Spatial patterns of wheat grain composition in relation to nutritional quality and processing properties

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Abstract

The quality of wheat-based food products, especially bread quality, is significantly affected by the characteristics of the wheat flour, both in term of processing quality and nutritional value. White flour, by far the most used flour in breadmaking, derives from grinding of the grain starchy endosperm. Several studies have demonstrated that the endosperm tissue shows significant inhomogeneity in its chemical composition, with both quantitative and qualitative patterns of tissue distribution observed for all its main storage components.

This project has focused on a detailed study of the spatial distributions of proteins and starch within the wheat endosperm, how they are influenced by genetic and environmental factors and how they affect the processing properties of the flour. Multiple technologies, including protein/starch chemistry, immunocytochemistry and microscopy, have been used to achieve the research aims.

The results show a clear deceasing gradient in total protein in the endosperm from the subaleurone toward the centre of the grain. Strong spatial gradients were also observed in the composition of gluten proteins. These patterns were best visualised in *vivo* by Immunofluorescence microscopy. The SE-HPLC profiles, which can be used to predict processing quality, also showed gradients across the grain.

The pattern of distribution of starch showed an opposite trend to that of proteins, being higher in the central endosperm cells than in subaleurone cells.

The amylose/amylopectin ratio also varied spatially in grain of some of the cultivars.

Strong genotype effects were found for all parameters that were measured. Nitrogen fertilisation was found to have a strong impact on the amount of protein (both quantity and quality) and their distributions. Moreover, it also influenced, albeit to a much smaller extent, the amount of starch.

The detailed information on protein and starch distribution within endosperm and effects of genotype and nitrogen fertiliser from this study provided knowledge that would lead to greater understanding of protein and starch synthesis as well as nutrition transport within grain. It is also potentially beneficial for improving milling technology and selecting breeding candidates for different purposes.

Declaration

I declare that this dissertation is my own work; all results are originally obtained from my experiments, except for total nitrogen content, SE-HPLC, starch damage and particle size distribution test, the prepared samples were tested by professional analytical teams of Rothamsted research and Campden BRI using confidential industrial protocols , all materials from other sources used in this dissertation are properly referenced.

Jibin He

Dedication

To my family,

without you, all of this would be impossible.

List of publications/presentations/posters

Publications:

Tosi, P., Gritsch, C. S., He, J.B., Shewry, P.R. (2011). "Distribution of gluten proteins in bread wheat (Triticum aestivum) grain." Annals of Botany **108**(1): 23-35.

Conference presentations:

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List of Abbreviations

ADPG: adenosine diphosphateglucose

AX: arabinoyxlans

BE: branching enzymes

BSA: bovine serum albumin

Con A: concanavalin A

DMSO: Dimethyl sulphoxide

DPA (d.p.a.): days after anthesis

DSC: differential scanning calorimetry

DTT: dithiothreitol

FAO: Food and Agriculture Organization

GBSS: granule-bound starch synthase

GI: gluten index

HMW-GS: high molecular weight gluten subunits

IMA: integrated Morphometry

L.S.D.: Least significant differences of means

LMW-GS: low molecular weight gluten subunits

NIR: Near-infrared reflectance

QTL: quantitative trait loci

PAGE: polyacrylamide gel electrophoresis

PDCAAS: Protein Digestibility Corrected Amino Acid Score

S.D.: standard deviation

SDS: sodium dodecyl sulfate

SE-HPLC: size-exclusion high performance liquid chromatography

SKCS: single-kernel characterization system

SS: starch synthase

TA: total area (in a SE-HPLC profile)

TBS: Tris-buffered saline

TKW: thousand kernel weight

TPC: total protein content

TSC: total starch content

TTBS: Tween-Tris buffered saline

Chapter 1: General Introduction

1.1 General introduction

Wheat is a major global source of human food and livestock feed. FAO figures (http://faostat.fao.org) show that, the annual production of wheat for the years 2004-2010 was over 600 million tonnes, equivalent to more than 25% of the total annual production of cereals, representing therefore a major source of carbohydrate and dietary protein for human nutrition. Based on FAO data, cereals contributed more than 45% to global food supply (kcal/capita/day) and more than 40% of the global protein supply (g/capita/day) in the past decade. In 2010, wheat production exceeded 650 million tonnes, and ranked as the third highest production after maize (840 million tonnes) and rice (696 million tonnes).

The term "wheat" is used to describe several species belonging to the genus *Triticum* in the *Gramineae* family. From a botanical perspective, wheat is classified as diploid, tetraploid or hexaploid, depending on the number of pairs of chromosomes present in wheat somatic cells. About 95% of modern wheat is hexaploid bread wheat; most of the remaining 5% is tetraploid durum wheat, which more is adapted to the dry Mediterranean climate and mostly used to produce pasta (Shewry, 2009). Commercially, hexaploid wheat is classified based on grain properties and plant *habitus*. Broadly, wheat can be classified as "spring" or "winter" wheat, based on the requirement for vernalisation; as "hard" or "soft" wheat based on grain texture; as "strong" or "weak" based on dough strength; as "red" or "white" based on bran colour (Orth and Shellenberger,

1988). In different countries, different classification systems are used for cultivation and trading proposes. In the USA, wheat is catalogued into six classes: durum, hard red spring, hard red winter, hard white, soft red winter, soft white (http://www.uswheat.org). In the UK, wheat is classified into four groups based on variety, cultivation, and end use properties (http://www.hgca.com). Group 1 varieties are high protein wheat with strong dough properties most suitable for breadmaking. Group 2 wheats are also breadmaking wheats, but command lower prices because of their inherent inconsistency or specific characteristics. Group 3 are mainly soft wheats with lower protein contents and are suitable for the production of biscuits, cakes and other products that require weak or highly extensible gluten. Group 4 wheats are generally used for livestock feed. A specific classification is also used in the UK for grading wheats for export: "ukp" is for semi-hard wheats to suit bread making worldwide and "uks" is for soft wheats used for biscuit making or blending for bread making (http://www.hgca.com).

Wheat has been very successful as a crop, partly due to its adaptability and high yields (Shewry, 2009). On the other hand, the unique visco-elastic properties of dough formed from wheat flour facilitate the wide use of wheat in the food industry. Based on its properties, wheat flour dough can be processed into various breads and other baked products including biscuits, cakes and pastries. The properties of dough are affected by many characteristics of the flour including protein content, starch damage, water absorption and enzyme activity level. These properties can be controlled by cultivar selection, agronomical practices, milling processes, the use of various additives and pre-treatment of flour.

1.2 Wheat quality and grading

There is no clear definition of wheat quality because the criteria for quality vary depending on different end uses (Halverson and Zeleny, 1988). The criteria are normally catalogued into "physical characteristics" and "chemical characteristics" (Halverson and Zeleny, 1988, Cornell and Hoveling, 1998). Various grading systems are used in different countries to classify wheat grain for trading. Although not all characteristics of wheat are considered as grading factors, they nonetheless may still influence the quality of wheat to some extent (for example, water content). The criteria for wheat quality are an extensive topic and include many aspects. In the following sections will focus on aspects of quality more directly related to breadmaking.

1.2.1 Physical characteristics

Test weight and grain weight

Test weight measures wheat density by weighing a known volume of the grain. Commonly used units for test weight include kilograms per hectoliter, pounds per Winchester bushel (2,250.42 in^3) and pounds per Imperial bushel (2,219.36 in^3). Test weight is one of the primary grading factors because it gives an indication of flour yield for wheat with density values below 73.4kg/hl (Halverson and Zeleny, 1988); above this value, however, the relationship between test weight and flour yield became insignificant. and other properties are more commonly used to predict flour yield (Carson and Edwards, 2009). Therefore, the requirement for test weight in grading systems is usually expressed as a

minimum test weight for each class of wheat. Test weight is greatly influenced by the environment and other characteristics such as grain shape, uniformity of grain size and shape, protein content and grain hardness. (Halverson and Zeleny, 1988, Carson and Edwards, 2009, Cornell and Hoveling, 1998). It can also be affected by class, but to a lesser extent (Carson and Edwards, 2009).

Grain weight, which can be expressed as thousand kernel weight (TKW), is also often used to describe wheat quality and considered as grading factor in some country such as Australia. It also could be expressed as single kernel weight when tested by Single Kernel Characterization System (SKCS), which was used in this project. Unlike test weight, grain weight is not a function of grain density, but simply indicates the average mass of one/thousand grains. Recent study found quantitative trait loci (QTLs) for TKW (Ramya et al., 2010), indicating that grain weight has an important genetic determinant. Also, it might be a better indicator than test weight for flour yield as grain weight/TKW is more independent than test weight from environmental factors.

Grain size and shape

Grain size is an important factor for wheat quality although it is not considered in most grading system. It is expected to influence the endosperm content and therefore affect flour yield. However, Evers (2000) reported that endosperm content varied little among different sized grains (Evers, 2000). He also concluded that grain size may influence protein quality and α- amylase activity, but the reasons for this remain unknown (Evers, 2000). In wheat, grain size varies greatly. However, modern bread wheats tend to have larger grains due to domestication as increasing grain size is considered as a major selection and

breeding target (Gegas et al., 2010). Grain size also can vary significantly among grains from a same plant and even within the same ear. Environmental factors may also influence the grain size (Evers, 2000).

Grain shape has become a breeding target in recently years as industry has started to recognise that rounder grain may give higher milling yields (Gegas et al., 2010). The mechanism behind this phenomenon is still unclear, although it is possibly related to differences in the distribution of the stresses applied to the grain during milling and their effect on the first break (Vincent et al., 2000). Although grain shape is often discussed together with grain size as aspects of wheat grain morphology, they have been shown to be independent traits in primitive and modern wheats (Gegas et al., 2010).

