BIOCHEMICAL AND RHEOLOGICAL PROPERTIES OF WAXY WHEAT FLOUR DOUGH

by

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Abstract

The rheological properties of two waxy and two normal wheat flours were investigated and the observed differences between them were explained by biochemical analysis. Protein analysis showed that waxy flour had lower polymeric to monomeric ratio (0.70 and 0.58 for waxy flour compared to 0.75 and 0.76 for normal flour) and higher gliadin content in waxy wheat dough (43.9 and 47.3 for waxy wheat dough compared to 41.0 and 41.7 for normal wheat dough). Waxy flour had high amounts of insoluble (IPP) and unextractable (UPP) polymeric protein despite the poor dough forming properties of the waxy flours, contrary to previous correlations made between IPP, UPP and dough strength. Gluten index determination showed a clear difference between waxy and normal flour; there was no gluten aggregation when the waxy samples were tested. The determination of gluten index done on a variety of water washed flour samples indicated that the water-extractable fraction may contain compounds that affect gluten aggregation. HPLC analysis coupled with arabinose/xylose ratio and viscosity determination of the water extractable portion of the flour indicated that water extractable arabinoxylans (WE-AX) in waxy wheat flour were different in composition and conformation. Further research is needed to determine if they could be responsible for the lack of gluten aggregation in waxy flour.

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Dedication

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1. Introduction

Starch is a storage carbohydrate of plants located in the endosperm of cereal grains, tubers, and roots (Graybosch, 1998; Copeland et al., 2009). Cereal grains are composed primarily of starch, and wheat is no exception. Starch is the principal component of wheat endosperm (Hung et al., 2006). Its properties and interactions with other food constituents influence food product properties (Copeland et al., 2009). Two glucose polymers together constitute starch in normal wheat endosperm: amylopectin (72-75%) and amylose (25-28%) (Hung et al., 2006). The ratio of amylose to amylopectin affects the physicochemical properties, granular structure, and quality of end-use products (Hung et al., 2006).

Amylopectin is a large and highly branched glucose polymer consisting of D-glucosyl units linked through α (1→4) bonds with α (1→6) branch points approximately every 20-25 residues (Graybosch, 1998; Hung et al., 2006). The degree of polymerization of amylopectin is very high, ranging from $3x10^5$ to $3x10^6$ anhydro glucose units (Hung et al., 2006). Amylopectin has been associated with pasting properties, granule crystallinity, as well as long-term retrogradation (Copeland et al., 2009). Amylose is essentially a linear chain of D-glucosyl units linked through α (1→4) bonds with a relatively low degree of polymerization ranging from 500-6000 glucose residues (Graybosch, 1998; Hung et al., 2006). Amylose is associated with water uptake properties, texture, and stickiness (Copeland et al., 2009). The ratio of these polymers defines the gel formation and thermal properties of the starch (Copeland et al., 2009).

The synthesis of starch occurs in amyloplasts (Graybosch, 1998). Amylopectin undergoes a more complicated synthesis than amylose, involving the collaborative action of starch synthases, and several branching and de-branching enzymes (Graybosch, 1998; Graybosch *et al*., 2003; Hung et al., 2006). Amylose synthesis takes place through the isoforms of the enzyme granule-bound starch synthase (GBSS), also called the 'waxy' protein (Wx protein) (Graybosch, 1998; Graybosch *et al*., 2003; Hung et al., 2006; Seib, 2000). Naturally occurring mutations to the GBSS isoforms have been identified (Graybosch, 1998; Sahlstrom et al., 2006). In wheat, these mutations can occur in one of three structural genes that encode for GBSS, and can result in the presence of little to no amylose in the starch (Graybosch, 1998).

Hexaploid wheat contains three genomes (A, B and D) each comprised of seven pairs of homologous chromosomes. The GBSS protein is encoded at three homologous loci found on chromosomes 7AS (*Wx-A1*), 4AL (*Wx-B1*) and 7DS (*Wx-D1*) (Jonnala *et al*., 2010; Nishio *et al*., 2009; Hung et al., 2006). Inactive or non-functional alleles at these loci result in the loss of GBSS isoforms (Sahlstrom et al., 2006). Depending on how many of the loci are inactive (null), the wheat can be classified as wild type (no null alleles), partial waxy (single or double null) or full waxy wheat (null alleles at all three waxy loci) (Jonnala *et al*., 2010; Chibbar and Chakraborty, 2005). The term 'waxy' originally comes from the waxy appearance of amylose-free mutants of maize, compared to the translucent appearance of normal kernels (Graybosch, 1998). Reports have shown that the effectiveness of reduction in amylose content varies between the three Wx genes (Hung et al., 2006). Null GBSS at the B and D loci cause the greatest reduction in amylose content compared to any other combination (Chibbar and Chakraborty, 2005).

The development of waxy and partial waxy wheat was first accomplished through the traditional breeding of wheat varieties Kanto 107 (*Wx-A1* and *Wx-B1* null alleles) and BaiHuo (null *Wx-D1* allele). Since then, cultivars with null alleles at Wx-A1 have been identified, mostly from Japan, Korea, and Turkey (Hung et al., 2006). Seib (2000) provides a detailed history of developments in waxy wheat. Cultivars with null alleles at Wx-B1 have been identified in Australia, and only cultivar BaiHuo had been originally identified to have a null allele at the Wx-D1 locus (Hung et al., 2006; Chibbar and Chakraborty, 2005). Later, a second cultivar from the same province in China (BaiHuoMai) was shown to have a null allele at the Wx-D1 locus (Graybosch, 1998). Two other methods have been used to develop full waxy lines: treatment of partially waxy lines with ethyl methanesulfonate (chemical mutagen), and the use of double-haploid breeding programs to speed up the introgression of null Wx alleles (Chibbar and Chakraborty, 2005; Hung et al., 2006).

The study of waxy wheat starch has been fueled by the desire to understand the relationship between the structure of waxy starch and its functional properties (Chibbar and Chakraborty, 2005; Hung et al., 2006). Studies have been done to characterize its different properties compared to normal wheat starch. X-ray diffraction studies have revealed that waxy wheat starch has a higher degree of crystallinity (Chibbar and Chakraborty, 2005; Hung et al., 2006; Abdel-Aal et al., 2002; Ma el al., 2013). Differential scanning calorimetry results have

shown higher gelatinization temperatures and enthalpy required for waxy wheat starch (due to the higher degree of crystallinity), and an absence of the amylose-lipid complex peak (Chibbar and Chakraborty, 2005; Hung et al., 2006; Abdel-Aal et al., 2002; Ma el al., 2013; Morita et al., 2002).

Studies on the pasting properties of waxy wheat starch have determined that waxy starch has the ability to take up water at a faster rate, swell faster, and at a lower temperature than does non-waxy starch, meaning it is characterized by lower pasting and peak temperature, higher peak viscosity and breakdown, and low setback (Chibbar and Chakraborty, 2005; Hayakawa et al., 2004; Hung et al., 2006; Takata et al., 2007). Waxy cereal starches swell very rapidly in hot water to form a thick paste and granules rupture easily during cooking, resulting in a low paste viscosity upon cooling (Hung et al., 2006; Garimella Purna et al., 2015). The pastes formed by waxy starch upon cooling are translucent, experience lower syneresis and have a higher resistance to retrogradation than do non-waxy starch gels (Chibbar and Chakraborty, 2005). Furthermore previous studies have shown that waxy starches have greater refrigeration and freeze-thaw stability (Abdel-Aal et al., 2002). Waxy starches are often chemically modified for use in food applications, and are very commonly used as thickeners. Waxy wheat starch in particular has higher thickening power with less modification than its waxy maize and normal wheat starch counterparts (Hung et al., 2006).

Information regarding quality characteristics of waxy wheat had been limited outside of starch, yet increasing information relating to waxy wheat flour has been gathered (Jonnala et al., 2010; Takata et al., 2007). Waxy wheat flour is generally characterized by higher protein, ash, dietary fiber, and lipid contents compared to normal wheat flour (Hung et al., 2006; Morita et al., 2002; Park and Baik, 2007; Takata et al., 2007). Waxy flour also exhibits higher starch damage, lower color brightness, and higher polyphenol content (Takata et al., 2007). Other properties of waxy wheat flour include high water absorption during dough mixing, lower starch content, low pasting temperature and setback viscosity, higher peak viscosity and breakdown, increased swelling power, and increased α-amylolysis (Abdel-Aal et al., 2002; Garimella Purna et al., 2015; Ma el al., 2013; Sahlstrom et al., 2006; Takata et al., 2007). It is possible that the lack of amylose-lipid complexes is what makes the starch more susceptible to α-amylase activity (Abdel-Aal et al., 2002). Waxy wheat flour creates dough of intermediate strength that rapidly reaches peak development, has very low tolerance to mixing and breaks down rather quickly

(Abdel-Aal et al., 2002; Park and Baik, 2007; Sahlstrom et al., 2006). The reasons for the weak dough properties observed in waxy wheat flour are not well understood and require further research. Risograph tests have shown up to 100% more gas production by waxy wheat than by normal flours during fermentation. Enzyme digestibility tests show that waxy starch is more easily digestible by enzymes. This would provide a higher sugar supply for yeast, resulting in higher gas production (Garimella Purna et al., 2011).

A number of studies address the effects of adding waxy wheat flour to pan bread. One such study found that bread baked from dough with a higher content of waxy wheat flour showed smaller differences in specific volume after frozen storage, were more extensible and had less damage to the gluten matrix after freezing, resulted in softer bread crumb, and darker crust color than did control samples. However yeast activity diminished above 15% substitution (Yi et al., 2009). Another study found that incorporation of 20% of waxy wheat flour in bread formulations causes significant improvements in product quality (Ma el al., 2013). Bread made with 100% waxy wheat flour showed higher initial volume than did the non-waxy controls, but had a porous and open grain and large air cells in the crumb, as well as a dark color and dull appearance of the crumb (Morita et al., 2002). Another study where loaves were baked with 100% waxy wheat flour showed poor appearance but increased loaf volume and softness (Morita et al., 2002). Optimum bread properties were seen with a maximum substitution of 40% waxy wheat flour (Jonnala et al., 2010).

In some cases, the addition of waxy wheat flour results in poor quality product. Hearth bread made with waxy wheat flour overall showed poor appearance characterized by a low weight, and an open pore structure (Sahlstrom et al., 2006). Another study showed that waxy wheat flour is not suitable for the production of acceptable steamed bread if its addition exceeds 10-15% of the flour in the product (Qin et al., 2007). The bread that was made with waxy wheat flour at this level retained its softness even after 3 days stored at -18°C, so substitution of 10-15% waxy wheat flour could be used to extend shelf life of frozen steamed bread without effects on the overall subjective scores for the product (Qin et al., 2007). French bread baked with waxy wheat flour collapsed after baking and could not hold the crumb structure during slicing (Park and Baik, 2007). The weakness could be because in the absence of amylose, there is no immediate retrogradation to hold the structure (Park and Baik, 2007). Open and porous crumb structure and high loaf volume immediately after baking have also been identified when waxy

wheat flour is included in formulation, yet excessive shrinkage and collapse is observed within the first 24 hours of storage (Garimella Purna et al., 2011; Morita et al., 2002).

Further studies have looked at addition of waxy wheat flour to pan bread formulas to study the effect on product shelf life. According to Qin *et al.* (2009) addition of over 20% waxy wheat flour to white bread results in sticky, lumpy and less crispy textures but addition below the 20% mark result in significant improvements in product shelf life. However, additions below 10-15% waxy wheat flour had no effect on bread staling. Bhattacharya *et al.* (2002) showed that addition of 20-30% waxy wheat flour to bread loaves slows down rate of staling, and that this phenomena is not due to moisture retention as the loaves made with waxy wheat had a comparable moisture content to those made using 100% normal wheat flour. The addition of 20% waxy wheat flour to bread formulation resulted in reduced crumb firmness after 5 days of storage compared to bread made using 3% shortening, suggesting that waxy wheat flour has the potential to substitute for shortening in bread formulations (Bhattacharya et al., 2002). The slow retrogradation of waxy wheat starch makes it desirable to use in the production of refrigerated and frozen products (Qin et al., 2009). Waxy wheat could be the key to high volume loaves with prolonged shelf life without the need to add shortening and dough conditioners (Bhattacharya et al., 2002). The combination of different studies show that the addition of different levels of waxy wheat flour to different products results in softer products for anywhere from 0-7 days of storage but then may become harder than their counterparts made with no waxy wheat flour (Morita et al., 2002; Acosta et al., 2011).

Guo et al. (2003) discuss studies of different products when using waxy wheat flour. For example, positive results seen in Asian noodles, cakes resulting in increased resistance to staling, gyoza with improved quality after cold storage, and fresh tortillas with greater dough extensibility. Beneficial effects have been observed when adding waxy wheat flour to 'same day' consumption tortilla products as it helps provide optimum amylose content for hot press processing (Qin et al., 2009). The swelling properties of waxy starch and flour are desirable for salt noodles (Seib, 2000). The addition of 40% waxy wheat flour improves the quality of dry white Chinese noodles and 20 to 30% substitution improves quality of white salted noodles (Qin et al., 2008). Inclusion of up to 30% of whole waxy wheat flour in a whole-wheat flour muffin formulation resulted in acceptable sensory characteristics, resulting in a moister and softer product even after 4 days of storage (Acosta et al., 2011). The substitution of normal wheat flour

with waxy wheat flour in sponge and butter cakes resulted in lower volume, and increased cell wall thickness, moistness, and heaviness of the cake. Eating quality in sponge cakes with less than 30% substitution showed a desirable increase in stickiness and elasticity, and butter cakes with 15% substitution showed higher scores for chewiness and palatability (Hayakawa et al., 2004).

A study by Nishio *et al*. (2009) showed poor quality cookies resulted from the addition of waxy wheat flour to the formulation. The increased water absorption of waxy wheat flour interferes with the hydration of sugar, thus maintaining dough viscosity high and limiting spread in the oven (Nishio et al., 2009). Salted noodles made with waxy wheat flour did not produce desirable results. The fast swelling of the starch makes it impossible to boil the noodles for optimum cooking time for best textural properties without sustaining cooking loss (Hayakawa et al., 2004).

The availability of waxy wheat flour allows millers to blend flours to specific amylose levels (Graybosch, 1998). Starches of different amylose contents are important to the food industry because they can produce different textures and end-use qualities in products (Blazek and Copeland, 2008). Potential uses for waxy wheat include use as a bakery ingredient or as an alternative to waxy maize in the production of modified starches (Graybosch et al., 2003). Partial substitutions of normal wheat with waxy wheat flour can be used to obtain a softer bite, slower retrogradation and increased shelf life in baked goods. The softening effect of waxy wheat flour makes it a good substitute for fat or oil. Furthermore, Guan et al. (2009) discuss how waxy flour imparts improved friability of expanded cereal snacks compared to the crunchiness of normal wheat flour.

The use of waxy wheat flour for other food applications is very limited, in part because little research has been done on the use of this flour in a wide variety of products. Further research regarding the properties, quality, composition, and application of waxy wheat is necessary to expand its use and to allow for effective commercialization (Hayakawa et al., 2004; Ma el al., 2013; Hung et al., 2006). Little information is available regarding the rheological properties of waxy wheat flour (Zhang et al., 2014). As reviewed earlier, waxy wheat flour acts differently than normal wheat flour. The mixing characteristics of waxy flour (shorter mixing time, lower mixing tolerance, associated to weaker doughs) cannot be tied to properties associated with changes in amylose content. The reasons why differences not commonly

associated with amylose properties are seen between waxy and normal have not been explained. For this reason, the objectives of the present study were to compare dough forming properties between waxy wheat flour and normal wheat flour and to identify possible explanations for the observed differences based on compositional attributes of the flour samples.

2. Materials and Methods

Two waxy hard wheat samples and two normal hard wheat samples grown in Nebraska and harvested with the 2013 crop were provided by Dr. Robert Graybosch (USDA-ARS, Lincoln, NE) and used for all studies. The two normal wheat samples used as controls were Nuplains (hard white winter wheat) and Wesley (hard red winter wheat). The two hard wheat waxy samples; were Mattern (NW98S061/99Y1442) and NX11MD2337 (NF98466/NWX03Y2450//NX02Y4549).

2.1 Wheat Milling

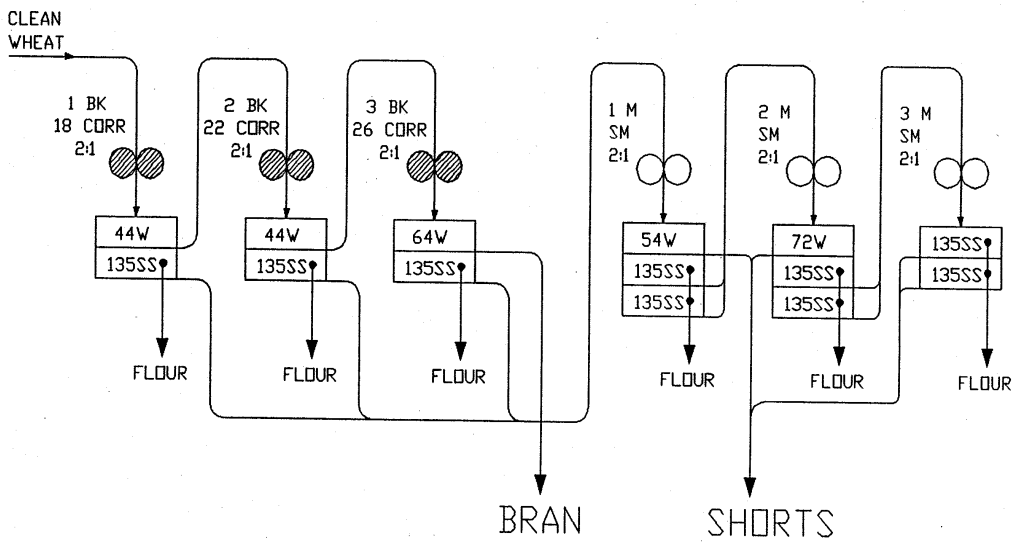
Wheat was tempered to 15% moisture content for 24 hours. The post-temper moisture was determined using the single kernel characterization system (SKCS4100 Perten Instruments Inc., Springfield, IL, USA). Tempered samples were milled into straight grade flour using a Buhler MLU 202 Experimental Mill (Buhler Inc., Uzwil, Switzerland) attached to a Syntron PowerPulse (FMC Technologies, Houston, TX) vibratory feeder. AACCI Method 26-21.02 (1999) was used as a reference for roll gap settings.

