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INVESTIGATION OF STRESS RELAXATION IN HYDRATED GLUTEN NETWORKS USING SPECTRAL ANALYSIS

OLUWATODIMU SAMUEL DAHUNSI

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Abstract

Gluten and its protein fractions were isolated from wheat flour. Stress relaxation in hydrated gluten, its protein fractions and varying percentage mixtures of the components were investigated by means of rheometry and confocal laser scanning microscopy. Stress relaxation was carried out over a period of 30 min at 20 °C for all samples under investigation. Samples exhibited relaxation modulus $G(t)$ ranging from 1 to about 1000 Pa with the gliadin fraction showing almost full decay. MATLAB with the combination of regularization algorithms were used to generate L-curves and calculate the relaxation spectra of the samples. Stress relaxation spectra identified five dominant relaxation modes with baseline resolution. Stress relaxation is somewhat independent of compositional differences, although, confocal microscopy showed the influence of protein composition on the morphology of the networks. Therefore, morphology and relaxation dynamics seem to be controlled by independent mechanisms for gluten networks. These findings provide a fundamental understanding of gluten dynamics and may allow controlling the industrial performance of flours and engineering novel dough formulations.

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Chapter 1

1.   Introduction

Wheat is one of the three most important crops in the world together with maize and rice (Fellers, 1979, Roussel et al., 2005, Shewry et al., 2009) and it is immensely significant in the global food supply (Atchison et al., 2010). It belongs to the grass family (*Gramineae*). Also belonging to that family is rice, barley, oats, rye, maize, sorghum, and millets (Belderok, 2000). Wheat grain contains mainly of starch, water and protein (see Table 1). When viewed from the economic point of view, it is regarded as a very important economic commodity across the world (Lv et al., 2013a). Wheat grain is also regarded as the oldest and most important of the cereal grains in world food supply chain and over the years, the role of wheat in the world food economy has increased substantially not only in the developed world but more importantly in the developing countries of the world (Byerlee and de Polanco, 1983). Approximately, over 600 million tonnes are harvested yearly with cultivation covering over a vast geographical area, from Scandinavia to Argentina and the higher altitudes in the tropics (Shewry, 2009, Shewry et al., 2002a). Of all the food crops known, it has had the largest area harvested for many years (Lv et al., 2013a).

In Europe, where wheat is the main staple, bread wheat (*Triticum aestivum*) covers the majority of land on which wheat is cultivated (Belderok et al., 2000). It is known to be the dominant crop in temperate countries used for human food and livestock feed (Shewry, 2009). In Canada, wheat is the largest crop with most of the production in the western Canadian prairie provinces of Manitoba, Saskatchewan and Alberta covering about 10 million hectares of land (Xue et al., 2012). Wheat is known to be the second principal crop in China and perceived to be an important constituent for national food security (Lv et al., 2013b) where food security is a major policy concern due to its large populace that needs to

be fed (Lu and Fan, 2013). The North China Plain (NCP) is regarded the most significant wheat production area in China and it produces about two-thirds of China's overall wheat output (Lu and Fan, 2013).

In the north of China, wheat grain provides 40% of the total protein needed in people's daily life (Jiang et al., 2008). In Australia, wheat is regarded as the main cash crop and wheat export from Australia is ranked fourth in the world (Luo et al., 2003). Maize is currently the main crop among cereal crops grown in sub-Saharan Africa while wheat is the most important crop in North Africa. The African region has conventionally played a small part in wheat production over the years and has also witnessed a rapid decline in its production since the 1980s (DeCapua, 2012). Extreme temperatures (heat) may have been the reason for low cultivation of wheat in Sub-Saharan Africa. While it is true that wheat is still outshined by maize in most African countries and predominantly among the poor in southern Africa, there is high demand for wheat in urban centres where people are developing an appetite for mass-produced, convenient foods containing processed wheat flour. Mason's study shows that consumers, on average, spend more on wheat than on other cereals in the cities of Lusaka and Kitwe in Zambia, Maputo in Mozambique and Nairobi in Kenya (IRIN, 2013). It is therefore evident that regardless of what part of the world, wheat is a major food constituent and a main contributor to the economy by means of exportation to other parts of the world where wheat is not cultivated in mass to meet the increasing needs of the people.

The ability of wheat to give high yield under a wide range of conditions has contributed to its success (Shewry, 2009). While wheat growers are mainly concerned about yield, wheat millers and bakers are concerned about the variability in the functional properties of flour (Dupont and Altenbach, 2003). The major importance of wheat is principally due to the fact that its seeds can be milled into flour, semolina, etc., which form the basic components of bread and other bakery foodstuffs, as well as pastas such as macaroni, spaghetti and noodles

(Belderok, 2000). With increase in world population and rise in demand for wheat and wheat based products, a scheme to increase the overall world production and wheat yield is therefore crucial.

Wheat flour is usually produced by the removal of the germ and the outer layer (often regarded as bran) from the endosperm of wheat during milling (Preston and Williams, 2003). Various types of wheat are milled and processed into a wide range of flours that are used for specific purposes and all wheat flours are capable of making some type of leavened bakery product (Pomeranz, 1968). For example, soft wheat varieties (because of its low protein content) are used for food products, such as cakes and cookies, whereas hard wheat flour (bread making quality) is suitable for bread (Lv et al., 2013a). However, cereals are not only needed for making bread and other baked foodstuffs but also very useful in the brewing of beer (Belderok, 2000).

Table 1: Wheat flour composition (Adapted from (Goesaert et al., 2005))

Component	Quantity (%)
Starch	70-75
Water	14
Proteins (mainly gluten)	10-12
Non-starch polysaccharides	2-3
Lipids	2

The nutritional value of wheat is extremely important as it takes an important place among the few crop species being extensively grown as staple food source in so many parts of the world (Sramkova et al., 2009) with the main component being starch (Goesaert et al., 2005). The unusual properties of the wheat are ascribed to the presence of gluten storage

proteins of the endosperm which are further composed of two fractions – the alcohol soluble gliadins and the alcohol insoluble glutenins (Khatkar et al., 2013, Dobraszczyk, 2004).

Wheat varies broadly in chemical composition as percentages of protein, minerals, vitamins, pigments, and enzymes can show up to a five-fold range among categories of wheat with such differences having extensive effects on processing and best use (Pomeranz, 1968). Wheat contributes essential amino acids, minerals, and vitamins, and beneficial phytochemicals and dietary fibre components to the human diet, and these are particularly enriched in the whole-grain products such as whole meal bread and pasta (Shewry, 2009). However, wheat products are also suggested to be responsible for a number of adverse reactions in humans, including intolerances (notably coeliac disease) and allergies (respiratory and food) (Shewry, 2009). As mentioned earlier, whole wheat is an important source of dietary fibre, the consumption of which is reputed to have lowered the risk of colon cancer, diabetes mellitus and cardiovascular disease. With regards to micronutrients, the average daily consumption of 200 g of bread covers about 12-31% of the daily requirements of important vitamins such as riboflavin or thiamine and is particularly high in fructo-oligosaccharides, while wheat germ is high in raffinose oligosaccharides (Koehler et al., 2007, Slavin, 2010).

The most important factor has been the unique mechanical properties of wheat dough that allows it to be processed into a range of foodstuffs. These viscoelastic properties which are largely determined by the structure and interaction of the storage proteins determine the end use quality (Shewry et al., 2002b, Dutta et al., 2011). The grain proteins determine the viscoelastic properties of dough, in particular, the storage proteins that form a network in the dough which is often known as gluten (Schofield, 1994). Functionality of wheat flour for different applications is governed by the starch and proteins present in it (Dutta et al., 2011). It is important to note that wheat quality is greatly influenced by genotype, growing

environment and the interaction between these factors. Also worthy of note is that the quality and quantity of wheat gluten is of great importance as this influences to a large extent the end use and the quality of the end product (Wieser and Kieffer, 2001, Shewry et al., 2002a, Lv et al., 2013a, Singh et al., 2010). However, due to interactions between the factors, precise effects on protein content and hardness may differ (Swanston et al., 2012). It has also been observed that extreme temperature and drought during grain filling have been recognised as a major basis of disparity in wheat flour quality characteristics (Singh et al., 2010). Investigation into the influence of extreme temperatures on durum wheat shows that flour protein is significantly increased in both cold and heat treatment and that the current trend experienced in relation to climate change suggests that global warming may be beneficial for wheat crop in some regions with the exemption of regions where optimal temperature already exist (Labuschagne et al., 2009, Ortiz et al., 2008). Presence of water could also play a major role in flour protein composition and technological quality. Exploring the influence of water deficit on durum wheat storage protein composition and technological quality, it was found that when a terminal water stress occurred in grain filling, an improvement in gluten strength was observed consistently with an increase in the amount of glutenin macropolymers. The study further shows that a combination of water deficit and high temperature stress that usually occur under Mediterranean conditions during grain filling may have encouraged the aggregation of the glutenin subunits which was reflected in an enhancement in technological value (Flagella et al., 2010).

The grain genotype, growing environment and the interaction of both on the antioxidant properties and chemical composition of wheat flour from soft wheat was studied. It was discovered that total carotenoids were primarily affected by growing environment. Also evident from the study is that the interaction of both the growing environment and genotype had a larger effect on the level of total tocopherols. The results show that the environment,

genotype and their interaction could influence the levels of lipophilic antioxidants and antioxidant activities of wheat flour. In addition, genotype had less influence on both chemical composition and antioxidant activities compared to environment and the interaction between genotype and the environment. It was concluded that it may be possible for wheat breeders to select optimal environment and genotype to improve the levels of selected health components and antioxidant activities of soft wheat flour. From these studies and submissions, it can be concluded that environmental conditions (e.g. water and temperature) and genotype do play a vital role in nutritional and technological quality of wheat grain and consequently influence to a great deal the end use (Lv et al., 2013a).

On the molecular and biochemical impacts of environmental factors on wheat grain development and protein synthesis, it is found that despite the several years of research, there still exists a crucial gap in the way the factors controlling yield and quality are understood and further proposes that new information regarding the molecular mechanism underlying the quality and response of the developing grain to environmental stress is needed (Dupont and Altenbach, 2003). Studying the role of water availability at post-anthesis on grain nutrition and quality, it was found that proper water conditions at the later growth and development stage of wheat grain can be favourable to more mineral concentrations, better nutrition, higher quality and yield in wheat grain (Zhao et al., 2009). It is therefore critical to comprehend the mechanisms that control the partitioning of carbon and nitrogen into the major grain storage compounds as well as into cell walls and other grain components if the intention is to go beyond these current genetic limits and extend the range of variation in wheat grain composition for specific end uses (Shewry et al., 2009). While it may be true that it is difficult to define 'gluten quality' because of the structural differences in wheat genotypes, crude protein value differs from one wheat sample to another (table 2) (Wieser and Kieffer, 2001).

The nutritional quality of a protein can be measured by a variety of criteria, but in essence, it is the relative amounts and the balance of essential amino acids in the dietary protein that determines its nutritional value (Sramkova et al., 2009). In trying to determine the appropriateness of using gluten index (GI) to measure wheat quality, it was found that while it is true that the GI might give some indications of the flour gluten quality, this cannot be used as the sole evaluation of wheat quality as results obtained are questionable (Bonfil and Posner, 2012). The problem of relating the chemical composition and structure of wheat flour components to the functional properties is complicated by the presence of large number of components, the high molecular weight and limited solubility of proteins, interaction of the various components, and the exertion involved in isolating pure components without changing them (Pomeranz, 1968). The desire to therefore isolate pure protein from wheat flour for accurate characterisation and study of its functional properties has been on the increase over the years. During the mixing of dough, gluten proteins are stretched which also leads to broken bonds and subsequently, new bonds are formed during dough resting. The result of all these chains of process is that a gluten matrix (network) that is much stronger than the gluten protein agglomerates is formed under more dispersed conditions in flour suspensions and with lower energy input. The strong agglomeration during dough making is referred to as 'gluten development'(Van Der Borght et al., 2005). An important step therefore in the isolation of gluten from wheat flour is adequate hydration time (usually 30 min or more) to allow suitable gluten development to occur. The amount of water used in the hydration is also considered to be very important because a good balance between the water/solid (flour) ratios is important for proper hydration and gluten development. It is well-known that if the isolated gluten is still able to form a network with viscoelastic properties, it is well-defined as 'vital gluten' which usually means concentrated protein with its functionality still intact (Van Der Borght et al., 2005). The ability of vital gluten to be

highly elastic when mixed with water sets it apart from other available plant proteins. It can rapidly absorb about twice its weight of water which gives rise to increased yield, strength and extended shelf life hence the name 'vital gluten'. Vital gluten is therefore the dry matter of the wet gluten. However, higher wet gluten content does not directly translate to higher vital gluten quality due to differences in protein fractions composition (Hu and Shang, 2007).

1.1 Wheat Grain Morphology

In an attempt to give a graphical picture of wheat, it can be described as an oval shaped grain (Fig.1) with slight variations observed in other wheat grains ranging from almost spherical to long, narrow and flattened shapes (Sramkova et al., 2009). The grain is usually between 5 and 9 mm in length and weighs between 35 – 50 mg. It has a crease down one side which is the point where it was originally connected to the wheat flower. The wheat grain contains 2-3% germ, 13-17% bran and 80-85% mealy endosperm in dry matter basis (Sramkova et al., 2009). In broad terms, starch accounts for about 70–80% of the mature grain and protein for 8–14% (Shewry et al., 2009). As the grain develops, wheat storage proteins are deposited as protein bodies which as the grain matures, lose their distinct structure and form a continuous matrix within the endosperm cells, in which starch granules are entrenched (Van Der Borght et al., 2005).

The bran which is the outer layer of the wheat grain (Fig.1) is rich in B vitamins and minerals and more than half of the bran consists of fibre components (53%) (Sramkova et al., 2009). The grain also contains the residue of many metabolic proteins that have been needed by the developing grain, together with the proteins providing those reputed mechanisms that must carry life on into the next generation of the wheat plant when the germination process begins (Shewry, 1999).

