of protein polymers, a Dn/Dc value for wheat proteins of 0.31 was used (Bean and Lookhart 2001).

Micro-scale Dough Extensibility Test

Micro-scale dough extension test was performed using a texture analyzer equipped with a Kieffer rig (model TA.XT.Plus, Texture Technology Corp., Scarsdale, NY). Dough was prepared using a 10 g mixograph (National Manufacturing Mixograph, Lincoln, NE), using water absorption and mixing time previously determined by mixograph (Table 3.2). A constant mixing time of 40 min. was used for transgenic flours exhibiting “no time”. Dough formulation included flour, 2% sodium chloride and water only, as recommended by the Kieffer rig manufacturer.

Following mixing, dough was kept in a closed container for 20 min. in a chamber at 35°C with 70% relative humidity (RH). Dough was gently rounded by hand, elongated and placed on top of the base form previously greased with paraffin oil. The top form was placed over the base and pressed down. Any excess dough protruding from the edges of the forms was removed, the forms holding the pressed dough in between was placed in a plastic bag and allowed to rest for 40 min. in a proof chamber set at the same conditions as described above. Dough strips were then recovered from the forms and placed across the Kieffer rig dough holder and the test performed immediately, using a trigger force of 5 g, pre-test speed of 2.0 mm/sec., test speed of 3.3 mm/sec., post-test speed of 10.0 mm/sec. and a maximum distance of 75 mm.

Dough samples were made in triplicates for each flour. From each dough sample, four dough strips were analyzed and the mean obtained. Values reported are the mean of triplicates. Extensibility (mm) and resistance to extension (Rmax, g) were the parameters measured.

Tortilla Formulation and Processing

Tortillas were made with transgenic or control flours by the hot-press method, adapted to a research laboratory setting (Akdogan et al. 2006). Briefly, wheat flour was mixed with other dry ingredients in a commercial mixer with a paddle (Kitchen-Aid, model KSM-90, St Joseph, MI) at low speed (speed setting 1) for 2 min. Dry ingredients included: 1.50% salt (Norton International, Chicago, IL), 0.50% sodium propionate (Caravan Ingredients, Lenexa, KS), 0.40% potassium sorbate (American Ingredients, Lenexa, KS), 0.58% sodium aluminum sulfate (Budenheim USA, Inc., Plainview, NY), 0.60% sodium bicarbonate (Baking soda, Arm & Hammer, Princeton, NJ), and 0.24% encapsulated fumaric acid (Balchem Corp., New Hampton, NY). All percentages are expressed as "baking percentage", therefore calculated based on weight of wheat flour. Vegetable shortening (Crisco, Orrville, OH) was added to 6.00% and mixing maintained for additional 6 min. Distilled water heated to 35°C was added slowly over 1 min. and the dough developed by mixing with a hook at higher speed (speed setting 2). Dough development time varied. A constant time of 4 min. was used for control flours, while doughs from transgenic flours were developed for 6-19 min. The amount of water used in the formulation was 10 ml less than the water absorption determined by the mixograph analysis for each 100 g of flour.

Dough samples were placed in a closed plastic container, rested for 5 min. at room temperature, divided into 40 g pieces and rolled into balls using an automatic rounder (Round O Matic dough rounder, AM Manufacturing, Dolton, IL). Additional resting in a proof chamber at 35°C with 70% RH was maintained for 30 min.

Dough balls were pressed using a tortilla dough press (TXA-SS Tortilla Press, DoughXpress, Pittsburg, KS) with both top and bottom platens set at 71°C for 10 sec. under the "thin" setting. Immediately after pressing, tortillas were baked on a griddle (DoughPro, model 1520) at 160°C, for 30 sec. on each side, followed by an additional 10 sec. on each side. Tortillas were allowed to cool on a metal baking rack for about 5 min, packaged into plastic bags and stored at room temperature, protected from light.

Tortilla Quality Tests

Tortilla quality parameters measured were weight, thickness, diameter and texture. Tortilla texture was measured subjectively by the rollability test and objectively using a texture analyzer. All tests were performed 2 hours after baking and this time point was designated d0. At days 2, 4, 7 and 14, only tortilla texture tests (rollability, stretchability and rupture force) were performed.

Weight measurements were conducted with an analytical scale (model HF2000G, A&D Company, Japan). Tortilla thickness was determined by an automatic caliper (model SC-6, Mitutoyo, China). Two diagonal diameters of each tortilla were measured with a ruler and averaged. Weight, thickness and diameter means of twenty-three tortillas were determined in each experimental group.

The subjective rollability test (Friend et al. 1995) was performed by individually wrapping tortillas around a 1.0 cm diameter wooden dowel and visually inspecting the wrapped tortilla. Rollability determinations were assigned according to a scale ranging from 1 (impossible to roll due to breakage) to 5 (no cracking or breaking). At d0, one tortilla from each experimental group was evaluated by this test. At d2, d4, d7 and d14, subjective rollability was determined in three tortillas from each experimental group and the scores were averaged.

Objective extensibility tests (Akdogan et al. 2006) were performed using a texture analyzer (model TA.XT.Plus, Texture Technology Corp., Scarsdale, NY). Values of rupture

force (Fr) and stretchability (distance at Fr) were derived from the force-distance graph. In all days of analysis, two tortillas from each experimental group were evaluated in this test. Each tortilla provided four strips and the mean of eight measurements was determined for each experimental group and for each day of analysis.

Statistical Analysis

Means, standard error of the means, plots and *t*-tests were derived with Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, WA). One way analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) at level of 0.05 were used to identify significant differences in comparisons involving more than two experimental samples (SAS statistical software package, SAS Institute, Cary, NC).

3.3 Results

Determination of Levels of Over-expression of HMW-GS 10 in Transgenic Samples

Analysis of HMW-GS from flours derived from groups 1, 2 and 3 using the Agilent Lab-on-a-chip bioanalyzer are shown in Figure 3.1. Data from these assays are digital simulations of protein gel electrophoresis and a clear distinction between controls and transgenic samples was detected. HMW-GS 10 has an approximate mobility of 142 kDa in this system. More intense staining of this band was observed in transgenic flours when compared to controls. This indicates a higher level of HMW-GS 10 expression in transgenic flours.

To quantitatively determine the relative expression of HMW-GS 10 in transgenic and control flour samples, RP-HPLC analysis was performed. Typical chromatograms derived from polymeric protein extracted from control and transgenic samples are shown in Figure 3.2A. The protein peak eluting at approximately 4.2 min. corresponds to HMW-GS 10. The area under HMW-GS 10 peak is substantially greater in transgenic flour than that of control. Integration of results revealed the transgenic flours to have a 2.3 to 5.8-fold increase in HMW-GS 10 expression, compared to control flours (Figure 3.2B). HMW-GS 10 levels in transgenic flours from groups 1A, 1B, 2, 3A and 3B was ~ 5 times greater than the respective control flours. The

transgenic flour in group 3B-1RS had a reduced expression of HMW-GS 10, determined to be 2.3-fold increase over that of its respective control.

Figure 3.1: Protein gel electrophoresis derived from Lab-on-a-chip bioanalyzer. Lanes depict bands resolved from protein extracted from control or transgenic flours (+10). The HMW-GS 10 band (~142 kDa, arrow) reveals more intense staining in transgenic flours.