Grain hardness

Grain hardness is the physical resistance to particle size reduction of the wheat grain performed by a crushing/shearing force (Carson and Edwards, 2009). As would be expected, grain hardness is strongly associated with energy input during milling process. Because harder forces are required, the milling of hard wheat results in a higher level of starch damage compared to milling of soft wheat (Barlow et al., 1973). Milling of soft wheat also generates a higher amount of smaller particles than milling of hard wheat (Devaux et al., 1998). More importantly, grain hardness is strongly associated with grain protein content (Pasha et al., 2010, Belderok et al., 2000) and consequently influences the end use of flour (Figure 1.1). One of the first methods widely used to measure grain hardness is the particle size index (PSI) (AACC Method 55-30.01), which measures the proportion of material that passes through a 75μm

pores sieve after milling. A more recent and simpler method for measuring grain hardness is represented by the single-kernel characterization system (SKCS) instrument which determines the force required to crash the grain. The outcome of this measurement is named Hardness Index (AACC Method 55-31.01) and is calibrated against PSI. Near-infrared reflectance (NIR) can also be used to determine hardness, but is again calibrated against PSI (AACC Method 39-70.02).

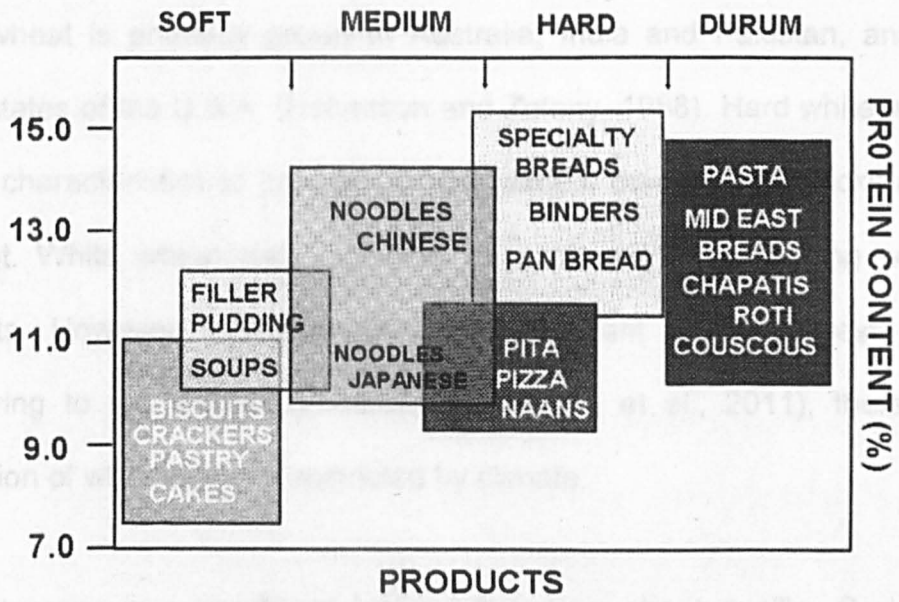

Figure 1.1 End uses of flours with various protein content and relationship to wheat hardness (Carson and Edwards, 2009)

Genetic differences in grain hardness largely result from differences in the strength of adhesion between the surface of the starch granules and the protein matrix (Pasha et al., 2010). A protein fraction present on the surface of the starch granule, termed friabilin, was recognised having a close association with grain hardness (Greenwell and Schofield, 1986). Further studies showed that grain texture is controlled by the *Hardness* (*Ha*) locus, which includes three

genes (*Pin a, Pin b* and *Gsp-1*) (Hogg et al., 2004, Beecher et al., 2002). Grain texture is also affected by environmental factors influencing protein content and deposition, such as N-fertilisation (Saint Pierre et al., 2008, Makowska et al., 2008).

Other physical characteristics affect wheat quality

Based on grain colour, wheat can be classified as either "red" or "white". Red wheat is mainly cultivated in Europe, North America and parts of Asia, while white wheat is primarily grown in Australia, India and Pakistan, and also in some states of the U.S.A. (Halverson and Zeleny, 1988). Hard white wheat has similar characteristics to hard red wheat, except that the bran lacks a reddish pigment. White wheat may therefore be preferred for producing wholemeal products. However, white wheat is less tolerant to pre-harvest sprouting comparing to red wheat (Belderok, 2000, Himi et al., 2011), therefore, the cultivation of white wheat is restricted by climate.

Grain damage is a significant factor influencing wheat quality. Grain can be damaged at several stages of crop development and in many different ways. For example, insect damage can result in loss of yield and quality. Wet weather at pre-harvest may result in sprouting, which causes reduced yield and quality due to increased enzyme activity in flour. Frost damage at different stages of grain development may result in small grain, poor protein quality, low flour yield and dark colour. Excessive heating during storage may lead to discoloration of the grain and reduced protein quality. (Halverson and Zeleny, 1988, Carson and Edwards, 2009)

Various types of contamination of the grain can occur, including contamination with other cereal grain, with mycotoxins, fertilisers, stones, inserts and metal. The contamination of wheat grain not only affects the yield and the end-use quality but can also be a significant safety issue, especially the contamination with mycotoxins such as ergot alkaloids.

1.2.2 Chemical characteristics

Moisture content

Moisture content is not a grading factor but is nevertheless important for wheat quality. As expected, moisture content significantly affects the price of wheat as only the dry matter of grain is of value. Also, excessive moisture can result in problems during storage such as spoilage, deterioration and fungal infections (Carson and Edwards, 2009). On the other hand, grains with too low a moisture content could cost more to condition because grain moisture need to be raised to 15-16.5% before milling (Cornell and Hoveling, 1998). The commonly accepted moisture level for harvesting is around 15%. Grain moisture content can be measured by numerous ways such as calculating the weight loss after dry in an oven, near-infrared reflectance/transmittance (NIR) spectroscopy and measuring the electric conductivity.

Protein content and quality

Protein content is one the most important quality characteristic of wheat, as different baking products require flours with different protein contents. Table 1.1 shows the broad differences in flour protein content required for different

products. For making yeast-leavened bread, the minimum requirement of protein content in flour is about 11%, which means a minimum protein content in grain at least 12%, as 1-1.5% protein would be lost during milling (Halverson and Zeleny, 1988). For pan bread and buns, more that 13% grain protein is recommended (Peña, 2002). Genotype is important factor for grain protein content (Monaghan et al., 2001). On another hand, environment conditions, especially nitrogen fertilisation also affect grain protein content with higher nitrogen inputs, particularly in post anthesis, resulting in higher grain protein contents. The precise application timing for optimum effect on protein concentration depends on several factors including variety, previous cropping, soil type, and rainfall/water availability (Gooding, 2009). However the benefit of high protein content are accompanied with negative effects, such as an increased risks of frost damage, lodging, foliar disease, and delayed maturation (Gooding, 2009). High nitrogen input also results in higher costs for farmers and may significantly impact on the environment causing soil and water pollution by nitrate leaching (Motavalli et al., 2008). However, the leaching problem can be minimised by carefully planning the nitrogen application.

The Protein content is most commonly determined on the basis of total nitrogen by the Kjeldahl and Dumas methods. The Kjeldahl method uses strong sulphuric acid to release nitrogen from protein by oxidation; this is then converted to ammonia gas and quantified by titration. There are two types of titration used in Kjeldahl method. The back titration uses a measured excessive standardized acid solution such as standard sulphuric acid as receiving solution. Then uses standardized alkaline base solution such as sodium hydroxide to quantify the ammonium sulfate, which is the product of reaction of ammonia and sulphuric acid. The direct titration uses boric acid as receiving

solution which would product ammonium-borate complex in reaction with ammonia, and then standard sulphuric acid can be used to quantify the ammonium-borate complex by neutralisation. The Dumas method is a combustion method in which high temperature and high oxygen concentration are used to decompose the sample to release gas composed of CO_2, H_2O, SO_2, NO_2 and N_2. The CO_2, H_2O and SO_2 are removed on a column and the nitrogen content measured by determining the thermal conductivity of the remaining gas. (Owusu-Apenten, 2002, Chang, 2010). In both methods, the values for N needed to be converted to protein content using a nitrogen-protein conversion factor. For wheat grain the most widely used conversion factor is Nx5.7 (Tkachuk, 1969, Lafiandra et al., 2000, Wrolstad et al., 2005). In terms of accuracy, many studies have been done to compare the Kjeldahl method and the Dumas method. Some suggested there are no significant differences between the two method in terms of accuracy (Thompson et al., 2004). Some studies suggested that Dumas method is more reliable for the determination of organic nitrogen (Miller et al., 2007) in particular in the case of low protein content food stuff (Wiles et al., 1998). On another hands, there is an agreement that Dumas method is faster and easier to use comparing to the Kjeldahl method.

Table 1.1 Wheat protein characteristics for different end uses (Peña, 2002)

Type	Grain protein (%)	Gluten (dough) strength type
Leavened breads		
Pan-type, buns	>13	Strong-extensible
Hearth, French	11-14	Medium-extensible
Steamed	11-13	Medium/Weak
Unleavened (flat) breads		
Arabic	12-14	Medium-extensible
Chapati, tortilla	11-13	Medium-extensible
Crackers	11-13	Medium
Noodles		
Yellow alkaline	11-13	Medium/Strong
White	10-12	Medium
Cookies, cakes, pastries	08-12	Weak/Weak-extensible

While protein content is important for the general classification of wheat, within classes protein quality is a more important determinant of the characteristics of flour and its performance. Wheat quality is a rather generic term as it depends on end use. For human nutrition, a method named the Protein Digestibility Corrected Amino Acid Score (PDCAAS) has been adopted by FAO to define protein quality. The score is based on the concentrations of essential amino acids and their digestibility (Schaafsma, 2000). For use in food production, protein quality describes the ability of protein to form the gluten network (dough) and the characteristics of the dough. As shown in figure 1.1, different gluten properties are required for producing various products; therefore, the description of "high" or "low" protein quality is only meaningful when discussing certain types of product. There are several ways to measure aspects of gluten properties. The gluten index (GI) measures the ratio of the weight of wet gluten remaining on a sieve after centrifugation to the total wet gluten content of the flour. It reflects gluten strength to some extent and correlates to dough extensibility (Curic et al., 2001). However, there are doubts about whether GI truly reflects the quality of wheat flour (Bonfil and Posner, 2012, Borkowska et al., 1999). SDS sedimentation is a test measuring gluten strength. The test involves mixing a flour sample in lactic acid containing sodium dodecyl sulphate

(SDS). A higher sedimentation volume indicates stronger gluten. This method is widely used in industry because the rapidness of the test. The Farinograph and Mixograph provide information on the mixing properties of flour, such as dough development time, stability, tolerance to over-mixing, and optimum water absorption, by recording the resistance of dough during mixing (Sahin and Sumnu, 2006). The Alveograph and Extensograph (or extensigraph) measure dough extensibility and its resistance to stretching. The Extensograph uses mechanical force to stretch the dough, while the Alveograph blows a bubble in a piece of dough to apply pressure. Both methods give information about dough strength and elasticity (Sahin and Sumnu, 2006), although Alveograph might provide more useful information for breadmaking as it simulates the gas expansion in the dough. Understandably, protein quality or rather protein characteristics are mainly determined by the gluten protein content and gluten protein structure/composition. More details of this topic will be given in section 1.3.3.