The protein content of flour is not only an indicator of direct nutritional value, but it also has an important impact on dough rheological properties (Payne et al., 1987). As seen in Table 2, a wide range of variability is observed in the protein content depending on the wheat flour source and this plays a major role in deciding the end use of the flour. Although, variation is also observed in the starch content of wheat flour from different origins but it is important to note that more variation is often observed in the protein content. This wide variation in protein content may be due to the availability of nitrogen, usually through application as fertilizer which has a greater effect on grain protein content than genotype (Shewry et al., 2013). The cell wall polymers also exhibit large variation in content and structure according to cultivars, therefore a greater ability to control cell wall variation for grain quality enhancement requires an improved understanding of the mechanisms controlling cell wall polymer biosynthesis (Saulnier et al., 2012).

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Fig. 1: Wheat grain (http://www.britannica.com)
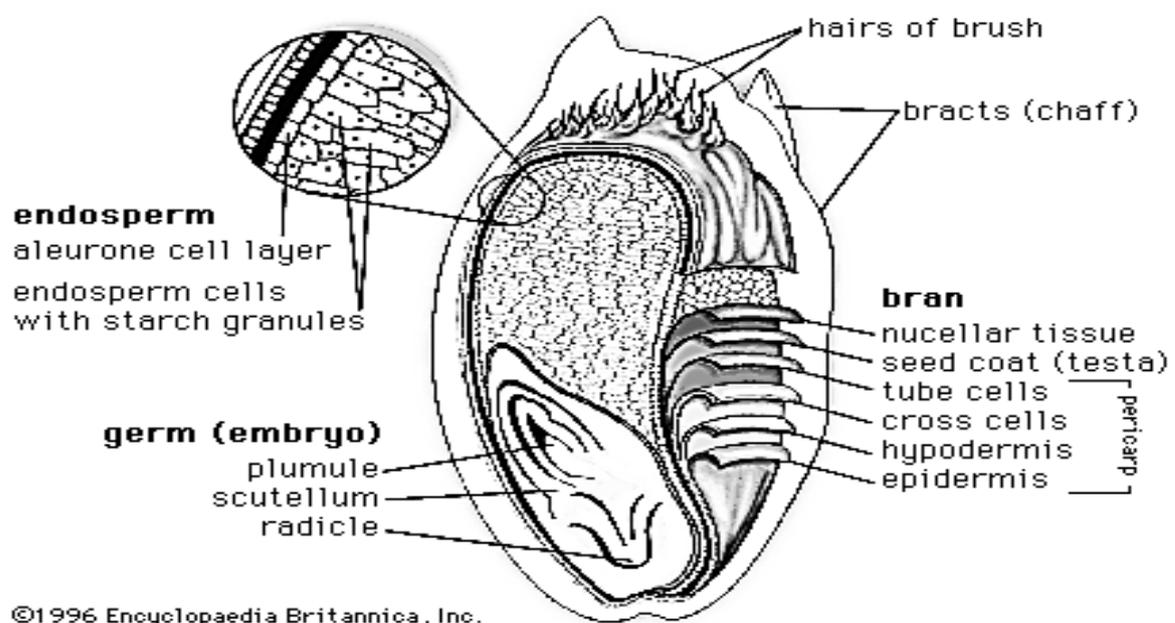
Therefore in general terms, the mature wheat grain comprises three groups of major components which include starch, proteins, and cell wall polysaccharides, which together account for about 90% of the dry weight, and minor components that include lipids, terpenoids, phenolics minerals, and vitamins. However, these components differ in their distribution within the grain (Shewry et al., 2013).

Table 2: Samples and crude protein contents (%) of various flours (Wieser and Kieffer, 2001).

Wheat Sample	Crude Protein (%)*
Bussard	10.9
Canadian Western Red Spring	11.8
Dutch commercial flour	10.5
Dark Northern Spring	12.0
Fresco	10.1
Gambrinus	9.4
Glenlea	11.4
Hereward	10.4
Kanzler	11.0
Kraka	8.8
Minaret	9.4
monopol	10.5
Obelisk	8.7
Soissons	9.3

*N x 5.7 (Nitrogen content determined multiplied by the conversion factor)

Wheat gluten is a highly complex mixture of proteins with at least 50 individual components that can be separated by SDS-PAGE (Shewry et al., 2002a). Wheat gluten proteins are of immense importance in the food industry as their properties underpin the processing of wheat flour to produce flour based products such as bread, pasta, noodles and a range of other foods (Shewry and Lookhart, 2003). This has led to a volume of work ranging from gluten isolation to studies on its properties and an attempt to characterise the protein. Consequently, the gluten proteins have been widely studied over a period more than 250 years (Anjum et al., 2007) in order to determine their structure and properties and to provide a basis for manipulating and improving end use quality. Evident from these studies is that gluten is a complex mixture of proteins which vary in their proportions, structures and properties.

The wheat gluten proteins correspond to the major storage proteins that are deposited in the starchy endosperm cells of the developing grain. These form a continuous proteinaceous matrix in the cells of the mature dry grain and are brought together to form a continuous viscoelastic network when flour is mixed with water to form dough (Shewry et al., 2002c).

In the past five decades, there has been a rise of gluten as a commodity in its own right, through the extensive industrial separation of wheat starch from gluten, and the specific drying of the gluten in order to retain its functional properties (Day et al., 2006).

The starch and gluten components of wheat flour can be separated by a number of processes (Van Der Borght et al., 2005). One of the methods is that gluten can be readily prepared by gently washing dough under a stream of running water. This process of washing helps to remove the bulk of the soluble and particulate matter to leave a proteinaceous mass that retains its strength and cohesiveness upon stretching (Shewry et al., 2002a, Belitz et al., 1986, Anjum et al., 2007). This method has therefore been used over the years in obtaining

gluten from wheat flour. The crucial step in this method is ensuring that the washing is sufficiently done such that almost all the starch content is removed in the process. One easy way to check that this has been achieved is to wash until a clear solution is observed. A careful approach is also engaged to ensure that the gluten is not washed off in the process as over washing can also weaken the gluten strength by reducing the disulphide bonds. Once the disulphide bonds are weakened, this is followed by the decrease in the amount of protein fractions (Belitz et al., 1986). An overview of the general process of obtaining gluten from wheat flour is described below in Figure 2.

Wheat flour + Water

↓

Dough

↓

Washing under water (until clear solution is observed)

↓

Wet Gluten

↓

Lyophilisation

↓

Milling

↓

Gluten Powder
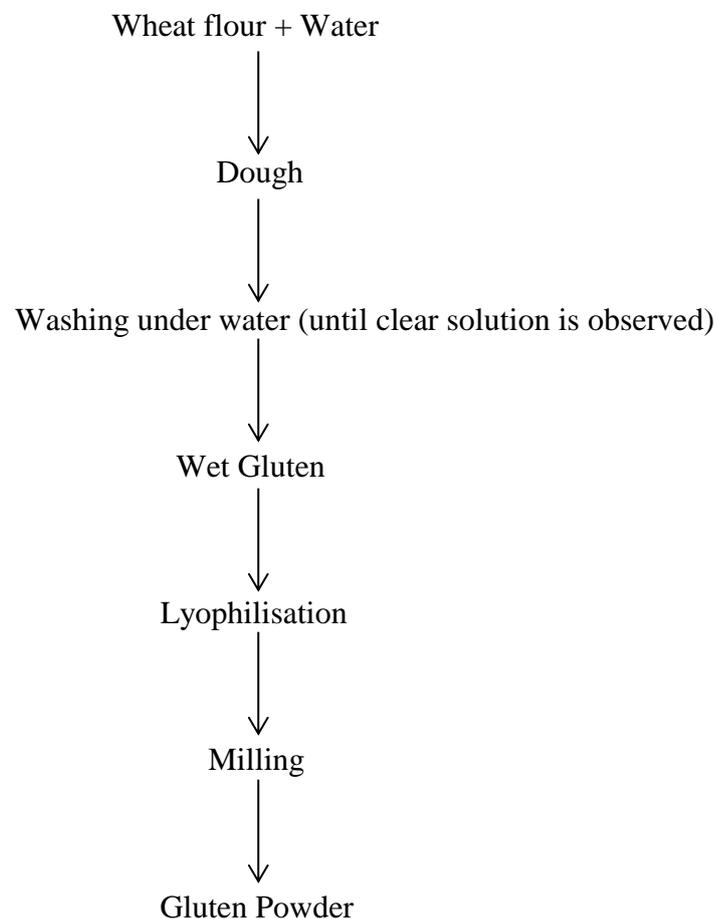
Fig 2: Overview of the process involved in gluten separation from wheat flour.

1.2   Gluten Chemistry

Depending on the thoroughness of washing, the dry solid contains about 75–85% protein and 5–10% lipids; most of what is left is starch and non-starch carbohydrates (Wieser, 2006). Additional washes will ultimately produce gluten with higher protein content (higher-grade protein) but the disadvantage in this is the fact that there will be larger volumes of water that needs to be removed (Georgopoulos *et al*., 2004). Regardless of what method is used in obtaining gluten (wet) of good quality from wheat flour, the drying step is crucial due to the sensitivity of proteins to high temperatures. Drying of gluten must therefore be carefully carried out at low temperatures so as not to alter the protein configuration. To achieve this aim, the freeze-drying method is usually employed. In freeze drying, the surrounding pressure is decreased and this further allows the frozen water in the food material to sublimate directly (avoiding the liquid phase) from the solid phase to the gas phase.

Gluten contains hundreds of protein components which are present either as monomers or linked by interchain disulphide bonds as oligomers- and polymers (Wrigley and Beitz, 1998). Both fractions consist of numerous but partially closely related protein components characterised by high glutamine and proline contents (Wieser, 2006). Gliadins and glutenins are storage proteins and cover about 75% of the total protein content while the albumin and globulin fraction covers about 25% (Belderok et al., 2000, Shewry et al., 2002a). The gluten proteins have unusual amino acid compositions with high percentages of proline and glutamine and low percentages of arginine and lysine (Vensel et al., 2011). The vast majority of the proteins are of single type called prolamins (Shewry et al., 2002a). Gliadin and glutenins are mainly located in the endosperm and are not found in the seed coat layer nor the germ (Belderok et al., 2000). Disulphide bonds which is formed when water is added and mixed (Fig. 3) plays a key role in determining the structure and properties of wheat gluten proteins (Shewry and Tatham, 1997).

Cereal proteins are usually categorized into three groups on the basis of their solubility: the water-soluble albumins, the salt- soluble globulins and the alcohol-soluble prolamins with the albumins and globulins widely distributed in dicotyledonous flowering plants and globulin-like proteins of the 11S family are a major component also of rice and oat endosperm (Tosi, 2012). These fractions account for at least 50% of the total grain nitrogen, and their amounts increase disproportionally when there are high levels of nitrogen (Tatham and Shewry, 2012). Prolamins which is the major class of storage proteins in cereals with preponderance of proline and glutamine, are synthesized at the endoplasmic reticulum during seed development and deposited into subcellular structures of the immature endosperm, the protein bodies (Zhang et al., 2013). Prolamins have diverged during the evolution of the grass family in their structure and their properties. The diversification of prolamin seed storage proteins is in part presumed to be accountable for the difference in functional properties of cereal products. Such variability in functional properties is that wheat is able to form a polymer of seed storage proteins (gluten), while maize does not. In spite of the divergence in seed storage proteins, however, prolamins from all species accumulate similar (endoplasmic reticulum) ER-derived protein bodies, suggesting that the basic functional interactions required for protein body formation have been preserved across species (Zhang et al., 2013). Different from the albumin and globulin fractions, which also contain metabolic proteins and are present in all plant tissues, prolamins are particular to the starchy endosperm cells of grasses. It is known that due to their unbalanced amino acid composition which is also characterised by very high contents of proline and glutamine, prolamins are considered to be poor in nutritional value (Tosi, 2012). Due to the presence of cysteine residues, most wheat prolamins are sulphur-rich the only exception to that are the ω-gliadins, which do not have any cysteine residues and are therefore regarded as sulphur-poor (Tosi, 2012).
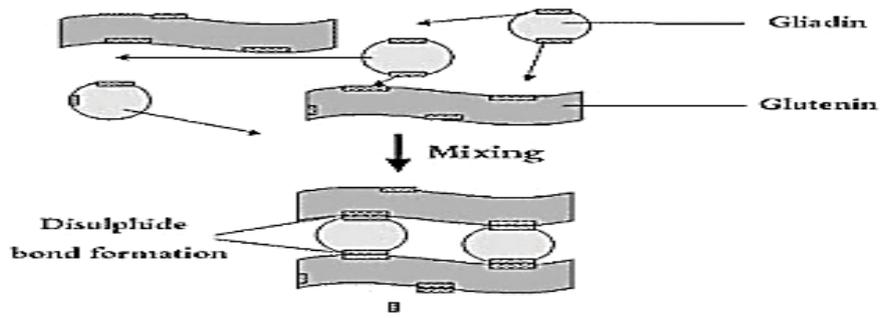
Fig 3: Disulphide bond formation within the gluten structure (Anonymous, 2012)

Hydration gives rise to the formation of an apparent continuous water phase between gluten particles and hydrated proteins form β-sheet structures that contribute to the network connectivity (Khatkar et al., 1995)
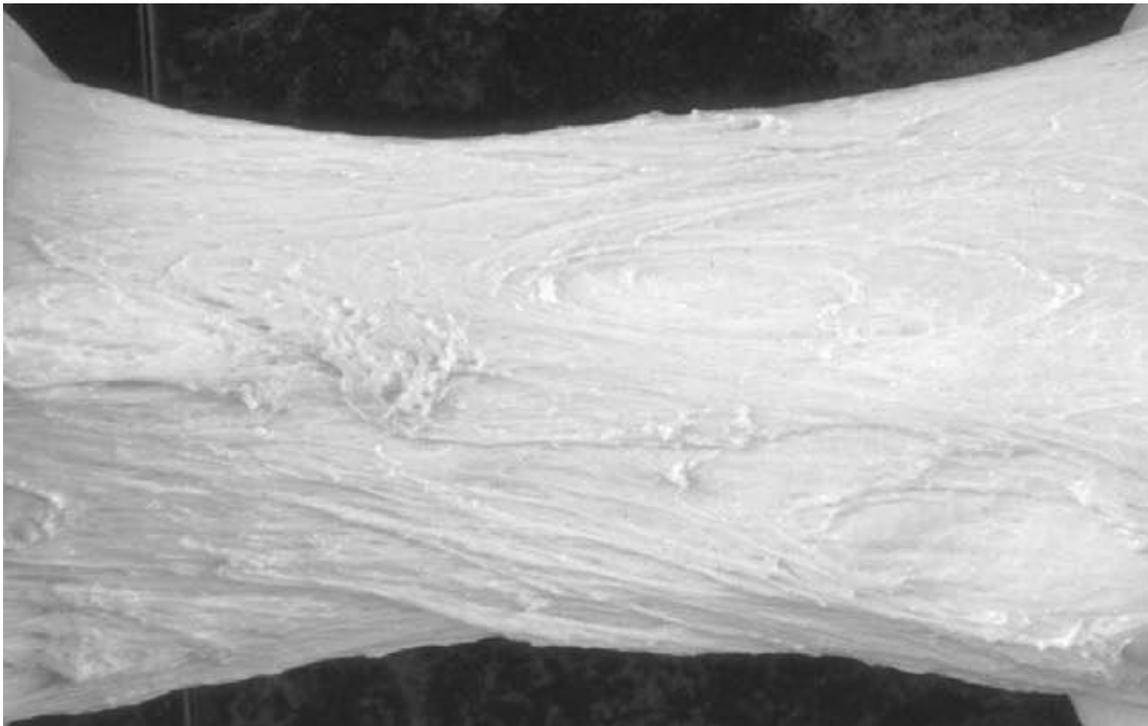
Fig 4: Gluten sheet showing its cohesiveness (Shewry et al., 2002a).