The roll gap for the first, second, and third break passes was set using a 0.076mm feeler gauge. The rolls were set to a loose 0.076mm gap, but tight enough that it was too tight to fit a 0.102mm gauge. The 1M, 2M, and 3M reduction roll gap settings were set to approximately 0.076mm by tightening the rolls using the gauge until it could barely be pulled out.

Figure 2.1 (courtesy of Andrew Mense, Grain Science and Industry) shows a flow diagram of the Buhler system. Two three-part screens were used for both the break and reduction rolls. The ground wheat from the first, second, and third breaks was first sifted over screens with openings of 0.71-RF, 0.60-RF, and 0.53-RF respectively.

The throughs were sent to a second screen that had openings of 150µm for first break, and 132µm for second and third breaks. The middlings from the 1M, 2M, and 3M passes were first sifted over screens with 132µm openings. Throughs were sent to a second screen that had openings of 132µm for first and second reductions, and openings of 118µm for third reduction. Feed rate was set at 2.0, which corresponded to approximately 26.0g/minute.

Figure 2.1 Flow diagram of a Buhler MLU 202 experimental mill (Mense, 2013)



2.2 Flour Composition

Milled samples were tested in triplicates for moisture, protein, ash, arabinoxylan content, and starch damage. AACCI Method 44-19.01 (1999) was followed for moisture determination. Protein content was determined by nitrogen combustion (LECO, using a factor 5.7 for conversion to protein content). Ash content was determined by AACCI Method 08-03.01 (1999). Total arabinoxylan content was done according to the Douglas (1981) colorimetric method. Starch damage was determined using a Megazyme International Ltd. (Wicklow, Ireland) kit following procedure K-SDAM 07/11 (2011) based on AACCI method 76-31.01.

2.3 Particle Size Analysis

The AACCI Method 55-60.01 (2011) was followed for particle size analysis. A RO-TAP RX-29 (W.S. Tyler, Mentor, Ohio) was used with round metal 250, 149, 125, 90, 45, and 25µm opening sieves. The machine was allowed to run with a 100 g flour sample for 5 minutes. The weight of flour over each sieve was determined and used to calculate percent (%) over each sieve. Each flour sample was tested in triplicate.

2.4 Iodine Staining of Flour Samples

An estimation of the purity of the starch in each of the normal and waxy wheat flours was obtained visually by iodine staining and observation under a microscope with bright-field illumination. The samples were prepared as follows: 0.03 g of flour was suspended in 1.5 mL of distilled water. In a separate container, 8.5 mL of distilled water was combined with 0.3 mL of 0.1N iodine solution and then rapidly added to the starch suspension. A drop of stained starch suspension was placed on a microscope slide and covered with a cover slip before observation. The stained samples were viewed with a light microscope (Olympus BX51, Melville, NY) under bright field illumination using a 40x objective lens. Samples were tested twice.

2.5 Dough Mixing

AACCI Method 54-40.02 (1999) was followed using a 10g mixing bowl (National Manufacturing Co., Lincoln, NE). The required flour sample size, absorption, and total amount of water needed were calculated according to Equations 1-3. Samples were tested at the calculated optimum absorption (Eq. 2), as well as 2%, 4%, 6%, and 8% higher absorption based on the results obtained. Wesley and NX11MD2337 were also tested at 10% higher absorption than the calculated optimum to ensure the true optimum absorption of each flour sample was identified. Calculated optimum absorptions were as follows: 61.32% for Nuplains, 61.89% for Wesley, 61.18% for Mattern, and 62.05% for NX11MD2337.

Equation 1 Calculation of the amount of flour required for mixograph using a 10g bowl

$$\boldsymbol{Flour\ (g) = \frac{100 - 14}{100 - flour\ moisture\ content} * 10}$$

Equation 2 Calculated optimum absorption based on flour protein content

$$\boldsymbol{Absorption\ (\%) = (1.5 * protein\ content) + 43.60}$$

Equation 3 Calculation of the amount of water required for mixograph using a 10g bowl

$$\boldsymbol{Water\ (g) = \frac{Absorption * 10}{100} + (10 - g\ of\ flour)}$$

2.6 Protein Analysis

Sample preparation and protein analysis testing of normal and waxy flour samples was completed as follows.

2.6.1 Dough Sample Preparation

Mixograph results were used to determine flour optimum absorptions and mixing times. These parameters were used to develop a flour/water dough using the 10g mixing bowl mixograph for each of the four flour samples. The doughs were collected individually, freeze dried and ground with a mortar and pestle prior to protein analysis.

2.6.2 Free Sulfhydryl Content

Free sulfhydryl content was determined according to the colorimetric method of Chan and Wasserman (1993). Flour and freeze-dried dough samples were both tested. The sample (30mg) was treated with 1.0 mL of a buffer composed of 8M urea, 3mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS), and 0.2M Trizma® hydrochloride (pH 8.0) and shaken for 1.0 hour. The sample was then treated with 0.1mL of a second buffer composed of 10mM Ellman's reagent (DTNB) and 0.2M Trizma® hydrochloride (pH 8.0) and centrifuged at 13,600x g for 10 minutes. The absorbance of the supernatant (A) was read at 412nm. Free sulfhydryl content was calculated according to Equation 4. The molar extinction coefficient (ε) was 13,600 $M^{-}cm^{-}$, the cell thickness of the spectrophotometer cell (b) was 1.2cm, and c corresponded to the free sulfhydryl concentration. Samples were tested in duplicate.

Equation 4 Determination of free sulfhydryl concentration

$$c = \frac{\varepsilon * b}{A}$$

2.6.3 Polymeric protein

Each sample was tested for its insoluble (IPP), total (TPP), extractable (EPP), and unextractable (UPP) polymeric protein. Flour and freeze-dried dough samples were both tested. All tests were performed at the United States Department of Agriculture grain testing facilities (USDA-ARS- CGAHR, Manhattan, KS). All samples were tested in duplicate. Detailed step-by-step instructions and diagrams for these procedures can be found in Appendix A.

2.6.3.1 Insoluble Polymeric Protein (IPP)

The method of Bean *et al.* (1998) was followed; 100 mg of each flour or freeze-dried dough sample was weighed out and vortexed for 5 minutes (Vortex Genie 2, Daigger, Vernon

Hills, IL) in a 50% 1-propanol solution. The tubes were centrifuged at 12,000x g for 5 minutes (Centrifuge 5424, Eppendorf, Enfield, Connecticut, USA) and the supernatant was discarded. The pellets were re-suspended in 50% 1-propanol and the process repeated two more times. The pellets were lyophilized and tested for protein content by nitrogen combustion (LECO FP-428, LECO Co., St. Joseph, MI). The following equations were used for the calculation of %insoluble polymeric protein (%IPP) and % soluble polymeric protein (%SPP) in the flour.

Equation 5 Calculation of flour % IPP

$$\%\, IPP = \frac{(Nitrogen\ value) * 5.7}{flour\ protein\ (db)}$$

Equation 6 Calculation of flour % SPP

$$\%\, SPP = 100 - \%\text{IPP}$$

2.6.3.2 Total Polymeric Protein (TPP)

This procedure followed the work by Gupta *et al*. (1993). Triplicate vials of each sample were prepared with 10.0mg ± 0.5 mg of flour. An appropriate volume of SDS buffer (0.05 M sodium phosphate, 0.5% sodium dodecyl sulfate buffer pH 6.9) to obtain a 10 mg flour/1mL buffer solution ratio was added to each replicate. Vials were vortexed for 5 minutes (Vortex Genie 2, Daigger), sonicated for 15 seconds with a power output at 6W using a Misonix Sonicator XL-2000 Series (QSonica, LLC., Newtown, CT), and centrifuged at 12,000 rpm for 20 minutes (Centrifuge 5424, Eppendorf). The supernatant was transferred to filter microtubes and centrifuged at 14,000 rpm for 5 minutes. The filtered supernatant was transferred to HPLC vials. The pellet was freeze-dried and tested for protein content by nitrogen combustion (LECO).

The ratio of polymeric to monomeric proteins was calculated according to Equation 7. The extracts from the TPP procedure were used to determine soluble polymeric protein (SPP; Equation 8), gliadin (Gli; Equation 8), and albumin/globulin (Alb/Glob; Equation 8) contents. Residual protein was calculated according to Equation 9 using %protein values obtained from nitrogen combustion by LECO FP-428 (LECO Co., St. Joseph, MI) done on the pellet left over after extraction. Figure 2.2 shows an example of the peaks used for calculations.

Equation 7 Calculation of total polymeric/ total monomeric proteins (TPP/TMP)

$$TPP/TMP = \frac{Area\ under\ peak\ 1\ of\ TPP\ analysis}{Area\ under\ peak\ 2\ of\ TPP\ analysis}$$

Equation 8 Calculation of SPP (peak 1), Gli (peak 2) and Alb/Glob (peak 3)

$$\%\ protein\ in\ flour = \frac{(\%\ Area\ under\ peak\ 1, 2, or\ 3) * (total\ protein\ wt - wt\ of\ \ residual\ protein)}{total\ protein\ wt\ in\ flour\ sample}$$

Equation 9 Calculation of residual protein

$$\%\ Residual\ protein\ in\ flour = \frac{\%\ protein\ from\ pellet\ nitrogen\ combustion * pellet\ wt}{total\ protein\ wt\ in\ flour\ sample}$$

2.6.3.3 Extractable Polymeric Protein (EPP)

This procedure followed the work by Gupta *et al.* (1993). Triplicate vials of each sample were prepared with 10.0mg ± 0.5 mg of flour. The appropriate volume of SDS buffer to obtain a 10mg flour/1mL buffer solution ratio was added to each replicate. Vials were vortexed for 5 minutes (Vortex Genie 2, Daigger) and centrifuged at 12,000 rpm for 10 minutes (Centrifuge 5424, Eppendorf). The supernatant was transferred to filter microtubes and centrifuged at 14,000 rpm for 5 minutes (Centrifuge 5424, Eppendorf). The filtered supernatant was transferred to HPLC vials. The pellet was saved for UPP analysis. Equation 10 gives the calculations for extractable polymeric protein (EPP).

Equation 10 Calculation of % EPP in total polymeric protein

$$\%EPP = \frac{\%\ Area\ under\ peak\ 1\ from\ EPP\ extraction\ curve * 100}{\%\ Area\ under\ peak\ 1\ from\ EPP\ curve + peak\ 1\ from\ UPP\ curve}$$

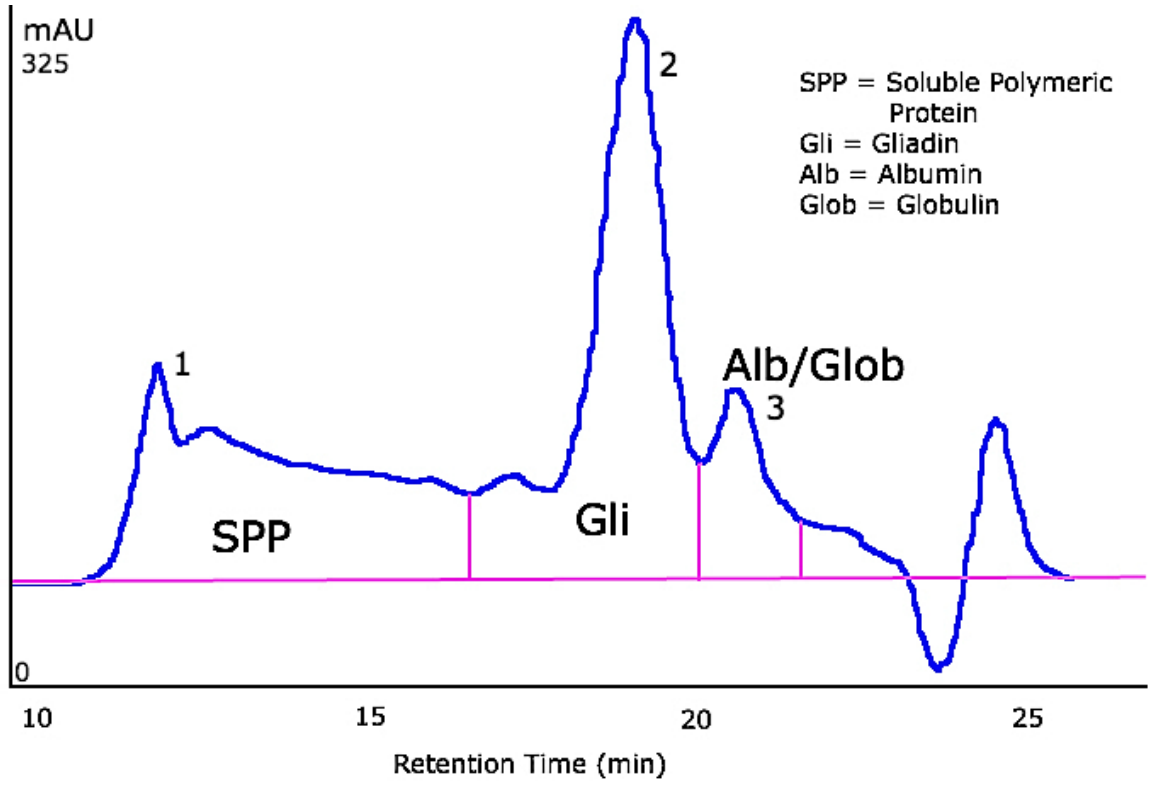
2.6.3.4 Unextractable Polymeric Protein (UPP)

Triplicate vials of each sample were prepared using the pellet remaining from the EPP assay (Gupta et al., 1993). SDS buffer (1.0 mL) was added to each replicate. The pellets were vortexed for 10 minutes (Vortex Genie 2, Daigger), sonicated for 25 seconds at a power output of 6W (QSonica, LLC., Newtown, CT), and centrifuged at 12,000 rpm for 10 minutes (Centrifuge 5424, Eppendorf). The supernatant was transferred to filter microtubes and centrifuged at 14,000 rpm for 5 minutes (Centrifuge 5424, Eppendorf). The filtered supernatant was transferred to HPLC vials. Equation 11 gives the calculations for unextractable polymeric protein (UPP). The precipitate was freeze-dried and analyzed by nitrogen combustion by LECO FP-428 (LECO Co., St. Joseph, MI) for determination of residual protein.

Equation 11 Calculation of % UPP in total polymeric protein

$$\%UPP = \frac{\%\ Area\ under\ peak\ 1\ from\ UPP\ extraction\ curve * 100}{\%\ Area\ under\ \ peak\ 1\ from\ EPP\ curve + peak\ 1\ from\ UPP\ curve}$$

Figure 2.2 HPLC chromatogram of Nuplains sample as measured at 210nm for peak identification



2.6.3.5 HPLC Analysis

Sample vials with treated supernatant from the TPP, EPP and UPP extractions were placed in a preheated 80°C water bath for 10 min to ensure no enzyme activity during testing. After cooling to room temperature, the vials were placed in the auto-sampler for HPLC analysis. The samples were analyzed using a BioSep SEC-S4000 (Phenomenex, Torrance, CA, USA) size exclusion chromatography column with a Variable Wavelength detector. The following parameters were used: 210nm wavelength, column temperature 30°C, flow rate 1mL/min, run time 30 min, post time 10 min, injection volume 20 μL, eluting solvent 50% of water (A) and acetonitrile (B) both with 0.1% trifluoroacetic acid according to the method by Batey et al (1991). ChemStation software was used for data analysis (Agilent Technologies, Santa Clara, CA).

2.7 Gluten Index

The gluten index for each flour sample was determined using a Glutomatic 2200 (Perten Instruments Inc., Springfield, IL, USA) according to AACC method 38-12 (2000). Equation 12 was used to calculate gluten index. Each sample was tested in duplicate.

Equation 12 Determination of gluten index based on Glutomatic weight values

$$\textbf{Gluten index} = \frac{\textbf{wt of gluten over centrifuge screen}}{\textbf{total wt of gluten mass}}$$

2.8 Free Elemental Sulfur Content

Elemental sulfur analysis was done according to the method of Garimella Purna (2010). One (1.0) gram of each sample was weighed in duplicate and exact weight recorded. Each sample was suspended in 10 mL of distilled water and shaken for 10 min. The flour-water mixture was centrifuged at 7000 rpm for 10 min and the supernatant discarded. The remaining pellets were frozen and freeze dried for analysis. Untreated flour samples as well as dried pellets were analyzed for elemental sulfur at the Kansas State University Soils Testing Laboratory by nitric-perchloric acid digestion followed by Inductively Coupled Plasma spectroscopy for sulfur analysis (Gieseking et al., 1935) (Appendix B). Samples were tested in duplicate.

2.9 Analysis of Water Extractable and Un-extractable Fractions

Flour-water suspensions (10% and 25% solids) were prepared by mixing calculated amounts of water and flour based on moisture content of the flour. The suspensions were shaken for 15 minutes and then centrifuged at 9000 rpm for 10 min. The water-extractable supernatant and water un-extractable pellet were collected and used for analysis as detailed in each section below. All samples were tested in duplicate.