Starch characteristics

Starch is the largest component of the wheat grain accounting for about 70% of the grain weight (Hucl and Chibbar, 1996). The characteristics of starch, such as the percentage of amylose, gelatinization and retrogradation properties, influence many aspects of wheat food production. In breadmaking, starch is the primary source of sugar for yeast fermentation. The characteristics of starch also influence the colour of the crust, crumb firmness and storage behaviour (staling). Milling results in breakage of starch granules and in the releasing of fragments with attached protein bodies (Barlow et al., 1973). Starch damage is important for breadmaking as damaged starch absorbs more water, and only

damaged starch can be attacked by α-amylase and produce sugars. The pasting and gelatinization properties of wheat starch are instead the most significant factors in determining noodles' texture. The ratio of amylose and amylopectin also plays a role in determining noodle quality, especially for the production of Japanese *udon* noodles (Carson and Edwards, 2009). More detailed information on the composition and characteristics of wheat starch are reported in section 1.3. The starch content, amylose to amylopectin ratio, and amount of damaged starch can be determined by methods based on enzymatic reactions. The gelatinization and retrogradation properties are commonly determined using differential scanning calorimetry (DSC). Details of the above mentioned methods can be found in Chapter 2.

Other chemical characteristics affect wheat quality

Lipids are minor components of wheat; however they have significant impacts on the production and nutritional quality of the food. Many bakery products, such as cakes, pastry and cookies, contain large amounts of lipids. Although most of the fat in these foods is added during production, the intrinsic grain lipids nevertheless have strong effects on the quality of the products (Ram et al., 2009). The role played in breadmaking quality by wheat protein-lipid interactions during mixing and dough formation have been of particular interest to researchers : wheat lipids can affect dough properties by increasing gas cell formation, and maintaining their stability during mixing and baking (Ram et al., 2009).

Enzymes in wheat grain may also have important effects on food processing. For example, a certain amount of α-amylase activity is necessary for breaking

down starch to provide sugar for yeast growth. However, excessive α-amylase can result in sticky dough which is difficult to handle. The Hagberg falling number is the most commonly used method to determine α-amylase activity and is an essential test for determining flour quality. The method was initially invented by Sven Hagberg in 1960 (Hagberg, 1960), and named "falling number" one year later by himself in a research note of simplified method (Hagberg, 1961). In a falling number test, the flour sample is weighted then dispersed by water in test tubes and shaken to form slurry. Stirring rods are then inserted into the test tubes, which are placed in a boiling bath. They are automatically stirred for 60 seconds, causing the starch-water slurry to thicken due to starch swelling or "gelatinization." After mixing and heating, the stirrers are released at the top of the slurry and fall under their own weight until reach the distance set by the protocol/instrument. The total time of falling in second plus the waiting and stirring time in second is recorded as falling number. A falling number value of 300 seconds or longer indicates low enzyme activity and very sound wheat.

1.3 Grain structure and chemical composition

1.3.1 Grain structure

The wheat grain or kernel is a one seeded fruit, which is botanically called a caryopsis. The wheat caryopsis is about 4-8 mm long, and its dimensions vary according to cultivar and growth conditions. The wheat grain consists of three main components: the bran, endosperm and germ (Figure 1.2). The germ or embryo represents the next generation of the plant. It consists of an embryonic axis and a scutellum, which lies between the embryonic axis and the endosperm, and acts as a storage, digestive and absorbing organ transferring nutrients from the endosperm to the seedling during germination. The bran consists of several layers including the epidermis, hypodermis, cross cells, tube cells, testa and nucellar tissue. These layers are of maternal origin and protect the grain from excessive moisture loss and pests. In terms of milling, the "bran fraction" also includes materials from aleurone layer, which is rich in dietary fibre, proteins, vitamins, and minerals. However, because the bran fraction gives an undesirable colour and flavour to food products, it is usually separated from flour during milling and used for livestock feed. Recent studies have suggested that the nutrients in the bran fraction may confer significant health benefits. Therefore, it is becoming increasingly popular to incorporate wheat bran fraction into food products.

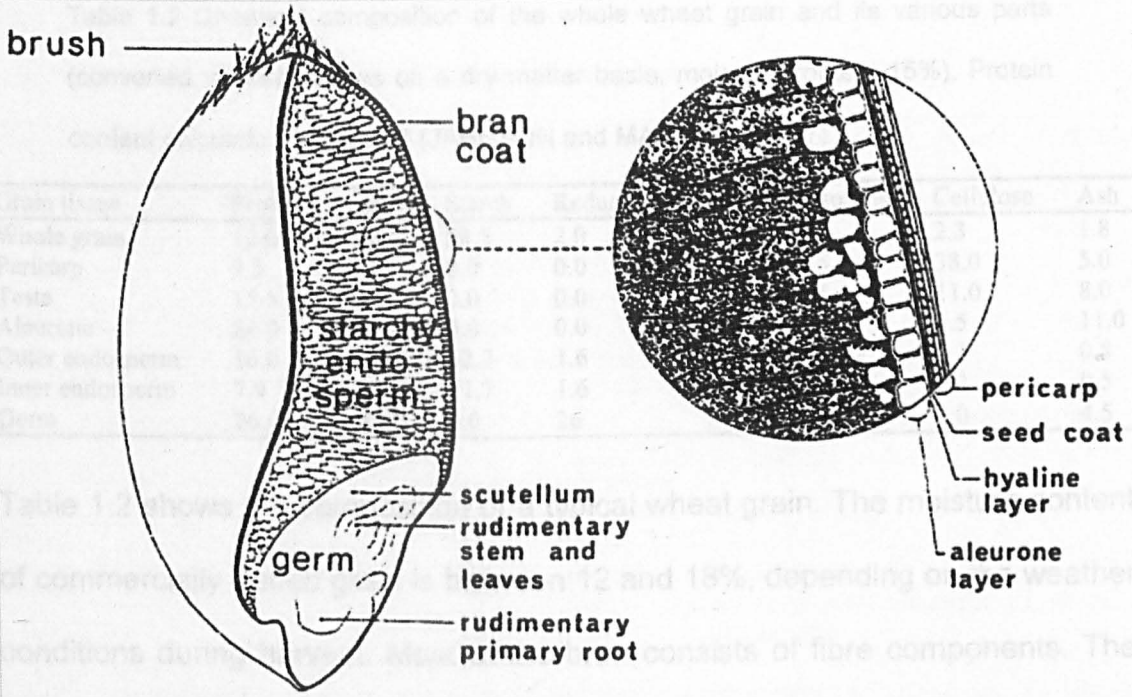

Figure 1.2 Wheat grain cut lengthwise through crease (Belderok, 2000)

Most of the mature wheat grain consists of a storage tissue called the "starchy endosperm". This comprises mainly of starch granules embedded in a protein matrix and upon milling produces the white flour. The starchy endosperm is surrounded by a single layer of cells with thickened walls--the aleurone layer. This is rich in proteins and produces enzymes that digest the storage reserves in the starchy endosperm cells during germination, acting as a source of carbon and nitrogen for the growing seedling. The starchy endosperm cells immediately below the aleurone layer, called the sub-aleurone cells, differ from the central cells of the starchy endosperm in in having much higher protein contents (33-34%, based on 14% moisture) (Kent, 1966) and fewer and smaller starch granules than the central endosperm cells (Kent, 1966).

Table 1.2 Chemical composition of the whole wheat grain and its various parts (converted to percentages on a dry matter basis, moisture content 15%). Protein content calculated by Nx5.83 (JAHNSON and MATTERN, 1987)

Grain tissue	Protein	Lipid	Starch	Reducing sugars	Pentosans	Cellulose	Ash
Whole grain	12.0	1.8	58.5	2.0	6.6	2.3	1.8
Pericarp	7.5	0.0	0.0	0.0	34.5	38.0	5.0
Testa	15.5	0.0	0.0	0.0	50.5	11.0	8.0
Aleurone	24.0	8.0	0.0	0.0	38.5	3.5	11.0
Outer endosperm	16.0	2.2	62.7	1.6	1.4	0.3	0.8
Inner endosperm	7.9	1.6	71.7	1.6	1.4	0.3	0.5
Germ	26.0	10.0	0.0	26	6.5	2.0	4.5

Table 1.2 shows the composition of a typical wheat grain. The moisture content of commercially traded grain is between 12 and 18%, depending on the weather conditions during harvest. Most of the bran consists of fibre components. The protein content is about 12%, including the contribution of the aleurone layer. However, most of these proteins are albumins and globulins, which are not functional in forming the gluten network. The starchy endosperm comprises about 80% carbohydrates (mainly starch) and 10-12% proteins, which play a central functional role in food processing.

1.3.2 Endosperm development and accumulation of protein and starch

Storage proteins and starch are synthesised and deposited in the starchy endosperm during grain development. Therefore, the final contents and compositions of protein and starch are determined by events during development and influenced by the environmental conditions and nutrition. Generally, endosperm development can be divided into several stages based on key events occurred during development (table 1.3).