1.3 Gliadins and glutenin

Gluten is rich in gliadins and glutenins but also contains 3.5–6.8% lipids, 0.5–0.9% minerals, and 7.0–16.0% carbohydrates (Khatkar et al., 1995). The lipid content of gluten is primarily determined by the lipid content of the flour from which it came, and it is usually not affected by additional washing (Day et al., 2006).

Gliadins and glutenins, accounting for 80–90% of the total wheat flour proteins, are the two principal classes of storage proteins, being essential for producing an appropriate equilibrium of viscous and elastic properties in gluten and flour dough (Song and Zheng, 2007, Khatkar et al., 1995). Wheat gliadins are a group of storage proteins that are extracted from the endosperm of the grain with aqueous alcohols (usually 70% ethanol). They are usually separated by electrophoresis at low pH under non-reducing conditions into four somewhat arbitrary groups of polypeptides called α-gliadins, β-gliadins, γ-gliadins and ω-gliadins (Shewry et al., 1983, Shewry and Lookhart, 2003) with individual bands within the groups being designated by numbers. The Gliadin content of wheat is highly variable, both in quality and quantity which is a function of the plant genetics and of the growing conditions (Prandi et al., 2012). Gliadin comprises mainly monomeric proteins with masses known to range from 30,000 to 50,000 (Shewry and Lookhart, 2003, Dutta et al., 2011). The gliadin fraction contributes mainly to the viscous properties of gluten (Xu et al., 2006, Shewry et al., 1986). Wheat glutenins are polymeric in nature and reduction of disulphide-bonds could result in the glutenin polymers being converted into monomers which imply that they are stabilized by the inter-chain disulphide bonds. (Shewry and Lookhart, 2003, Dutta et al., 2011). Because glutenins are mainly aggregated proteins, they have varying sizes ranging from about 500,000 to more than 10 million Da (Wieser, 2006).

Glutenin can be divided into two groups according to their electrophoretic mobility: high molecular weight subunits (HMW-GS) and low molecular weight subunits (LMW-GS)

(Lafiandra et al., 2004). They are linked together by covalent disulphide bonds and form very large polymeric structures. It is commonly acknowledged that the glutenin fraction contributes mainly to the elastic properties of gluten (Xu et al., 2006, Shewry et al., 1986). Low-molecular-weight glutenin subunits (LMW-GS) are polymeric protein components of wheat endosperm and like all seed storage proteins, are digested to provide nutrients for the embryo during seed germination and seedling growth. Due to structural characteristics, they exhibit features important for the technological properties of wheat flour. Their ability to form inter-molecular disulphide bonds with each other and/or with high-molecular-weight glutenin subunits (HMW-GS) is important for the formation of the glutenin polymers, which are among the biggest macromolecules present in nature, and they determine the processing properties of wheat dough (D'Ovidio and Masci, 2004). LMW-GS are a highly polymorphic protein complex, including proteins with gliadin-type sequences.

Difficulty in separating single components, arising from the complexity of the group, has limited the characterisation of the individual proteins and the establishment of precise relationships with quality parameters (D'Ovidio and Masci, 2004). The high molecular weight glutenins (HMWs) are minor components in terms of quantity, but they are key factors in the process of bread making. This is so because they are a major determinants of gluten elasticity, plays a major role in gluten functionality and the end use quality is mainly due to their presence (Anjum et al., 2007, Mimouni et al., 1998) . Due to the high level of polymorphism that exist between structures and properties both within and between genotypes, it is difficult to classify gluten proteins using classical biochemical approaches (Shewry and Lookhart, 2003).

The classification of wheat gluten proteins discussed is therefore based on differences in their solubility which is largely determined by their availability as monomers or as subunits of disulphide-stabilized polymers and their electrophoretic properties either in the

native state (gliadin) or as reduced subunits (glutenins) (Shewry and Lookhart, 2003). Conventionally, gluten proteins have been divided into equal fraction of gliadin and glutenin, but it is worthy of note that both are important contributors to the rheological properties of gluten (Wieser, 2006).

1.4   Viscoelastic behaviour of Materials

Polymers are commonly described as viscoelastic which is aimed at reflecting their ability to display both viscous and elastic behaviour. It has been shown that depending on the temperature and time scale of measurement, such materials may exhibit properties of a viscous liquid, an elastic rubber or a brittle glass (Turi, 1981). Consequently, for a satisfactory measurement and understanding viscoelastic properties of polymers (bio or synthetic), data needs to be acquired over a range of time and temperature, this is because as force is applied polymers can exhibit different behaviours over a period of time and at different temperatures (Ward and Sweeney, 2004, Turi, 1981). A time-temperature relationship is usually employed in achieving this. The classical demonstration of viscoelastic nature of polymers is therefore based on a series of measurements of the stress-relaxation modulus as a function of time (Turi, 1981). This is further discussed in detail later in this chapter. Creep and stress relaxation are complimentary aspects of plastic behaviour and in many cases, it may provide equivalent information for studies of both fundamental viscoelastic properties and performance in practical applications (Billmeyer, 1984).

Consequently, if we consider the term *rheology* from a broad sense, it refers to both studies of deformation as well as flow of materials under the influence of applied force. Thus the rheological behaviour of polymers covers a range of minuscular occurrences which includes the flow of viscous liquids, mechanical properties of elastic solids and viscoelasticity i.e., time dependent properties of polymers (Cowie and Arrighi, 2008, Billmeyer, 1984). One of the most interesting features of polymers to note is that a given

polymer can possibly display all the intermediate range of properties between an elastic solid and a viscous liquid depending on the temperature and the experimental time chosen (Ward and Sweeney, 2004). Therefore, the response of polymers to mechanical stresses (deformation) can vary widely and depends on the particular state the polymer is in at a given temperature as earlier mentioned (Cowie and Arrighi, 2008). In the description of the behaviour of a linear elastic solid by Hooke's law and Newton's law of linear viscous liquid, a constitutive relationship for the behaviour of a linear viscoelastic solid can be obtained by combining the two laws:

Elastic behaviour $(\sigma_{xy})_E = Ge_{xy}$……………………(i) where G is the shear modulus

Viscous behaviour $(\sigma_{xy})_V = \eta(\partial e_{xy}/\partial t)$………………(ii)

The combination of both equations for viscoelastic behaviour is given by

$\sigma_{xy} = (\sigma_{xy})_E + (\sigma_{xy})_V = Ge_{xy} + \eta\ (\partial e_{xy}/\partial t)$ …………...(iii)

The initial work on viscoelasticity that was performed on silk and rubber shows that these materials exhibited a delayed elasticity manifested in the observation that the imposition of stress resulted in instantaneous strain, which continued to increase more slowly with time. It is this delay between cause and effect that is fundamental to the observed viscoelastic response and the hysteresis effect are (a) *creep*, where there is a delayed strain response after the rapid application of stress and (b) *stress-relaxation* in which the material is quickly subjected to a strain and a subsequent decay of stress is observed (Cowie and Arrighi, 2008).

The velocity gradient established within the system during rheological measurement as a result of an applied shear stress is referred to as shear rate which is expressed in units of reciprocal seconds (s^{-1}). The shear stress is defined as the force applied divided by the area of application usually expressed in force per unit area (Pa) (Rao, 2007). A proper experimental design is critical in rheological measurement of any material including foods, in order to

acquire reliable and reproducible data. In addition to a well-designed experimental protocol, further precautions should also be taken to ensure that rheological data obtained on a sample are reliable. One of such is allowing the food sample to rest after loading as food structure is altered during sample loading. One major problem however is moisture loss during experiment which can also greatly alter the result obtained. Silicone or mineral oil can be placed around the sample edge to avoid dehydration but caution must be exercised to avoid the oil penetrating into the food. Furthermore, under conditions of high strain, deviation from the mechanical behaviour predicted by the low strain analysis may occur due to slip at the matrix-filler interface. In addition, non-uniform distribution of stress and strain throughout the material may result in a more complex mechanical response to deformation at high strains (Rao, 2007). All the above discussed emphasizes the importance of a proper experimental design for a meaningful rheological measurement of any food sample and how reliable data can be generated. In summary, deformation of an object can therefore be defined as a kind of permanent change either gain or loss in its existing symmetries and the viscoelastic behaviour of a network through its evolution therefore denotes how much rigidity (i.e. solid characteristics) and fluidity (i.e. viscous characteristics) it develops as force is applied (Gunduz, 2009). Knowledge from previous study shows that for gliadins, the storage moduli (G′), loss moduli (G′′), and phase shifts dramatically change within a narrow concentration range, indicating that gliadin suspension properties can change from viscous to viscoelastic while glutenins exhibits viscoelastic solid-like behaviour (Xu et al., 2006).

1.5   Application of Rheological Testing In Food Systems

Rheology is the study of the flow and deformation of materials (Dobraszczyk and Morgenstern, 2003). Furthermore, rheology attempts to define a relationship between the stress acting on a given material and the consequential deformation and/or flow that takes place (Tabilo-Munizaga and Barbosa-Cánovas, 2005, Fischer et al., 2009). Typical flow

processes in food processing include mixing/stirring, dispersing, extrusion, spinning, coating, injection moulding and spraying (Fischer and Windhab, 2011). Generally, to measure rheological behaviour, a controlled, well-defined deformation or strain is applied to a material over a given time and the resulting force response is measured (or vice versa) to give an indication of material parameters such as stiffness, modulus, viscosity, hardness, strength or toughness of the material (Dobraszczyk and Morgenstern, 2003, Rao, 2007, Tabilo-Munizaga and Barbosa-Cánovas, 2005). The science of rheology grew considerably due to research work done on synthetic polymers. Nevertheless, because of the biological nature of foods and the fact that many foods are composed mainly of biopolymers (proteins, polysaccharides and lipids), rheology has become important in the food system terrain (Rao, 2007). Rheological measurements are quite relevant in the food industry as a device for physical characterization of raw material before processing, for intermediate products as manufacturing progresses, and for finished products (Tabilo-Munizaga and Barbosa-Cánovas, 2005). Rheological properties vary from viscous fluids and elastic solids, defining the spectrum of possible material responses to applied stress σ, strain γ , or shear rate $\dot{\gamma}$ (Fischer et al., 2009).

Simply put in another term, rheological tests attempt to measure the forces required to produce given controlled deformations, such as squashing (compression), bending or pulling apart (tension), and to present them in such a way as to be independent of sample size, geometry, and mode of testing. A small test piece of the material is usually deformed in a controlled way, normally on a motor driven machine, and the force is measured as well as the distance moved or displacement of the object. The force is then usually plotted against the displacement to give a force-displacement curve. (Dobraszczyk and Morgenstern, 2003).

1.6   Stress relaxation

To obtain information regarding stress relaxation, the decay of stress as a function of time is recorded at a fixed magnitude of deformation (Rao, 2007). The method of stress relaxation as mentioned earlier has been widely used to test polymers as data generated lead to numerous material functions including the relaxation spectrum system (Kontogiorgos et al., 2008). The properties of viscoelastic materials are usually measured by creep, stress relaxation or dynamic oscillatory test to obtain fundamental information which could be helpful for improvement in quality control and processing (Hayta and Schofield, 2005). The most common types of fundamental rheological tests used in cereal testing are: (i) small deformation dynamic shear oscillation; (ii) small and large deformation shear creep and stress relaxation; (iii) large deformation extensional measurements; and (iv) flow viscometry (Dobraszczyk and Morgenstern, 2003). When studying the rheological behaviour of a food, the knowledge of the composition of food, the structuring components, the structure of the food itself and the processing and storage conditions is helpful as they all often affect the behaviour (Rao, 2007). For processed food, the composition and the addition of ingredients to obtain a particular food quality and product performance involves deep rheological understanding of individual ingredients, their relation to food processing, and their final perception (Fischer and Windhab, 2011).

In stress relaxation measurements, deformation is held constant and the force response is measured, whilst in creep the stress is held constant and the deformation is measured (Dobraszczyk and Morgenstern, 2003). Stress relaxation experimentation is a direct and fundamental approach in the elucidation of molecular dynamics of relaxation processes (Kontogiorgos et al., 2008). The method of stress relaxation and rheological testing has it is been used in polymer science also has been employed in investigating and defining structures and properties of biological materials such as flour dough and gluten and information

resulting from these rheological testing can therefore be used to predict the processiliblity and quality of food (Yang et al., 2011). Dynamic rheological testing has therefore become a powerful and preferred approach for examining the structure and the fundamental properties of wheat flour dough and proteins because of its characteristic and sensitive response to the structure variation of wheat flour dough and proteins (Song and Zheng, 2007). Information on the rheological properties of dough is useful for predicting the potential application of the wheat flour and also the quality of the end product (Mohammed et al., 2012). It is these rheological properties that form the basis of functional uses of gluten. It is these properties that permit breads, cakes, biscuits and noodles and all other flour based products to be made from wheat-flour dough (Day et al., 2006). The aim of rheological characterization of food is therefore to quantify the functional relationships between deformation, stresses, and the resulting rheological properties such as viscosity, elasticity, or viscoelasticity (Fischer and Windhab, 2011). Studies on the rheological properties in relation to dough and gluten are often regarded as very challenging and this is due to its wide range variance in nature and the fact that it depends on so many factors (Zaidel et al., 2010).