2.9.1 Viscosity Measurement of Water-Extractable Fraction

The water-extractable portion of the flour/water suspension (both 10% and 25% solids) was collected immediately after centrifugation. The viscosity was measured using a Brookfield DV-II + Pro Viscometer (Middleboro, MA, USA) equipped with a S21 spindle. The test ran at 100 rpm, 67.7 s^{-1}shear rate, for 0.5 minutes. Each sample was run in duplicate.

2.9.2 Gluten Index of Water Un-extractable Pellet and Treated Washed Flour

Testing and sample preparation for gluten index determination was done in three stages. Stage I refers to the flour washing step, stage II details treatments for sample preparation, stage III is the gluten index testing.

2.9.2.1 Stage I: Flour Washing

A 25% solids suspension was prepared by adding the proper amount of flour and water according to the moisture content of the flour. Approximately 27.70 g of flour and 72.30 mL of water must be mixed to result in the 25-30g of solids needed to run duplicates of each sample treatment. The suspension was then shaken for 15 minutes and centrifuged at 9000 rpm for 10 minutes. Stage II details sample treatment from this point forward.

2.9.2.2 Stage II: Sample treatments

The samples were subjected to seven treatments/ procedures in final preparation for gluten index determination. The steps taken after the tubes are removed from the centrifuge are detailed below.

1. Washed Flour (wet): the supernatant was discarded and the water un-extractable pellet was used as is.
2. Washed Flour (dried): the supernatant was discarded and the water un-extractable pellet was collected, frozen, freeze dried, and ground.
3. Reconstituted Flour: the supernatant (water-extractable) and water unextractable fractions were frozen, freeze dried, and ground together.
4. Enzyme Treated Control: the water-extractable fraction was placed in a 70°C water bath for 1.0 h followed by 10 min in a boiling water bath. The water-extractable and water un-extractable pellet were recombined, frozen, freeze dried, and ground. This treatment was used as a control for treatments 5 and 6.
5. Protease Treated: Protex 14L (Genencor, Rochester, NY, USA) was added to the water-extractable fraction at a 0.5% dosage level based on manufacturer recommendations. The dosage was calculated according to the total amount of protein in the sample by multiplying the % protein in the flour by the amount of flour weighed before washing. Enzyme activity was 160 U/g. One unit was defined as the amount of enzyme that will produce 200 μg of tyrosine-equivalent trichloroacetic acid soluble peptides per minute

from a casein substrate at pH 6.5 and 35°C (Genencor, 2014). The protease treated water-extractable fraction was then placed in a 70°C water bath for 1.0 h followed by 10 min in a boiling water bath. After protease treatment, the water-extractable and water un-extractable fractions were recombined, frozen, freeze dried, and ground.

6. Hemicellulase Treated: Multifect CX 13L (Genencor, Rochester, NY, USA) was added to the water-extractable fraction at a 0.5% dosage level based on manufacturer recommendations. Multifect CX 13L is described by the manufacturer as a "food grade complex of enzymes [that] exhibits significant activity towards cellulose, hemicelluloses, beta-glucans, and arabinoxylans" (Genencor, 2014). The dosage was calculated according to the total amount of arabinoxylans in the sample by multiplying the % arabinoxylans in the flour by the amount of flour weighed before washing. Enzyme activity was 3900 CMC-DNS U/g. One unit is defined as the amount of enzyme required to generate 1 μmol of glucose reducing sugar equivalents per minute by the action of cellulase using a carboxymethyl cellulose (CMC) substrate reacted with 3,5 dinitrosalicylic acid (DNS) (Genencor, 2014). The hemicellulase treated water-extractable fraction was then placed in a 70°C water bath for 1.0 hr followed by 10 min in a boiling water bath. After hemicellulase treatment, the water-extractable and water un-extractable fractions were recombined, frozen, freeze dried, and ground.
7. The final test combined the freeze dried un-extractable pellet of Wesley with the water-extractable fraction from Mattern and the freeze dried un-extractable pellet from Mattern with the water-extractable fraction from Wesley.

2.9.2.3 Stage III: Gluten index determination

The gluten index of samples from each of the seven treatments/procedures was determined using a Glutomatic 2200 (Perten Instruments Inc., Springfield, IL, USA) according to AACCI Method 38-12 (2000). Equation 12 above was used to calculate gluten index.

2.9.3 HPLC Analysis of Water Extractable Fraction

Water-extractables from water washed, protease treated and hemicellulase treated flour (treatments 1, 5 and 6) were analyzed by HPLC (Agilent 1100 Series, Agilent Technologies, Palo Alto, CA). The samples were eluted using 0.1 M sodium nitrate with 0.03% sodium azide as a mobile phase, an injection volume of 20 μm and eluting flow rate of 1.0 mL/min. Ultraviolet,

refractive index, right angle light scattering and viscometry detection were used. Data was analyzed using OmniSEC (Malvern Instruments, Worcestershire, UK) and Chemostation software (Agilent Technologies, Santa Clara, CA).

2.9.4 Determination of Arabinose/Xylose Ratio in Water Extractable Fraction

The 25% solids suspension was prepared for this test. Directly after centrifugation, the water-extractable fraction (supernatant) was collected, frozen, freeze dried and ground. Sulfuric acid (2.0 mL, 12 M) was added to 0.02 g of freeze dried water-extractables. The solutions were placed in a 35°C water bath for 30 minutes and then diluted to a 2 M sulfuric acid concentration by adding 10 mL of deionized water. The test tubes were then placed in an oven set at 100°C for 2 hours. After cooling to 25°C, the samples were diluted 100x with distilled water and filtered for analysis by high-performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) (Dionex ICS-3000, Thermo Scientific, Sunnyvale, CA, USA). A Carbo Pac™ PA1 column (Thermo Scientific, Sunnyvale, CA, USA) was used for monosaccharide separation. The sample was eluted with 15 mM sodium hydroxide at an eluting flow rate of 1.0 mL/min.

2.9.5 Protein Content in Water Un-extractable Pellet

The 25% solids suspension was prepared for this test. Directly after centrifugation, the water un-extractable fraction (pellet) was collected, frozen, freeze dried and ground. The freeze dried water un-extractable pellet was analyzed at the Kansas State University Soils Testing Laboratory by nitrogen combustion using a LECO FP-428 (LECO Co., St. Joseph, MI) to determine the % protein remaining in the pellet.

2.10 Dough Rheological Properties

An ATS RheoSystems (Bordentown, NJ) temperature/stress controlled rheometer, equipped with a parallel plate measuring system (25mm diameter serrated plate, gap 2.0mm), and plate temperature held constant at 30°C was used to measure the small deformation rheology of the doughs. Each of the samples was tested in duplicate.

2.10.1 Sample Preparation

Dough was mixed using a 10g mixing bowl mixograph at different absorption levels (65 and 70%) and mixing times (2.5, 4.0, and 5.5 minutes) based on the optima found from mixograph data. The dough was divided into 2.0g pieces and manually rounded. It was then allowed to rest (covered) for 30 min before measurement of rheological properties. A single dough ball was then placed on the bottom plate of the parallel plate measuring system and the gap adjusted to 2.10mm. Excess dough was trimmed with a bent metal spatula being careful to avoid excess deformation. Silicon oil was used to cover the edges of the dough to avoid drying. After trimming, the gap was adjusted to the target of 2.0mm, and the sample was allowed to reach final equilibrium (10 min rest) before testing.

2.10.2 Stress Sweep (Linear Viscoelastic Region)

Stress sweep tests were performed to determine the linear viscoelastic response region of the four wheat flour dough's response. The instrument operated with a 2.0mm gap and 30°C, the sample loading method "To Gap" was used. The maximum loading force was 8.149E+4 Pa. Testing proceeded when the residual force was below 4.074E+4 Pa or when waiting more than 1.000E+3 s. Final equilibrium time was 10 minutes. The rest of the settings were as follows: number of measurements 1, measurement interval at 2.000E+1 s, constant frequency set at 1.000E+0 Hz, delay time 1.000E+0 s, integration periods 1.00, fast Fourier transformation (FFT) size 512, and stress range between 0.1 and 10 000 Pa.

2.10.3 Frequency sweep

Frequency sweep tests were performed within the previously determined linear viscoelastic region of the wheat dough samples. Two replicates were tested at frequencies ranging from 0.01 to 100.0 rad/s and a constant stress of 15.0 Pa in LVR at 30°C. Equilibrium time was 10 minutes, maximum loading force 8.149E+4 Pa. Testing proceeded when the residual force was below 4.074E+4 Pa or when waiting more than 1.000E+3 s. The remaining settings were as follows: number of measurements 1, measurement interval 2.000E+1 s, delay time 1.000E+0 s, integration period 1.00, and FFT size 512.

2.11 Statistical Analysis

Data was analyzed using SAS software (SAS Institute Inc., Cary, NC). ANOVA and least significance difference (LSD) was applied to the data. Level of significance was set at $p < 0.05$ for all the tests.

3. Results and Discussion

3.1 Wheat Milling

The waxy samples exhibited lower flour yields compared to the normal wheat samples (Table 3.1). Chibbar and Chakraborty (2005) discussed several studies where dry milling of waxy wheat resulted in yields up to 20% less than its non-waxy counterpart. The characteristic low flour yield during dry milling could be a result of blinding of the sieves for the samples tested. Past studies have also attributed lower milling yield to higher grain fat, arabinoxylan, or beta-glucan content (Chibbar and Chakraborty, 2005; Guan et al., 2009; Jonnala et al., 2010; Takata et al., 2005; Ma et al., 2013).

Table 3.1 Milling post-temper moisture and final extraction for the waxy and normal wheat

Sample	Post-temper Moisture (%)	Final Extraction (%)
Nuplains $_{N}$	15.4±0.4	72.2
Wesley $_{N}$	15.6±0.4	77.0
Mattern $_{W}$	15.2±0.5	68.7
NX11MD2337 $_{W}$	14.9±0.4	69.7

*Subscript identifies waxy (W) and normal (N) samples

Ma et al. (2013) discussed past studies that showed that extractions similar to those from normal wheat can be obtained if the feed rate is lowered during milling. This was the reason why the feed rate here was slowed down to approximately 26.0g/minute. Even this reduced feed rate failed to increase waxy flour yields to those of their normal counterparts.

3.2 Flour Composition

Table 3.2 shows the composition of all four flour samples. The waxy samples had higher starch damage but no other trends were observed. Waxy wheat starch granules are known to have lower tolerance to mechanical shear, which results in higher levels of starch damage. A possible fundamental explanation is the effect of high starch crystallinity, characteristic of waxy wheat, during milling (Chibbar and Chakraborty, 2005; Graybosch et al., 2003). Higher arabinoxylan contents for waxy samples have been reported in previous studies (Jonnala et al., 2010; Takata et al., 2007) although these samples show no significant difference between them and the normal samples. The similarity in arabinoxylan content between these samples makes them a good sample set for the evaluation of differences in the types of arabinoxylans present in waxy flour compared to normal flour. The protein contents of all four samples are statistically similar, again

suggesting that comparisons can be made between the samples knowing that protein content is not a variable that needs to be considered. This allows for better conclusions to be drawn regarding sample protein quality.

Table 3.2 Flour composition of normal and waxy flours

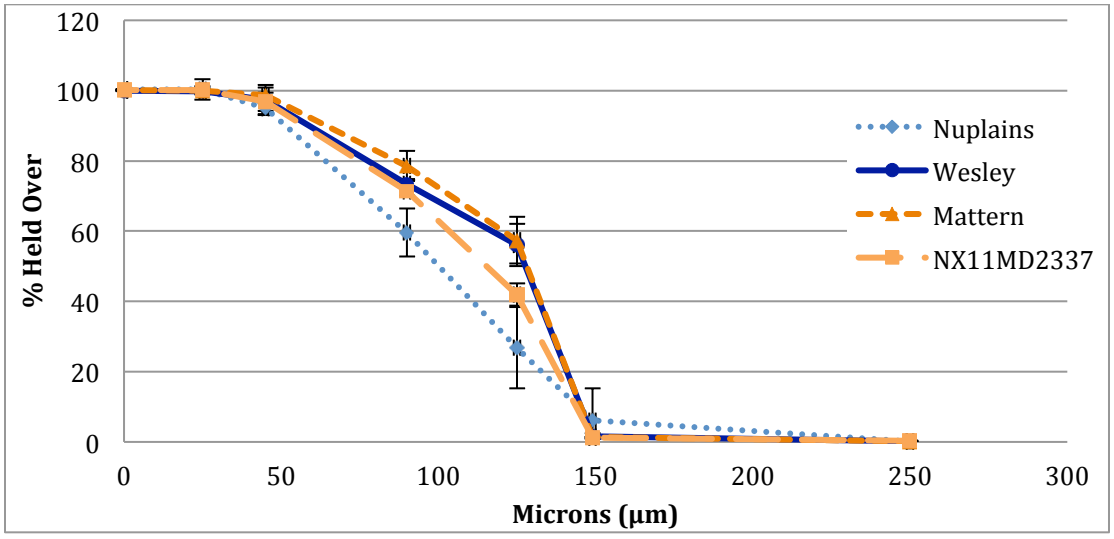
Test	$Nuplains_N$	$Wesley_N$	$Mattern_W$	NX11MD2337
Moisture (%)	11.00±0.00	10.51± 0.05	10.20± 0.10	9.96± 0.10
Protein (% db)	13.22± 0.19[a]	13.48± 0.21[a]	12.97± 0.41[a]	13.64± 0.02[a]
Total Starch (%db)	78.94± 2.03	74.79± 1.04	73.12± 1.86	74.60± 1.28
Ash (% db)	1.18± 0.05[a]	0.85± 0.03[b]	0.92± 0.02[b]	0.76± 0.02[c]
Arabinoxylans (% db)	2.10± 0.15[a]	1.33± 0.22[a]	2.73± 0.82[a]	2.01± 0.55[a]
Starch Damage (%)	9.85± 0.06[c]	10.61± 0.13[b]	12.74± 0.23[a]	12.66± 0.19[a]

[a] Different letters in a row indicate significant differences at $p < 0.05$

3.3 Particle Size Analysis

Flour particle size influences chemical and physical properties of any particular flour (Sakhare et al., 2013). Dry gluten content, optimum mixing time, and water absorption increase and dough extensibility/ elasticity balance improves with reduced particle size (Sakhare et al., 2013). Figure 3.1 shows the particle size distributions of the four flours. There is no clear trend between particle size of waxy (orange/ triangle and square lines) and normal (blue/ circle and rhombus lines) flour samples. Given the effect that particle size may have on dough rheology, the lack of a significant trend in particle size between waxy and non-waxy samples should allow for better comparison of mixing and compositional properties, as particle size is not a factor.

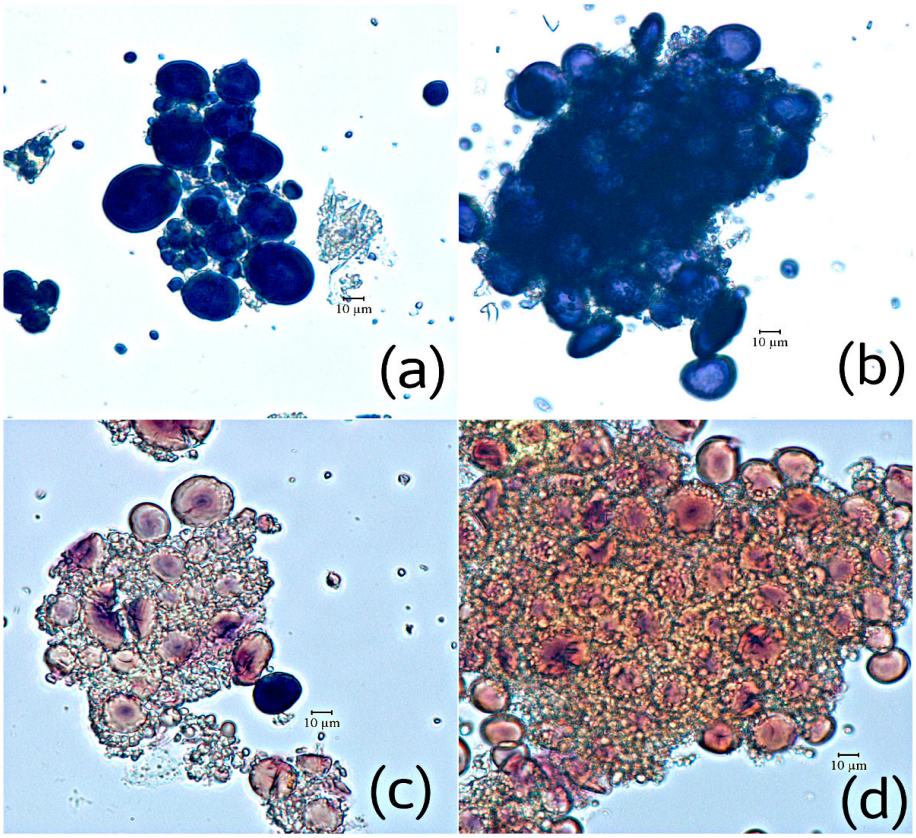
Figure 3.1 Particle size distribution for test samples (ROTAP method)



3.4 Iodine Staining of Flour Samples

Figure 3.2 shows the starch of normal and waxy wheat samples. The starch of normal wheat stained blue-black while the waxy samples stained red-brown. This is consistent with what has been previously reported (Graybosch et al., 2003; Guan et al., 2009). The only waxy sample to show any normal starch contamination was Mattern with 2.9% contamination (determined by granule count). The waxy samples were purple-blue at the hilum. This phenomenon is believed to be a result of sufficient activity from GBSS protein to produce amylose in early development yet not present or in levels below the necessary threshold to continue amylose synthesis later in development and resulting in amylose-free starch (Kuipers et al., 1994). It is possible that the increasing size of the granule as development continues has an influence on the capability of a limited amount of GBSS to continue to produce amylose. Kuipers *et al.* (1994) found that despite the purple-blue core at the hilum, there was no detectable amylose in the granules. Another possible explanation is the presence of amylopectin chains in the hilum that are long and linear enough to bind iodine and create that stained core.