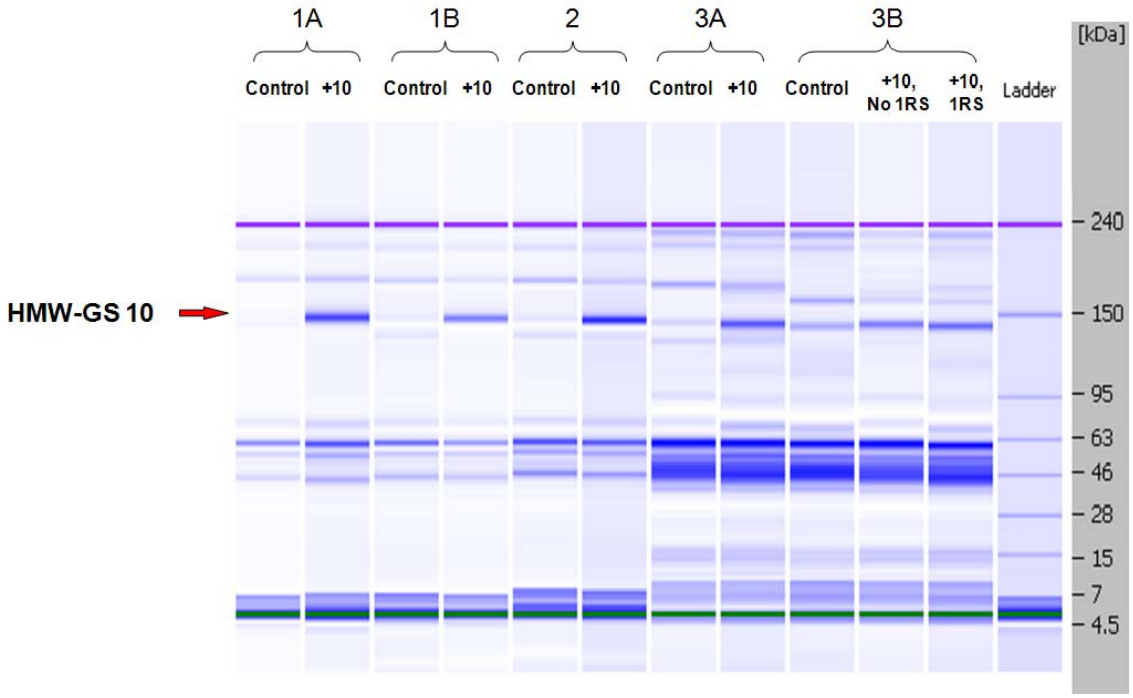
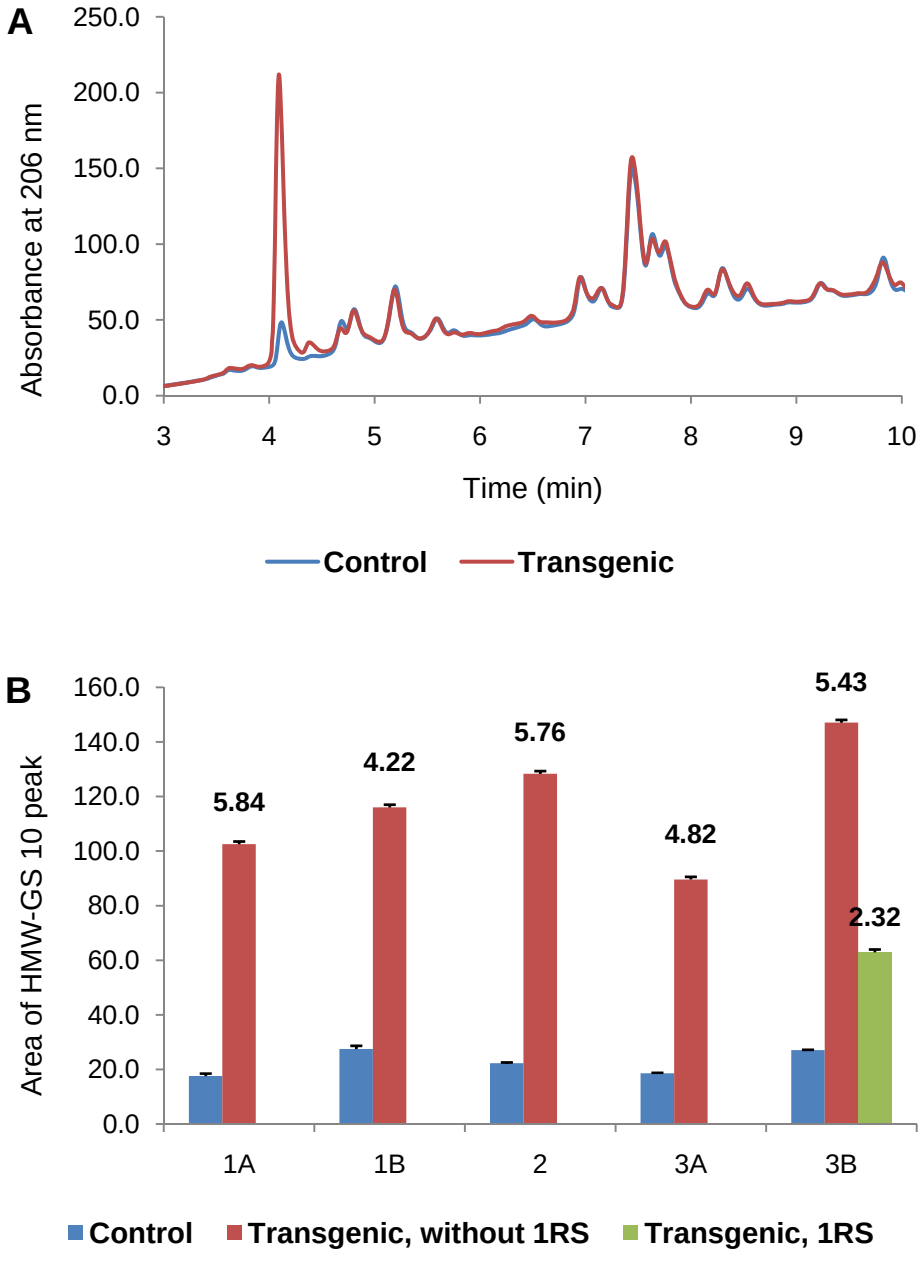


Figure 3.2: (A) Typical RP-HPLC chromatogram of polymeric protein extracted from control (blue) and transgenic flours (red). (B) Area under HMW-GS 10 peak obtained from RP-HPLC, normalized to protein content. Numbers above bars indicate the mean relative HMW-GS 10 expression in transgenic, compared to controls.



Insoluble Polymeric Proteins (%IPP) Analysis

This test was conducted to determine the extractability of flour proteins in control and transgenic samples. The %IPP was determined in each flour sample by LECO analysis following extraction of monomeric proteins. Control samples from groups 1A, 1B, 2, 3A and 3B had 59.37 to 63.61% IPP, while transgenic flours exhibited 66.01-74.48% IPP (Table 3.3). Although %IPP was consistently greater in transgenic samples, *t*-tests performed within each experimental group revealed that this pattern was significantly different in group 1B only ($P<0.05$). However, a *t*-test comparing all outcomes derived from the transgenic groups versus controls, revealed statistical significance ($P<0.05$). Flour 3B-1RS stood out of this trend as it revealed smaller %IPP than the 3B control flour.

Table 3.3: Insoluble polymeric protein determined by LECO analysis. Values are expressed as mean ± standard error of the mean. Mean values within a row with the same letter are not significantly different ($P<0.05$).

Groups	Control	Transgenic, without 1RS	Transgenic, with 1RS
1A	$61.78^{a} \pm 0.96$	$72.11^{a} \pm 0.28$	
1B	$63.61^{a} \pm 0.22$	$74.48^{b} \pm 0.25$	
2	$59.37^{a} \pm 0.58$	$73.77^{a} \pm 0.60$	
3A	$60.56^{a} \pm 1.15$	$67.99^{a} \pm 0.22$	
3B	$62.47^{a} \pm 2.61$	$66.01^{a} \pm 1.34$	$57.29^{a} \pm 2.66$

Determination of the Mw of Protein Polymers in the Insoluble Fraction

The size distribution of protein polymers in the insoluble fraction of flours was determined by SE-HPLC and MALLS analysis. The goal of this experiment was to determine whether over-expression of HMW-GS 10 affects the size distribution of polymeric proteins of flour. Therefore, only the largest polymers, which eluted first from the SE-HPLC column, were of interest. A typical size exclusion tracing combined to the molecular weight (M_w) curve of a flour sample is depicted in Figure 3.3. Based on the pattern of M_w curve, the excluded peak from SE-HPLC was split into 2 peaks, designated IPPE1 and IPPE2. The IPPE1 correspond to the

polymers with largest molecular weight and IPPE2 correspond to the average polymer size. Results from these assays are shown in Table 3.4. M_w of protein polymers in the IPPE1 region of control samples ranged from 10.00 x 10^7 to 16.3 x 10^7 Da, while transgenic samples exhibited values from 11.85 x 10^7 to 25.1 x 10^7 Da. Although transgenic samples exhibited greater M_w than control samples in this region, significant differences were observed only in groups 1B and 2 ($P<0.05$). The M_w of protein polymers from transgenic samples were also greater than control in the IPPE2 region, however significant differences were observed in group 1B only ($P<0.05$).

Figure 3.3: Typical SE-HPLC graphic of unextractable proteins and M_w curve determined by MALLS. Orange vertical lines delineate the IPPE1 and IPPE2 regions in the excluded peak from SE-HPLC.

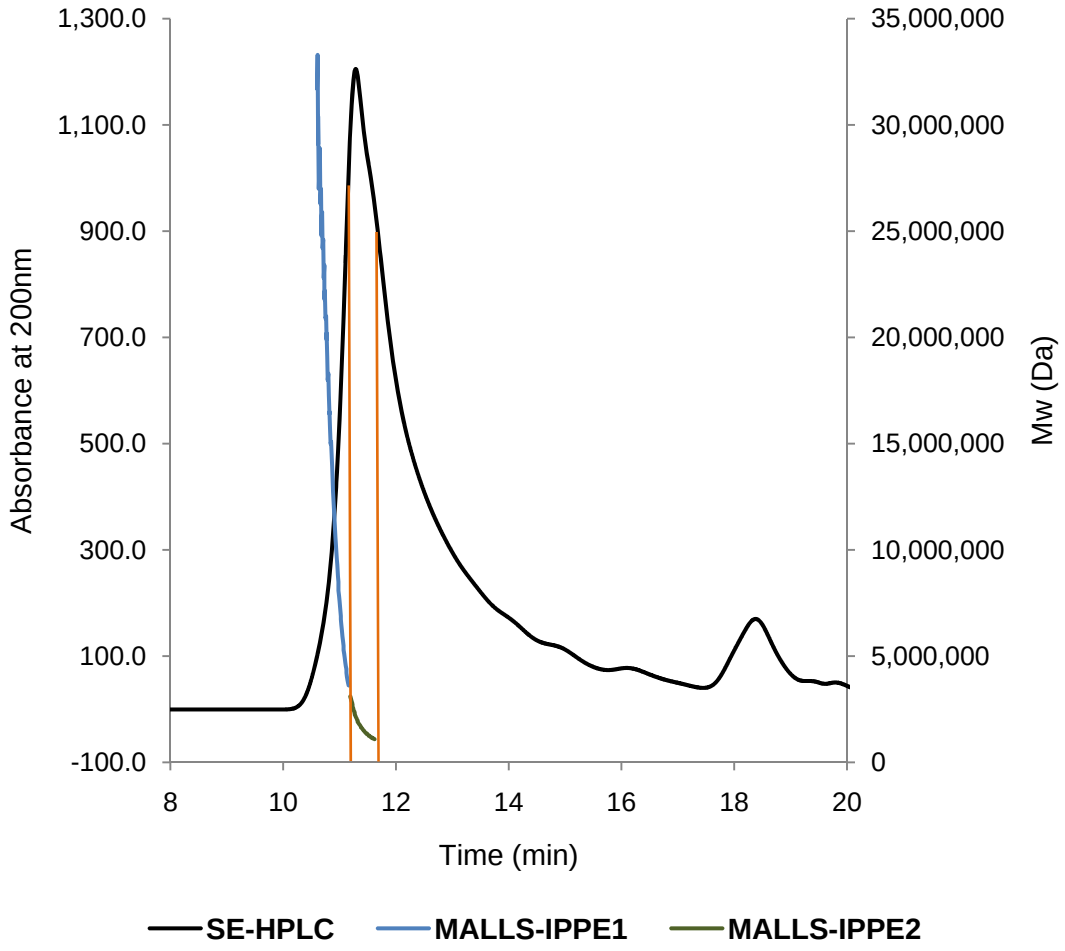


Table 3.4: Molecular weight of protein polymers in the insoluble fraction of control and transgenic flours.