This testing simultaneously measures the viscous and elastic characters expressed in storage and loss moduli, G′ and G″ (Khatkar et al., 1995). As earlier mentioned, protein quality can be measured by variety of criteria, dynamic rheological parameters of glutens are able to indicate the wheat quality as glutens from poor quality wheat are rheologically characterized as less elastic and more viscous than those from good quality wheat (Khatkar et al., 1995). Among the cereal flours, only wheat flour can form three-dimensional viscoelastic dough when mixed with water and rheological testing in the linear viscoelastic region has been used to study its structure and properties (Yang et al., 2011). The ability to successfully characterize and ideally understand the rheology of food materials is essential for several aspects of food science and technology, such as the standardized characterization of raw

materials and innovative products, or for enhanced industrial processing (Fischer and Windhab, 2011).

The relaxation of hydrated gluten network in which Tikhonov regularization, in conjunction with the L-curve criterion for optimal calculation of the regularization parameter was used to generate the relaxation spectrum from stress relaxation measurements on shear shows that six molecular events with baseline resolution which can be grouped into fast and slow relaxation regimes was observed (Kontogiorgos and Kasapis, 2010). Findings from this work also gave fundamental understanding and new insight into the complexity of interactions and relaxation mode of hydrated gluten.

Considering the mechanical properties of gluten fractions, the non-linear shear viscoelastic properties' of gliadin and glutenin depend on concentration and varying the gliadin/glutenin ratio by adding isolated gliadin or glutenin sub-fractions to a parent gluten exhibits a trend to show that G´ (storage moduli) value decreases as the gliadin/glutenin ratio increases (Xu et al., 2006, Khatkar et al., 1995). This confirms that there is a relationship between G´ and amount of gluten fractions present.

Also crucial in rheological measurement and worthy of note is the amount of water content present in the food material to be tested. Investigating the effect of water content on the rheology of gluten and dough, it was found that the values of G´ and G´´ for gluten decreased slightly with an increase in water content and in dough it was observed that there was a major decrease in the values of G´ and G´´ with greater effect on the storage moduli (G´) (Georgopoulos et al., 2004). This result agrees with the fact that the overall dynamic moduli of gluten decreases as water content is increased (Janssen et al., 1996).

Just as quantity of water added for hydration is crucial for gluten development, water also plays a major role in obtaining correct rheological information. This is crucial because if less water than required is added, proper mixing and hydration is not achieved and if too

much water is added, this will affect the geometry within the sample making relaxation measurement challenging.

1.7   Regularization of data and the L-Curve

From the point of view of modern day mathematics, all problems can be classified as being either correctly posed (well-posed) or incorrectly posed (ill-posed) (Yildiz et al., 2000). With the knowledge that inverse problems are usually ill-posed it is essential that some methods are used to reduce their deficiencies (Santos and Bassrei, 2007). Generally, an attempt to solve ill-posed problems will always lead to numerical problems. Therefore, to solve such problems numerically, it is vital to find appropriate stable algorithms. Such methods are often referred to as "regularization" methods (Yildiz et al., 2000).

To analyse data from relaxation experiments, a computational methodology to treat relaxation spectra is often developed. By using MATLAB and employing different regularization algorithms such TIKHONOV or CONTIN, the utilization of the L-curve criterion is possible and hence the location of the optimum regularization parameter for accurate data inversion (Kontogiorgos et al., 2008).

MATLAB which is a high-level language and interactive environment for numerical computation allows the analysis of data, development of algorithms and the creation of model and application. The language has in-built maths functions that enables the exploration of multiple approaches and reach a solution more quickly than other traditional programming languages such as C/C^{++} or Java (MathWorks, 2013).

Since the system matrix in any ill-posed problem is usually presumed to have some noise, regularization is then very important in helping to stabilize the computed solution (Lampe and Voss, 2013). Discretization of linear inverse problems generally gives rise to very ill-conditioned linear systems of algebraic equations and typically, the linear systems obtained have to be regularized to make the computation of a meaningful approximate

solution possible (Calvetti et al., 2000). When solving inverse problems, because the "naïve" least square solution formally given by $x_{LS} = A^+ b$ is dominated by contributions from data errors and rounding errors, regularization is always necessary and suggests that by adding regularization, it is possible to dampen these contributions and keep the norm $\|L(x - x0)\|2$ of reasonable size. (Hansen, 2000, Agarwal, 2003) Tikhonov regularization is one of the most popular regularization methods. Other regularization method includes the Singular Value decomposition (SVD) and Generalised Cross-Validation (GCV) (Calvetti et al., 2000, Hansen and O'Leary, 1993, Neuman et al., 2012). This philosophy underlies Tikhonov regularization and most other regularization methods. However, if the regularization is too much, the regularized solution does not fit the given data properly as the residual error $\|Ax-b\|2$ is too large and if the regularization is too small, the fit will be good but data error will be more (Agarwal, 2003, Hansen, 2000). Different regularization techniques differ on the basis on how they minimize this trade off which can be controlled by the selection of proper regularization parameter (Agarwal, 2003). Practically, regularization methods used in computation of stable solutions to inverse problems involve a trade-off between the "size" of the regularized solution and the quality of the fit that it provides to the given data (Hansen, 2000, Agarwal, 2003).

What distinguishes the various regularization methods is how they measure these quantities, and how they decide on the optimal trade-off between the two quantities, in finding a regularization parameter that gives a good balance, filtering out enough noise at the same time without losing too much information in the computed solution (Hansen, 2000, Hansen and O'Leary, 1993). Consequently, methods for selection of regularization parameters, L-curve method has gained attention in recent years. The log-log plot between the squared norm of the regularized solution and the squared norm of the regularized residual for a range of values of regularization parameter is usually defined as the L-curve (Agarwal,

2003). It can also be explained as a plot for all valid regularization parameters of the size of the regularized solution versus the size of the corresponding residual (Hansen and O'Leary, 1993).

The L-curve criterion for Tikhonov regularization gives a very robust estimation of the regularization parameter, while the GCV method occasionally fails to do so (Hansen, 2000). As the name suggests, the L-curve is usually a L shaped curve (see fig 5) with the general acceptance that the good regularization parameter and the corresponding solution is located at the corner of the L- curve and the rationale behind using the corner to find a regularization parameter is that the corner corresponds to a solution in which there is a fair balance between the regularization and perturbation errors because the corner separates the horizontal part of the curve from the more vertical part (Hansen and O'Leary, 1993). Especially, for cases when (regularization parameter) λ is very large (over-regularization), the residual norm is very sensitive to small changes in λ while the solution norm is relatively constant, so the curve is essentially a horizontal line and on the other hand, when λ is very small (under-regularization), changes in the solution norm occur much faster than changes in the residual norm, and the curve is essentially a vertical line and thus giving the characteristic L shape plot (Rezghi and Hosseini, 2009).
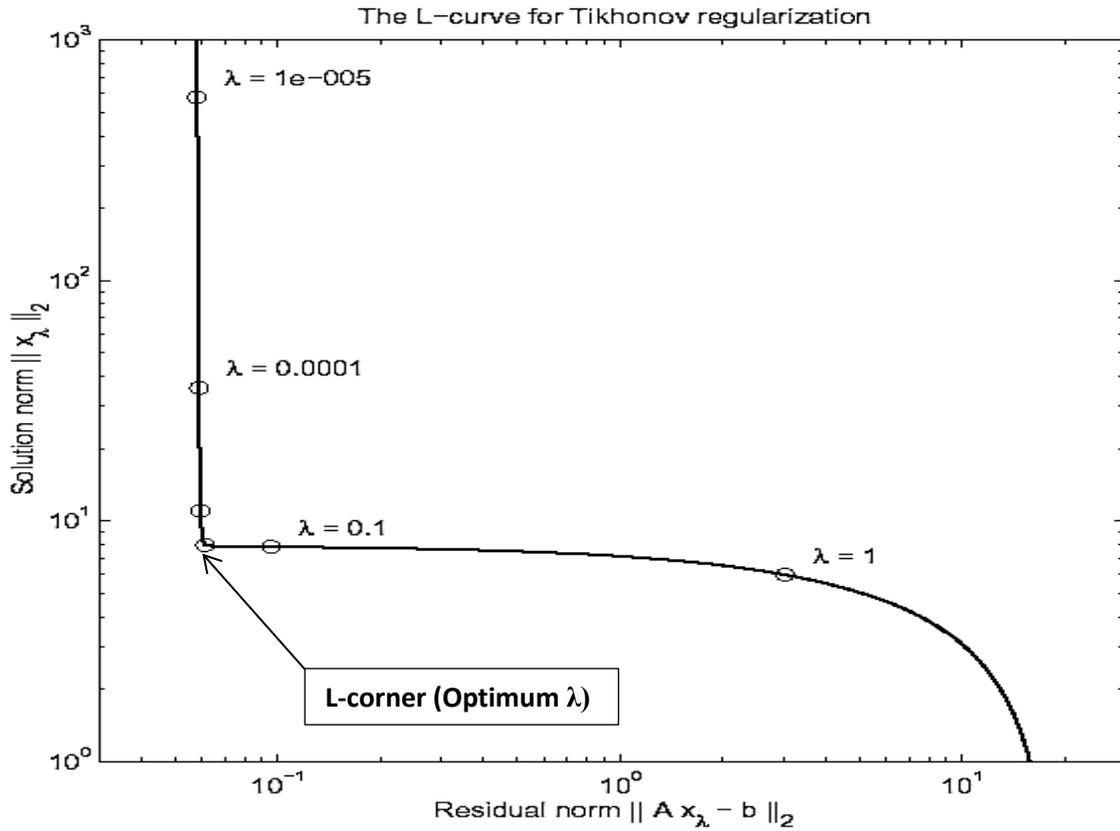
Fig 5: Theoretical L-curve for Tikhonov Regularization (Agarwal, 2003).

Since the crucial issue in regularization is the correct selection of the regularization parameter (λ) (Calvetti et al., 2000), a wrong selection of the regularization parameter will consequently void any result. However, when the regularization parameter is chosen correctly, the noise in the system tends to zero (Burger and Neubauer, 2003). For most scenarios it is observed that the L-curve criterion eventually leads to some over-regularization (i.e., a too large regularization parameter) as the problem size (*n*) increases. However, the amount of over-smoothing depends on the decay of the singular values: the faster they decay the less severe the over-smoothing (Hansen, 2000).

1.8 Aims

The aims of this investigation are therefore to:

a. To isolate gluten from wheat flour and further isolate its two major protein fractions (gliadin and glutenin).

b. Adopt the type of analysis afore-mentioned in identifying the relaxation and structural properties in order to characterise the mechanical properties of gluten and its components: gliadin and glutenin.

c. Investigate the effect of the concentration of each fraction on the mechanical properties of gluten.

d. To employ the use of CLSM to investigate the microstructure of gluten and its protein fractions.

The research work was therefore undertaken with the objective of unveiling the relaxation behaviour of hydrated gluten networks in order to give a fundamental description of the related phenomena.

Chapter 2

2.   Materials and Method.

2.1   Materials

General-purpose wheat flour was purchased from the local market to isolate gluten. Sodium chloride, acetic acid, dialysis membrane tubing (MwCO 12000) and formaldehyde (37-40%) were purchased from Sigma-Aldrich (St. Luis, MO) whereas ethanol from Fisher Scientific (Loughborough, UK). Chemicals (primary and secondary antibodies) used in the staining of samples for microscopy were purchased from Sigma-Aldrich (St. Luis, MO).

2.2   Gluten isolation

General-purpose plain flour (Heygates, UK) (600g) was mixed with water (400ml) with salt (5g) added to the mixture and made into dough (McCann et al., 2009). The importance of adding salt to the mixture is to aid the elimination of albumins and globulins (van Eckert et al., 2006). The dough was wrapped with cling film and allowed to hydrate for 30min for proper gluten development. The hydrated dough was washed under running water until clear water solution was observed which indicates the removal of starch and other water soluble materials. The rubbery mass left after washing was lyophilized (freeze dried) and made into powder (gluten powder). The choice of freeze drying the sample was imperative as to preserve the chemical configuration of protein as high temperature can alter the protein morphology and structure.

2.3   Isolation of gliadin and glutenin fractions

To isolate gliadin fraction, gluten powder that was obtained in the previous step was dispersed (5% w/v) in 0.5 M NaCl for 30 min at room temperature to remove the remaining albumins and globulins and the resulting dispersion was centrifuged at 5000g for 15 min.

Supernatant was discarded and the resulting pellet was rinsed with deionized water and dispersed in 70% v/v ethanol (1:1 ratio) overnight at room temperature. The resulting solution was centrifuged at 5000g for 15 min and the supernatant that contains the gliadin fraction was concentrated using a rotary evaporator at 35°C. The concentrated gliadin fraction was freeze-dried and ground into powder (gliadin). The pellet from the centrifugation step was rinsed with deionised water and dispersed in 0.1 M acetic acid (1:1 ratio) and stirred overnight at room temperature. The resulting supernatant that contains the glutenin fraction was centrifuged at 5000g for 15 min and after extensive dialysis (3 days) to remove acetic acid; it was freeze-dried and ground into powder (glutenin). Fig.6 gives a detailed explicit image of the fractionation procedure. Samples obtained from the above isolation procedures were subjected to proximate analysis (International Laboratory Services, Shadlow, UK). Nitrogen was analysed for using Leco FP 2000 (combustion) and the protein content was calculated from the nitrogen using a factor of 6.25. Ash content was determined using Gravimetric after ashing at 520 ºC. The moisture was determined by Gravimetric after drying at 105 ºC and the Fat content was determined using Werner-Schmid Gravimetric determination. The Carbohydrate content was determined by calculation (100-protein-ash-fat-moisture).