Table 1.3 Major events during endosperm development, directly taken from Stone and Morell, 2009 (Stone and Morell, 2009)

Stage	Stage name	Timing (d.p.a)	Development events	Major Events in Starch Granule Development
1	Syncytial	0-5	Fertilization occurs. Coencytial nuclei proliferate. Vacuoles form in cytoplasm Proplastids are found	Spherical A granules are initiated
2	Cellularization	6-8	Cell walls form Amyloplasts differentiate and divide rapidly	Equatorial plate on A granules develop
3	Cell division	9-14	Cells divide rapidly	B granules are initiated Equatorial plate surrounding A granules is completed
4	Differentiation	15-21	Aleurone layers forms. Endosperm cells divide rapidly.	Deposition of starch on A granule lateral facts continues Further initiation of B granules occurs Radial growth of existing B granules occurs
5	Maturation	22-35	Endosperm and aleurone cell divisions have ceased. Seed storage products are deposited	A granules complete growth through lateral deposition of starch. Equatorial groove is less prominent. B granules continue to be initated and expand radially. C granules are initiated and develop radially.
6	Desiccation	35 to maturity	Desiccation occurs.	Starch granules are compressed into the protein matrix. Amyloplast membrane integrity is lost

Gluten proteins are first detected as early as 6 d.p.a. (Greene et al., 1985). Skerritt et al, reported high molecular weight glutenin subunits (HMW-GS) and some α-gliadins at 8 d.p.a. (Skerritt et al., 1988). Albumin and globulin proteins are synthesised earlier than gluten protein, and playing a metabolic role in young developing grains but become storage proteins at later stages of development (Vensel et al., 2005) . After initiation, grain protein accumulates in a linear fashion until the onset of apoptosis which occurs at around 32 d.p.a. under a 24/17°C day/night regimen (Altenbach et al., 2003), to cease at the end of maturation. Although protein synthesis is no longer active after maturation,

the ratio of high M_r polymers to low M_r polymers increases sharply during desiccation, which suggests a conversion of low M_r polymers to high M_r polymers driven by grain dehydration (Shewry et al., 2009b). Different gluten protein subgroups also differ in their accumulation patterns. Gliadins are synthesised faster at earlier stages of development than glutenins (Panozzo et al., 2001) while HMW-GS accumulate more slowly during the early and mid-stages of development and may continue to accumulate after 42 d.p.a. (Shewry et al., 2009b).

The synthesis of starch in wheat grain follows a more specific temporal pattern correlated to developmental stages (Table 1.3). Among the two major types of starch granules (large lenticular type-A granules, 15-30μm; and smaller spherical type-B granules, <10μm (Karlsson et al., 1983)), the large A granules are initiated at the syncytial stage while the small B granules are initiated at the cell division stage. C granules are smallest starch granules (equivalent diameters <5.3μm) not always resented in endosperm, and are only initiated after 21 d.p.a. (Bechtel et al., 1990). The rapid accumulation of starch occurred from 12 d.p.a. until 42 d.p.a., and only little increase occurring after that (Shewry et al., 2009b). Meanwhile, the proportion of amylose increased consistently throughout the development of Hereward (Shewry et al., 2009b).

The patterns of synthesis of protein and starch can be significantly affected by environment factors such as temperature, water and fertiliser (Altenbach et al., 2003, Dupont and Altenbach, 2003). For example, sulphur deficiency can lead to an increase in the proportion of gliadin polypeptides in the early stages of development (Skerritt et al., 1988). The application of fertiliser (N, P and K)

post-anthesis do not affect the timing of starch accumulation (Altenbach et al., 2003).

1.3.3 Protein

Proteins are the most important components of wheat since they largely control grain processing properties. High protein content may contribute to good breadmaking quality, but the quality of the protein is also important. The most widely used classification of wheat proteins is based on their solubility, and on the sequential extraction method developed and described by T. B. Osborne, the so called "Osborne fractionation". Based on this system, wheat proteins can be classified into four groups: albumins, globulins, gliadins and glutenins, which are soluble in water, salt solution, 70% (v/v) aqueous ethanol solution and dilute acid or alkali, respectively (Osborne, 1907, Osborne, 1924).

Albumin and globulin

Many seeds of dicotyledonous plants contain storage albumins, called 2S albumins based on their sedimentation coefficients (Youle and Huang, 1981). They have typical M_r around 10-15kDa and have high levels of cysteine residues. Some 2S albumins also have high methionine contents (Moreno and Clemente, 2008). However, in wheat, most albumins are not storage proteins but enzymes and play metabolic roles (Singh et al., 2001, Merlino et al., 2009). Some albumins belong to the family of α-amylase/trypsin inhibitors which are most important wheat allergen in food allergy (Pastorello et al., 2007). Storage globulins can be divided into 7S globulins and 11S globulins based on their sedimentation coefficients. They are typically trimetric proteins of M_r 150-

190kDa for 7S globulins and hexametric proteins of M_r 300-450kDa for 11S globulins (Shewry, 2004). Globulins are generally deficient in cysteine and methionine (Shewry et al., 1995). Storage globulins are characteristic of dicot seeds, but 7S globulins occur in cereal aleurone cells and embryos and a modified form of 11S globulin (triticin) in present in wheat starchy endosperm. Albumins and globulins generally have a very limited role in gluten formation. However, albumins and globulins are necessary for normal baking characteristics although they are not responsible for differences in baking quality (Hoseney et al., 1969). Albumins and globulins are important in terms of nutritional value, because compared to gluten proteins they have higher contents of lysine (Cornell and Hoveling, 1998), which is the limiting essential amino acids in wheat (Shewry, 2007).. However, in industrial practice, a high proportion of the albumins and globulins are removed by milling, being contained mostly in the bran and aleurone.

Gluten proteins

The gluten proteins account for 80-85% of total wheat protein. They can be classified into two groups, called gliadins and glutenins. Gliadins are monomeric proteins and have molecular weight between 30,000 and 80,000 (Veraverbeke and Delcour, 2002). They are rich in proline (about 20%) and glutamine (about 35%) but poor in arginine, lysine and histidine (Cornell and Hoveling, 1998). Depending on their electrophoretic mobility at low pH, gliadins can be separated into three groups: α-, γ-, and ω-gliadins (Lafiandra et al., 2000, Rayment et al., 1999). The α- and γ- gliadins are often referred to as S-rich prolamins (Shewry and Miflin, 1985, Shewry et al., 1994), because they are rich in cysteine and methionine residues which form intra-molecular disulphide bonds. The ω-

gliadins contain no cysteine residues so are often referred to as S-poor prolamins and three major N-terminal types can be distinguished: ARQ, KEL, and SRL-type. (Kasarda et al., 1983). However, some α-, γ-, and ω-gliadins contain extra cysteine residuals and form inter-molecular disulphide bonds (D'Ovidio and Masci, 2004). These gliadins subunits which are present in glutenin polymers are called "bound" α-type (α- and β-gliadins) and γ-type gliadins. The gliadins were recognised having different roles in gluten/dough comparing to glutenins as glutenins provide elasticity and gliadins provide extensibility and viscosity (Shewry et al., 2009a). This functional role of gliadins may relate to the interactions between gliadins and glutenins through non-covalent interactions and hydrogen bonds (Van Der Borght et al., 2005). In general, high gliadin content results in lower dough strength and lower loaf volume (Uthayakumaran et al., 2001, Hussain and Lukow, 1997, Fido et al., 1997). However different gliadin protein may have different effects on dough quality and baking quality, with γ-gliadins being reported as having a positively effect on breadmaking (Vanlonkhuijsen et al., 1992). Branlard and Dardevent (1985) reported multiple correlations between gliadin proteins and several baking quality parameters, with some being positive and others negative. They suggested that the differences were related by the strength of interactions between individual gliadin proteins with glutenins or other gliadins (Branlard and Dardevet, 1985).

Glutenins are large polymers with molecular weights ranging from 80,000 to several million (Veraverbeke and Delcour, 2002). These polymers comprise subunits linked by disulphide bonds (intra- and inter- molecular bond), and further associated by hydrogen bonds and hydrophobic interactions and are the most important contributors to the elasticity and strength of dough. The glutenin

polymers can be dissociated into subunits by disulphide reducing agents such as dithiothreitol (DTT). The subunits are classified into two groups based on their molecular masses: high molecular weight subunits (HMW subunits) and low molecular weight subunits (LMW subunits). LMW subunits have molecular weights ranging from 30,000 to 60,000, and comprise three groups: D, B and C. The B-group of LMW subunits are S-rich prolamins with two cysteine available for intermolecular disulphide bonding (Shewry and Miflin, 1985, Shewry et al., 1994). They can be divided further into LMWm ,LMWs and LMWi-types on the basis of their N-terminal amino acids (methionine for LMWm , isoleucine for LMWi and serine for LMWs) (Lew et al., 1992). The D-group consists of "bound" ω-gliadins and C-group of "bound" α- and γ-gliadins (as discussed above).