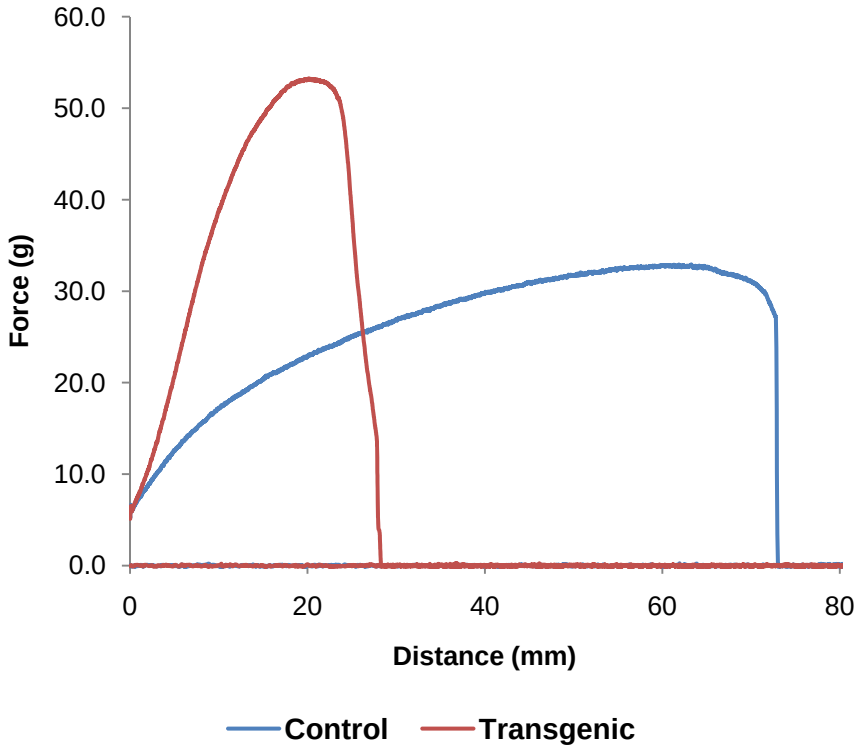
Groups	Mean of Molecular Weight (Da) IPPE1	IPPE2
1A		
Control	10.00×10^7	1.69×10^7
Transgenic	11.85×10^7	1.92×10^7
1B		
Control	13.30×10^7	2.01×10^7
Transgenic	24.15×10^7	3.42×10^7
2		
Control	14.95×10^7	2.04×10^7
Transgenic	20.15×10^7	2.87×10^7
3A		
Control	13.45×10^7	1.83×10^7
Transgenic	25.1×10^7	2.66×10^7
3B		
Control	16.3×10^7	1.93×10^7
Transgenic, without 1RS	18.3×10^7	2.53×10^7
Transgenic, 1RS	16.65×10^7	2.27×10^7

Dough Extension Properties

Figure 3.4 shows typical dough extensibility curves derived from control and transgenic samples. Results of extension properties (resistance to extension (Rmax) and extensibility) tests from doughs made with control or transgenic flours are shown in Table 3.5. Although doughs made with flours from groups 1A, 3A and 3B had a similar pattern of Rmax for control and transgenic, variability from group to group was substantial. Rmax ranged from 23.27 to 31.93 g in control doughs and from 55.76 to 82.36 g in transgenic doughs. Transgenic flours produced doughs with significantly greater resistance to extension than control flours in these three

experimental pairs (P<0.05). In groups 1B and 2, transgenic flours showed lower resistance to extension, which was statistically significant in group 2 only (P<0.05). In group 3B, the 1RS-transgenic flour produced dough with Rmax of 33.16 g, while its control had 30.50 g. This difference was not statistically significant (P<0.05; Figure 3.5A).

Figure 3.4: Typical dough extensibility curves from control (blue) and transgenic (red) samples.

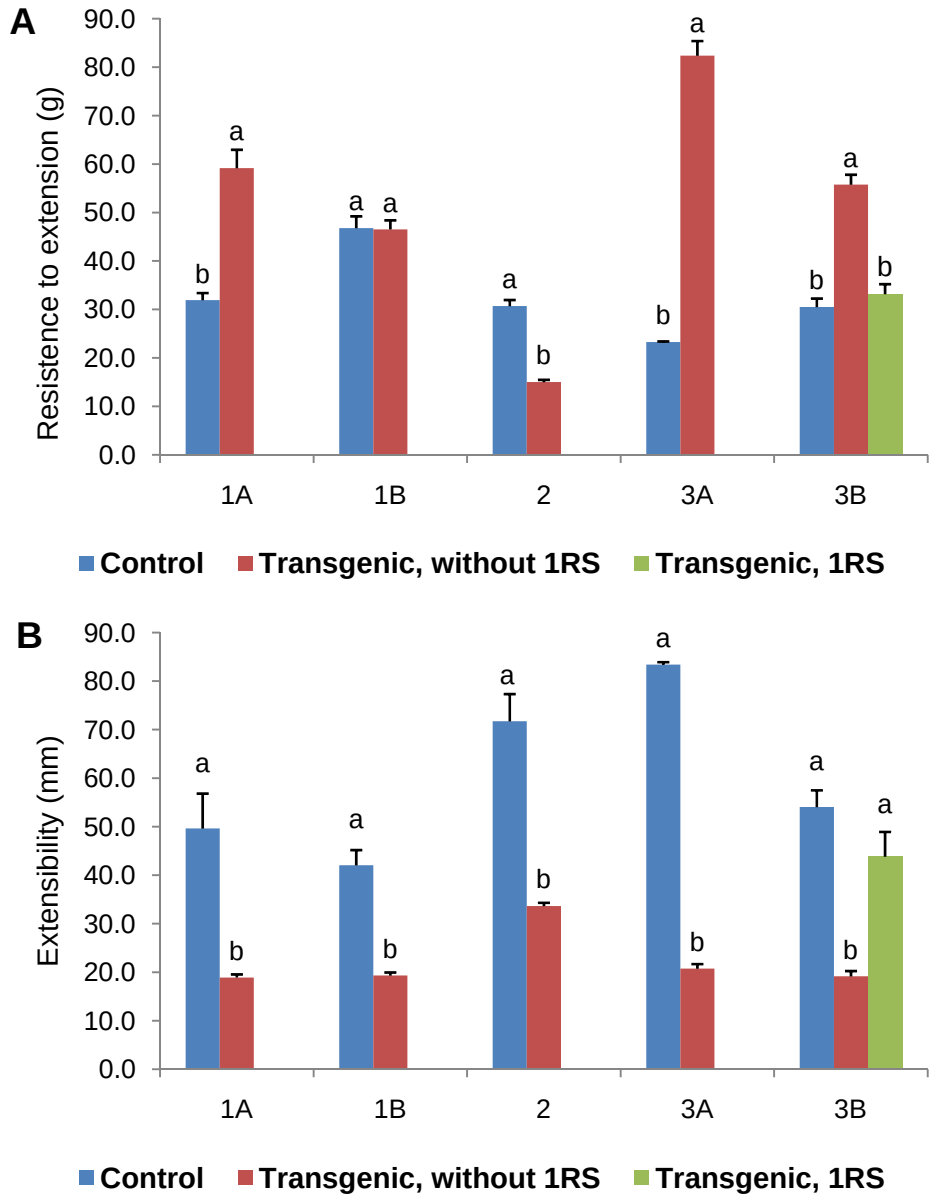


Extensibility of doughs made with control flours ranged from 42.05 to 83.39 mm, while doughs made with transgenic flours exhibited 18.91 to 33.64 mm, in groups 1A, 1B, 2, 3A and 3B. These results revealed that transgenic flours produced doughs significantly less extensible than controls in all groups (P<0.05). In group 3B, the extensibility of the 1RS-transgenic dough was 43.84 mm, while its control was 54.06 mm. This apparent difference did not prove statistically significant (P<0.05; Figure 3.5B).

Table 3.5: Dough properties derived from control and transgenic flours. Values are expressed as mean ± standard error of the mean. Within a column and each group, mean values that exhibit the same letter are not significantly different (P<0.05).