2.4   SDS PAGE.

SDS-PAGE following the (Laemmli, 1970) protocol with few modifications was performed using NuPAGE gradient precast gel (4-12%) gradient Bis-Tris (10x10cm^2) in a novex Xcel mini cell (Invitrogen, USA). MES buffer containing 50mM MES, 50mM tris base, 0.1% SDS, 1mM EDTA, pH 7.3, was used as the electrophoresis running buffer. The Protein samples were dissolved in sample buffer containing 11.25mM tris-HCl, pH 8.5, 3.6% SDS, 50mM dithiothreitol, 18% glycerol and 0.0025% bromophenol blue and heated at 95°C for 10min. Small amount of Ethanol (0.1ml) was added to also help in dissolving the protein samples. The experiment was carried out in the reduced form; therefore samples were heated

in a water bath at 85°C for 10min. The protein samples (20µl) and the protein standard markers (3.5 to 260 kDa) (Invitrogen, UK) were loaded onto the gel. Electrophoresis was performed at 200V for 35min. Proteins were visualized by staining with Coomassie Brilliant Blue for 1h and de-stained overnight with methanol.

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                        ┌─────────────────┐
                        │   Dry gluten    │
                        └─────────────────┘
                                │
                  ┌──────────────────────────────┐
                  │ Dispersion in 0.5M NaCl (400ml)│
                  │    2hrs, Room Temperature      │
                  └──────────────────────────────┘
                                │
  ┌───────────────────────────────┐        ┌──────────────────────┐
  │ Centrifugation (4000g for 15mins)│─────▶│ Supernatant Discarded │
  └───────────────────────────────┘        └──────────────────────┘
                                │
        ┌────────────────────────────────────────────┐
        │ Pellets dispersed in 70% w/v Ethanol (400ml), 2hrs,│
        │            Room Temperature                 │
        └────────────────────────────────────────────┘
                                │
  ┌─────────────────────┐          ┌────────────────────────┐
  │ Centrifugation (4000g│─────────▶│ Supernatant gliadins   │
  │     for 15mins)      │          └────────────────────────┘
  └─────────────────────┘                      │
                                │               │
        ┌───────────────────────────────┐       │
        │ Pellets washed with distilled water│   │
        └───────────────────────────────┘       │
                                │          ┌──────────────────┐
                                │          │ Rotary Evaporation│
                                │          └──────────────────┘
                                │                    │
        ┌──────────────────────────────┐      ┌───────────────┐
        │ Pellets dispersed in 0.1M acetic acid│  │ Freeze Drying │
        │  (400ml), 2hrs, Room Temperature │      └───────────────┘
        └──────────────────────────────┘
                                │
  ┌─────────────────────┐          ┌────────────────────────┐
  │ Centrifugation (4000g│─────────▶│ Supernatant glutenins  │
  │     for 15mins)      │          └────────────────────────┘
  └─────────────────────┘                      │
                                │          ┌──────────┐
        ┌──────────────────┐               │ Dialysis │
        │ Pellets discarded│               └──────────┘
        └──────────────────┘                    │
                                          ┌───────────────┐
                                          │ Freeze Drying │
                                          └───────────────┘
```

Fig 6: Gluten fractionation protocol

2.5 Sample preparation and stress relaxation measurements

Isolated gluten (Glut), glutenin-enriched (Gln), gliadin-enriched (Gli) fractions and their mixtures in ratios of 3:1, 1:1 and 1:3 (Gln:Gli) were prepared for stress relaxation measurements to yield samples with 40% w/w total protein solids in 60% w/w deionized water and kept in the fridge for 30 min to allow proper hydration. Stress relaxation measurements were performed between 0 - 70°C using a Bohlin Gemini 200HR-nano rotational rheometer (Malvern Instruments, Malvern, UK) equipped with serrated plate-serrated plate geometry (25 mm diameter and 1000 μm gap). Experimental protocol of the present investigation included the following steps:

(i)   Shear strain amplitude sweep experiments were performed between 0-70°C using angular frequency of 6.28 rad/s to determine the linear viscoelastic region (LVR) of the samples. 2% strain was found to be within the LVR of all samples and give satisfactory S/N ratio and it was therefore used for the next set of experiments.

(ii) Time sweeps in dynamic oscillation on shear were executed at 6.28 rad/s and 2% strain for 60 min revealing that storage (G') and loss moduli (G'') reach pseudo-equilibrium within 10 min. Therefore, samples were left to equilibrate for 10 min before measurements to dissipate stresses that were created during loading.

(iii) Stress relaxation tests were carried out using 2 % instantaneous strain for each sample. Reproducible and highly resolved relaxation spectra can be obtained with 30 min relaxation following application of the instantaneous strain. Data of stress relaxation modulus ($G(t)$) were collected in a logarithmic mode with respect to the timescale of observation. Strain rise time was 20 ms and data point collection started after 30 ms.

A thin layer of low viscosity silicone oil (dimethylpolysiloxane, Sigma-Aldrich, St. Louis,MO) was also applied to minimize moisture loss during the course of experiments. Mechanical measurements were performed ten times for each sample and average curves

were reported. Nonlinear regression was performed with GraphPad Prism v.6 (Graph-Pad Software, San Diego, USA).

2.6   Numerical computation

Numerical computation was performed in MATLAB (v7.0 R14 Service Pack 2, The Mathworks Inc., MA) as described previously. The first step involves discretization of stress relaxation function to create matrix A and was performed with the discr.m. Following that step, algorithms csvd.m for calculation of:

(i)   Discretization of kernel K(s,t) to create matrix A

(ii)   Creation of the L-plot and calculation of the optimum regularization parameter

(iii)   Calculation of the spectrum using Tikhonov regularization.

2.7   Confocal laser scanning microscopy

Samples prepared as described for stress relaxation measurements were embedded at ambient temperature in CRYO-M-BED resin (Bright, Huntingdon, UK), quench frozen and sectioned at -20 ℃ using a cryostat (Bright Starlet 2212, Huntingdon, UK) to obtain sections with thickness between 8-12 μm. Sectioned samples were collected on a slide and fixed immediately using 10% v/v PBS-buffered (phosphate buffer saline) formalin solution for 15 min. Slides were then rinsed three times for 5 min in 1xPBS and left to dry at room temperature. Sections were imaged using protein self-fluorescence without further treatment in a confocal laser scanning microscope (Zeiss LSM510 META Upright, Heidelberg, Germany) at an excitation wavelength of 488 nm and emission wavelengths above 515 nm equipped with argon laser (488 nm) operating at 5% output. z-Stacks with resolution 1024x1024 pixels were captured using Zen 2009 software. z-Projected images have been produced using "maximum intensity" projection in ImageJ v1.74b.

Chapter 3

3.   Results and Discussion

3.1   Material Characterisation

3.1.1   Content Determination

Determining the actual content of samples will help in being able to attribute results from further tests to the presence of constituents within the sample (in this case, protein) and also to eliminate any doubt that may arise. As mentioned in the previous chapter, sample characterisation was carried out at International Laboratory Services, Shadlow, UK and results given in wet basis for protein, fat, carbohydrate and moisture.

The isolation procedure that was employed for this research work results in samples with the following compositional characteristics in wet basis:

Table 3: Gluten composition

Constituent	Amount (%) in Wet Basis
Protein	83
Fat	3.05
Moisture	8
carbohydrate	5
Ash	0.95

Table 4: Gliadin composition

Constituent	Amount (%) in Wet Basis
Protein	84
Fat	2.07
Moisture	10
Carbohydrate	3
Ash	0.93

Table 5: Glutenin composition

Constituent	Amount (%) in Wet Basis
Protein	84
Fat	2
Moisture	9
Carbohydrate	4
Ash	1

Sample characterisation is often very important in investigation relating to mechanical properties of materials. Since the aim is to observe how these protein fractions respond to stress and deformation, it is important to verify the materials being studied. From the results obtained (Tables 3, 4 and 5), it can be seen that the method used for the isolation is appropriate. This is further corroborated in a very high quality material especially from the protein content values of the samples with low carbohydrate and fat content. It is important to be able to isolate samples with this level of purity so as to confirm that results from other

tests (especially rheological measurements) is a function of the protein and not fat, carbohydrates or any other constituent present. It is a possibility that even though the isolation was done properly, the drying method may change alter the chemical composition but these results show that the drying method employed (lyophilisation) may not have altered the chemical structure of the proteins as the bands from the SDS PAGE showed corresponding weight of proteins expected.

3. 1.2   SDS-PAGE Electrophoresis

The SDS-PAGE protocol employed in this work was carried out in the reduced state where separation of protein bands is chiefly by molecular weight.
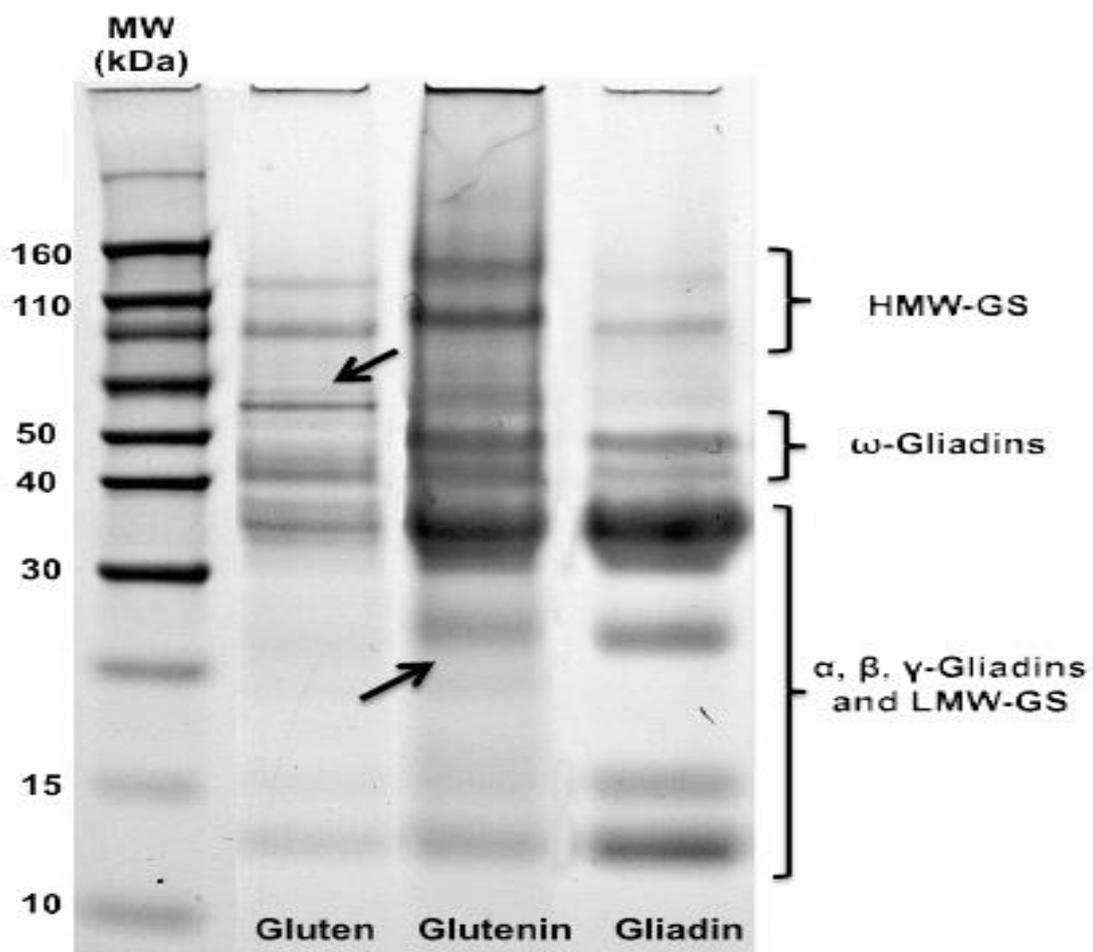

Fig. 7: SDS PAGE of gluten, gliadin and glutenin.

Electrophoresis revealed typical protein bands for gluten proteins with the enrichment of glutenin and gliadin fractions in the respective proteins being evident. It should be noted from the SDS PAGE that protein bands of the enriched fractions were not identical to the original material (gluten). This was anticipated as enrichment involves treatment with ethanol and acid where preferential partitioning into the solvents and partial hydrolysis results in changes in the overall electrophoretic patterns.

Nevertheless, extraction procedures created glutenin and gliadin enriched fractions containing the characteristic protein composition for these materials. Having established the protein compositional characteristics of the samples it was then sensible to proceed with the stress relaxation measurements that are described later in this work.

3.2   Stress Relaxation Measurements