Figure 3.2 Images of iodine stained normal and waxy wheat flour at 400x magnification



* Nuplains_N (a), Wesley_N (b), Mattern_W (c) (2.9% contamination), and NX11MD2337_W (d)

3.5 Dough Mixing

The mixograph results in Table 3.3 show clear differences between the waxy and the hard red winter normal (Wesley) wheat sample. The normal hard white wheat sample, Nuplains, displays characteristics consistent with very weak doughs. The difference in mixing properties between the two normal samples makes it difficult to determine trends between normal and waxy samples. Mixograms (shown in Appendix C) show higher absorption than the calculated optimum described in Equation 2 for all wheat samples. High water absorption for waxy wheat flour agrees with previous studies. Guan *et al.* (2009) claims that waxy wheat starch can absorb 10-20% more water than normal starch. Increased water absorption has been linked to higher protein content, dietary fiber, starch damage, or arabinoxylan content (Jonnala et al., 2010; Hung et al., 2007). The samples tested in this particular study showed higher starch damage for waxy samples; which is consistent with the observed high absorption. However, the wide range of factors that affect water absorption, including some measured for the present samples (such as protein and arabinoxylan content) showed no significant difference between normal and waxy samples; which could explain why no clear trend is seen between waxy and normal wheat flours.

Waxy wheat doughs have been characterized by lower tolerance to overmixing, and sticky, unstable doughs (Chibbar and Chakraborty, 2005). The higher absorption, shorter time to peak and lower stability seen in this sample set is consistent with these previous findings. The mixing characteristics observed limit the use of waxy wheat dough in a commercial operations. The observed behavior of the dough would cause handling difficulty during makeup due to dough softness and stickiness (Hung et al., 2007; Zhang et al., 2014). Previous studies have shown that the addition of hemicellulase reduces waxy wheat dough stickiness, and could help improve the mixed dough handling properties (Guan et al., 2009). Other characteristics of waxy wheat dough include higher gas production but lower retention, faster fermentation, open crumb, shrinkage of the loaf when cooling, and poor appearance (Chibbar and Chakraborty, 2005).

Table 3.3 Envelope time analysis of optimum absorption dough from flour samples

Sample	Optimum absorption (%)	Left of Peak (min)	Peak (min)	Right of Peak (min)
$Nuplains_N$	65.3	1.2	2.7	4.5
$Wesley_N$	69.9	3.5	5.5	7.8
$Mattern_W$	67.2	1.4	2.5	5.5
$NX11MD2337_W$	70.1	2.8	3.2	6.0

3.6 Protein Analysis

The free sulfhydryl and polymeric protein analyses were performed to determine if the differences in gluten development observed during mixograph testing can be explained by the protein composition, specifically disulfide bond formation. Test results were also compared to study the differences between flour and dough. The optimum absorption and mix time used for the preparation of the dough samples to be tested for protein analysis corresponds to the optimum absorption and time to peak columns displayed in Table 3.3. The parameters were chosen based on mixograph results of flour tested using increasing water absorptions to determine optima (data shown in Appendix C).

3.6.1 Free Sulfhydryl Content

Table 3.4 shows no significant differences in the sulfhydryl content between samples, yet a decrease in free sulfhydryl is seen for all samples when comparing flour to dough. Weak flour has been reported to have high levels of glutathione and related thiol compounds (Li et al., 2003). Free sulfhydryls (-SH groups) are suspected to play an important role in disulfide exchange between gluten proteins during dough formation (Garimella Purna, 2010). Free –SH groups can participate in inter-chain bonds between high molecular weight gluten subunits or can be contributed to reduced glutathione (GSH) (Garimella Purna, 2010). The inter-polymer bonds between gluten subunits act as chain extenders and promote dough formation during mixing. The –SH group in glutathione is easily oxidized to form an S-S bond, therefore it can easily form bonds with proteins acting as a chain terminator that interferes with the sulfhydryl-disulfhydryl exchange during mixing, ultimately inhibiting the formation of polymeric gluten (Garimella Purna, 2010; Li et al., 2003).

The levels of free sulfhydryl groups in the waxy samples could be acting as chain terminators such as GSH, which may help explain the poor dough forming properties of waxy wheat flour as low concentrations of glutathione can significantly weaken dough and increase its extensibility (Goesaert et al., 2005). The levels of free –SH groups in the Nuplains sample may be acting as chain terminators as well meaning that the effect of free –SH and chain terminators in relation to mixing properties of dough is not an exclusive trait of the waxy gene. On the other hand, it is possible that a greater percentage of the free –SH present in Wesley (strong dough) is interacting to form disulfide bonds rather than acting as a chain terminator. The decrease seen

from flour to dough is to be expected as some of those free –SH groups have been engaged in the gluten protein disulfide exchange that occurs during mixing. Further exploration and testing would be needed to determine if these theories are correct.

Table 3.4 Free sulfhydryl content in waxy and normal flours and doughs

nmol/mg	Flour	Dough
$Nuplains_N$	0.68±0.15[a]	0.65± 0.19[a]
$Wesley_N$	0.62± 0.15[a]	0.51± 0.12[a]
$Mattern_W$	0.77± 0.05[a]	0.63± 0.15[a]
$NX11MD2337_W$	0.67± 0.07[a]	0.62± 0.13[a]

[a] Different letters in a column indicate significant differences at $p < 0.05$

3.6.2 Polymeric Protein

Treatment with 1-propanol separates proteins into soluble and insoluble polymeric fractions. Results (Table 3.5) show that waxy wheat samples had a significantly higher IPP content than did the normal wheat flour counterpart. A 50% 1-propanol solution can extract all monomeric proteins (albumins, globulins, and gliadins) as well as the smallest polymeric proteins from flour effectively (Bean et al., 1998). Thus the majority of the protein left for nitrogen combustion analysis corresponds to glutenin. The molecular size distribution of polymeric proteins is key in the prediction of breadmaking properties of a given wheat line and IPP has been identified as a strong indicator of dough strength, mixing properties and bake mix time (Bean et al., 1998). However, waxy wheat displays qualities of a weak dough contrary to what their high IPP levels predict.

Table 3.5 Insoluble Polymeric Protein (IPP) and Soluble Polymeric Protein (SPP) content in waxy and normal wheat flours and doughs

Sample	IPP (%)		SPP (%)	
	Flour	Dough	Flour	Dough
$Nuplains_N$	38.3± 0.9[c]	39.5± 0.8[c]	61.7± 0.9[c]	60.5± 0.8[c]
$Wesley_N$	45.2± 0.2[b]	43.5± 0.6[b]	54.8± 0.2[b]	56.5± 0.6[b]
$Mattern_W$	47.6± 0.5[a]	46.6± 1.3[a]	52.4± 0.5[a]	53.4± 1.3[a]
$NX11MD2337_W$	48.0± 0.3[a]	47.0± 1.0[a]	52.0± 0.3[a]	53.0± 1.0[a]

[a] Different letters in a column indicate significant differences at $p < 0.05$

Table 3.6 shows significantly lower total polymeric to monomeric ratios for the waxy samples compared to the normal wheat samples. This reflects a higher monomeric protein

content for the waxy flours. Large molecular size proteins govern dough strength, expressed as longer time to peak mixing time and greater elasticity. In fact polymer science reveals that proteins have a molecular weight threshold value below which, they cannot participate in entanglement and cannot support the formation of a gluten matrix (Gupta et al., 1993).

Gliadins, monomeric proteins of 30-80 kDa molecular weight, are responsible for the viscous nature of dough (Song and Zheng, 2007; Don et al., 2003). Glutenins are polymeric proteins that range either between 12-60 or 60-120 kDa for low or high molecular weight glutenin subunits respectively and provide strength and elasticity to dough (Song and Zheng, 2007; Don et al., 2003). Due to its large size, glutenin forms a continuous network that provides strength to the dough while gliadin acts as a plasticizer and provides viscosity (Goesaert et al., 2005). The HPLC gliadin results for flour show a significantly lower gliadin content in Wesley, a significantly higher gliadin content in NX11MD2337, and similar gliadin contents between the normal Nuplains and waxy Mattern. These results would suggest that the flour with the strongest dough forming properties is Wesley, followed by Nuplains and Mattern, and NX11MD2337 as having the poorest dough forming properties. However, looking at the gliadin contents in dough samples reveals that both the normal samples have significantly lower gliadin contents than the waxy samples. These results suggest that the gliadins present in Nuplains participate in the gluten matrix more easily than those in Mattern. This is in agreement with the previous mixograph data and the gluten index data below.

The gliadin/glutenin ratio is key in the dough forming properties of a flour sample because, as a viscoelastic material, dough requires balance between its elastic and viscous elements. Increase in the gliadin/glutenin ratio result in lower dough elasticity (Song and Zheng, 2007). The ratio of monomeric to polymeric proteins thus defines the bread-making qualities of a flour sample (Flaete et al., 2005). The TPP/TMP ratio is the inverse of the gliadin/ glutenin ratio so given the lower TPP/TMP ratio of waxy wheat flour samples; it is not a surprise to encounter weak dough properties with these waxy flours.

Proteins have been classified into soluble and insoluble polymeric fractions. The differences in extractability are believed to be a result of molecular size. Large glutenin proteins cannot be extracted without the use of sonication, reducing agents, or acid-base hydrolysis (Bean et al., 1998). The waxy wheat flour samples show a significantly higher residual protein content than does Nuplains and significantly lower than does Wesley (Table 3.6), yet the dough samples

show no significant difference in residual proteins across all samples. The lower levels of residual protein in Nuplains suggest the presence of few high molecular weight proteins; in turn, this might explain the poor dough forming properties exhibited by this flour. The higher residual protein content seen in Wesley is consistent with the strong dough forming properties of this flour. The fact that the waxy flour samples have a significantly higher residual content than Nuplains is inconsistent with the lower TPP/TMP ration shown. However, the lack of a significant difference in dough residual protein may indicate that the large proteins present in waxy samples are less extractable by sonication but will interact during mixing. Increase in extractability is seen for all samples (SPP) when comparing flour to dough because protein aggregates are broken down and become more extractable during mixing.

Table 3.6 Protein composition in waxy and normal wheat flours and doughs as determined by TPP procedure

Sample		TPP/TMP ratio	SPP %	Gli %	Alb/ Glob	Residual %
Nuplains$_N$	Flour	0.75± 0.02^a	31.19± 0.41^a	41.45± 0.93^a	11.04± 0.10^a	7.90± 0.80^a
	Dough	0.78± 0.01^b	32.55± 0.83^a	41.69± 0.47^a	10.96± 0.16^a	7.07± 1.06^a
Wesley$_N$	Flour	0.76± 0.02^a	29.87± 0.28^b	39.39± 1.21^b	9.33± 0.24^b	13.97± 1.46^b
	Dough	0.81± 0.01^a	33.18± 0.45^a	41.00± 0.60^a	10.09± 0.34^b	7.87± 1.63^a
Mattern$_W$	Flour	0.70± 0.01^b	28.59± 0.86^c	41.12± 0.60^a	10.09± 0.15^c	11.50± 1.58^c
	Dough	0.71± 0.01^c	25.94± 0.27^b	43.91± 0.17^b	10.06± 0.07^b	6.81± 0.39^a
NX11MD2337$_W$	Flour	0.58± 0.01^c	31.35± 0.36^d	44.65± 0.75^c	10.36± 0.23^c	10.90± 1.08^c
	Dough	0.61± 0.00^d	28.77± 0.32^c	47.32± 0.29^c	10.61± 0.17^a	5.81± 0.96^a

*TPP/TMP = total polymeric/total monomeric protein; SPP = soluble polymeric protein

*Gli = gliadins; Alb/Glob = albumins and globulins

[a] Different letters in a column between flour results indicate significant differences at $p < 0.05$

[a] Different letters in a column between dough results indicate significant differences at $p < 0.05$

* There is no statistical information comparing flour to dough shown on this table

Table 3.7 shows a dramatic increase in protein extractability after dough mixing and higher dough UPP values for waxy wheat dough samples. During mixing, the gluten proteins are hydrated and form a continuous protein network; during this process, the solubility of proteins increases significantly (Goesaert et al., 2005). According to Song and Zheng (2007) and Gupta *et al*. (1993) the molecular size of UP proteins is greater than that of EP proteins and the size distribution of polymeric protein largely defines the strength of a particular flour. Furthermore, the unextractable and larger size proteins have been correlated with dough strength in the past and indicate the selection of high UPP flour for good dough forming properties (Bean et al.,

1998; Gupta el al., 1993). The theory is that only protein polymers over a certain size can contribute to the elasticity of the protein network in dough. This is why flour with higher UPP levels has been associated with stronger doughs (Goesaert et al., 2005). However, the higher UPP values for waxy wheat dough found here disagree with previous conclusions regarding this relationship between dough strength and UPP quantity. A possible explanation could be the role that the higher levels of gliadin play in the dough mixing process.

Gliadins can have a weakening effect on the gluten network during mixing (Garimella Purna, 2010). A possible explanation for the above results could be the presence of more ω-gliadin in the waxy samples (as reported by Garimella Purna, 2010). This gliadin subunit lacks the cysteine containing residues that can interact in the formation of disulfide bonds and thereby increase protein polymerization (Garimella Purna, 2010). Disulfide bonds hold together the glutenin subunits, therefore any compounds that affect the thiol system affects the polymerization of glutenin. This can result in significant changes to the rheological properties of dough (Goesaert et al., 2005). Gluten polymers with at least two cysteine residues can form intramolecular disulfide bonds and extend the gluten network during mixing, gluten polymers with just one or an odd number of cysteine residues could form intermolecular bonds thus acting as a chain terminators (Garimella Purna, 2010). Isolating the gliadins in the samples and adding them back in varying levels would give a more complete picture of how gliadins are affecting the gluten formation.

Table 3.7 Extractable and unextractable polymeric protein, and residual protein content in waxy, and normal wheat flour and dough

Sample		EPP %	UPP %	Residual %
Nuplains$_N$	Flour	32.41± 1.44^a	39.35± 3.54^b	5.81± 2.19^a
	Dough	84.63± 3.76^a	15.37± 3.76^a	4.69± 2.13^a
Wesley$_N$	Flour	28.23± 0.89^b	47.27± 2.80^a	7.91± 3.28^a
	Dough	83.84± 0.31^a	16.16± 0.31^a	6.44± 0.49^a
Mattern$_W$	Flour	29.98± 0.26^c	42.76± 1.80^{ab}	7.36± 2.99^a
	Dough	83.23± 6.48^{ab}	16.77± 6.48^{ab}	5.34± 0.25^a
NX11MD2337$_W$	Flour	25.38± 0.65^d	46.93± 2.79^a	7.32± 3.69^a
	Dough	76.56± 1.57^b	23.44± 1.57^b	5.37± 0.37^a

[a] Different letters in a column between flour results indicate significant differences at $p < 0.05$

[a] Different letters in a column between dough results indicate significant differences at $p < 0.05$

The amount of cysteine residues available for cross-linking has the potential to determine the molecular size distribution of glutenin based on whether they are acting as chain terminators

or extenders, and therefore this can determine flour properties (Goesaert et al., 2005; Gupta et al., 1993). LMW-GS increases the number of physical crosslinks, improving dough elasticity yet the amount and composition of HMW-GS has been previously correlated to dough and final product properties (Don et al., 2003; Song and Zheng, 2007). The increased gliadin content in the dough and poor dough forming properties of waxy wheat flour would suggest that this type of flour has higher amounts of low molecular weight proteins yet we see high insoluble, unextractable, and residual protein values (Tables 3.5, 3.6, and 3.7) that also suggest the presence of very high molecular weight proteins. This observation indicates that it may be the structure of the proteins (anywhere from primary to quaternary) that affects the dough forming properties in waxy wheat flour rather than the quantity and general molecular size alone.

The variations between replicates for some of the samples are most likely a result of differences in mixing during dough sample preparation or due to differences in sonication. Variations in exposure to the sonicator, either in time or output, may easily occur due to human handling depending on: the exact position of the probe within the tube, reaction time removing the probe and shutting the sonicator off after the timer was set off, and speed with which the output setting reached 6W. The significant effect of sonication was previously explained by Singh *et al* (1990).