Groups	Dough properties	
	Resistance to extension (g)	Extensibility (mm)
1A		
Control	31.93^{b} ± 1.47	49.62^{a} ± 7.22
Transgenic	59.15^{a} ± 3.81	18.91^{b} ± 0.66
1B		
Control	46.79^{a} ± 2.44	42.05^{a} ± 3.14
Transgenic	46.53^{a} ± 1.88	19.31^{b} ± 0.65
2		
Control	30.71^{a} ± 1.26	71.72^{a} ± 5.62
Transgenic	15.01^{b} ± 0.48	33.64^{b} ± 0.69
3A		
Control	23.27^{b} ± 0.14	83.39^{a} ± 0.54
Transgenic	82.36^{a} ± 3.04	20.73^{b} ± 0.97
3B		
Control	30.50^{b} ± 1.76	54.06^{a} ± 3.46
Transgenic, without 1RS	55.76^{a} ± 2.05	19.15^{b} ± 1.10
Transgenic, 1RS	33.16^{b} ± 2.08	43.84^{a} ± 5.10

Figure 3.5: Dough properties of control and transgenic samples, measured by the micro-scale extensibility test. (A) Resistance to extension. (B) Extensibility. Within each group, mean values depicting the same letter are not significantly different (P<0.05).



Tortilla Quality Parameters

Tortilla weight, thickness and diameter were measured and the respective results are summarized in Table 3.6. Tortillas made with control flours in all groups weighed between 35.28 and 35.70 g, while tortillas made with transgenic flours over-expressing HMW-GS 10 had

weights between 35.72 and 36.24 g. ANOVA analysis indicated that tortillas made with transgenic flours from groups 1B, 3A and 3B were significantly heavier than control ($P<0.05$). Tortillas made with transgenic flours from groups 1A, 2 and 3B-1RS were also heavier than control, however these weight differences were not statistically significant ($P<0.05$).

Table 3.6: Tortilla quality properties derived from control and transgenic flours. Values are expressed as mean ± standard error of the mean. Within a column and each group, mean values exhibiting the same letter are not significantly different ($P<0.05$).

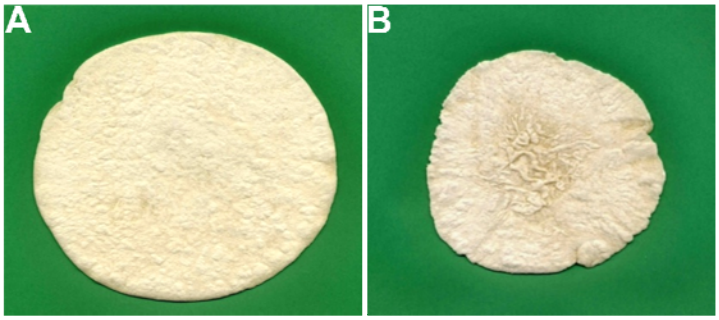
Groups	Weight (g)	Thickness (mm)	Diameter (cm)
1A			
Control	$35.70^{a} \pm 0.08$	$2.5^{b} \pm 0.05$	$15.9^{a} \pm 0.12$
Transgenic	$35.76^{a} \pm 0.07$	$2.7^{a} \pm 0.07$	$13.7^{b} \pm 0.11$
1B			
Control	$35.69^{b} \pm 0.08$	$2.3^{b} \pm 0.07$	$16.0^{a} \pm 0.11$
Transgenic	$36.24^{a} \pm 0.04$	$2.6^{a} \pm 0.07$	$13.5^{b} \pm 0.10$
2			
Control	$35.58^{a} \pm 0.10$	$2.2^{b} \pm 0.07$	$16.1^{a} \pm 0.11$
Transgenic	$35.79^{a} \pm 0.05$	$2.8^{a} \pm 0.04$	$13.8^{b} \pm 0.08$
3A			
Control	$35.28^{b} \pm 0.08$	$2.2^{b} \pm 0.07$	$16.9^{a} \pm 0.10$
Transgenic	$36.09^{a} \pm 0.09$	$2.6^{a} \pm 0.09$	$14.1^{b} \pm 0.16$
3B			
Control	$35.7^{b} \pm 0.08$	$2.1^{b} \pm 0.05$	$16.2^{a} \pm 0.12$
Transgenic, without 1RS	$35.95^{a} \pm 0.03$	$2.3^{a} \pm 0.04$	$14.8^{c} \pm 0.07$
Transgenic, 1RS	$35.72^{b} \pm 0.08$	$2.3^{a,b} \pm 0.07$	$15.9^{b} \pm 0.13$

Tortilla thickness from control flours in all groups ranged from 2.1 to 2.5 mm (Table 3.6). On average, tortillas from transgenic flours were 15% thicker than control tortillas. ANOVA analysis indicated that tortillas made from flours over-expressing HMW-GS 10 were

significantly thicker than their respective controls in all groups, except in group 3B-1RS ($P<0.05$).

Tortilla diameter from control samples ranged from 15.9 to 16.9 cm, while tortillas made from transgenic flours ranged from 13.5 to 14.8 cm (Table 3.6; Figure 3.6). Tortillas made with flours over-expressing HMW-GS 10 exhibited significantly smaller diameter than controls in all groups, including 3B-1RS ($P<0.05$).

Figure 3.6: Tortilla made with control (A) and transgenic flours (B).



Pearson's correlation analysis was carried out to investigate the relationship between diameter and %IPP, as well as diameter and dough properties (Figure 3.7; Table 3.7). Results indicated that diameter had a high negative correlation (R^2=0.84) with %IPP (Figure 3.7A) and a positive correlation (R^2=0.75) with dough extensibility (Figure 3.7B).

Figure 3.7: Degree of linear correlation between tortilla diameter and respective flour insoluble polymeric protein fraction (A) and, otherwise, between tortilla diameter and respective dough extensibility (B).

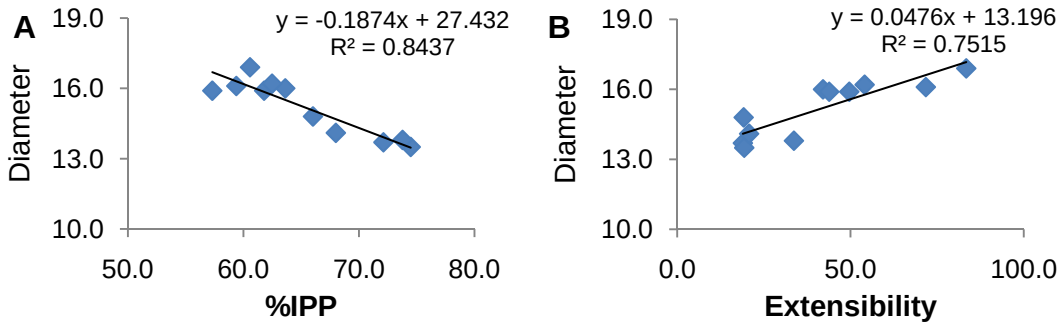
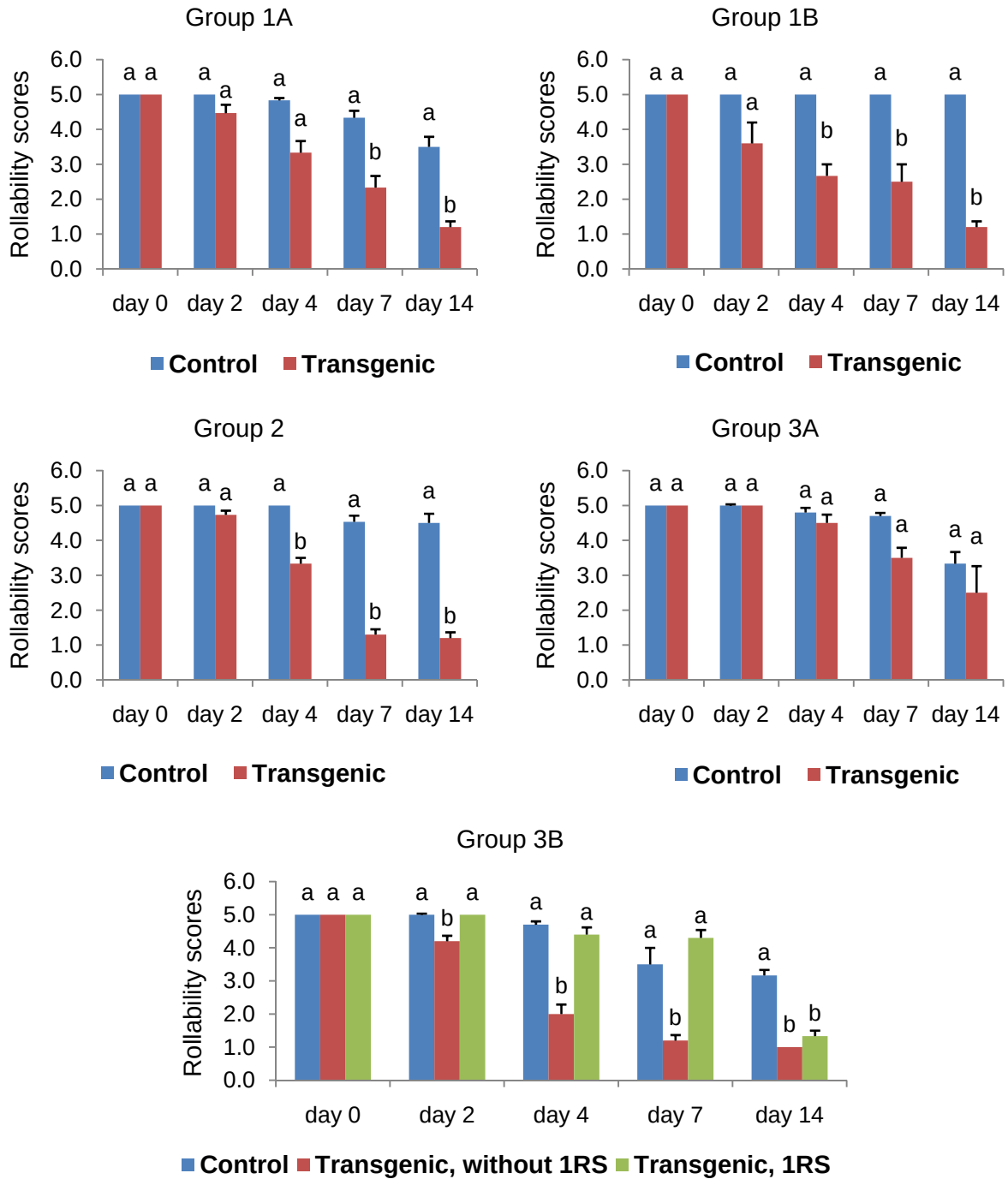


Table 3.7: Pearson's correlation of tortilla diameter with insoluble polymeric protein (%IPP) and dough properties (extensibility and resistance to extension).