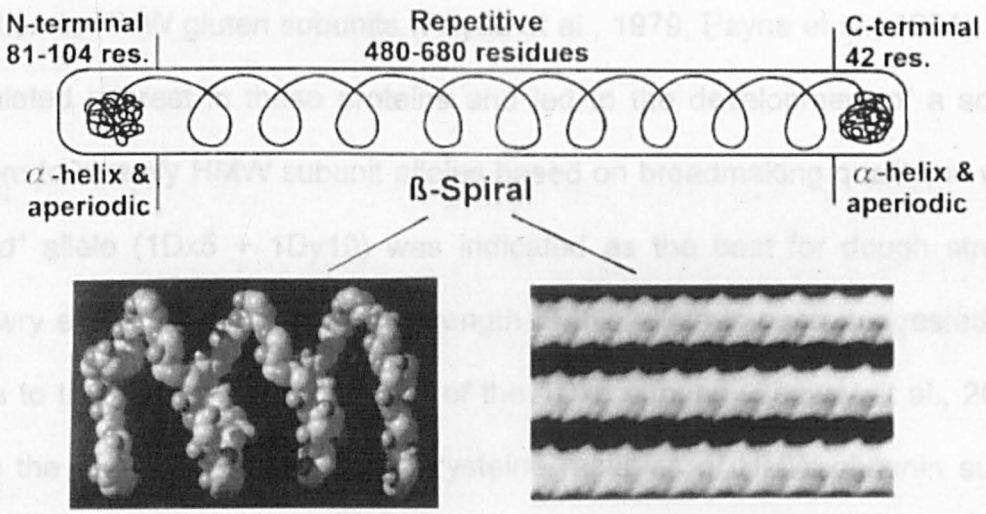

Figure 1.3 A structure model of HMW gluten subunits, adopted from Shewry et. al., 2001 (Shewry et al., 2001)

HMW subunits have molecular weights ranging from 60,000 to 90,000, and are rich in glycine, glutamine and proline. An HMW subunit comprises a long central domain consisting of repeated sequence motifs and shorter non-repetitive residues at N- and C- terminal domains. The motifs are suggested to form a β-

spiral structure (figure 1.3), which shows intrinsic elasticity and may contribute to the elastomeric properties of glutenin (Shewry et al., 2001). However, this structure has not yet been proven to exist *in vivo* (Shewry et al., 2009a). The HMW subunits are considered to form an "elastic backbone" in the gluten network. This backbone may comprise HMW subunits linked by disulphide bonds with the LMW subunits forming branches (also linked by disulphide bonds). A summary of this structure is showed in figure 1.4. Although disulphide bonds are considered to be the most important structures that stabilise the gluten network, conferring dough strength, non-covalent interactions such as hydrogen bonds may also contribute to elasticity (Shewry et al., 2001).

Payne reported an association between breadmaking quality and allelic variation in HMW gluten subunits (Payne et al., 1979, Payne et al., 1981). This stimulated interest in these proteins and led to the development of a scoring system to classify HMW subunit alleles based on breadmaking quality, in which the "d" allele (1Dx5 + 1Dy10) was indicated as the best for dough strength (Shewry et al., 2003). The great strength of this allele is been suggested may relate to the extra cysteine residue of the 1Dx5 subunit (Shewry et al., 2009a) since the number and position of cysteine residues of HMW glutenin subunit seem to play an important role in gluten polymer elasticity (Shewry et al., 2009a).

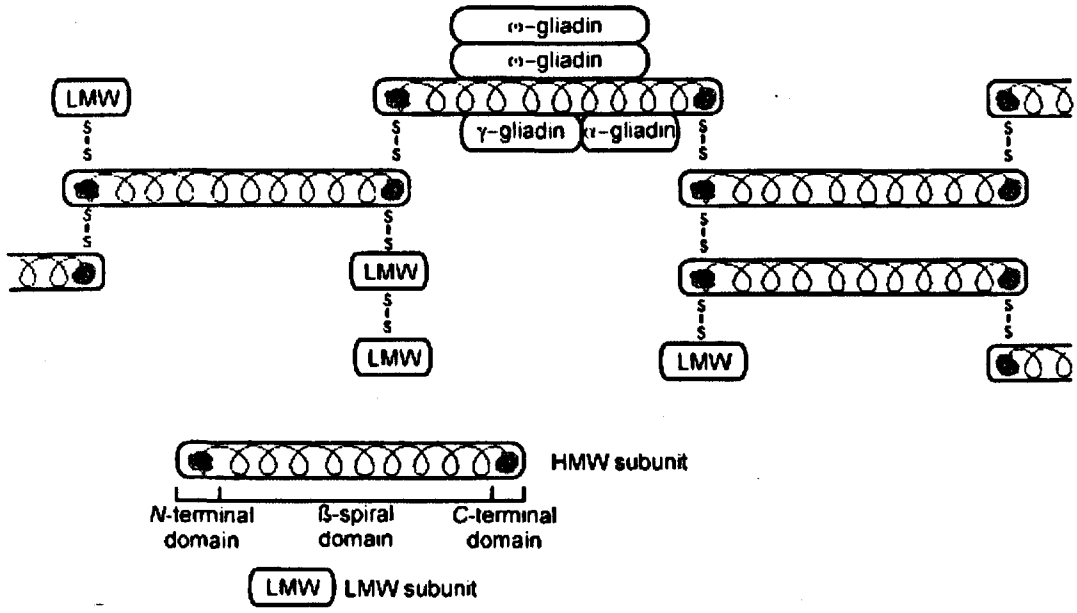

Figure 1.4 A structure model of wheat gluten network, adopted from Shewry et. Al.. 2001 (Shewry et al., 2001)

1.3.4 Starch

Starch is the most abundant component of the wheat grain. It consists of two types of glucose polymers: amylose and amylopectin accounting usually for about 25% and 75%, respectively. These proportions vary little between cultivars, with the exception of waxy type wheat which have level of amylose as low as 0-1%. Amylose is a linear polymer consisting of glucose residues linked by α-(1-4) bonds. Amylopectin has a highly branched structure, and consists of glucose residues linked by α-(1-4) bonds and α-(1-6) bonds. The degree of branching of amylopectin is positively correlated with its solubility.

Gelatinisation is a change in the molecular order of starch that occurs on heating in the presence of water. The starch granules keep swelling after the initiation of gelatinisation and soluble components (majority amylose) start to leach, leading eventually to breakdown and the formation of a starch paste

consisting of a continuous phase of solubilised amylose and/or amylopectin and a discontinuous phase of granule remnants, which consist of granule ghost (insoluble portion of gelatinised granules) and granule fragments (BeMiller and Whistler, 1996). Cooling of such a paste causes the paste to become less soluble and eventually results a firm but viscoelastic gel. This process is called retrogradation and results from amylose and linear fragments of amylopectin molecules rearranging themselves and trying to re-crystallise. The gelatinisation and retrogradation properties of starch are significant for all wheat-related food processing since starch is the largest component of the flour. In bread making, the starch is only partly gelatinised in baking (Yasunaga et al., 1968) and the quality of bread is mostly determined by protein quality. However, the retrogradation properties of starch do affect the staling of bread which is important for the shelf life. Starch gelatinisation and retrogradation properties are more important in the food processes that depend less on gluten properties, such as short dough biscuits and cakes. For these products, the gelatinisation of starch, rather than the gluten network, provides structural support to the end product.

1.3.5 Other chemical compositions in wheat grain: lipids and cell wall polysaccharides

Lipid accounts for 2% of wheat flour weight. Wheat total lipids are generally classified as non-polar and polar lipids (Van Der Borght et al., 2005, Ram et al., 2009). Many studies have showed that protein-lipid interactions in the dough system are important for bread making quality (McCann et al., 2009) and polar lipids in particular were shown to have a positive effect on loaf volume of bread

(Macritch and Gras, 1973) and on the volume and softness of steamed bread (Pomeranz et al., 1991). However, it has also been suggested that most of the lipids in flour are unavailable for protein-lipid interactions, mostly because they are strongly bound in the starch granule until the starch is gelatinised (Van Der Borght et al., 2005). Lipids are more important for the production of pastries, short-dough biscuits and cakes, because lipids contribute to the layer structure and oral texture (Papantoniou et al., 2004).

Cell wall polysaccharides commonly constitute less than 10% of whole wheat grain (Fincher and Stone, 2004) and are concentrated in bran and aleurone. Arabino-(1 → 4)-β-D-xylans (arabinoyxlans, AX), (1 → 3, 1 → 4)-β-D-glucans (β-glucans) and cellulose are the major components of cell wall polysaccharides. The bran is in rich in cellulose and arabinoyxlans, which comprise 30% and 65% of the total cell wall polysaccharides in bran, respectively (Fincher and Stone, 2004). β-glucans and arabinoyxlans are the major components of the starchy endosperm and aleurone, accounting for about 30% and 70% of total cell wall polysaccharides, respectively, in these tissues (Fincher and Stone, 2004). Arabinoyxlans have exceptionally high water absorption capacitates (Shewry and Morell, 2001), and it is therefore expected that arabinoyxlans will affect dough formation by competing with the gluten network for water and will also affect the viscosity of the dough. In addition to affecting processing, the cell wall polysaccharides also affect the health benefits of wheat products as they act as dietary fibre and have benefits in reducing the risk of several diseases including cardio-vascular disease, type-II diabetic and forms of cancer (Shewry and Morell, 2001).

1.4 Project aim: the spatial gradients of protein and starch quantity and composition within endosperm

Previous studies indicated that different tissues of the wheat grain have different chemical compositions and differences also exist between cells within these tissues (Mills et al., 2005, Evers, 1970, Lending and Larkins, 1989).., The subaleurone cells, corresponding to the two to three layers of cells immediately below the aleurone layer , are richer in protein and contain less starch granules than cells in the central starchy endosperm (Bradbury et al., 1956, Kent, 1966, Kent and Evers, 1969). Gradients also exist in the proportions of different types of gluten protein across the wheat endosperm (Lamacchia et al., 2001, Mills et al., 2005, Piston et al., 2009, Tosi et al., 2009, Tosi et al., 2011). These previous studies were based on microscopy observations. However it remained unknown whether these gradients differed between genotypes, and in particular between high protein bread making wheat and low protein feed wheats, and whether they are affected by the level of nitrogen fertilisation. And, although it would be predictable that flours from different parts of the grain would differ in their functional properties, this has not yet been provided. Analysis of starch amount in different cell layers of the wheat endosperm has been previously reported (Ugalde and Jenner, 1990a), but such study was limited to the dorsal area of the grain.. Also, the composition gradient and functionality gradient of starch has not yet been reported.

This project therefore was designed to answer these questions. A pearling mill was used to prepare fractions from grain samples of different cultivars grown at

two nitrogen levels. The fractions were tested using various technologies to test following main objectives:

- Provide detailed information of total protein content gradients across the grain
- Provide detailed information of gluten composition gradients across the grain
- Determine the quality (in terms of functionality in breadmaking) differences of pearling fractions
- Provide detailed information on total starch content gradients across the grain
- Determine whether the starch composition (amylose/amylopectin ratio) differs across the grain
- Determine whether the starch functionality (including gelatinization properties, starch damage and particle size distribution) differs across the grain
- Determine the effects of genotype and N fertiliser on all of above gradients.

Also, immunofluorescence microscopy was used to gather a comprehensive understanding of spatial distribution of gluten compositions in grain.