3.7 Gluten Index

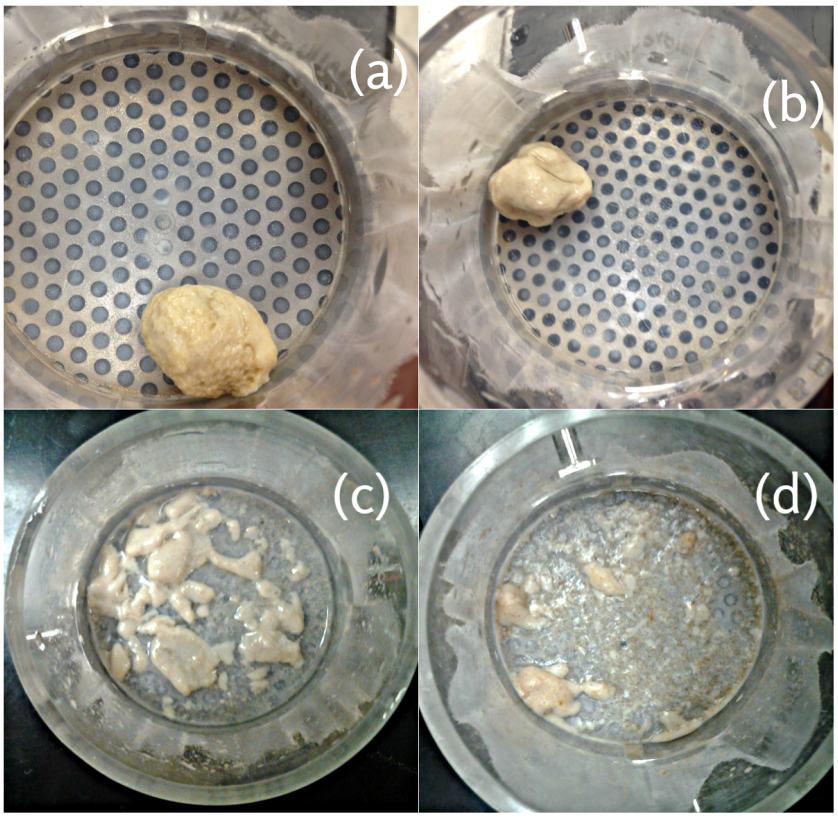
Table 3.8 presents the gluten indices for the normal wheat flour samples. A coherent gluten ball could not be obtained from the waxy samples so it was not possible to get a gluten index measurement (Figure 3.3). This suggests that certain components in those flours interfere with the formation of a continuous gluten matrix. The presence of non-starch polysaccharides in waxy wheat flour may dilute the protein and cause a poor matrix interaction with the starch granules rather than the continuous gluten matrix characteristic of normal wheat flour dough. If so, the dough would fail to form an elastic matrix resulting from cross-linked gluten molecules (Hung et al., 2007). A second possible explanation relates to the protein characteristics described above. The increased level of gliadins in waxy wheat flour (Table 3.6) could explain the lack of protein aggregation during the Glutomatic test of waxy samples.

Table 3.8 Gluten index obtained for normal wheat flour samples

Sample	Gluten index
Nuplains	68± 2[a]
Wesley	86± 3[b]

[a] Different letters in a column indicate significant differences at $p < 0.05$

Figure 3.3 Gluten aggregation Nuplains (a), Wesley (b), Mattern (c) and NX11MD2337 (d)



3.8 Free Elemental Sulfur Content

The results in Table 3.9 show an increase in sulfur content in the waxy samples after washing and a decrease in that of the normal wheat flour samples. The waxy and Nuplains flours have lower sulfur content than the strong Wesley flour. Increased levels of sulfur in flour have been associated with reduced time to peak development as measured by mixograph, increased dough extensibility and increase bread loaf volume (Jez, 2008). As the sulfur content is reduced, the TPP/TMP ratio decreases (Flaete et al., 2005). Therefore one would expect to see lower sulfur content in the waxy samples based on the results in Table 3.6 (Flaete et al., 2005). The increased levels of insoluble sulfur containing compounds in the weak flours could be related to a possible shift from sulfur rich proteins (LMW-GS and α and γ gliadins) to sulfur poor proteins

(ω-gliadins and HMW-GS) (Flaete et al., 2005). The increased levels of sulfur seen in the waxy samples after flour washing suggest that waxy wheat flour contains a higher amount of LMW-GS, α and γ gliadins than normal wheat flour that are somehow more insoluble than the HMW-GS of normal wheat flour.

Table 3.9 Free elemental sulfur content in native and washed flour samples

Sample		% S
$Nuplains_N$	Flour	0.138 ± 0.001^b
	Washed	0.130 ± 0.009^a
$Wesley_N$	Flour	0.141 ± 0.000^a
	Washed	0.131 ± 0.002^a
$Mattern_W$	Flour	0.137 ± 0.001^b
	Washed	0.144 ± 0.003^a
$NX11MD2337_W$	Flour	0.138 ± 0.001^b
	Washed	0.147 ± 0.014^a

[a] Different letters in a column between flour results indicate significant differences at $p < 0.05$

[a] Different letters in a column between washed results indicate significant differences at $p < 0.05$

3.9 Analysis of Water Extractable and Un-extractable Fractions

Wang *et al.* (2004b) studied the effects of the water-extractable fraction on gluten aggregation. Their research showed that the addition of water-extractables decreased gluten aggregation in the normal flour used for their analysis. Wang *et al.* (2004b) proposed that the reduced aggregation could be a result of the arabinoxylan content in the water-extractable portion. Wang *et al*. (2004a and b) and Song and Zheng (2007) proposed two mechanisms by which arabinoxylans interfere with gluten aggregation during mixing: viscosity increase (physical interference) and interference by arabinoxylan bound ferulic acid (chemical interference). The flour samples in the present study were subjected to a series of tests to test these previous findings and apply them to the interactions in normal versus waxy flour samples.

3.9.1 Viscosity Measurement of Water Extractable Fraction

Table 3.10 shows the viscosity of the water extractable fractions of the four flour samples. The viscosity of the supernatant obtained from the waxy samples is significantly higher than that of normal wheat flour samples, suggesting that there are compounds that are water soluble in waxy wheat flour that are either not present or present in smaller quantities in normal wheat flour. The increased viscosity could be a result of water-extractable arabinoxylans or

proteins. Water extractable (WE) arabinoxylans can form highly viscous solutions depending on their chain length, substitution pattern, and substitution degree. About one third of the intrinsic viscosity of flour is a result of water-extractable arabinoxylans, even though they only compose about one forth to one third of the 1.5-2.5% arabinoxylan content in the flour (Goesaert et al., 2005).

Table 3.10 Viscosity of water extractable fraction measured at 67.7 s^{-1} constant shear rate

	Viscosity (cP)	
Sample	**10% solids**	**25% solids**
$Nuplains_N$	1.00± 0.00[a]	4.00± 0.71[a]
$Wesley_N$	2.25± 0.35[b]	2.75± 2.47[a]
$Mattern_W$	3.75± 0.35[c]	14.00± 2.12[b]
$NX11MD2337_W$	5.00± 0.71[d]	9.00± 0.00[c]

[a] Different letters in a column indicate significant differences at $p < 0.05$

3.9.2 Gluten Index of Water Extracted Pellets

Table 3.11 shows aggregation of gluten in normal and waxy samples subjected to different treatments. The normal samples form gluten balls under all treatment conditions, while the waxy samples could only form a gluten ball when the flour was washed and tested before drying (washed flour wet). Even though a high gluten index value was initially obtained from the washed waxy wheat flour, no gluten ball was formed when the experiments were repeated (data not shown). The experiment was not reproducible.

Furthermore, the total weight for all the samples under the washed flour (wet treatment) was much lower than for the samples from the other treatments. The difference could be because the washed flour (wet) samples had a lower solids content than the other treatment samples. The actual dry weight of the sample would be much closer to about 6-7 g compared to 10g for the other samples tested. When measuring the required 10g of sample for the Glutomatic test, a large portion of that weight was water, which was easily removed during centrifugation.

The results for the washed flour (dry) treatment in Table 3.11 show no trend. This would suggest that the removal of the water-extractable fraction does not have an effect on the gluten aggregation of the samples. The reconstituted flour treatment serves as a control for the effect of the extraction procedure and the freeze-drying on the gluten aggregation properties of the samples. Table 3.11 shows little difference between the gluten index of the untreated and reconstituted normal flours and still no gluten aggregation for the waxy samples. This would

suggest that the extraction procedure itself followed by freeze-drying does not affect the aggregation properties characteristic of any of the four flour samples.

The results of the enzyme treated control compared to those of the reconstituted flour treatment suggest that exposing the water-extractables to heat and boiling water had little to no effect on the gluten index of Nuplains but had a small negative effect on Wesley. However, this treatment done to the water extractable fraction does not affect the overall trend of gluten aggregation in normal samples and lack of aggregation for the waxy samples. This means that the effect of protease and hemicellulase treatment on gluten index results can be observed with the security that any conclusions drawn were not affected by the conditions of the sample preparation but are only a result of the effect the enzymes themselves had on the water-extractable fraction.

The lack of gluten aggregation by waxy samples after the protein was degraded by treatment with protease suggests that protein in the water-extractable fraction is not the factor responsible for the poor aggregation properties of waxy wheat flours. The treatment of the water extractable fraction with hemicellulase resulted in poor dough washing during the gluten index determination for Mattern. A possible explanation is what Wang *et al*. (2004a) defined as 'the overdose effect'. The overdose effect is related to the release of water caused by WE-AX when hemicellulase is added to a dough sample resulting in inefficient mixing. Inefficient mixing during testing could explain the poor washing.

The gluten index results from the normal samples together with their HPLC analysis, and the viscosity test of the water-extractable fraction suggest that the normal samples have compounds that inhibit gluten aggregation similar to those present in the waxy samples but they are present in greater amounts in the waxy samples. The higher viscosity of the water-extractable fraction in waxy samples coupled with the lack of gluten aggregation bring to mind the results by Wang *et al* (2004a) describing that physical interference to gluten aggregation is associated with arabinoxylans competing for the available water, and how the increase in viscosity limits the movement of proteins toward each other. Chemical interference could also be a factor. Chemical interference is related to the reaction of arabinoxylan-bound ferulic acid (AX bound FA) (Wang et al., 2004a). AX bound FA links to gluten forming proteins, therefore limiting their interaction with each other to aggregate into a gluten ball during the gluten index determination (Wang et al., 2004a). In light of these findings, it is possible that WE-AX present in higher amounts in the

waxy water-extractable fractions are partially responsible for the poor aggregation seen in waxy wheat flour.

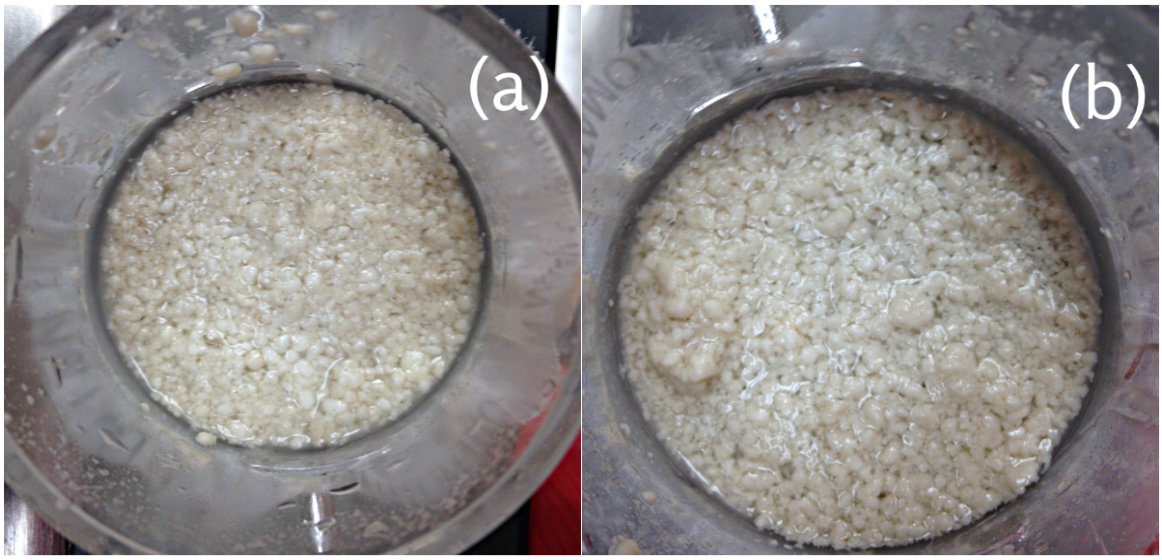
Figure 3.4 shows the poor aggregation that resulted when adding the water-extractable fraction of Mattern to the Wesley un-extractable pellet and when adding the water-extractable fraction of Wesley to the Mattern un-extractable pellet. The poor aggregation by the $Mattern_W$ solubles/$Wesley_N$ pellet combination agrees with the suggestion that the waxy water-extractables are responsible for the poor gluten aggregation in waxy flour. The poor aggregation seen in the $Mattern_W$ pellet/ $Wesley_N$ solubles combination suggests that the water-extractable fraction is not the only factor responsible for the poor aggregation properties characteristic of waxy flours. The Mattern pellet/ Wesley extractables data suggests a greater sensibility of the waxy wheat gluten to the effects of certain compounds that are present in the water extractable fraction of both normal and waxy samples (such as WE-AX). Further experimentation is necessary to confirm this theory.

Table 3.11 Gluten index obtained for the washed flour pellets and reconstituted flours

	Through wt	Total wt	Gluten index
Untreated Flour (Table 3.8)			
$Nuplains_N$	1.08±0.14	3.39± 0.20	68.16± 2.25
$Wesley_N$	0.49± 0.10	3.47± 0.11	85.81± 3.29
$Mattern_W$		no gluten ball	
$NX11MD2337_W$		no gluten ball	
Washed Flour (wet)			
$Nuplains_N$	0.40± 0.02	2.08± 0.08	80.46± 1.80
$Wesley_N$	0.05± 0.03	1.93± 0.03	97.42± 1.43
$Mattern_W$	0.47± 0.03	1.77± 0.01	73.44± 1.81
$NX11MD2337_W$	0.04± 0.04	1.59± 0.12	97.34± 2.93
Washed Flour (dry)			
$Nuplains_N$	1.00± 0.06	4.01± 0.14	75.05± 0.74
$Wesley_N$	0.50± 0.05	4.22± 0.04	88.14± 1.00
$Mattern_W$		poor gluten ball	
$NX11MD2337_W$		no gluten ball	
Reconstituted Flour			
$Nuplains_N$	1.23	4.26	71. 07
$Wesley_N$	0.62± 0.01	4.01± 0.00	84.47± 0.30
$Mattern_W$		no gluten ball	
$NX11MD2337_W$		no gluten ball	

Enzyme Treated Control			
$Nuplains_N$	1.90± 0.07	6.28± 0.07	69.72± 0.71
$Wesley_N$	1.47± 0.09	6.64± 0.07	77.93± 1.18
$Mattern_W$		no gluten ball	
$NX11MD2337_W$		no gluten ball	
Protease Treated			
$Nuplains_N$	1.39	5.32	73.79
$Wesley_N$	1.14± 0.13	5.51± 0.45	79.32± 0.72
$Mattern_W$		no gluten ball	
$NX11MD2337_W$		no gluten ball	
Hemicellulase Treated			
$Nuplains_N$	1.36	4.82	71.73
$Wesley_N$	0.89± 0.06	4.76± 0.43	81.31± 0.49
$Mattern_W$		slack dough	
$NX11MD2337_W$		no gluten ball	

Figure 3.4 Gluten aggregation: Wesley unextractable pellet plus Mattern water extractables (a) and Mattern unextractable pellet plus Wesley water extractables (b)



3.9.3 HPLC Analysis of Water Extractable Fraction

The HPLC ultraviolet Absorbance shown in Figure 3.5 indicates the presence of water-soluble proteins in the water solubles. However there are no particular differences observed when comparing the normal samples to the waxy samples. The HPLC patterns using refractive index detection allow for the identification of four peaks (Figure 3.6). Peak 1 represents the high

molecular weight compounds present in amounts so small that they are barely detected by the RI detector but are clearly identified by the light scattering detector (not shown). Peak 2 was the only one to show a measurable response by all detectors. The molecular weight analysis of that peak calculated by the OmniSEC software is presented in Table 3.12. The results in Table 3.12 indicate that both waxy and normal wheat flour samples have similar compounds that are soluble in water. The quantity of those compounds is greater in waxy wheat flour samples. Mark-Houwink a values (Table 3.12) indicate that the molecules in Peak 2 (very likely arabinoxylans) in normal flours have a more rigid structure than do the molecules of waxy samples (Lapasin and Pricl, 1999). Possibly, the water extractable arabinoxylans (WE-AX) are of higher molecular weight but different molecular arrangement in waxy samples compared to normal wheat flour. Peak 4 indicates the presence of a low molecular weight compound in much greater amounts for the waxy samples than the normal samples.

Hoseney *et al*. (1969) reported the composition of flour water extractable fractions and identified four major compounds: globulins, albumins, arabinoxylans (pentosans), and glycoproteins. Protein solubility is dependent on the flour to water ratio; upon the addition of water, the ash in the flour creates a salt solution that solubilizes certain proteins of different concentrations depending on the amount of water added (Hoseney et al., 1969). The comparison between the four detectors used in this study indicates the presence of those low molecular weight proteins (albumins and globulins) reported by Hoseney *et al*. (1969). Water extractable arabinoxylans of high molecular weight have a greater effect increasing dough absorption and decreasing optimum mix time than do their lower molecular weight counterparts (Goesaert et al., 2005). The mixing properties of waxy flour and the molecular weight distribution of the waxy water extractable fraction suggests the possibility that waxy wheat flour may have a greater amount of WE-AX and that there is a shift towards a higher proportion of high molecular weight WE-AX out of the total.

Figure 3.7 shows the degree of hydrolysis achieved by the enzyme treatments of the water extractables (treatment with protease or hemicellulase). The results show a clear shift in the HPLC curve towards lower molecular weight compounds as would be expected with both treatments.