	Diameter	*% IPP*	*Extensibility*	*Resistance to Extension*
Diameter	1			
% IPP	**-0.919**	1		
Extensibility	**0.867**	**-0.726**	1	
Resistance to Extension	-0.466	0.294	-0.659	1

Tortilla texture was determined subjectively by the rollability test over a period of 14 days. Tortillas made from both transgenic and control flours from groups 1A, 1B, and 2 showed similar rollability scores over time (Figure 3.8). In these groups, freshly baked tortillas and tortillas assessed on d 2, made from both control and transgenic flours, had high rollability scores with no significant differences between them ($P<0.05$). However, significantly lower rollability scores were measured in tortillas made with transgenic flours after d4 (groups 1B and 2) and after d7 (group 1) ($P<0.05$). At the end of 14 days, control tortillas exhibited rollability scores of 3.5, 5.0 and 4.5 for groups 1A, 1B and 2, respectively, while tortillas made with transgenic flour had rollability score 1.2 for all three groups. In group 3A, freshly baked tortillas and tortillas at d2 also had high rollability scores for both control and transgenic flours (Figure 3.8). At subsequent time points, although control tortillas exhibited better rollability scores than tortillas made with transgenic flour, the differences in rollability were not statistically significant ($P<0.05$). At the end of 14 days, control tortillas and tortillas made with transgenic flour exhibited average rollability scores of 3.3 and 2.5, respectively. In group 3B, control tortillas and tortillas made with transgenic flour (both with and without 1RS translocation) had rollability scores 5 at d0 (Figure 3.8). After d2, tortillas made with 3B-transgenic flour had significantly lower rollability scores than control tortillas and tortillas made with 3B-1RS-transgenic flour at all time points, except at d14 ($P<0.05$). No significant differences were observed between control and tortillas made with 3B-1RS transgenic flour in most days of analysis, except at d14 ($P<0.05$). At the end of the 14 days, control tortillas exhibited rollability score 3, tortillas made with 3B-transgenic flour had score 1 and tortillas made with 3B-1RS transgenic flour revealed score 1.3.

Figure 3.8: Subjective rollability of tortillas made with control and transgenic flours. Within each time point, mean values exhibiting the same letter are not significantly different (P<0.05).



Tortilla texture was analyzed objectively using a texture analyzer over the period of 14 days. One of the parameters measured by the extensibility test was stretchability. Control tortillas and tortillas made with transgenic flours from groups 1A, 1B, 2 and 3A had a similar pattern of stretchability, with small differences in mean values (Figure 3.9). In these groups, tortillas exhibited the highest stretchability at d0, with values ranging from 4.21 to 5.31 mm for control tortillas and 3.20 to 3.97 mm for tortillas made with transgenic flours. The greatest changes were observed from d0 to d2, in which control and transgenic tortillas decreased their stretchability by ~73%. After d2, stretchability still decreased in both groups, but to a much lesser extent. ANOVA analysis demonstrated that at all time points, control tortillas had significantly greater stretchabilities than tortillas made from flours over-expressing HMW-GS 10 in groups 1A, 1B and 2 ($P<0.05$). In group 3A, there was no significant differences in stretchability between control tortillas and tortillas made with transgenic flours at all time points, with exception of d14 ($P<0.05$). In group 3B, tortillas had the greatest stretchability at d0, with average values of 3.8 mm. This was followed by a substantial decrease at d2, reaching stretchabilities of ~1.08 mm. Changes in stretchability after d2 were minimal (Figure 3.9). Significant differences in stretchability between control and transgenic tortillas (with and without 1RS translocation) were not observed at d0, d4 and d14. At d2, tortillas originated from group 3B-1RS-transgenic flour were significantly more stretchable than the control tortillas and tortillas made with 3B-transgenic flour. At d7, control tortillas were more stretchable than tortillas made with transgenic flours ($P<0.05$).

Rupture force (Fr) was the second parameter measured by the extensibility test at days 0, 2, 4, 7 and 14. Freshly baked tortillas had the lowest values of Fr, followed by a steep increased at d2 and smaller increments as tortillas aged after d2. Fr of tortillas made from flours in groups 1A, 1B, 2 and 3A were also similar, with small differences in mean values (Figure 3.10). At d0, control tortillas exhibited variation in Fr of 4.86 to 6.36 N, while transgenic tortillas had values from 7.24 to 10.91 N. At d2, a two fold increase in Fr was observed for both control and transgenic tortillas. Tortillas made with transgenic flours had significantly greater rupture forces than control tortillas at all time points ($P<0.05$). In group 3B, the same pattern of Fr as in the previous groups was detected, in which tortillas exhibited the lowest Fr at d0 and increases in Fr as tortillas aged (Figure 3.10). Control tortillas in this group revealed Fr of 6.16 to 14.62 N from d0 to d14, respectively, while tortillas made with 3B-transgenic flour exhibited values from

Figure 3.9: Stretchability of tortillas made with control and transgenic flours. Within each time point, mean values exhibiting the same letter are not significantly different (P<0.05).