The objective of this set of measurements was to explore the dependence of gluten stress relaxation curve on its constituent protein factions and temperature. To accomplish this purpose, networks of hydrated gluten, two protein fractions enriched in gliadin or glutenin and their mixtures were subjected to stress relaxation measurements. These measurements were carried out within the LVR (which was determined earlier) for the samples at temperature 20°C.
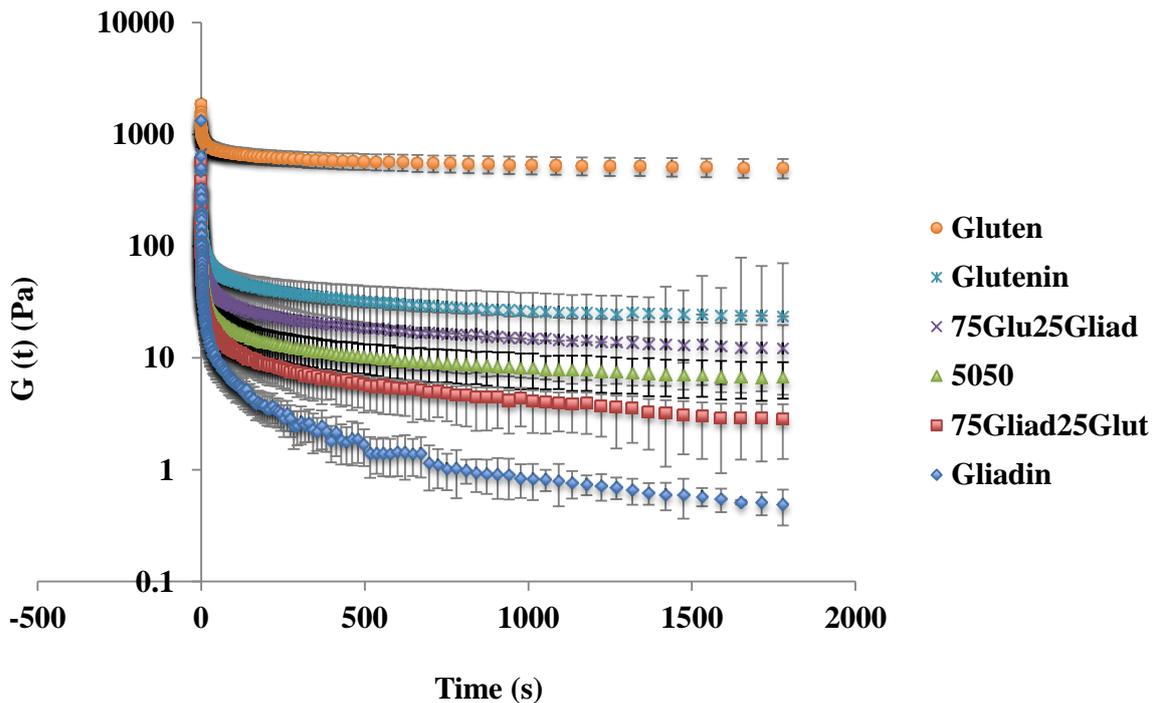
Fig 8: Stress relaxation curves for gluten, its constituent protein fractions and their mixtures (n=10 per sample)

As mentioned earlier, the samples were allowed to rest for 15mins after loading onto the rheometer. During sample loading, the sample is slightly stressed as it is being pressed to the surface of the geometric plate. The 15 min wait is therefore crucial to reduce noise, ensure that sample has returned to original position and stress exerted during loading is eliminated before any measurement is taken. For gluten a minimum decay with the relaxation modulus G(t) of just below 1000 Pa was observed. Gliadin exhibited almost complete decay with a relaxation modulus of about 0.5 Pa. This is somewhat expected because of the viscous nature of the material (see Figure 8)

Because of the elastic property of glutenin, even though it exhibited quite significant decay before reaching equilibrium, the decay is not as much as the decay observed in gliadin. This further established the difference in the protein composition of the two fractions of gluten. Different ratios of the fraction were also investigated. It was expected that the

relaxation curve for the ratio 1:1 mixture of the fractions will bear a resemblance to the relaxation curve observed in gluten due to the understanding that gliadin and glutenin is present in gluten in equal amount. It is however not unanticipated that there could be a change and this will be discussed later. Relaxation curve from mixture of 75% gliadin and 25% glutenin was also studied. Because of the higher percentage of gliadin in this particular mixture, the relaxation curve is almost the same as for gliadin. The presence of 25% glutenin however stops the decay from reaching G(t) = 0.5 Pa as seen in gliadin fraction. The lowest G(t) for this mixture ratio is 2.85 Pa. Just as seen in the mixture with the more gliadin, in the mixture containing 75% glutenin and 25% gliadin, because of higher percentage of glutenin, the curve resembles that of glutenin but with a distinct effect of gliadin fraction which then brought about further decay. The figure 8 is the curves for gluten, the individual fractions and the different mixture ratios of the fractions. This puts into clear and direct perspective the behaviour of these constituents.

The curves as seen from Figure 8 follow the expected trend of relaxation. Gluten exhibited minimum decay while the maximum decay was observed in gliadin. As previously mentioned, all samples investigated are of high purity with minimal amount of fat and carbohydrates which is very important for the purpose material characterization. Therefore the relaxation behaviour that was observed in the relaxation measurement can be comfortably accredited to proteins in the network and not to starch or lipids or any other substance that may be present as contaminants during the isolation steps. The presence of starch is of particular importance as it could act as non-interacting filler and change substantially the mechanical responses of the samples. Absence of starch was also confirmed with microscopic imaging that will be discussed later.

From the relaxation experiment it was observed that there was decrease in relaxation modulus with increase in the gliadin ratio as seen in the 75%glaidin/25%glutenin mixture. A

rise in elasticity G(t) was observed with increase in the glutenin ratio as seen in 75%glutenin/25%gliadin mixture which is a strong inverse to the 75%glaidin/25%glutenin mixture. The decrease in G(t) with increase in gliadin ratio may be attributed to a plasticising effect of gliadin. The result is an indication that the relative proportions of gliadin and glutenin is of significance in governing the rheological properties of whole gluten.

At rest before application of the step strain protein chains are in a non-deformed state forming macromolecular entanglements that interact with non-covalent forces creating transient binding partners (mostly by hydrogen bonding) and are further cross-linked with disulphide bridges. The system attempts to reach a similar energetic state during relaxation in an effort to reach thermodynamic equilibrium. During application of step strain internal stresses are generated immediately in the individual protein chains as well as in the entire network due to their interconnectivity. Differences in rigidity are reflected in the difference observed in the shear modulus values G(t) of the various samples. However, it was very difficult to establish the differences that exist between the samples judging from the shape of the relaxation curves as G(t) ranges within one logarithmic cycle at the same temperature. As mentioned earlier, gliadin-enriched samples show somewhat lower G(t) values when compared to the rest of the samples, something that was generally expected due to its viscosity. Two notable common features can be distinguished in all samples studied in this work. Primarily, in the transient phase of the measurement there is a continuous gradual decrease in shear modulus failing to reach equilibrium value (G_e). Double logarithmic plots of relaxation modulus vs. time usually show a plateau the extent of which increases with molecular weight for most synthetic polymers whereas for low Mw counterparts G_e virtually disappears. It should be noticed however that unlinked macromolecules may show complete relaxation to $G_e = 0$ if they are left to relax long enough (Mezger, 2011).

To gain further insights into the relaxation processes of gluten superstructures it was crucial to progress from a qualitative to quantitative description of the relevant relaxation events. This method will allow us to better understand the contribution of various relaxation mechanisms of gluten as this helps in calculating exact times and magnitudes at which a relaxation event occurs. This can be achieved with the generation of the L-curve and calculation of the relaxation spectra of the materials which is described below in fig 9.
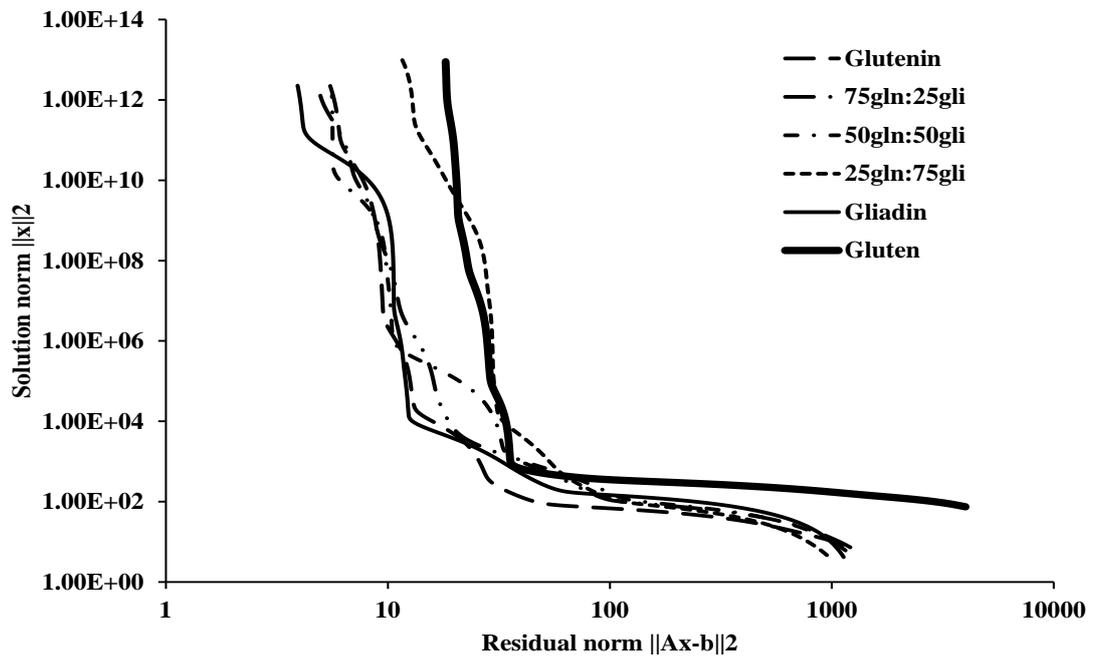
**Fig.9:** L – curves for gluten, its protein fractions and their mixtures

Relaxation spectra cannot be directly measured but can be calculated from stress relaxation data. A first-kind Fredholm integral gives the generalized function that describes stress relaxation curve:

$$g(s) = \int_o^a K(s,t)f(t)dt, 0 \leq s \leq a \text{ ……….. (iv)}$$

Where, K(s, t) is the exponential kernel exp (- t/s) that describes the decay, g(s) is the measured signal i.e., G(t), and f(t) is the unknown integral solution that represents the

relaxation spectrum of the material (Kontogiorgos et al., 2008). The limits of integration are between zero and α which is the beginning and end of the experimental time respectively. Understanding that this is an ill-posed problem, numerical computation and solution of such an integral is a demanding task and often requires a special mathematical approach. The subsequent numerical analysis of the curves requires fully decayed transients in order to calculate the relaxation spectra. Relaxation curves obtained in the previous step of the experimentation were analysed with the L-curve criterion and Tikhonov regularization using the MATLAB software. The discretization was performed between the minimum and maximum experimental time points; discretization outside the experimental timeframe will yield peaks with no physical interpretation. Calculation of the optimum regularization parameter (λ) is a necessary step as it controls the interplay between the regularization error and the loss of resolution. The optimum regularization parameter that needs to be used in the numerical analysis is located at the corner of the L-curves. As mentioned in chapter 1, identifying the right optimum regularization parameter is crucial to meaningful result. The x-axis of the curve corresponds to solutions where the calculation error controls the solution whereas the y-axis corresponds to solutions that are sensitive to experimental noise. That is, if we select λ that is greater than the optimum (to the right of the curve) the solution will be smooth with remarkable loss in resolution. On the other hand, if λ is smaller than the optimum it will result in a noisy spectrum with several artificial peaks (Hansen and O'Leary, 1993).

The resulting spectra calculated from the stress relaxation curve after numerical computation using MATLAB is represented in the figure 10. The major points of observation in the spectra are the peaks formed, the point (time) of the peak and the magnitude as well. This is useful in the interpretation of molecular rearrangements within the samples under investigation.
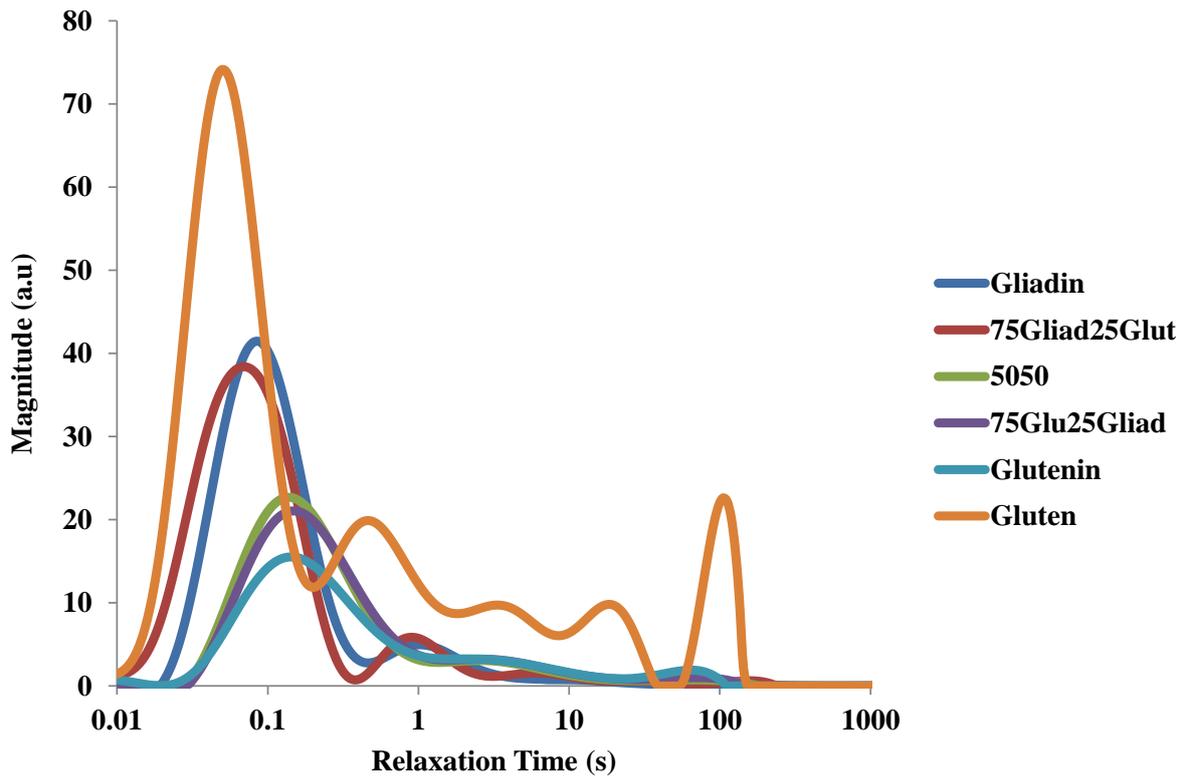
Fig.10: Relaxation spectra for gluten, its constituent protein fractions and their mixtures

In the short relaxation times regime (t < 100 s) spectral analysis reveals three relaxation mechanisms in our materials at about 0.01, 0.1 and 1 s. In the intermediate relaxation times (10^1<t<10^4 s) there is not evident any measurable relaxation response whereas two weaker final events appear at long times (>10^4 s) the intensity of which remains relatively unaffected by compositional differences.

Data handling using regularization tools reveals some interesting new features about the mechanical behaviour of gluten. Primarily, as proven by the intensity of the peaks, stress mostly relaxes in the early stages signifying that macromolecules rearrange to new configurations in less than a second. It should be stressed that intensity of peaks decreases with the rigidity of the material (i.e., gliadin content), which indicates that intensity will also vary with moisture, presence of cross-linking agents (e.g., ascorbic acid) or with composition

as in the present investigation. However, the same relaxation modes appear in all different samples and are maintained regardless of compositional differences.

Knowledge about the microstructure of biopolymers may be a clue to further understanding their mechanical properties and could answer questions about their behavioural pattern during the application of stress or in the course of deformation. Therefore having a clear insight to the microstructural differences that may exist between gluten and its protein fractions (gliadin and glutenin) is important in being able to properly account for the behavioural pattern and consequently understand its rheology. A seemingly slight alteration to their microstructure either by the addition of another component or by changing the ratio of individual fraction (as seen in the study) could mean a great change to how they behave. On that premise the microstructure of gluten and its enriched constituent fractions: gliadin and glutenin was investigated using confocal laser scanning microscope. Figure 11 shows maximum intensity z-projected images of the morphology of the specimens as protein composition changes in the mixtures. This process creates a compound image that contains the maximum value of each pixel over all images in the z-stacks at the specific pixel position.