Table 3.12 Molecular weight analysis of Peak 2 (Figure 3.6) from water extractable HPLC analysis

	Mn (KDa)	Mw (KDa)	Mz (KDa)	Mp (KDa)	Mark-Houwink a
Nuplains$_N$	38.39± 0.36[a]	40.83± 1.05[a]	43.34± 1.77[a]	42.69± 0.15[a]	2.00± 0.10[a]
Wesley$_N$	36.48± 1.87[a]	39.23± 2.42[a]	42.12± 3.06[a]	44.29± 1.54[a]	1.56± 0.06[b]
Mattern$_W$	31.06± 9.98[a]	34.48± 11.48[a]	38.37± 12.91[a]	35.15± 14.67[a]	1.24± 0.07[c]
NX11MD2337$_W$	36.71± 1.17[a]	38.86± 1.63[a]	41.55± 2.25[a]	39.05± 1.33[a]	1.11± 0.02[c]

[a] Different letters in a column indicate significant differences at $p < 0.05$

Figure 3.5 Ultraviolet response of the water extractable fraction analysis by HPLC

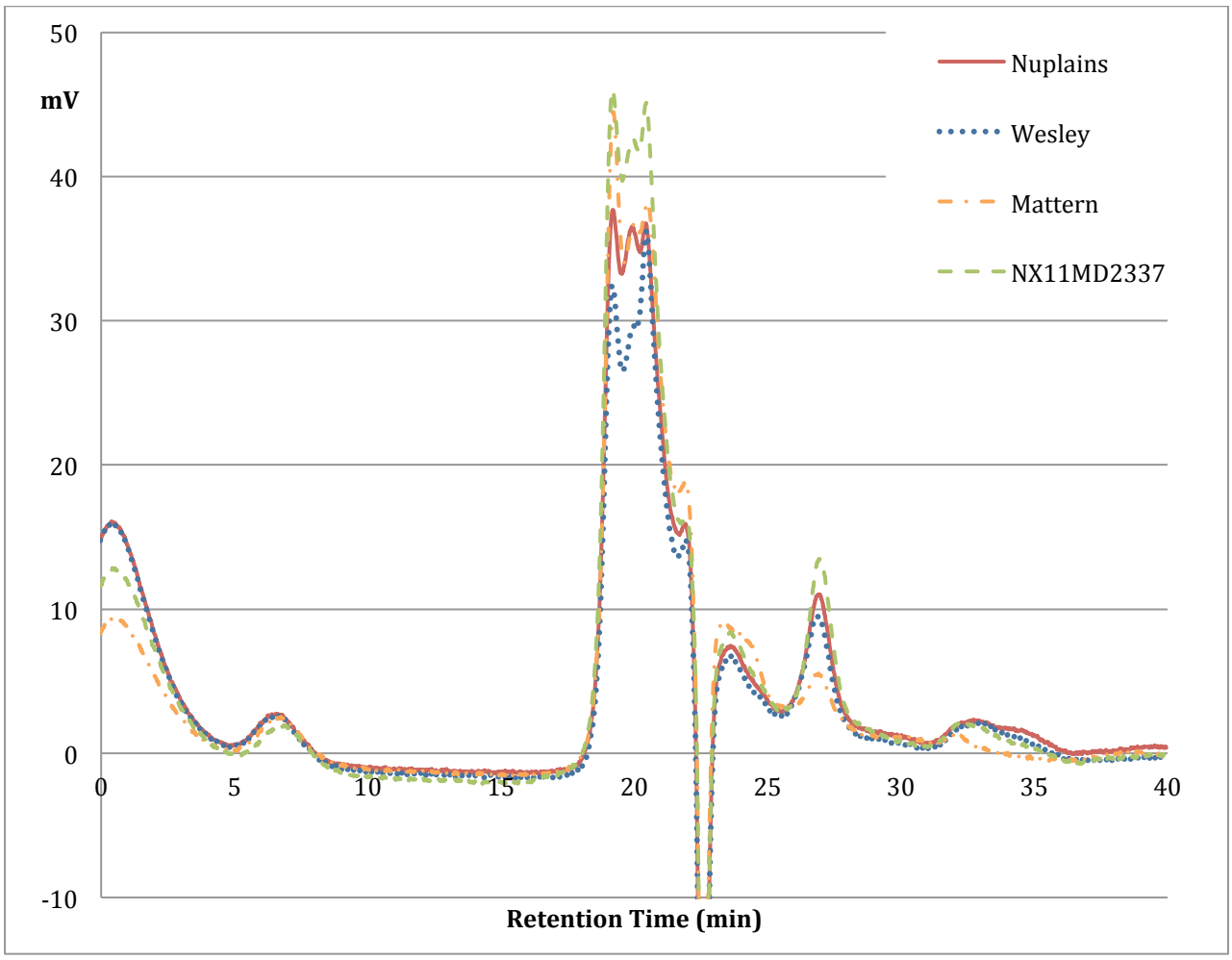


Figure 3.6 Refractive Index response of the water extractable fraction analysis by HPLC

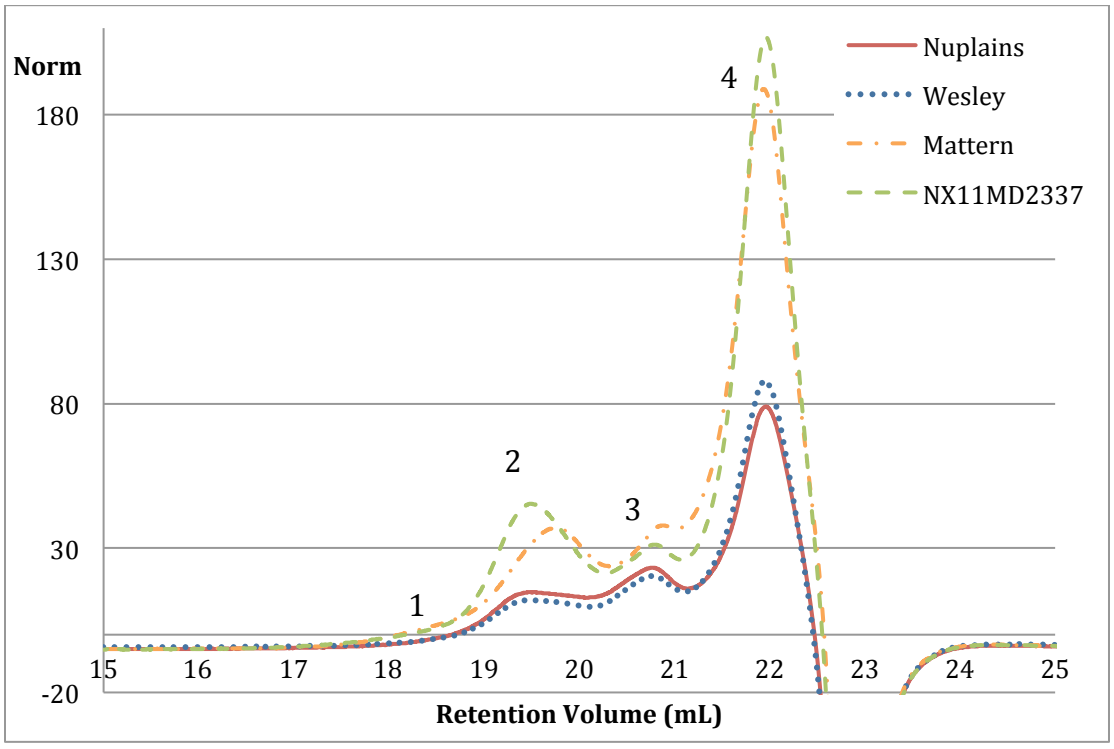
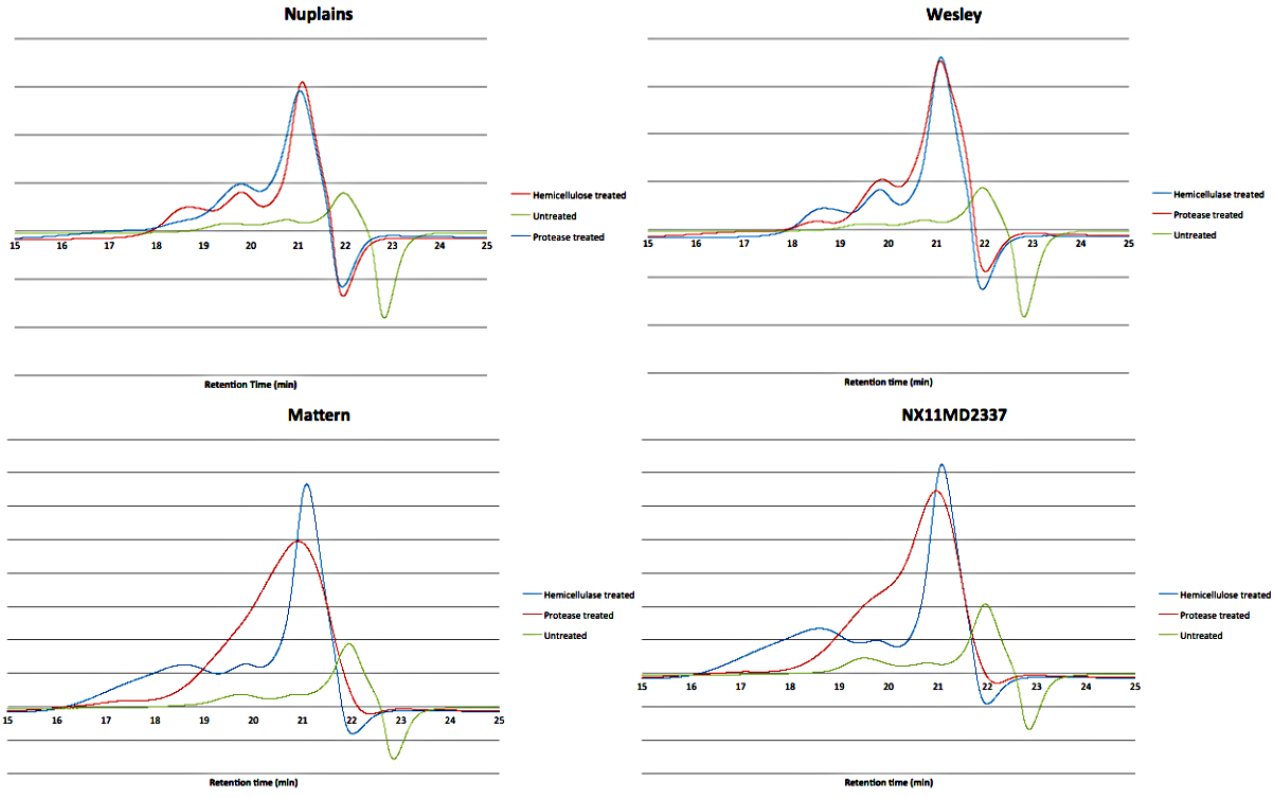


Figure 3.7 Refractive Index response of protease, hemicellulase and untreated water extractables



3.9.4 Determination of Arabinose/Xylose Ratio in Water Extractable Fraction

Table 3.13 shows a clear trend of lower arabinose/ xylose ratios for waxy and higher arabinose/ xylose ratios for normal wheat water extractables. The difference in arabinose/xylose ratio suggests a difference in the structures of WE-AX between normal and waxy samples. The lower ratio seen in the waxy samples indicates less branching. This data together with the Mark-Houwink a value further support this conclusion and hint at a possible factor affecting the differences in gluten aggregation between the waxy and normal wheat flours.

Table 3.13 Arabinose/Xylose ratio in the water extractable fraction of waxy and normal wheat flours

Sample	Arabinose/Xylose (w/w)
$Nuplains_N$	0.72±0.00
$Wesley_N$	0.65±0.02
$Mattern_W$	0.45±0.04
$NX11MD2337_W$	0.50±0.05

3.9.5 Protein Content in Water Un-extractable Pellets

Table 3.14 shows no clear pattern in between waxy and normal samples in the amount of protein left in the water washed flour pellets. These results support the results in Figure 3.4 (presence of water soluble proteins without a clear separation between waxy and normal samples). The lack of such a trend further suggests that the key component that creates a difference in characteristics of waxy and normal flours is not the protein but other flour components.

Table 3.14 Protein content of 25% solids pellet after flour washing

Sample	% Protein	Protein wt (g)
$Nuplains_N$	$13.372±0.041^a$	$1.258±0.004^a$
$Wesley_N$	$12.900±0.065^b$	$1.215±0.006^b$
$Mattern_W$	$13.524±0.179^a$	$1.244±0.016^{ab}$
$NX11MD2337_W$	$14.413±0.186^c$	$1.304±0.017^c$

[a] Different letters in a column indicate significant differences at $p < 0.05$

3.10 Dough Rheological Properties

Fundamental rheology studies of dough in the viscoelastic region allows for determination of the structure and properties of dough as well as the effects of different ingredients (Song and Zheng, 2007). Stress sweeps were done on all four samples at 65 and 70% absorption after mixing for 2.5, 4.0, and 5.5 minutes. Figure 3.8 presents the stress sweep curves for all samples at the different test conditions. The linear viscoelastic response region was approximately 10-25 Pa stress. This determination is necessary to analyze any dynamic rheological measurements as the underlying mathematical theory relies on the assumption that the dough is being tested in its linear behavior region (Oshikiri, 2013). Loss in the elastic modulus (G') began after approximately 25 Pa stress and a large drop began above approximately 100 Pa of stress. The linear viscoelastic region (below 25 Pa) is similar to previous results found for wheat flour dough (Oshikiri, 2013).

At 70% absorption and 5.5 min mix time clear differences in the elastic modulus (G') between waxy and normal wheat flour samples were observed. Under these conditions the waxy wheat and Nuplains dough samples are overmixed while Wesley is near to optimum absorption and development. The results are contrary to what would be expected, with Wesley (strong dough) showing lower G' than the weaker Nuplains and waxy flours. It is possible that the levels of arabinoxylans could increase G' values for the weaker doughs (Song and Zheng, 2007).

Frequency sweeps were done for all four samples at 65 and 70% absorption after mixing for 2.5, 4.0, and 5.5 minutes. Figure 3.9 presents the results. Mixing for 2.5 minutes resulted in small differences between waxy and normal flours at 70% absorption. Still at 70% absorption, extended mixing (4.0 minutes) showed a separation between the strong Wesley flour and weak waxy and Nuplains flours while mixing to 5.5 min eliminated that difference between the different types of flour. Mixing for 4.0 or 5.5 minutes at 65% absorption showed the same separation between weak and strong flours.

The proteins in strong flours are highly cross-linked and therefore should show lower frequency dependence of G' (Song and Zheng, 2007). Nuplains and the waxy samples have low quality gluten according to the mixograph and gluten index results and so their rheological behavior would be expected to show greater frequency dependence. However, the slope of all the samples is similar indicating comparable frequency dependency between the samples (Oshikiri, 2013).

G’ increased steadily with increasing frequency under all test conditions. The dough does not have time to recover as much as the test frequency increases. The shorter recovery time the dough has between measurements as the frequency increases results in an increase of G’ at higher frequencies (Oshikiri, 2013).

Tan δ is the ratio between G”/G’ and so gives an idea of the shifts between liquid-like and solid-like behavior dough undergoes in response to frequency with time. A higher tan δ value means the dough is acting more as a viscous material rather than an elastic material. Figure 3.10 shows the relationship between frequency and tan δ. The dough samples mixed for 2.5 and 5.5 minutes show a slight separation between waxy and normal samples at higher frequencies, those mixed for 4.0 minutes show a separation between weak (waxy and Nuplains) and strong (Wesley) flours. The three weak dough forming flour samples show higher tan δ values at the different conditions, meaning these particular flour samples form more viscous doughs. This translates to a weaker and difficult to handle dough. Gluten from high quality flour is characterized as being more elastic than viscous (Song and Zheng, 2007).

Weak gluten shows a shift from solid-like to liquid-like behavior with increasing frequency (Song and Zheng, 2007). The results obtained agree with this previous study, as the tan δ values for Nuplains and the two waxy flours increase more sharply than do the tan δ values for Wesley (shown to be stronger by mixograph and gluten index). Studies also show a correlation between higher dough strength and lower moduli values under small deformation testing (Oshikiri, 2013). The higher tan δ for the waxy and Nuplains frequency sweep data confirms the weaker gluten of these flours as seen in the mixograph results.