Chapter 2: Material and Methods

2.1 Plant Material

2.1.1 Plant material for chemical composition experiments

Seven wheat cultivars (table 2.1) were selected for this project from the 2009 harvest of Rothamsted Research WGIN [1]field trial (More information of this trial can be found on http://www.wgin.org.uk/information/objectives/objective8.php). All varieties were grown at three nitrogen fertilisation levels (100, 200 and 350 kg/ha). All plots were randomised with three replicates. Grains of N level 100 kg/ha and 350 kg/ha were used for analysis in this project. In the context of this thesis, specific plant samples will be identified by abbreviation of cultivar name, followed by nitrogen level and field biological replicate number (R1, R2 or R3). For example, MA350R2 represent Malacca, nitrogen fertilisation levels 350 kg/ha, bio-replicate 2.

Table 2.1 Selected cultivars for chemical composition experiments

Name	Abbreviations	Description
Hereward	HE	UK good quality bread-making variety
Paragon	PA	popular UK Spring breadmaking variety
Malacca	MA	good breadmaking variety
Xi19	XI	breadmaking wheat
Cordiale	CO	early maturing breadmaking variety, good response to added N application
Masksman	MA	new UK breadmaking variety, good response to added N application
Istabraq	IS	Low protein variety used for animal feed, soft non-breadmaking wheat

[1] WGIN: wheat Genetic Improvement Network: http://www.wgin.org.uk/about.php

2.1.2 Materials for microscopy

Experiment setup

Seven wheat cultivars (table 2.2) were grown in the glasshouse at Rothamsted Research at two nitrogen fertilisation levels (100 and 350 kg/ha). Each cultivar was grown in three replicates, separated into three blocks each containing two nitrogen fertilisation levels. The locations of cultivars within each block were randomised. The experiment used 10" pots with10 plants per pot. Each nitrogen fertilisation level of each replicate of each cultivar has two pots (20 plants).

Table 2.2 Cultivars for microscope investigations

Name	Abbreviations	Description
Hereward	HE	UK good quality bread-making variety
Istabraq	IS	Low protein variety used for animal feed, soft non-breadmaking wheat
Atlas 66		High protein content (20%)soft wheat
Yumai 34		High soluble fibre and strong gluten wheat
Kanto 107		Low amylose (21%) wheat
N11 SGP-1		High amylose 37% wheat
N11 WX-1		Waxy (100% amylopectin) wheat

Note: The N11 lines were sourced from Prof Domenico Lafiandra at University of Tuscia, Italy. Other lines were from the WGIN project mentioned in previous page

Plant growing conditions

Vernalisation of plants was carried out in a cold room at 6°C, for 12 weeks with an 8h light cycle per day. Plants were then moved to a glasshouse. The glasshouse conditions were: minimum temperature day 18°C/night 14°C, minimum light per day 16h, manual watering.

The growing medium and nutrition supply

The plants were moved to glasshouse in May 2009 and used a mixture of Rothamsted prescription soil (RPM, consist of 1.75kg/m^3 Osmocote[2] and 0.25kg/m^3 PG-MIX[3]), medium grade perlite, peat, and 2EW sand[4]. The composition ratio (in volume) is one part RPM with two parts perlite, four parts peat, and two parts 2EW sand. The concentrations of nutrients in the soil mixture were equivalent to: 100kg/ha N, 75kg/ha K_2O and 76.5kg/ha P_2O_5. Additional 100ml/pot of 0.66g/l KH_2PO_4 liquid fertiliser were added to all pots and 100ml/pot of 3.55g/l NH_4NO_3 liquid fertiliser were added to the high N pots weekly for 10 weeks. The final concentrations of nutrients were equivalent to: 120kg/ha K_2O and 145kg/ha P_2O_5 for all pots, 100kg/ha N for low N pots, and 350kg/ha N of high N pots.

Sample collection

Two developing seeds of each c.v. and treatment were collected at 15 dpa (days post-anthesis) and 21 dpa, then sectioned and fixed (method described in section 2.7) for fluorescence microscopy.

[2] Osmocote: a slow release fertiliser, supplied by Everris International B.V.
[3] PG-MIX: brand name of peat substrates fertilizers produced by Hydro Agri
[4] 2EW sand: comes from fine sterilised sands from Leighton Buzzard, Beds, sold by Garside Sands

2.2 Sample preparation methods

2.2.1 Physical properties of grains

The weight, size, hardness index and moisture content were measured using the Single Kernel Characterisation System 4100 (SKCS 4100) by Perten Instruments, Inc. The system separates and weights individual grains, then forces the grain through a progressively narrowing gap between a rotor and a crescent equipped with sensors (Figure 2.1). During this process, grain crush force, diameter and moisture content (through grain's conductivity) are measured, and then calculated to give weight (mg), size (diameter mm), moisture (%), and Hardness Index of the grain. The Hardness Index value is based on algorithms which separate on a numerical scale from 75 (hard wheat) to 25 (soft wheat) (Gaines et al., 1996). Each of the parameters is an average of 300 grains calculated automatically by SKCS system.

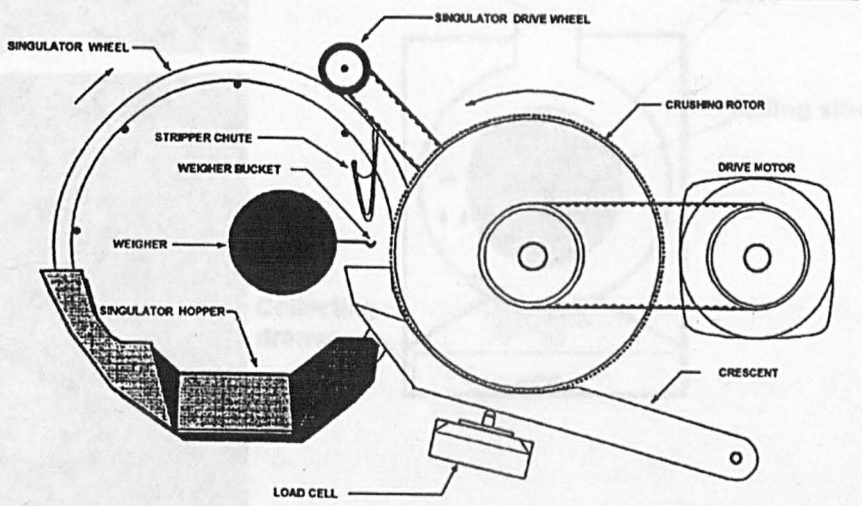

Figure 2.1 Singulator and Crushing Mechanism (A copy from Single Kernel Characterisation System 4100 Manual)

2.2.2 Milling by pearling and fractions collection

Pearling was conducted using a laboratory pearling mill, manufactured by Streckel & Schrader (Germany). Further milling of pearled grains was conducted using a SPEX laboratory ball mill. Materials were as described in section 2.1.1.

Pearling Working principle

Gains are trapped between a stone mill wheel and a wheel shaped sieve. When the mill wheel rotates at high speed, the frictional force applied to the seeds when they come in contact with the mill wheel abrades the seeds from the outer layer and also move the seeds around the mill wheel. The fine flour falls through the sieve while the cores remain in the trap for further pearling (Figure 2.2).

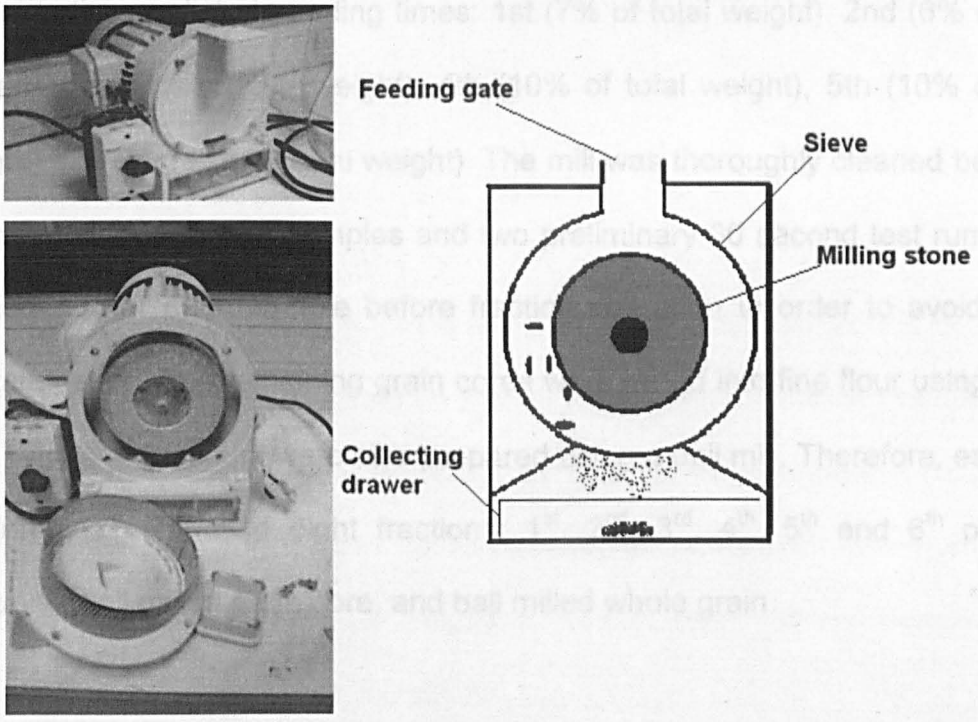

Figure 2.2 Streckel & Schrader laboratory pearling mill and structure indication

Method

Before pearling to collect fractions, pearling efficiency tests were carried out to determine the correct pearling times for each fraction. 50g of seeds from each line (selected to rule out broken and small seeds and contamination) were pearled to obtain the shell rates ($Shell\ rate = \frac{Fraction\ weight\ (g)}{Total\ seeds\ weight\ (g)} \times 100$) at 0.5, 1, 2, 3, 4, 5 and 6 min (6 min only apply to lines that had Hardness Index higher than 60). According to the shell rates obtained in the efficiency test, a cubic interpolative[5] curve was built using GenStat (14th edition) to predict the times required to remove 7, 13, 20, 30, 40 and 50% of the total seed weight (accumulative weight). After the efficiency test, 50g samples of selected seeds from each line were pearled to collect fractions. Six fractions were removed representing flour fractions enriched in bran, aleurone, sub-aleurone and three different endosperm layers, respectively (Pomeranz, 1988, Barron et al., 2007), by using the predicted pearling times: 1st (7% of total weight), 2nd (6% of total weight), 3rd (7% of total weight), 4th (10% of total weight), 5th (10% of total weight) and 6th (10% of total weight). The mill was thoroughly cleaned between the pearling of different samples and two preliminary 30 second test runs were carried out for each sample before fraction collection in order to avoid cross contamination. The remaining grain cores were milled into fine flour using a ball mill. Whole grain flours were also prepared using a ball mill. Therefore, each set of samples comprised eight fractions: 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th} and 6^{th} pearling fractions, ball milled grain core, and ball milled whole grain.