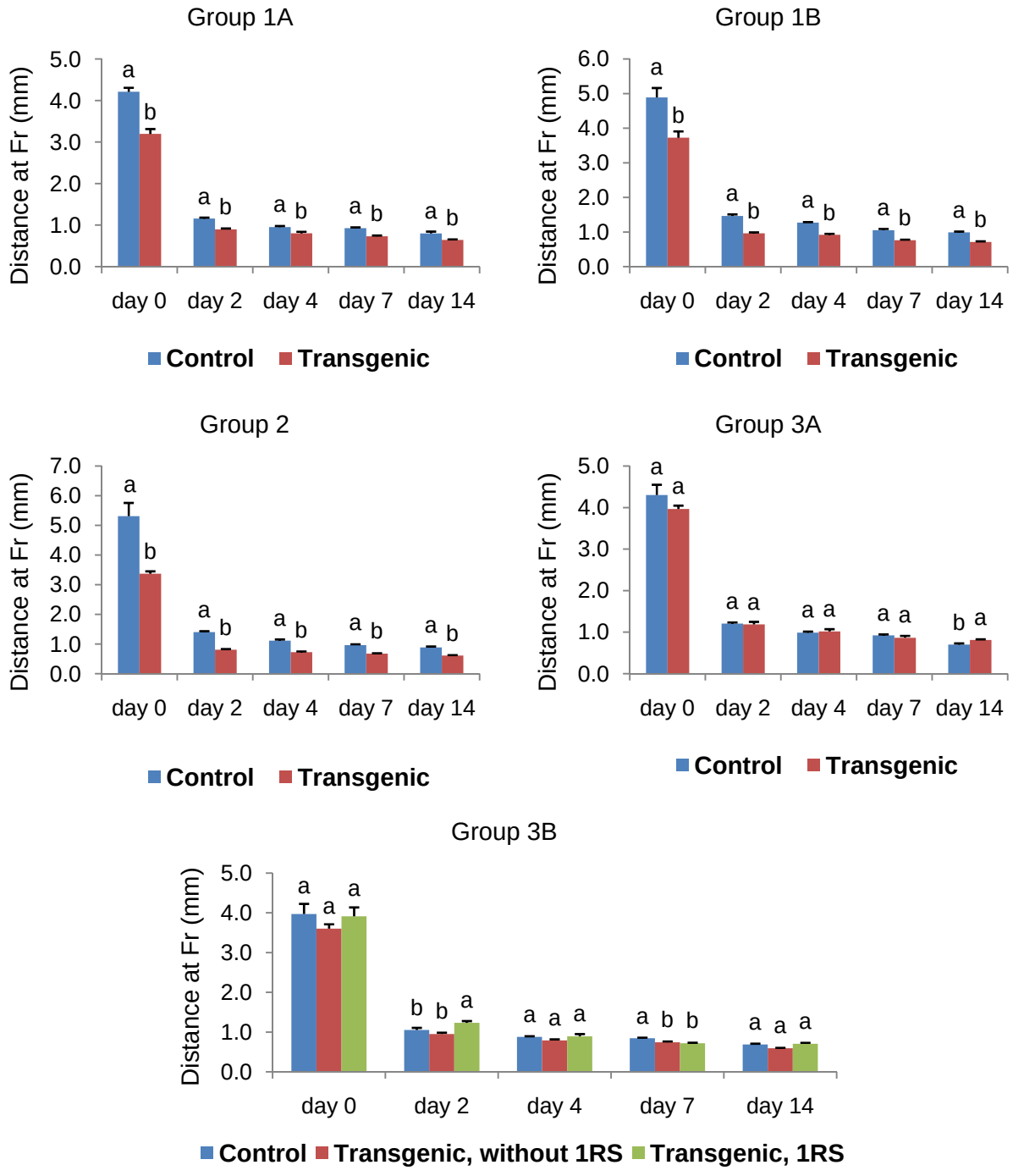
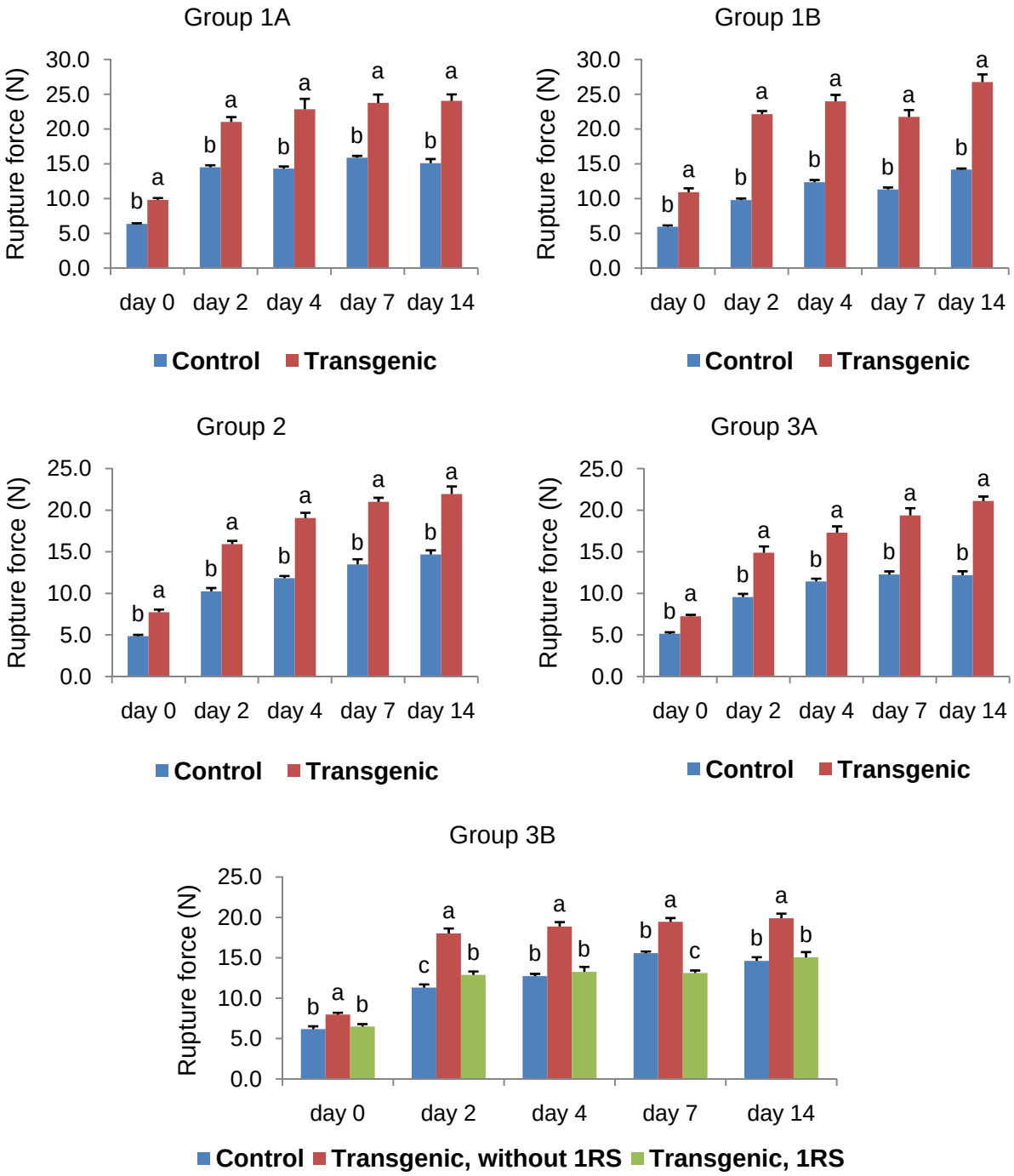


Figure 3.10: Rupture force of tortillas made with control and transgenic flours. Within each time point, mean values exhibiting the same letter are not significantly different (P<0.05).



7.97 to 19.90 N and tortillas made with 3B-1RS-transgenic flour had 6.48 to 15.05 N, respectively. ANOVA analysis revealed that tortillas from transgenic flour without 1RS translocation had significantly greater Fr than control tortillas and tortillas made with 3B-1RS-transgenic flour at all time points. Rupture forces of tortillas made with 3B-1RS-transgenic flour were not significantly different from control tortillas, except for d2 and d7 ($P<0.05$).

Figure 3.11 shows typical texture profiles of tortillas in groups 1A, 1B, 2 and 3A. Likewise, Figure 3.12 depicts typical texture profiles of tortillas in group 3B.

Figure 3.11: Typical texture profiles of tortillas in groups 1A, 1B, 2 and 3A. (A) Rollability scores; (B) Stretchability and (C) Rupture force. Dashed lines indicate the trend of changes in tortilla texture.

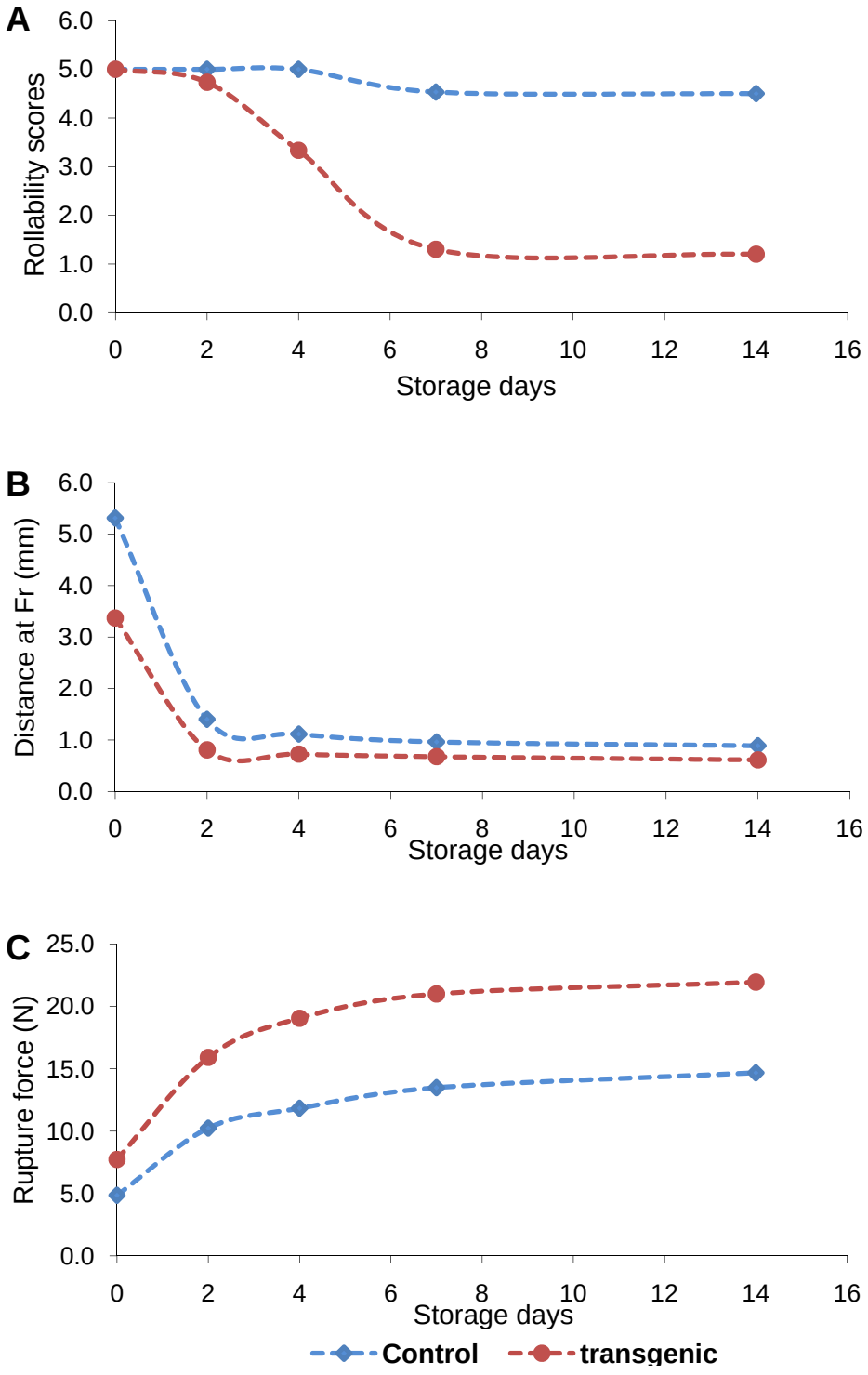
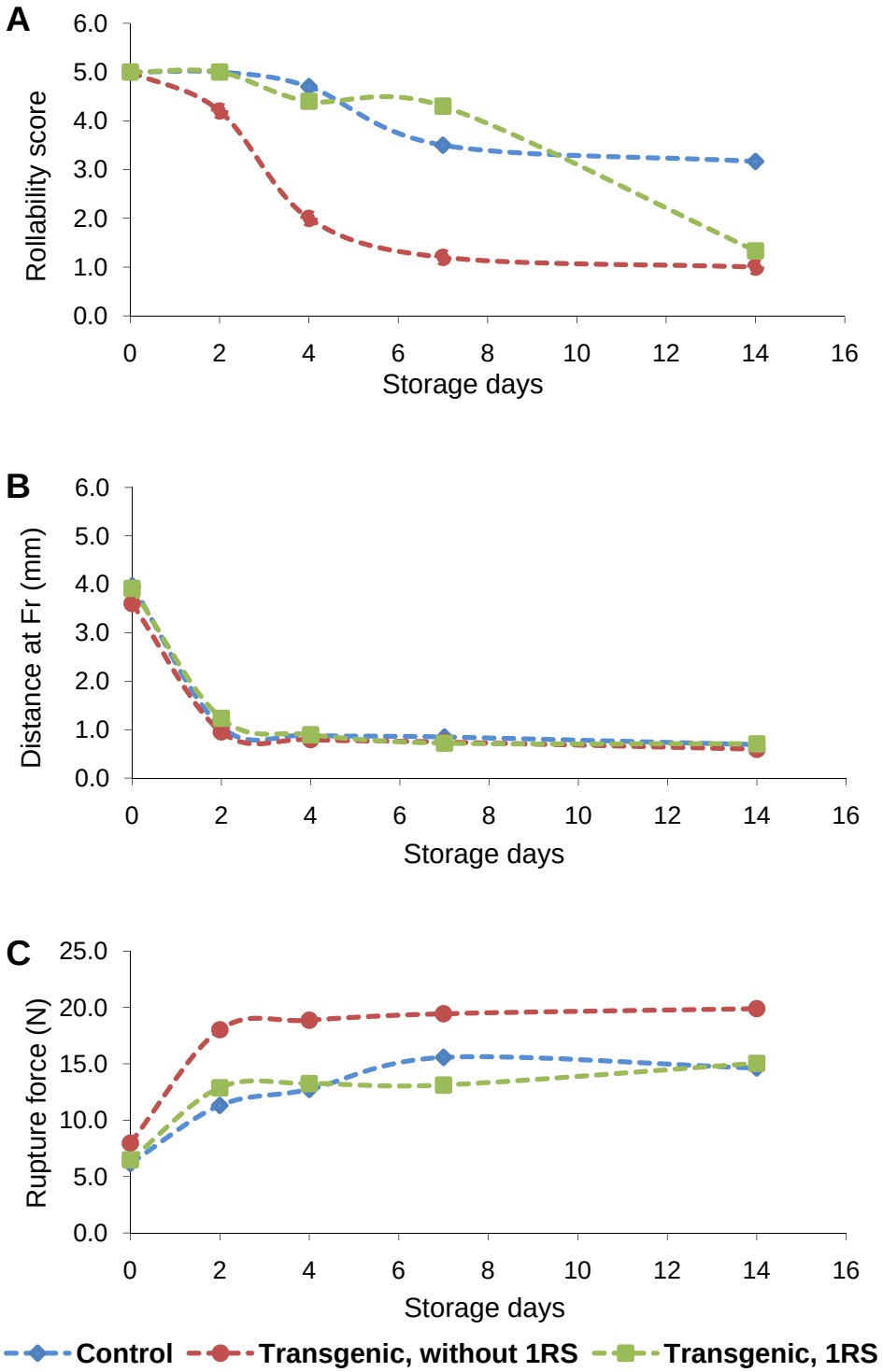