Figure 3.8 Stress sweep G' curves for the four samples tested at 65 and 70% absorption for 2.5, 4.0 and 5.5 minutes of mix time

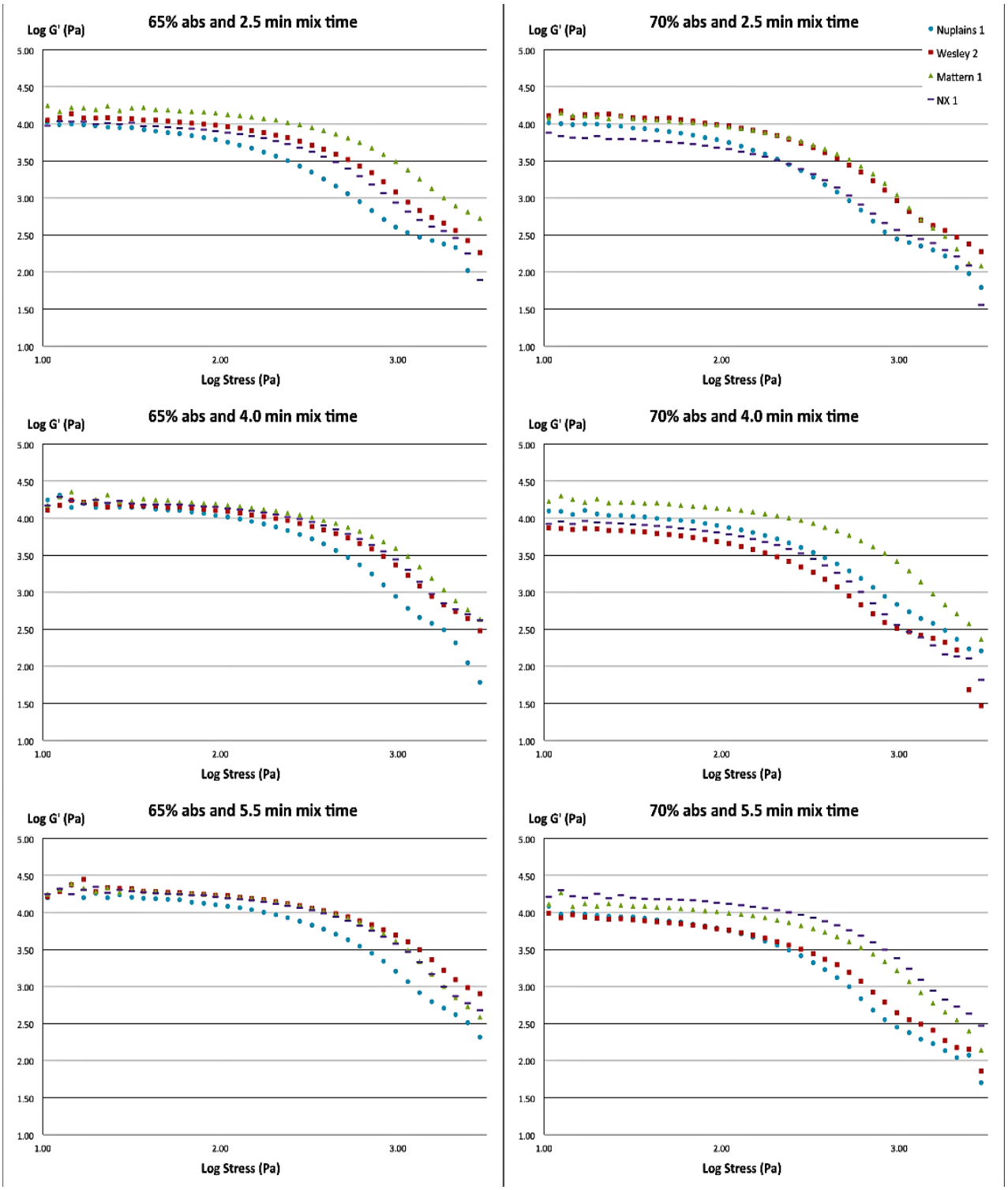


Figure 3.9 Frequency sweep G' curves for the four samples tested at 65 and 70% absorption for 2.5, 4.0 and 5.5 minutes of mix time

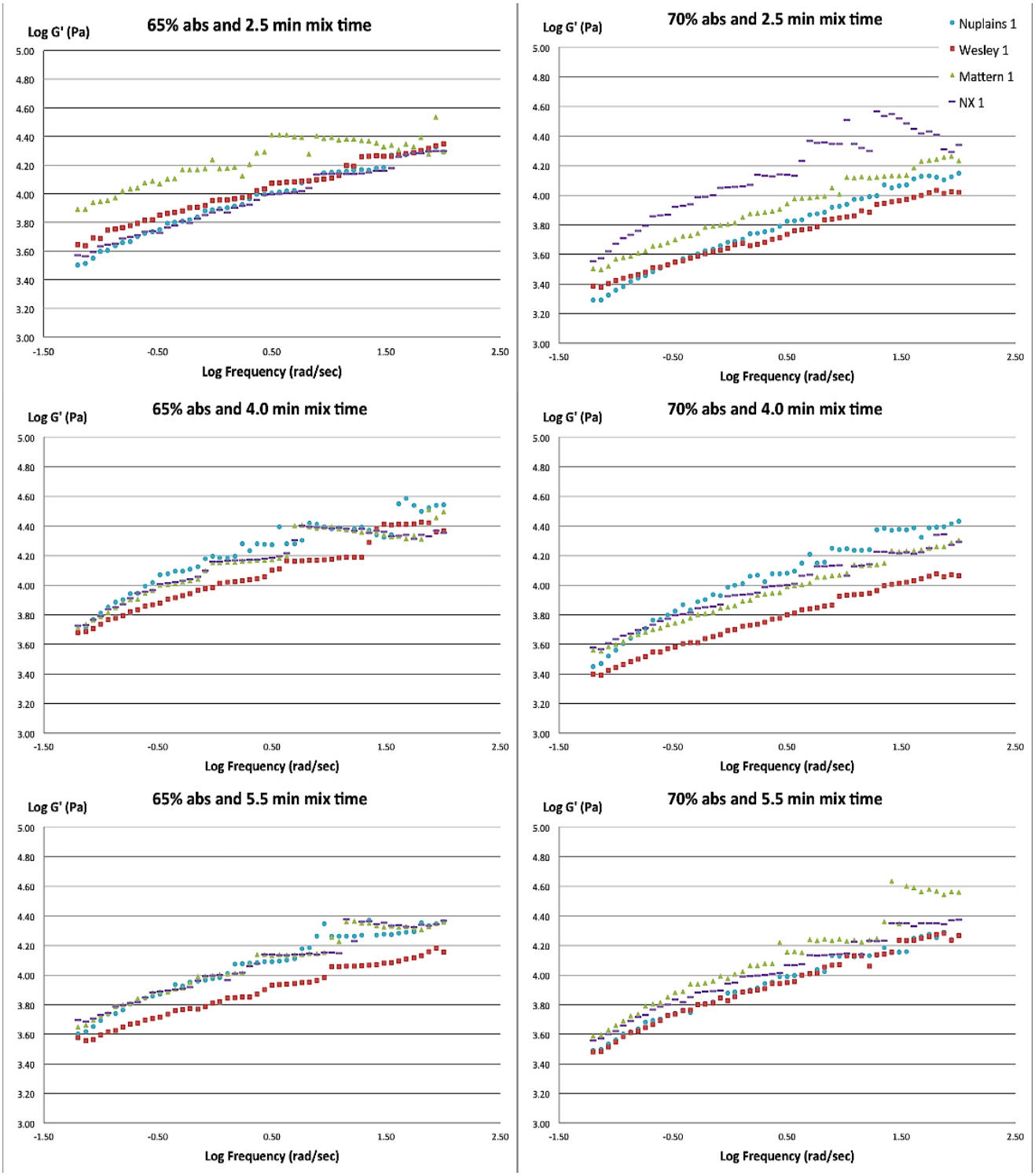
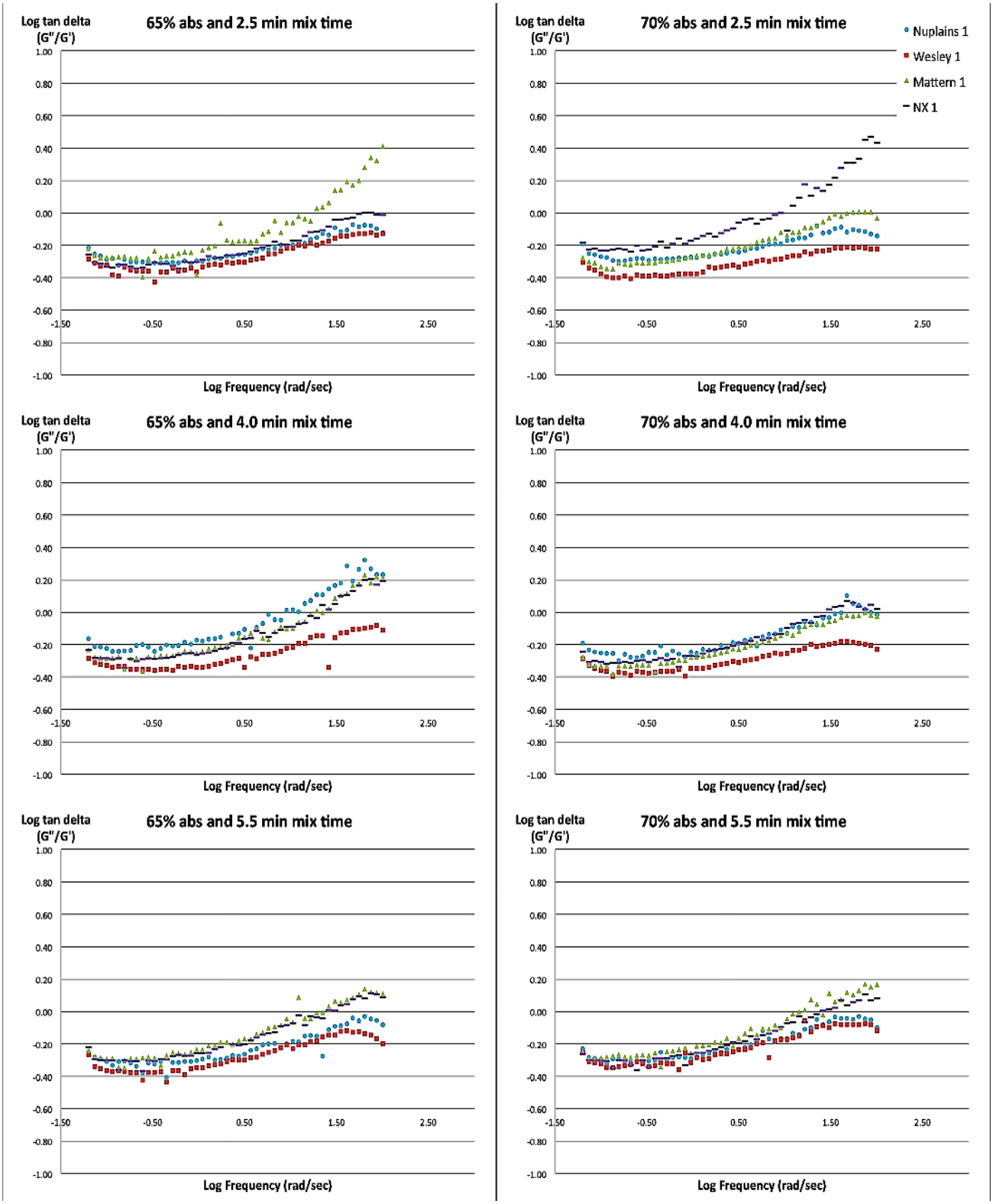


Figure 3.10 Frequency sweep tan δ curves for the four samples tested at 65 and 70% absorption for 2.5, 4.0 and 5.5 minutes of mix time



4. Conclusions

Mixograph testing shows weak dough forming properties and high water absorption for both waxy wheat samples and for Nuplains. Rheological study revealed similar linear viscoelastic regions for all test samples, frequency dependence for all samples and higher tan δ values consistent with weak gluten networks for the waxy and Nuplains samples. Waxy wheat had a slightly lower milling yield, yet acceptable extraction if the feed rate was reduced. Gluten index determination was the only macromolecular test to show a clear separation between the normal and waxy wheat flours.

Polymeric protein analysis showed high IPP and UPP polymeric protein values in waxy samples. This data is contrary to the conclusions previous studies have made regarding a relationship between dough strength and IPP/UPP values. Total polymeric protein analysis showed a significantly lower polymeric to monomeric protein ratio for waxy wheat flour as well as a significantly higher gliadin content in waxy wheat dough compared to normal wheat flour and dough respectively. This breakdown of total polymeric proteins could in part explain the poor dough forming properties seen for waxy wheat flour.

Gluten index of the washed flour (wet) pellets suggests that the factor responsible for the lack of gluten aggregation in waxy wheat flour is present in the water extractable portion of the flour, yet the irreproducibility of the test and the gluten index for the washed flour (dry) samples suggest that the gluten aggregation may be a function of the moisture content in the sample before testing or not related to the extraction of water solubles. The gluten index of the combinations (Mattern solubles/Wesley pellet and Mattern pellet/ Wesley solubles) would suggest that the poor gluten aggregation (hence low index) of waxy flours could be a result of greater susceptibility to the effects of certain water extractable compounds that are found in both normal and waxy samples (such as WE-AX).

HPLC analysis coupled with the higher viscosity of the water-extractable fraction of waxy wheat flour suggests the presence of a greater amount of water-extractable arabinoxylans (WE-AX) in waxy wheat flour. Furthermore, the Mark-Houwink a values and the arabinose/xylose ratio suggest that not only are these WE-AX present in higher amounts but may have a different chemical conformation and composition to those of the normal wheat flour.

Gluten index determination of washed flour with protease treated water extractables indicates that soluble proteins are not responsible for poor gluten aggregation.

The use of the Glutomatic is an effective way to evaluate the factors that affect gluten aggregation since specific factors can be isolated and the capacity to form a gluten ball or not is easily determined. Further analysis of the water-extractable and un-extractable fractions of waxy flour, particularly the arabinoxylan composition and chemical structure as well as associated molecules such as ferulic acid is necessary to fully understand the molecular interactions that impede gluten aggregation in waxy wheat flour. Detailed experimentation designed to test the susceptibility of the gluten in normal compared to waxy wheat flour to any level of WE-AX and UE-AX is also an area for further research.

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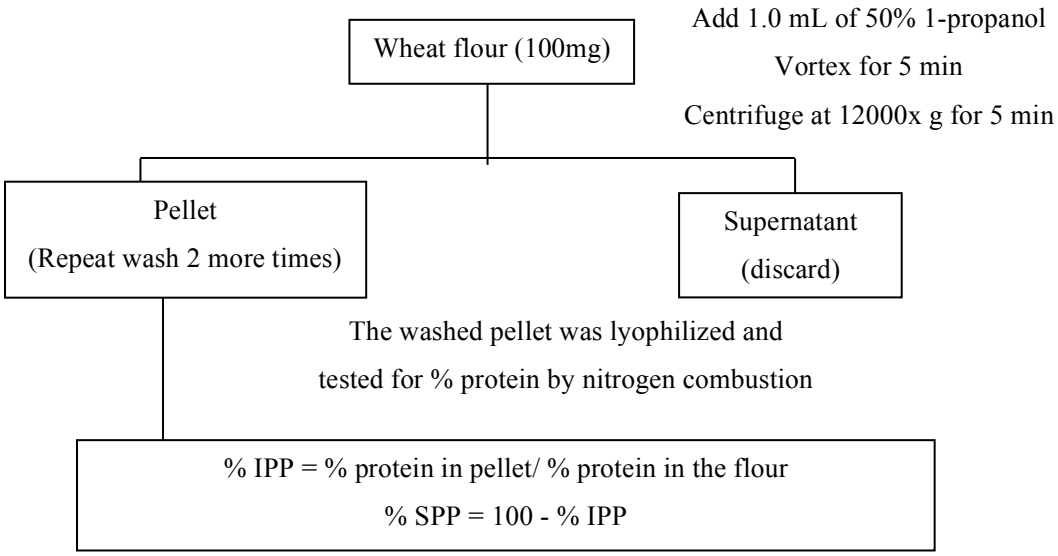
Appendix A - Detailed Procedures for Polymeric Protein Analysis

Three procedures were followed for the analysis of polymeric protein in the flours and corresponding doughs. The following flow charts describe each procedure in a detailed manner.

Detailed step by step procedure for the IPP/ SPP (Bean et al., 1998) determination:

1. Weigh 100.0mg of flour into a microfuge tube per assay
2. Add 1.0 mL of 50% 1- propanol, vortex 5 min, and centrifuge 12000x g for 5 min
3. Discard the supernatant and re-suspend the pellet in 1.0 mL of 50% 1-propanol
4. Repeat steps 2-4 two more times
5. Freeze dry the pellet and analyze by nitrogen combustion

Figure A.1 Insoluble and soluble polymeric protein (IPP/ SPP) method by Bean *et al.* (1998)



Detailed step by step procedure for the TPP, EPP and UPP determination:

A.1 Sample & reagent preparation

1. Prepare SDS SE-HPLC buffer: 7.1g Na2HPO4 + 5g SDS were dissolved in 1L water and the pH is adjusted to 6.9 with HCl
2. HPLC starting up.
3. Weight approximately 10 mg flour into a microfuge tube per assay
4. Set water bath temperature to 85 °C and insure there is ice available

A.2 Total protein analysis (Gupta et al., 1993)

1. Add appropriate volume of SDS buffer to obtain a 10mg/mL solution

2. Vortex on setting 5 for 5 min, sonicate for 15 s with the power output at 6 W, centrifuge at 12000 rpm for 10 min.
   Note: the sonicator probe tip should be in the tube center and 1/3 of the distance up from the bottom. Clean the probe tip in between samples.
3. Transfer half of supernatant to a filter microtube, centrifuge at 14000 rpm for 5 min. Then add the rest of the supernatant and centrifuge at 14000 rpm for 5 min again
4. Transfer the filtered supernatant into a HPLC vial

A.3 Extractable protein analysis (Gupta et al., 1993)

1. Add appropriate volume of SDS buffer to obtain a 10mg/mL solution
2. Vortex on setting 5 for 5 min.
3. Transfer half of supernatant to a filter microtube, centrifuge at 14000 rpm for 5 min. Then add the rest of the supernatant and centrifuge at 14000 rpm for 5 min again
4. Transfer the filtered supernatant into a HPLC vial
5. Save precipitate for UPP analysis

A.4 Unextractable protein analysis (Gupta et al., 1993)

1. Add the same volume of SDS buffer to the precipitate, resuspend the pellet and vortex hard for 10 min. sonicate for 25 s with 6 W output. Centrifuge at 12000 rpm for 10 min
2. Transfer half of supernatant to a filter microtube, centrifuge at 14000 rpm for 5 min. Then add the rest of the supernatant and centrifuge at 14000 rpm for 5 min again
3. Transfer the filtered supernatant into a HPLC vial

Figure A.2 Total polymeric protein (TPP) method following method by Gupta *et al.* (1993)