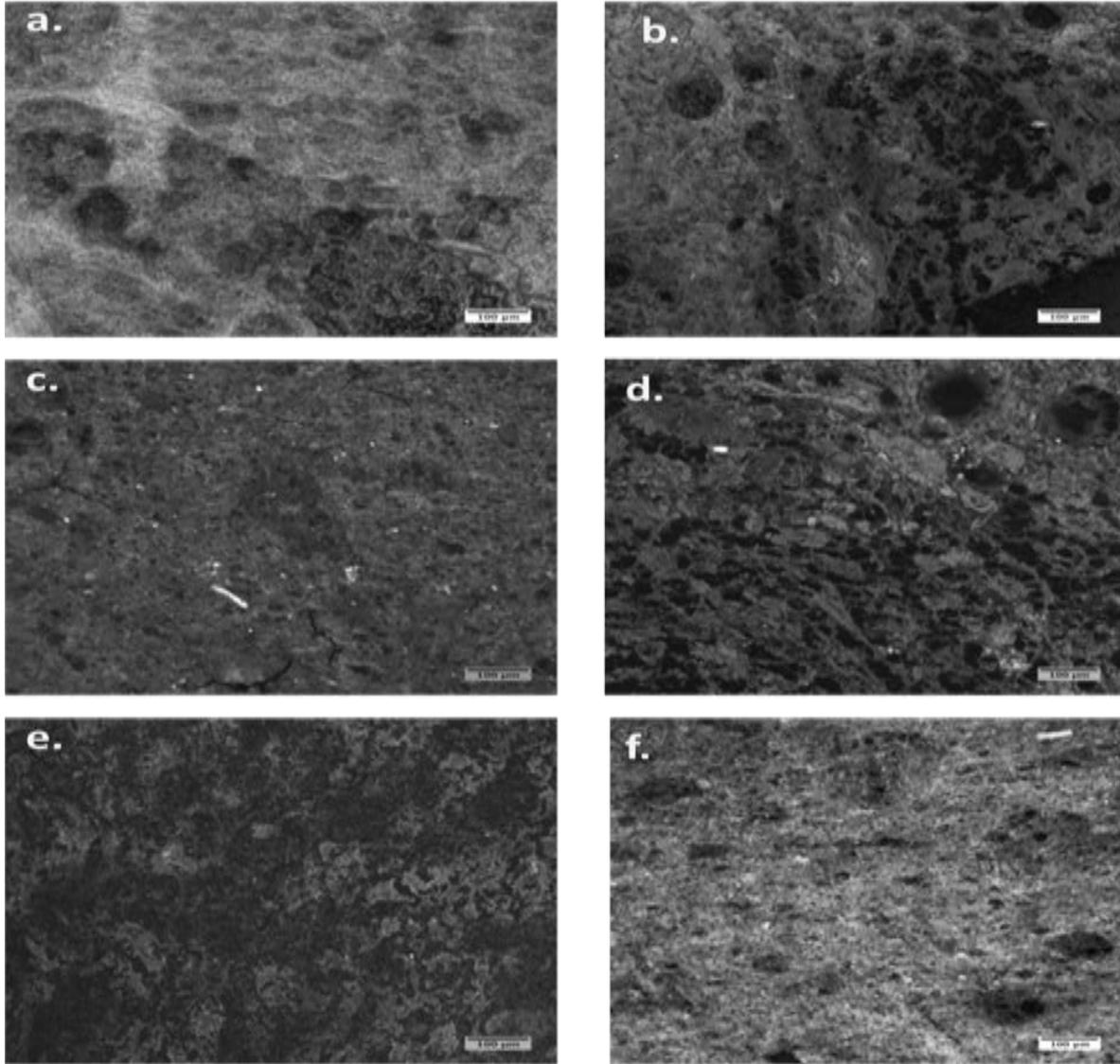
Fig. 11: CLSM images of gluten, its constituent protein factions and their mixtures. a-enriched glutenin, b- 1:3 (gliadin: glutenin respectively), c- 1:1, d- 3:1, e- enriched gliadin and f- gluten. (Scale bar: 100μm).

Glutenin exhibits dense network structure that gradually becomes sparse as the gliadin ratio increases in the mixtures. It should be emphasized that because these images are z-projected they show the network density from the top to the bottom of the image plane. Therefore, the dark areas in the images correspond to void spaces throughout the structure and they get larger with higher gliadin concentration. This results in weakening of the superstructures which to some degree was also verified by the rheological data. Gluten

morphology (Fig. 11f) and relaxation spectrum (Fig.10) are quite similar to the sample with 1:1 Gln-Gli ratio something that can be easily rationalized by the fact that gluten is composed of approximately equal quantities of glutenin and gliadin. Combining network morphology and rheological data it appears that although network microstructure varies dramatically with composition it does not contribute to changes in the relaxation modes of the biopolymers. It is important however to note that this argument stands for short relaxation times considered in this work and it may be important to investigate what happens during a longer relaxation time.

Chapter 4

4.   Conclusion and future work

4.1   Conclusions

Stress relaxation behaviour of hydrated gluten and its constituent protein fractions were investigated over the period of 30 min at 20°C. The Shear strain amplitude sweep experiments were performed using angular frequency of 6.28 rad/s to determine the linear viscoelastic region (LVR) of the samples which showed that 2% strain will give satisfactory S/N ratio. This was used in the relaxation experiments. Samples exhibited relaxation modulus $G(t)$ ranging from 1 to about 1000 Pa with the gliadin fraction showing almost full decay and gluten showing minimum decay. Gliadin is thought to have exhibited such decay because of its high level of viscosity and the elasticity of glutenin was seen to have given a bit more stability to deformation when compared with gliadin. A very interesting interplay is observed in the relaxation behaviour of the different mixtures as seen in figure 8. The 50:50 mixture of individual protein fraction which was expected to have a relaxation pattern similar to gluten showed a bit of variation. This is thought to have been the effects of the fractionation procedure which may have slightly alter the original texture of the proteins.

Data from the relaxation experiment were treated using MATLAB programming language in combination with regularization algorithms to generate the regularization parameter (λ) and hence the spectral analysis which identified in total five dominant relaxation modes with baseline resolution.

However as observed from the analysis, stress relaxation is somewhat independent of compositional differences, although, confocal microscopy showed the influence of protein composition on the morphology of the networks. Therefore, morphology and relaxation dynamics seem to be controlled by independent mechanisms for gluten networks. These

findings provide a fundamental understanding of gluten dynamics and may allow controlling the industrial performance of flours and engineering novel dough formulations.

4.2   Future Work

With results and observations from this present investigation, more interesting investigations can be carried out to further give critical insight into the relaxation behaviour of gluten and its protein constituents. Such works may include:

a. The first major area of further work may include carrying out the experiments at different temperatures to determine the effect of temperature on relaxation.

b. This work was carried out using flour from only one particular wheat source. Understanding that protein content and quality may differ between wheat sources, it may therefore be worth investigating to find out if there will be difference in stress relaxation and relaxation spectra between different types of wheat flour.

c. This work has only looked at the effect of different mixing ratios on the behavioural patter of gluten fractions but kept the water content constant throughout the experiment. It will also be important to investigate the effect of different water content to see how this affects the rheology and mechanical properties of gluten and its components.

References

AGARWAL, V. 2003. Total Variation Regularization and L-curve method for the selection of regularization parameter. ECE599

ANJUM, F. M., KHAN, M. R., DIN, A., SAEED, M., PASHA, I. & ARSHAD, M. U. 2007. Wheat Gluten: High Molecular Weight Glutenin Subunits—Structure, Genetics, and Relation to Dough Elasticity. *Journal of Food Science,* 72**,** R56-R63.

ANONYMOUS 2012. Disulphide bond formation. *http://celiac2051.wordpress.com/about/* [Accessed 24[th] April,2013]

ATCHISON, J., HEAD, L. & GATES, A. 2010. Wheat as food, wheat as industrial substance; comparative geographies of transformation and mobility. *Geoforum,* 41**,** 236-246.

BELDEROK, B. 2000. Developments in bread-making processes. *Plant Foods for Human Nutrition,* 55**,** 1-14.

BELDEROK, B., MESDAG, H. & DONNER, D. A. 2000. *Bread-Making Quality of Wheat,* New York, Springer.

BELITZ, H. D., KIEFFER, R., SEILMEIER, W. & WIESER, H. 1986. Structure and Function of Gluten Proteins1. *Journal of Cereal Chemistry,* 63**,** 336-341.

BILLMEYER, F. W. 1984. *Textbook of Polymer Science,* New York, John Wiley & Sons.

BONFIL, D. J. & POSNER, E. S. 2012. Can bread wheat quality be determined by gluten index? *Journal of Cereal Science,* 56**,** 115-118.

BURGER, M. & NEUBAUER, A. 2003. Analysis of Tikhonov regularization for function approximation by neural networks. *Neural Networks,* 16**,** 79-90.

BYERLEE, D. & DE POLANCO, E. H. 1983. Wheat in the world food economy: Increasing role in developing countries. *Food Policy,* 8**,** 67-75.

CALVETTI, D., MORIGI, S., REICHEL, L. & SGALLARI, F. 2000. Tikhonov regularization and the L-curve for large discrete ill-posed problems. *Journal of Computational and Applied Mathematics,* 123**,** 423-446.

COWIE, J. M. G. & ARRIGHI, V. 2008. *Polymer: Chemistry and Physics of Modern Materials,* USA, CRC press.

D'OVIDIO, R. & MASCI, S. 2004. The low-molecular-weight glutenin subunits of wheat gluten. *Journal of Cereal Science,* 39**,** 321-339.

DAY, L., AUGUSTIN, M. A., BATEY, I. L. & WRIGLEY, C. W. 2006. Wheat-gluten uses and industry needs. *Trends in Food Science & Technology,* 17**,** 82-90.

DECAPUA, J. 2012. Boosting Africa's Wheat Production. Voice of America.

DOBRASZCZYK, B. J. 2004. The physics of baking: rheological and polymer molecular structure–function relationships in breadmaking. *Journal of Non-Newtonian Fluid Mechanics,* 124**,** 61-69.

DOBRASZCZYK, B. J. & MORGENSTERN, M. P. 2003. Rheology and the breadmaking process. *Journal of Cereal Science,* 38**,** 229-245.

DUPONT, F. M. & ALTENBACH, S. B. 2003. Molecular and biochemical impacts of environmental factors on wheat grain development and protein synthesis. *Journal of Cereal Science,* 38**,** 133-146.

DUTTA, T., KAUR, H., SINGH, S., MISHRA, A., TRIPATHI, J. K., SINGH, N., PAREEK, A. & SINGH, P. 2011. Developmental changes in storage proteins and peptidyl prolyl cis–trans isomerase activity in grains of different wheat cultivars. *Food Chemistry,* 128**,** 450-457.

FELLERS, D. A. 1979. WHEAT AND WHEAT FOODS IN THE TROPICS. *In:* GEORGE, I. (ed.) *Tropical Food: Chemistry and Nutrition.* Academic Press.

FISCHER, P., POLLARD, M., ERNI, P., MARTI, I. & PADAR, S. 2009. Rheological approaches to food systems. *Comptes Rendus Physique,* 10**,** 740-750.

FISCHER, P. & WINDHAB, E. J. 2011. Rheology of food materials. *Current Opinion in Colloid & Interface Science,* 16**,** 36-40.

FLAGELLA, Z., GIULIANI, M. M., GIUZIO, L., VOLPI, C. & MASCI, S. 2010. Influence of water deficit on durum wheat storage protein composition and technological quality. *European Journal of Agronomy,* 33**,** 197-207.

GEORGOPOULOS, T., LARSSON, H. & ELIASSON, A.-C. 2004. A comparison of the rheological properties of wheat flour dough and its gluten prepared by ultracentrifugation. *Food Hydrocolloids,* 18**,** 143-151.

GOESAERT, H., BRIJS, K., VERAVERBEKE, W. S., COURTIN, C. M., GEBRUERS, K. & DELCOUR, J. A. 2005. Wheat flour constituents: how they impact bread quality, and how to impact their functionality. *Trends in Food Science & Technology,* 16**,** 12-30.

GUNDUZ, G. 2009. VISCOELASTIC PROPERTIES OF NETWORKS. *International Journal of Modern Physics C: Computational Physics & Physical Computation,* 20**,** 1597-1615.

HANSEN, P. C. 2000. The L-curve and its use in the numerical treatment of inverse problems. In: Johnston P, editor. Computational inverse problems in electrocardiology. Southampton: WIT Press; 2001. p 119–142

HANSEN, P. C. & O'LEARY, P. D. 1993. THE USE OF THE L-CURVE IN THE

REGULARIZATION OF DISCRETE ILL-POSED PROBLEMS. *SIAM Journal on Scientific Computing* 14**,** 1487-1503.

HAYTA, M. & SCHOFIELD, J. D. 2005. Dynamic rheological behavior of wheat glutens during heating. *Journal of the Science of Food and Agriculture,* 85**,** 1992-1998.

HU, X. & SHANG, Y. 2007. A new testing method for vital gluten swelling index. *Journal of the Science of Food and Agriculture,* 87**,** 1778-1782.

IRIN 2013. FOOD: We want wheat - Africa's growing cereal demand. http://www.irinnews.org/report/96510/ [Accessed 12[th] May,2013].

JANSSEN, A. M., VAN VLIET, T. & VEREIJKEN, J. M. 1996. Rheological Behaviour of Wheat Glutens at Small and Large Deformations. Comparison of Two Glutens Differing in Bread Making Potential. *Journal of Cereal Science,* 23**,** 19-31.

JIANG, X.-L., TIAN, J.-C., HAO, Z. & ZHANG, W.-D. 2008. Protein Content and Amino Acid Composition in Grains of Wheat-Related Species. *Agricultural Sciences in China,* 7**,** 272-279.

KHATKAR, B. S., BARAK, S. & MUDGIL, D. 2013. Effects of gliadin addition on the rheological, microscopic and thermal characteristics of wheat gluten. *International Journal of Biological Macromolecules,* 53**,** 38-41.