Figure 3.12: Typical texture profiles of tortillas in groups 3B. (A) Rollability scores; (B) Stretchability and (C) Rupture force. Dashed lines indicate the trend of changes in tortilla texture.



3.4 Discussion

Gluten proteins, specifically the HMW-GS, have been the focus of studies dedicated to wheat quality improvement tailored to baked products, especially bread. The main goal of breeding programs is to develop wheat cultivars with strong gluten and increased mixing tolerance, which are characteristics desirable for good quality bread. Traditional breeding programs use natural variability in protein composition to produce cultivars that are more suitable for bread making. Subsequently, such cultivars are tested for their protein functionality and its effects on final quality of bread. Traditional breeding is also the approach to develop cultivars used in research to study protein functionality, such as the development of near-isogenic lines. This breeding method makes use of crossing between different wheat lines to develop a subset of lines differing in HMW-GS composition but with a common genetic background. The development of wheat cultivars with a specific variation in HMW-GS composition cannot be achieved by traditional breeding. Therefore, other techniques were implemented to overcome this problem, such as the development of transgenic wheats. Such wheat lines are developed for research only, since cultivation of transgenic wheat is not allowed in the US.

Modification of wheat proteins by genetic engineering has largely targeted the HMW-GS, due the importance of these in determining end-use quality (Payne et al. 1987, Shewry et al. 2003). Through the development of transgenic wheats, it was possible to study the effect of HMW-GS 5 on dough and bread properties in the absence of subunit 10. It was demonstrated that transgenic expression of HMW-GS 1 and 5 (alone or in combination) in wheat lines that did not express neither proteins originally, caused a step-wise increase in both peak dough resistance and mixing time (Barro et al. 1997). Over-expression of HMW-GS 5 has been linked to excessive dough strength that is unsuitable for bread making, as demonstrated by small volume loaves (Darlington et al. 2003, Rooke et al. 1999).

Transgenic wheat over-expressing HMW-GS 10 has also been developed and mixograph analysis from those lines indicated that increased levels of this protein increased both the mixing time and tolerance to over-mixing (Blechl et al. 2007). It has been suggested that over-expression of HMW-GS 10 produces more extensible dough than of HMW-GS 5, as indicated by thicker bandwidths at peak resistance than dough made with flour derived from a wheat line over-expressing HMW-GS 5 (Blechl et al. 2007, Butow et al. 2003). Currently, understanding the

effects of individual HMW-GS on tortilla quality is a subject of increased interest, because demand for this product is high and the development of ideal cultivars for tortilla production has not been achieved.

Transgenic wheat flours were used in this study to investigate the effects of increased expression of HMW-GS 10 on dough and tortilla quality. Five sets of experimental samples, each composed of control and transgenic flours, originating from 3 different genetic backgrounds were used. Experimental sets were paired by similar total protein content, as it is known that protein content modulates tortilla quality (Qarooni et al. 1994, Suhendro et al. 1993, Wang and Flores 2000, 1999, Waniska et al. 2004).

Over-expression of HMW-GS 10 promoted significant negative effects on mixing behavior, as well as on dough and final product properties. Transgenic flours exhibited longer times to achieve dough peak development than controls, as observed by mixing times (Table 3.2). Transgenic samples from groups 1B and 2 did not achieve dough development in the mixograph analysis, samples exhibited extremely long mixing time and interruption of mixing had to be conducted before a dough peak was achieved. This is in agreement with a study (Blechl et al. 2007) in which the major effect of over-expression of subunit 10 on mixing behavior was the extension of mixing time. Over-expression of HMW-GS 5 also causes an increase in mixing time and depending on the level of this protein in the sample, an increase in work input by the mixing speed may be necessary for appropriate mixing (Barro et al. 1997, Blechl et al. 2007, Rooke et al. 1999). Data presented here suggests a similar trend. Lines with the greatest level of HMW-GS 10 (5.8 fold increase in groups 1A and 2) showed the longest mixing times. The line with the lowest level of HMW-GS 10 (2.5 fold in the transgenic with 1RS translocation in group 3B) showed the shortest mixing time. A previous report demonstrated that increasing levels of HMW-GS 10 in flour led to increasing mixing times (Blechl et al. 2007). However, data derived from the remaining lines in this study, do not point to a positive correlation between increased levels of HMW-GS 10 and increased mixing time.

Data from micro-extension tests showed that HMW-GS 10 over-expression influenced dough properties. In all groups, except 3B-1RS, doughs from transgenic flours were less extensible than control doughs. In addition, resistance to extension was greater in doughs prepared with transgenic flours in all groups, except 1B and 2. These observations are in agreement with a study in which incorporation of isolated HMW-GS 10, or all HMW-GS, to

flour lead to decrease in dough extensibility and increase in resistance to extension (Uthayakumaran et al. 2000). On the other side, the lack of all HMW-GS in wheat flour has been linked to a decrease in both dough resistance to extension and extensibility (Uthayakumaran et al. 2003). The greater resistance to extension and lower extensibility observed in doughs containing increased amount of HMW-GS 10 as presented here, suggest the development of a very strong gluten network in these doughs. A mixograph curve typical of strong doughs, but with increasingly longer mixing time has been previously reported for flour with increased amount of HMW-GS 10 (Blechl et al. 2007). Over-expression of other HMW-GS, such as 5 and 1, also caused production of stronger doughs (Barro et al. 1997, Popineau et al. 2001, Rakszegi et al. 2005, Rooke et al. 1999). Therefore, it is likely that the negative effects observed on dough properties are derived from the formation of very strong gluten network, which in turn, affected final tortilla quality.