[5] Interpolation: is a mathematic method to produce new data points within the range of known data set. The advantage of such method is it could give precise predictions of data points without knowing the model behind a data set; the disadvantage is it cannot function outside the range of known data set.

2.2.3 Collection of milling fractions

Milling and collection

The same cultivars (described in section 2.1.1) were also used to prepare conventional milling fractions. Milling was carried out by the Cereal and Milling Department of Campden BRI using a Buhler MLU-202 mill. Figure 2.3 shows the process of milling: cleared grains were crushed sequentially between three break rollers: the flour produced during each break was sieved twice then collected. The flour particles rejected by the 1st sieve were sent to the next break stream. The flour particles rejected by 2nd sieve from each of the three break streams were combined and sent to the reduction streams. The process consisted, therefore, of three break streams and three reduction streams and eight fractions were collected: 1st break, 2nd break, 3rd break, 1st reduction, 2nd reduction, 3rd reduction, bran, and offal flour. The production rates can be found at appendix 3.

Ash content test

The ash contents of milling fractions were tested by Campden BRI to determine the total minerals contents using an industry-standard test method derived from British Standard Method No. BS 4317, part 10 (British-Standards, 1993). The sample was incinerated in an oxidising atmosphere until combustion of organic matter was complete and the weight of residue obtained and calculated as percentage of flour based on dry weight.

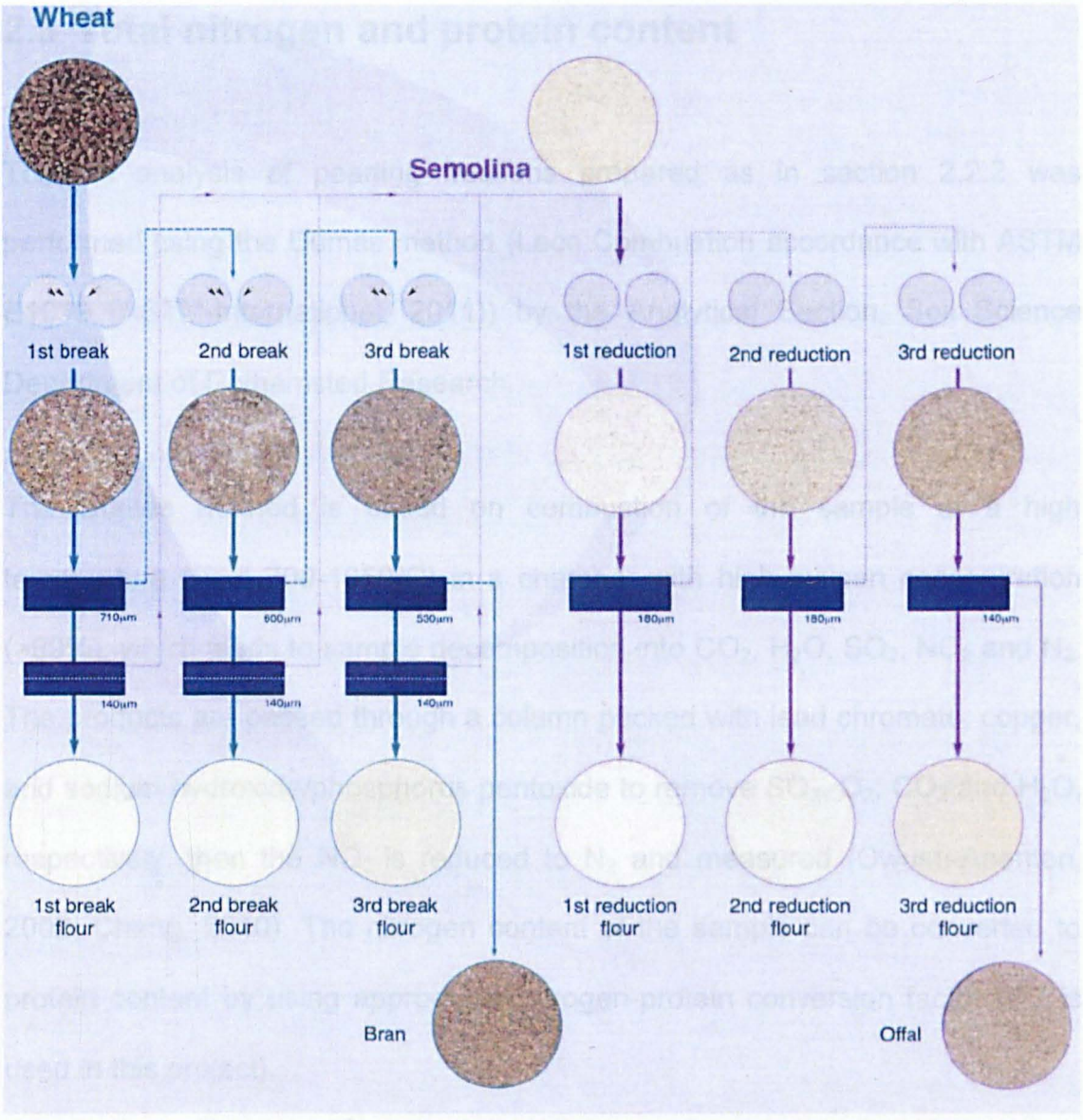

Figure 2.3 Buhler MLU-202 mill flow chart, provided by Campden BRI

2.3 Total nitrogen and protein content

Total N analysis of pearling fractions prepared as in section 2.2.2 was performed using the Dumas method (Leco Combustion accordance with ASTM E1019 (ASTM-International, 2011)) by the Analytical Section, Soil Science Department of Rothamsted Research.

The Dumas method is based on combustion of the sample at a high temperature (from 700-1050°C) in a chamber with high oxygen concentration (>99%), which leads to sample decomposition into CO_2, H_2O, SO_2, NO_2 and N_2. The products are passed through a column packed with lead chromate, copper, and sodium hydroxide/phosphorus pentoxide to remove SO_2, O_2, CO_2 and H_2O, respectively; then the NO_2 is reduced to N_2 and measured (Owusu-Apenten, 2002, Chang, 2010). The nitrogen content of the sample can be converted to protein content by using appropriate nitrogen-protein conversion factor (5.7 is used in this project).

2.4 Gluten protein profile by SDS-PAGE

2.4.1 Introduction

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) uses polyacrylamide gels to separate proteins based on their electrophoretic mobility, which is determined by the protein size. A combination of SDS and dithiothreitol (DTT) or 2-mercaptoethanol is commonly used in extraction buffers to denature and reduce wheat proteins prior to electrophoresis. Also, SDS impart uniform negative charges on polypeptides, therefore their electrophoretic mobility would be solely depending on the length of the polypeptide chains (molecular weight). However, this method also extracts non-gluten proteins (total protein extraction lanes in Figure 2.4). After exploring the literature (Fu and Sapirstein, 1996, van den Broeck et al., 2009) and trying several protein extraction procedures, an extraction protocol using a combination of 1-propanol, SDS and DTT was selected to exclusively extract gluten proteins and give clear bands on gels (50% 1-propanol extraction lanes in Figure 2.4). The gels were scanned by a photo scanner and the images processed by gel analysing software.