KHATKAR, B. S., BELL, A. E. & SCHOFIELD, J. D. 1995. The Dynamic Rheological Properties of Glutens and Gluten Sub-Fractions from Wheat of Good and Poor Bread Making Quality. *Journal of Cereal Science,* 22**,** 29-44.

KOEHLER, P., HARTMANN, G., WIESER, H. & RYCHLIK, M. 2007. Changes of folates, dietary fiber, and proteins in wheat as affected by germination. *Journal of Agricultural and Food Chemistry,* 55**,** 4678-4683.

KONTOGIORGOS, V., JIANG, B. & KASAPIS, S. 2008. Numerical computation of relxation spectra from mechanical measurements in biopolymers. *Food research international,* 42**,** 130-136.

KONTOGIORGOS, V. & KASAPIS, S. 2010. Temperature dependence of relaxation spectra for highly hydrated gluten networks. *Journal of Cereal Science,* 52**,** 100-105.

LABUSCHAGNE, M. T., ELAGO, O. & KOEN, E. 2009. The influence of temperature extremes on some quality and starch characteristics in bread, biscuit and durum wheat. *Journal of Cereal Science,* 49**,** 184-189.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227**,** 680-685.

LAFIANDRA, D., MASCI, S. & D'OVIDIO, R. 2004. *The Gluten Proteins,* Cambridge, The Royal Society of Chemistry.

LAMPE, J. & VOSS, H. 2013. Large-scale Tikhonov regularization of total least squares. *Journal of Computational and Applied Mathematics,* 238**,** 95-108.

LU, C. & FAN, L. 2013. Winter wheat yield potentials and yield gaps in the North China Plain. *Field Crops Research,* 143**,** 98-105.

LUO, Q., WILLIAMS, M. A. J., BELLOTTI, W. & BRYAN, B. 2003. Quantitative and visual assessments of climate change impacts on South Australian wheat production. *Agricultural Systems,* 77**,** 173-186.

LV, J., LU, Y., NIU, Y., WHENT, M., RAMADAN, M. F., COSTA, J. & YU, L. 2013a. Effect of genotype, environment, and their interaction on phytochemical compositions and antioxidant properties of soft winter wheat flour. *Food Chemistry,* 138**,** 454-462.

LV, Z., LIU, X., CAO, W. & ZHU, Y. 2013b. Climate change impacts on regional winter wheat production in main wheat production regions of China. *Agricultural and Forest Meteorology,* 171–172**,** 234-248.

MATHWORKS. 2013. *MATLAB- The Language of Technical Computing* [Online]. Available: http://www.mathworks.co.uk/products/matlab/ [Accessed 11th April,2013].

MCCANN, T. H., SMALL, D. M., BATEY, I. L., WRIGLEY, C. W. & DAY, L. 2009. Protein-lipid interactions in gluten elucidated using acetic acid fractionation. *Food Chemistry,* 115**,** 105-112.

MEZGER, G. T. 2011. *The Rheology hand book,* Hanover, Vincentz Network.

MIMOUNI, B., ROBIN, J. M., AZANZA, J.-L. & RAYMOND, J. 1998. Wheat flour proteins: isolation and functionality of gliadin and HMW-glutenin enriched fractions. *Journal of the Science of Food and Agriculture,* 78**,** 423-428.

MOHAMMED, I., AHMED, A. R. & SENGE, B. 2012. Dough rheology and bread quality of wheat–chickpea flour blends. *Industrial Crops and Products,* 36**,** 196-202.

NEUMAN, A., REICHEL, L. & SADOK, H. 2012. Implementations of range restricted iterative methods for linear discrete ill-posed problems. *Linear Algebra and its Applications,* 436**,** 3974-3990.

ORTIZ, R., SAYRE, K. D., GOVAERTS, B., GUPTA, R., SUBBARAO, G. V., BAN, T., HODSON, D., DIXON, J. M., IVÁN ORTIZ-MONASTERIO, J. & REYNOLDS, M. 2008. Climate change: Can wheat beat the heat? *Agriculture, Ecosystems & Environment,* 126**,** 46-58.

POMERANZ, Y. 1968. Relation Between Chemical Composition and Bread-Making Potentialities of Wheat Flour. *In:* C.O. CHICHESTER, E. M. M. & STEWART, G. F. (eds.) *Advances in Food Research.* Academic Press.

PRANDI, B., BENCIVENNI, M., FACCINI, A., TEDESCHI, T., DOSSENA, A., MARCHELLI, R., GALAVERNA, G. & SFORZA, S. 2012. Composition of peptide mixtures derived from simulated gastrointestinal digestion of prolamins from different wheat varieties. *Journal of Cereal Science,* 56**,** 223-231.

PRESTON, K. R. & WILLIAMS, P. C. 2003. FLOUR | Analysis of Wheat Flours. *In:* EDITOR-IN-CHIEF: BENJAMIN, C. (ed.) *Encyclopedia of Food Sciences and Nutrition (Second Edition).* Oxford: Academic Press.

RAO, M. A., . 2007. *Rheology of Fluids and Semisolid Foods,* New York, Springer.

REZGHI, M. & HOSSEINI, S. M. 2009. A new variant of L-curve for Tikhonov regularization. *Journal of Computational and Applied Mathematics,* 231**,** 914-924.

ROUSSEL, V., BRANLARD, G., VÉZINE, J. C., BERTRAND, D. & BALFOURIER, F. 2005. NIRS analysis reveals temporal trends in the chemical composition of French bread wheat accessions cultivated between 1800 and 2000. *Journal of Cereal Science,* 42**,** 193-203.

SANTOS, E. T. F. & BASSREI, A. 2007. L- and Θ-curve approaches for the selection of regularization parameter in geophysical diffraction tomography. *Computers & Geosciences,* 33**,** 618-629.

SAULNIER, L., GUILLON, F. & CHATEIGNER-BOUTIN, A.-L. 2012. Cell wall deposition and metabolism in wheat grain. *Journal of Cereal Science,* 56**,** 91-108.

SCHOFIELD, J. D. 1994. Wheat Proteins: structure and functionality. *In:* BUSHUK, W. R., F.V. (ed.) *Wheat: Production, Properties and Quality.* USA: Springer.

SHEWRY, P. R. 1999. The synthesis, processing, and deposition of gluten proteins in the developing wheat grain. *Cereal Foods World,* 44**,** 587-589.

SHEWRY, P. R. 2009. Wheat. *Journal of Experimental Botany,* 60**,** 1537–1553.

SHEWRY, P. R., HALFORD, N. G., BELTON, P. S. & TATHAM, A. S. 2002a. The structure and properties of gluten: an elastic protein from wheat grain. *The Royal Society,* 357**,** 133-142.

SHEWRY, P. R., HALFORD, N. G., BELTON, P. S. & TATHAM, A. S. 2002b. The structure and properties of gluten: an elastic protein from wheat grain. *The Royal Society, London.* 357, 133-142.

SHEWRY, P. R., HAWKESFORD, M. J., PIIRONEN, V., LAMPI, A.-M., GEBRUERS, K., BOROS, D., ANDERSSON, A. A. M., ÅMAN, P., RAKSZEGI, M., BEDO, Z. & WARD, J. L. 2013. Natural Variation in Grain Composition of Wheat and Related Cereals. *Journal of Agricultural and Food Chemistry,* 61**,** 8295-8303.

SHEWRY, P. R. & LOOKHART, G. L. 2003. *Wheat gluten protein analysis,* USA, AACC International.

SHEWRY, P. R., MIFLIN, B. J., LEW, E. J. L. & KASARDA, D. D. 1983. The preparation and characterization of an aggregated gliadin fraction from wheat. *Journal of Experimental Botany,* 34**,** 1403-1410.

SHEWRY, P. R. & TATHAM, A. S. 1997. Disulphide bonds in wheat gluten proteins. *Journal of Cereal Science,* 25**,** 207-227.

SHEWRY, P. R., TATHAM, A. S., FORDE, J., KREIS, M. & MIFLIN, B. J. 1986. The classification and nomenclature of wheat gluten proteins: A reassessment. *Journal of Cereal Science,* 4**,** 97-106.

SHEWRY, P. R., UNDERWOOD, C., WAN, Y., LOVEGROVE, A., BHANDARI, D., TOOLE, G., MILLS, E. N. C., DENYER, K. & MITCHELL, R. A. C. 2009. Storage product synthesis and accumulation in developing grains of wheat. *Journal of Cereal Science,* 50**,** 106-112.

SINGH, S., GUPTA, A. K., GUPTA, S. K. & KAUR, N. 2010. Effect of sowing time on protein quality and starch pasting characteristics in wheat (Triticum aestivum L.) genotypes grown under irrigated and rain-fed conditions. *Food Chemistry,* 122**,** 559-565.

SLAVIN, J. 2010. Whole grains and digestive health. *Cereal Chemistry,* 87**,** 292-296.

SONG, Y. & ZHENG, Q. 2007. Dynamic rheological properties of wheat flour dough and proteins. *Trends in Food Science and Technology,* 18**,** 132-138.

SRAMKOVA, Z., GREGOVA, E. & STURDIK, E. 2009. Chemical composition and Nutritional quality of wheat grain. *Acta Chimica Slovaca,* 2**,** 115-138.

SWANSTON, J. S., SMITH, P. L., AGU, R. C., BROSNAN, J. M., BRINGHURST, T. A. & JACK, F. R. 2012. Variation, across environments within the UK, in grain protein and grain hardness, in wheat varieties of differing distilling quality. *Field Crops Research,* 127**,** 146-152.

TABILO-MUNIZAGA, G. & BARBOSA-CÁNOVAS, G. V. 2005. Rheology for the food industry. *Journal of Food Engineering,* 67**,** 147-156.

TATHAM, A. S. & SHEWRY, P. R. 2012. The S-poor prolamins of wheat, barley and rye: Revisited. *Journal of Cereal Science,* 55**,** 79-99.

TOSI, P. 2012. Trafficking and deposition of prolamins in wheat. *Journal of Cereal Science,* 56**,** 81-90.

TURI, E. A. 1981. *Thermal Characterisation of Polymeric Materials,* London, Academic Press.

VAN DER BORGHT, A., GOESAERT, H., VERAVERBEKE, W. S. & DELCOUR, J. A. 2005. Fractionation of wheat and wheat flour into starch and gluten: overview of the main processes and the factors involved. *Journal of Cereal Science,* 41**,** 221-237.

VAN ECKERT, R., BERGHOFER, E., CICLITIRA, P. J., CHIRDO, F., DENERY-PAPINI, S., ELLIS, H. J., FERRANTI, P., GOODWIN, P., IMMER, U., MAMONE, G., MÉNDEZ, E., MOTHES, T., NOVALIN, S., OSMAN, A., RUMBO, M., STERN, M., THORELL, L., WHIM, A. & WIESER, H. 2006. Towards a new gliadin

reference material–isolation and characterisation. *Journal of Cereal Science,* 43**,** 331-341.

VENSEL, W. H., DUPONT, F. M., SLOANE, S. & ALTENBACH, S. B. 2011. Effect of cleavage enzyme, search algorithm and decoy database on mass spectrometric identification of wheat gluten proteins. *Phytochemistry,* 72**,** 1154-1161.

WARD, I. M. & SWEENEY, J. 2004. *An Introduction to Mechanical properties of Solid Polymers,* England, Wiley.

WIESER, H. 2006. Chemistry of gluten proteins. *Food Microbiology,* 24**,** 115-119.

WIESER, H. & KIEFFER, R. 2001. Correlations of the amount of gluten protein types to the technological properties of wheat flours determined on a micro-scale. *Journal of Cereal Science,* 34**,** 19-27.

WRIGLEY, C. W. & BEITZ, J. A. 1998. *Proteins and amino acids. In:Pomeranz Y. (Ed), Wheat: Chemistry and Technology,* USA, American Association of Cereal Chemistry.

XU, J., BIETZ, J. A. & CARRIERE, J. C. 2006. Viscoelastic properties of wheat gliadin and glutenin suspensions. *Food Chemistry,* 101**,** 1025-1030.

XUE, A. G., CHI, D. T., SHU-ZHEN, Z. & ZUO-FU, L. 2012. Wheat Production and Wheat Rust Management in Canada. *Journal of Northeast Agricultural University (English edition),* 19**,** 1-14.

YANG, Y., SONG, Y. & ZHENG, Q. 2011. Rheological behaviors of doughs reconstituted from wheat gluten and starch. *Journal of Food Science and Technology,* 48**,** 489-493.

YILDIZ, B., SUBASI, M. & SEVER, A. 2000. On a regularization problem. *Applied Mathematics and Computation,* 109**,** 67-72.

ZAIDEL, D. N. A., CHIN, N. L. & YUSOF, Y. A. 2010. A review on rheological properties and measurements of dough and gluten. *Journal of Applied Sciences,* 10**,** 2478-2490.

ZHANG, W., SANGTONG, V., PETERSON, J., SCOTT, M. P. & MESSING, J. 2013. Divergent properties of prolamins in wheat and maize. *Planta*, 1-9.

ZHAO, C.-X., HE, M.-R., WANG, Z.-L., WANG, Y.-F. & LIN, Q. 2009. Effects of different water availability at post-anthesis stage on grain nutrition and quality in strong-gluten winter wheat. *Comptes Rendus Biologies,* 332, 759-764.