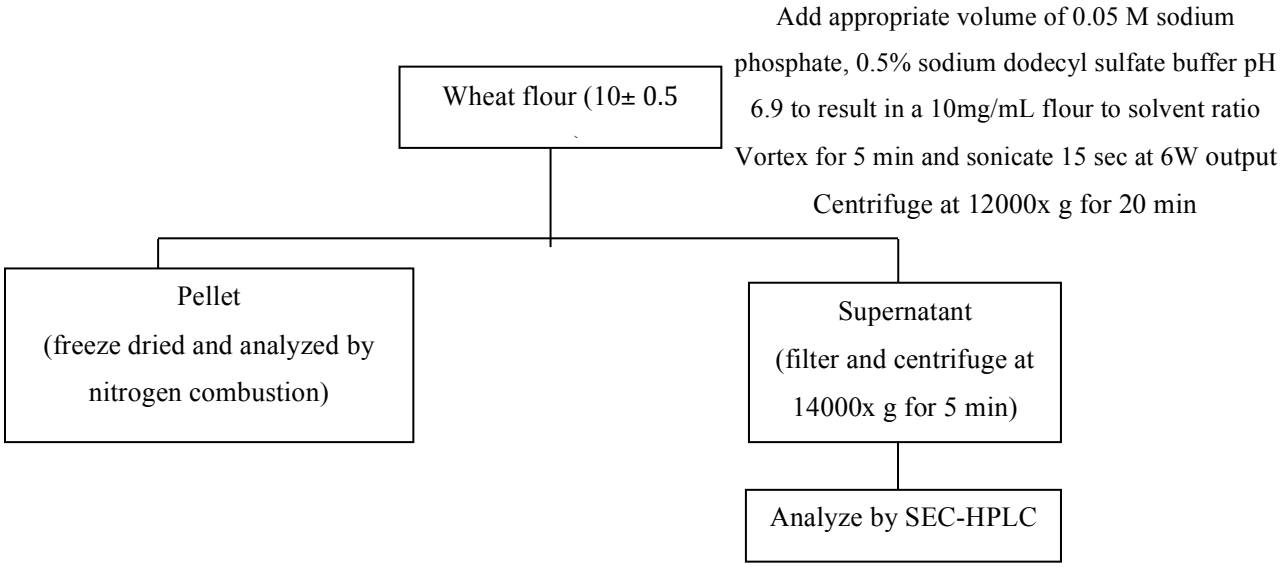
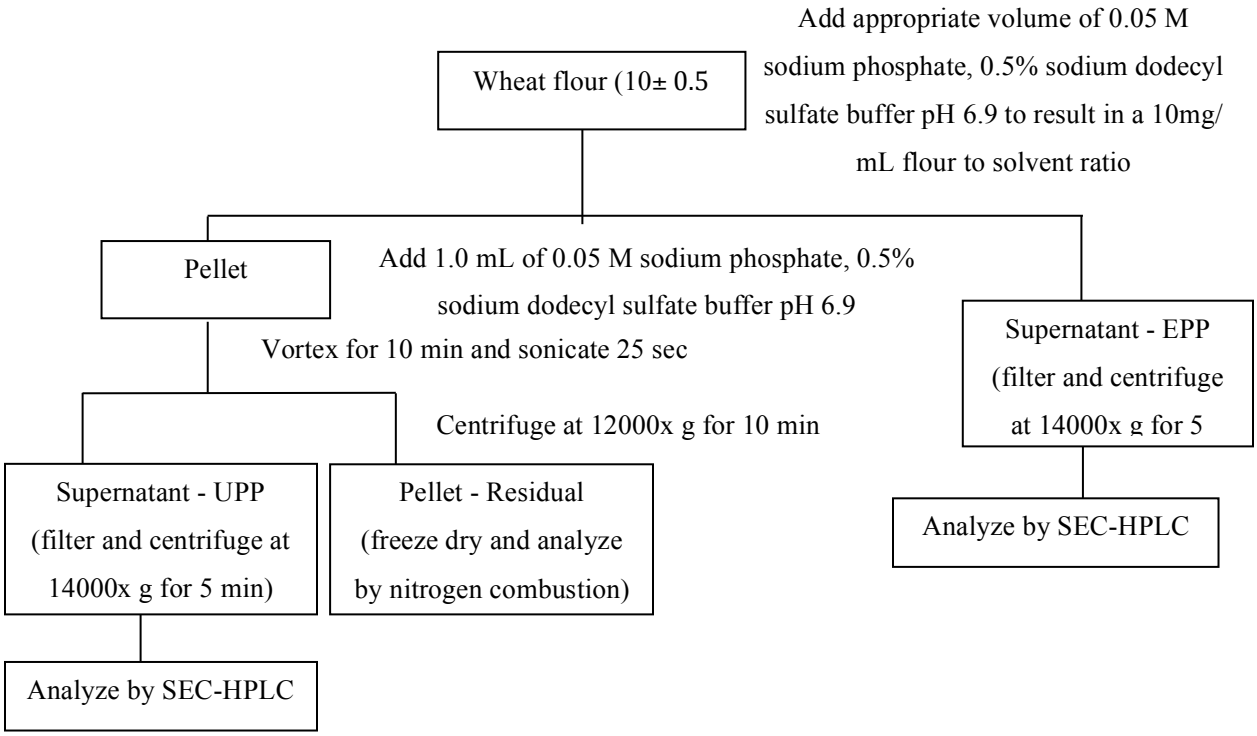


Figure A.3 Extractable and unextractable polymeric protein (EPP/UPP) method following method by Gupta *et al.* (1993)



Appendix B - Nitric-Perchloric Acid Digestion

Method by Gieseking *et al.* (1935):

1. Using an analytical balance, weigh about 0.25 grams of plant tissue or grain (use the Balance Talk program to collect the exact weight for each sample – to the fourth decimal)
2. Place sample in 50 ml Kimax digestion tube (using a metal funnel)
3. Under a fume hood, add 5 ml of Nitric Acid to the tube using a bottle top dispenser.
4. Cover with saran wrap and let stand overnight in the hood
5. Under a Perchloric Acid fume hood, add 5 ml of Perchloric Acid to the tube using a bottle top dispenser
6. Place rack of tubes in digestion block and turn temperature on to 200C
7. Digest for 1 hour. The nitric fumes (orange in color) should be burned off at this time and the rack should be rotated in the block to ensure even heating.
8. Continue digesting for 2 more hours, rotating the rack every 30 minutes or so.
9. After a total time of 3 hours, remove the rack from the block and allow to cool for 20 minutes.
10. Dilute to the 50 ml mark on the digestion tube and mix the sample by inverting the tube several times. Cover the rack with saran wrap until ready to analyze.
11. Samples can be analyzed on the ICP (Inductively Coupled Plasma Spectrometer, Model 720-ES ICP Optical Emission Spectrometer, manufactured by Varian Australia Pty Ltd, Mulgrave, Vic Australia).

Appendix C - Mixograph Data at Varying Absorption Levels

Figure C.1 Mixograph results of increasing water absorption for Nuplains normal wheat flour

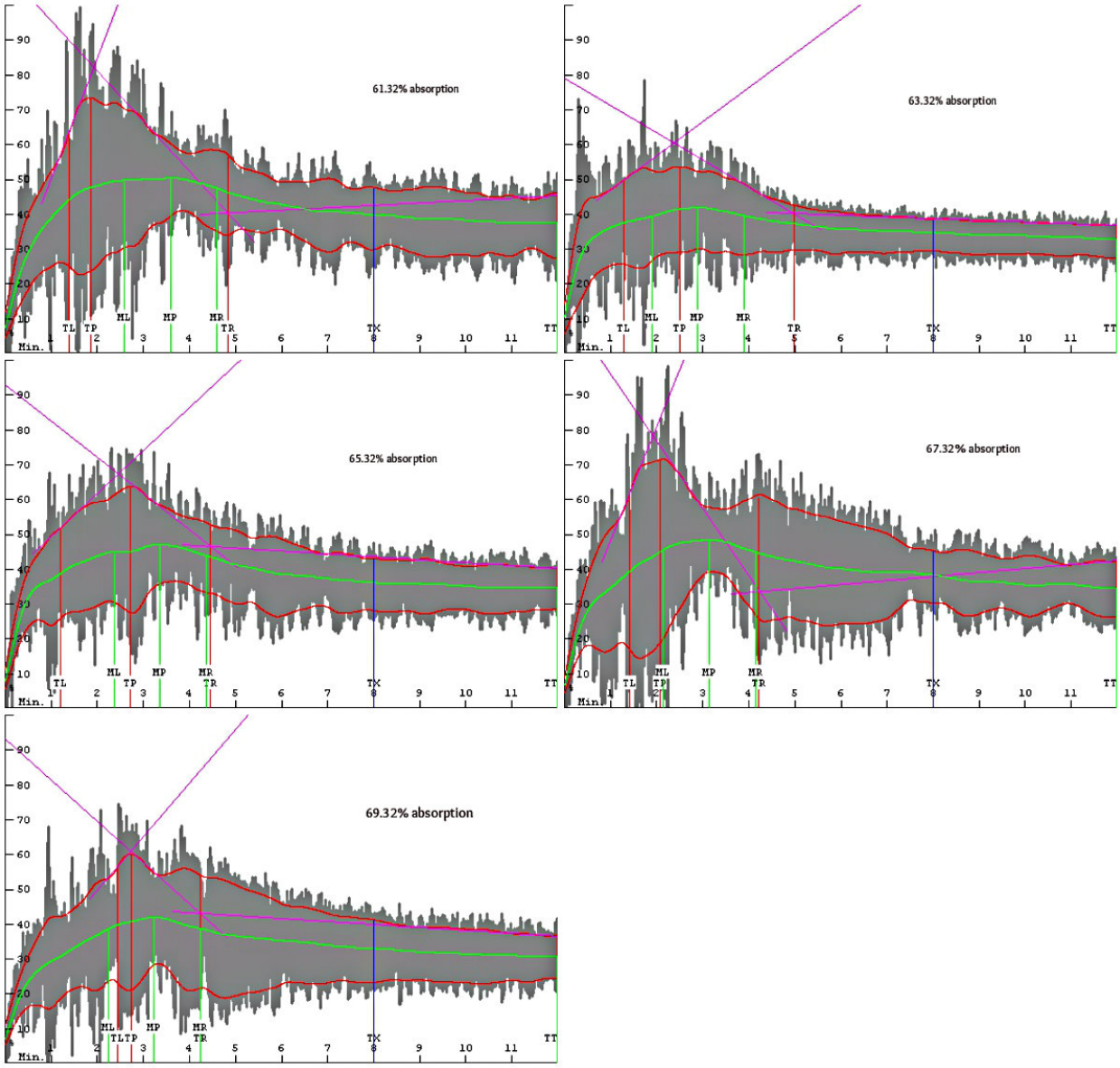


Figure C.2 Mixograph results of increasing water absorption for Wesley normal wheat flour

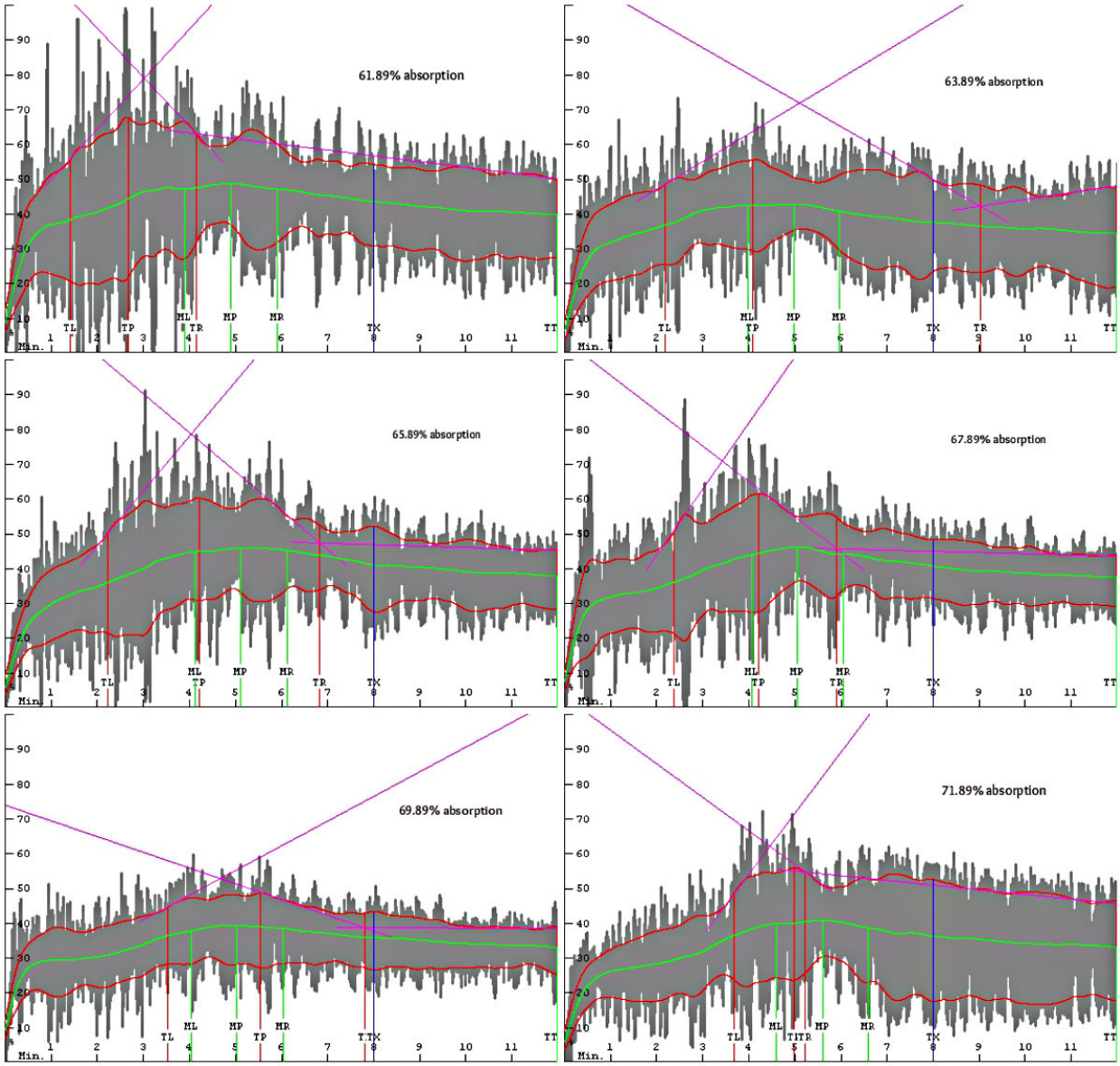


Figure C.3 Mixograph results of increasing water absorption for Mattern (waxy) wheat flour sample

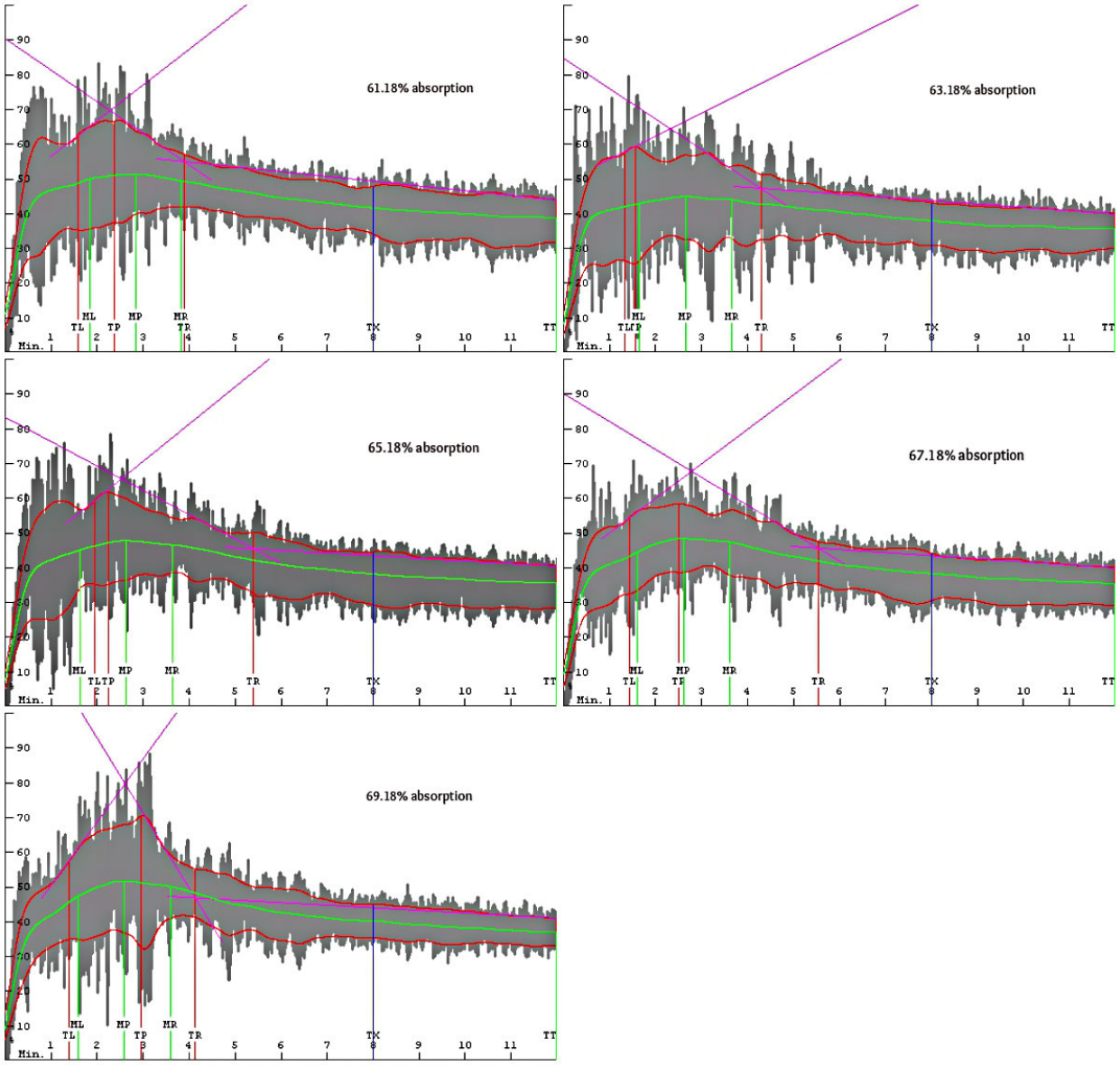


Figure C.4 Mixograph results of increasing water absorption for NX11MD2337 (waxy) wheat flour