The diameter of tortillas from transgenic flours was smaller when compared to controls in all groups tested. Dough with viscous and elastic characteristics is formed by hydration of flour and subsequent application of mechanical force (Pyler 1988). The balance between the viscosity and elasticity modulates the final product properties. In order to obtain large diameter tortillas, extensible dough with low elasticity is required. Data presented here shows that dough extensibility is highly and positively correlated with diameter (R^2=0.75). Since dough originated from transgenic flours exhibited lesser extensibility, tortillas from those flours had reduced diameters. In addition, since glutenins modulate dough elasticity (MacRitchie 1987), increased amount of HMW-GS 10 caused excessive elasticity and shrinkage of tortillas after hot-pressing, resulting in tortillas with decreased diameter and irregular shape (Figure 3.6). This is in agreement with the findings of Uthayakumaran et al (2003), who demonstrated that flours lacking all HMW-GS produced dough with very low elasticity, tortillas did not shrink after hot-press and large diameters were obtained. However, flour lacking all HMW-GS produced dough with very low extensibility and tortillas still exhibited large diameters, an observation that contradicts the high and positive correlation between dough extensibility and diameter derived from data presented here. Therefore, results presented here combined with previously published data (Uthayakumaran et al. 2003), suggest that a balance in HMW-GS is important to obtain desirable dough extensibility and elasticity, and consequently, tortillas with increased diameter.

Tortilla thickness was significantly affected by HMW-GS 10 over-expression in all groups, except 3B-1RS, as transgenic flours produced thicker tortillas than control flours. Adequate tortilla thickness ranges from 1 to 5 mm (Waniska 1999). Under normal conditions, an increase in tortilla thickness is largely derived from increased moisture retention and puffing, which occurs when air bubbles incorporated by mixing expand during baking (Waniska 1999). However, data presented here reveals that transgenic tortillas exhibited greater thickness and a rough, non desirable appearance. These observations associated with high dough elasticity and reduced tortilla diameter, suggest that increased moisture retention was not the underlying factor. Most likely, greater thickness derived from shrinkage. Evidence to support this statement is such that doughs from transgenic flours had higher gluten strength than control flours. It was demonstrated that stronger doughs produced by addition of vital wheat gluten (VW gluten) produced small diameter tortillas due to high elasticity (Pascut et al. 2004). Therefore, the greater thickness observed in transgenic tortillas might have originated from highly elastic dough that shrank after hot-press.

Tortilla rollability, stretchability and rupture force were negatively affected by over-expression of HMW-GS 10. More specifically, tortillas made with transgenic flours in groups 1A, 1B, 2 and 3B exhibited significantly decreased rollability scores, decreased stretchabilities and increased Fr when compared to control tortillas over time. Such subset of physical characteristics is related because they derive from a common biochemical factor: the gluten protein network. The presence of HMW-GS in gluten was shown to be very important for tortilla shelf stability, since tortillas made from flours lacking some of the HMW-GS, or all of them, exhibited low rollability (Mondal et al. 2008, Uthayakumaran et al. 2003). However, data presented here demonstrated that excess of HMW-GS 10 also caused a decrease in rollability scores. This outcome may be associated with the formation of an inadequate gluten protein structure, caused by excessive incorporation of HMW-GS 10 into the protein polymer. Evidence supporting that HMW-GS 10 was excessively added to protein polymers and formed a highly cross-linked structure, is the data presented here that reveals greater %IPP and larger M_w polymer size in transgenic flours when compared to controls. Increased incorporation of HMW-GS 10 among other gluten proteins promoted an increase in the size of protein polymers, resulting in decreased protein solubility. These findings are in agreement with a previous report in which over-expression of HMW-GS 5 caused an increase in cross-linking of gluten proteins

(Popineau et al. 2001). Flours over-expressing HMW-GS 5 produced dough with limited expansion potential and with very low volume bread. These findings were also linked to high cross-linkings among proteins (Darlington et al. 2003). In addition, it has been demonstrated that larger protein polymers are formed and decreased protein solubility is observed when the number of HMW-GS increases in flour (Popineau et al. 1994). In accord to data presented here, excessive polymerization formed in tortillas made with transgenic flours could also justify the significantly higher rupture force and decreased stretchability observed in transgenic tortillas.

Results derived from group 3A revealed that dough resistance to extension, dough extensibility, tortilla diameter, thickness and rupture force were negatively affected by HMW-GS 10 over-expression, but no significant differences were found in tortilla rollability and stretchability as tortillas aged. The reason for this is not clear. Transgenic flour in group 3A had levels of HMW-GS 10 expression similar to other groups (~5-fold increase). However, this group revealed the lowest absolute amount of HMW-GS 10 in both transgenic and control flours, as demonstrated by RP-HPLC analysis. In addition, group 3A's transgenic flour showed lower %IPP than transgenic flours in groups 1A, 1B and 2. These results could have derived from a lesser incorporation of HMW-GS 10 into the gluten structure, as HMW-GS 10 was expressed in a smaller absolute amount in group 3A, when compared to other groups. However, data from SEC-MALLS assays indicated that group 3A's transgenic flours exhibited the greatest M_w of insoluble polymeric proteins when compared to transgenic flours in other groups. This could have derived from differences in HMW-GS and LMW-GS composition in this group and from intrinsic properties of the flour obtained from the parental line Jagger, which is considered strong wheat.

Over-expression of HMW-GS 10 in group 3B-1RS did not cause negative effects on dough resistance to extension, dough extensibility, tortilla thickness, rollability, stretchability and rupture force. Tortilla diameter originated from 1RS-transgenic flour was significantly smaller than control tortillas, but was significantly larger than tortillas made with transgenic flour without 1RS translocation. The 1RS-transgenic line exhibited the lowest %IPP among all experimental samples tested. The M_w of protein polymers in the insoluble fraction of 3B-1RS transgenic was very similar to its control line. In addition, the relative level of HMW-GS 10 in 3B-1RS-transgenic flour was 2.5-fold of control, while transgenic flours without 1RS translocation revealed ~5-fold increase. These results most likely derive from the fact that 1RS

translocations change the protein composition of flour. Wheat-rye translocation lines were originally produced in order to introduce beneficial genes to wheat, such as those conferring resistance to pests and pathogens and to enhance grain yield (Bartos and Bares 1971, Bartos 1973, Rajaram 1983). However, 1RS translocation also introduced genes to the wheat genome that promote deleterious effect on dough and bread properties, as observed by low loaf volumes, production of sticky doughs, reduce dough strength, lack of tolerance to overmixing and low SDS sedimentation volumes (Burnett et al. 1995, Dhaliwal et al. 1987, Dhaliwal et al. 1988, Graybosch et al. 1990, Martin and Stewart 1986, Moonen and Zeven 1984, Zeller et al. 1982). The most sensitive problems are low dough strength and stickiness. Genes that contribute to the negative effects on dough properties include those present in the complex *Sec-1* locus of the 1RS chromosome, which codes for several secalins of γ- and ω-type (Graybosch 2001). Those proteins are monomeric with a hydrophilic character, especially the ω-type. Most importantly, wheat lines holding 1RS translocation contain a section of the rye chromosome that replaces the short arm of the wheat chromosome that codes for gliadins and LMW glutenins. Gliadins are monomeric proteins with a hydrophobic character. LMW glutenins are part of the polymeric glutenin matrix. Therefore, flours derived from wheats containing a 1RS translocation have an increase in hydrophilic monomeric proteins and a decrease in hydrophobic monomeric and polymeric proteins (Dhaliwal et al. 1987, Graybosch et al. 1990, Graybosch et al. 1993, Graybosch et al. 1996, Lee et al. 1995). A combination of factors such as lower over-expression (2.5-fold instead of 5-fold) of HMW-GS 10, differences in protein composition in the 1RS-transgenic flour and the association of 1RS translocation with low dough strength, might form the basis for the similarities between dough and tortilla properties in group 3B-1RS. Incorporation of HMW-GS 10 into excessively large gluten polymers during development of dough made with 1RS-transgenic flour might not have been as intense as in transgenic without 1RS translocation, provided the lower amount of HMW-GS 10 and the absence of some LMW glutenins in the mixture. Two data sets presented here support this statement. First, the %IPP in 1RS-transgenic flour and its respective control were comparable. In addition, the M_w of IPP proteins in the 1RS-transgenic line was very similar to its control.

3.5 Conclusions

HMW-GS 10 over-expression in transgenic wheat plants caused a broad negative effect on dough properties and tortilla quality. Doughs produced from transgenic flours exhibited greater resistance to extension and lesser extensibility than control doughs. Tortillas derived from transgenic flours exhibited an undesirable rough appearance with decreased diameter and greater thickness. In addition, tortillas made from flours containing greater levels of HMW-GS 10 exhibited lower rollability scores, lower stretchability and greater rupture force over time. Data presented here supports that the changes in the dough and tortilla properties induced by increased amounts of HMW-GS 10 derive from an inappropriate formation of the gluten network.

Over-expression of HMW-GS 10 in a wheat line containing 1RS rye translocation did not promote the same deleterious effects on dough and tortilla properties as it did in transgenic lines without 1RS translocation. This finding might derive from a lower level of HMW-GS 10 over-expression in this line and the defined protein composition differences in 1RS-translocated lines.

Future Research

The studies presented here could be further developed in order to advance on understanding of protein functionality in tortilla production. Three immediate fronts of experimentation are proposed.

First, additional studies using wheat cultivars with similar genetic background, but different HMW-GS compositions should allow for an evaluation of the performance of other HMW-GS that were not included in this study. Wheat lines expressing HMW-GS 2*, 1 or not expressing protein at all from *Glu-A1* loci and lines expressing HMW-GS 17+18 or 20 from *Glu-B1* loci would be valuable samples to compare those HMW-GS.

Data described in the second chapter were derived from flours originated from wheat crop cultivated in one single year and location. To exclude the possibility that environmental factors may have biased the outcomes described in chapter 2, the same experiments should be conducted with flours derived from additional harvests.

Finally, wheat lines containing 1RS wheat-rye translocation could be used to evaluate the potential of this type of flour for tortilla production.

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