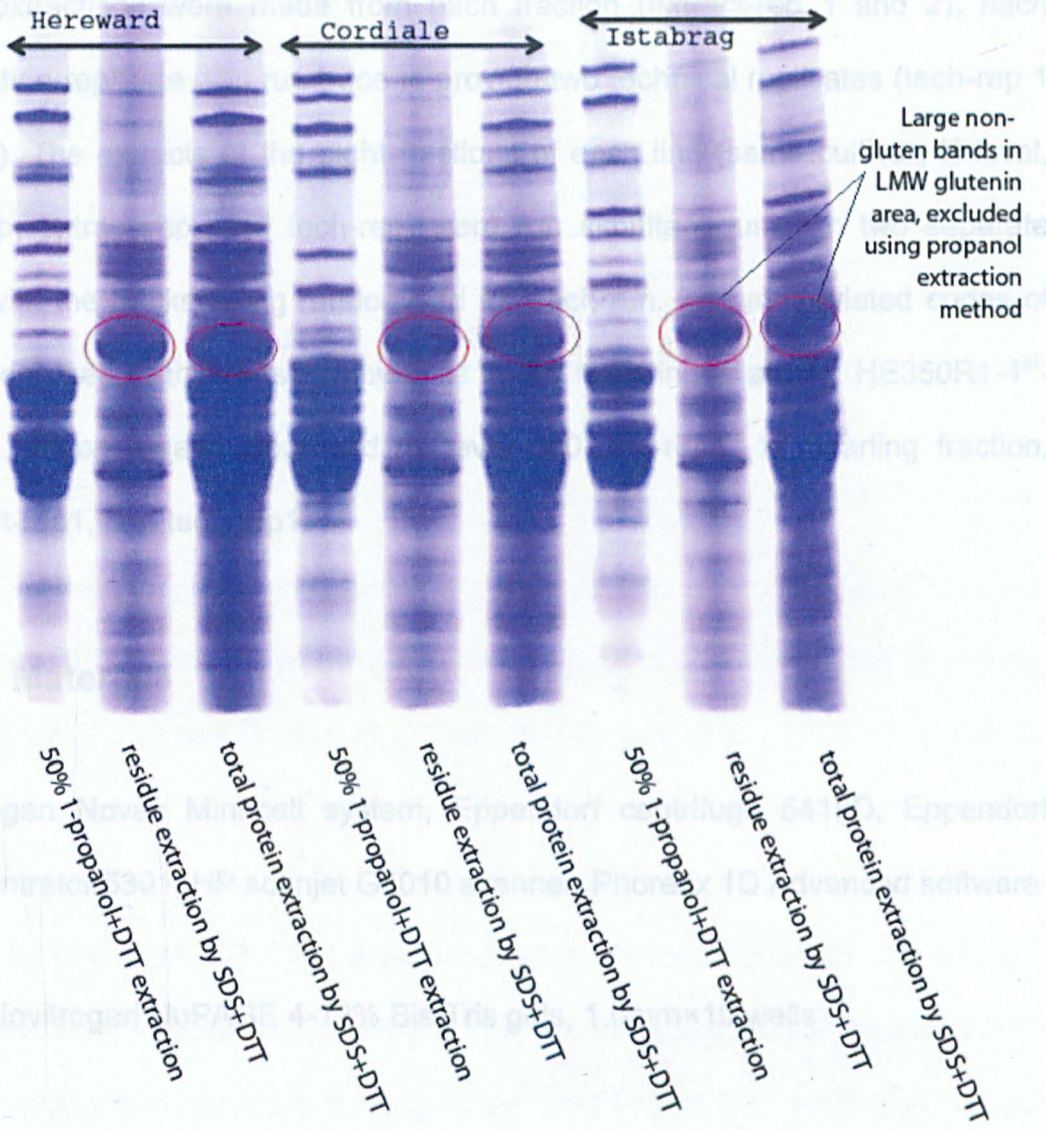

Figure 2.4 2nd pearling fractions of HE350R1, CO350R1 and IS350R1

2.4.2 Statistical analysis of SDS-PAGE gels: experimental design

This experiment included four (Hereward-HE, Cordiale-CO, Malacca-MA and Istabraq-IS) of the seven cultivars described in section 2.1.1, two nitrogen fertilisation levels for each cultivar (N350 and N100), and three biological replicates (Bio-rep 1-3). Each replicate consists of eight fractions (the six pearling fractions milled core, and whole grain flour, described in section 2.2.2).

Two extractions were made from each fraction (extract-rep 1 and 2); each extraction-replicate was run twice to provide two technical replicates (tech-rep 1 and 2). The extracts of the eight fractions of each line (same cultivar, N level, bio-rep, extract-rep, and tech-rep) were run simultaneously on two separate gels with the tracks being randomised for each run. The abbreviated codes of samples used in this thesis appear as in the following example: HE350R1-1st-E1-T1, which means Hereward, N level 350, bio-rep1, 1st pearling fraction, extract-rep1, and tech-rep1.

2.4.3 Materials

Invitrogen Novex Mini-cell system, Eppendorf centrifuge 5415D, Eppendorf concentrator 5301, HP scanjet G4010 scanner, Phoretix 1D Advanced software

Gels: Invitrogen NuPAGE 4-12% Bis-Tris gels, 1.0mm×10 wells

Running buffer: NuPAGE MES SDS running buffer

Total protein extraction buffer: 0.0625M Tris-HCL, pH 6.8, 2% (w/v) SDS, 1.5% (w/v) dithiothreitol (DTT), 10% (v/v) glycerol, 0.002% (w/v) Bromophenol Blue

Gluten protein extraction buffer: 50% 1-propanol, 0.0625M Tris-HCL, pH 6.8, 10% (v/v) glycerol, 0.002% (w/v) Bromophenol Blue

Loading buffer: 0.0625M Tris-HCL, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.002% (w/v) Bromophenol Blue

Stain solution: 40% (v/v) methanol, 10% (v/v) trichloroacetic acid, 0.1 % (w/v) Coomassie Brilliant Blue R-250

Destain solution: 10% (v/v) trichloroacetic acid

2.4.4 Extraction method: extraction of gluten protein using (v/v) 50% 1-Propanol

Figure 2.5 shown the protocol used for gluten protein extraction using 50% (v/v) 1-propanol. The protocol was modified from a two-step gluten extraction method described by van den Broeck et al. (van den Broeck et al., 2009). The sample was extracted at first using 50% 1-propanol without reducing agent in order to selectively extract gliadins. The residue was then extracted twice with 50% 1-propanol plus 4.5% DTT to extract most of the reduced glutenin subunits and the remaining gliadins. The extracts were pooled, dried to remove the 1-propanol and then diluted in loading buffer (1mg flour: 10µl buffer). The proteins remaining in the pellet were then extracted with total protein extraction buffer (1mg flour: 10µl extraction buffer). Both the propanol extracts and the residual total protein extracts were analysed as a considerable portion of HMW gluten subunits (10-20%) remained in the residual pellet.

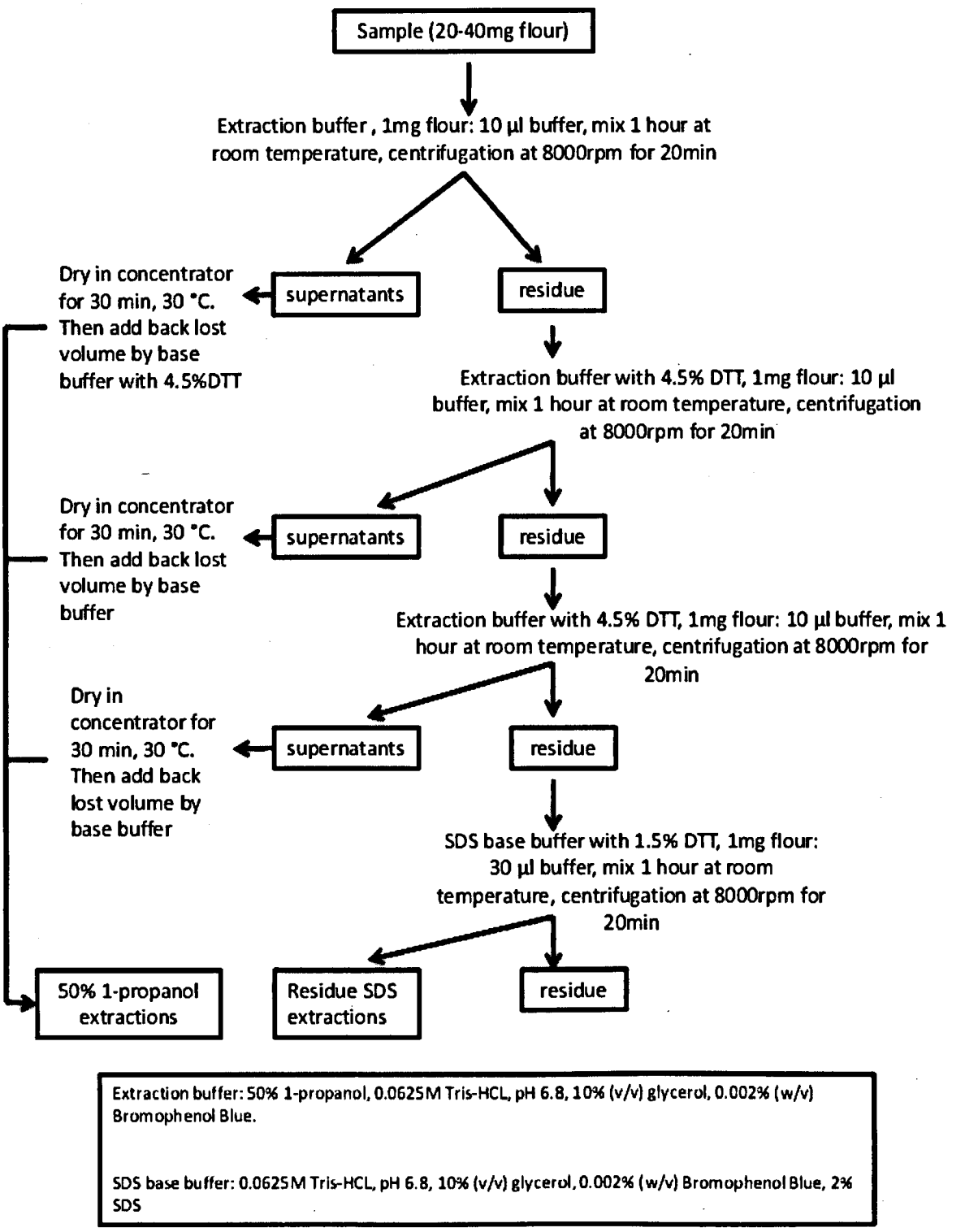

Figure 2.5 50% 1-Propanol protein extraction protocol

2.4.5 Gel Electrophoresis

The gel apparatus was assembled according to the manufacturer's instructions (http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Protein-Expression-and-Analysis/Protein-Gel-Electrophoresis/Electrophoresis-Instruments-Power-Supplies-Accessories/Protein-Gel-Electrophoresis-Chamber-Systems/Xcell-Surelock-Mini-Vertical-Electrophoresis-Chamber.html). 8 µl sample per well were loaded after denaturation at 80°C for 3 min. Gels were run at a power setting of 200v for 57min

Stain and destain: Gels were stained in stain solution in for 24h, then destained with destain solution for 24h.

2.4.6 Gel scan and analysis

The gels were scanned immediately after destaining using a modified HP Scanjet G4010 scanner and the following settings: saved as TIF file; resolution: 300 ppi; 256 grey shades; Highlight: 70-90 (adjusted within range to achieve similar lightness); Shadows: 0; Midtones: 0; Sharpen: Medium. The images were processed using Phoretix 1D advanced software.

Background subtraction: Minimum Profile was used for the propanol extract lanes as the backgrounds were relatively low; the Rolling Ball method was used (radius 30 unit—internal unit of Phoretix 1D advanced software) in lanes corresponding to SDS extracts as the backgrounds were strong, particularly in the HMW glutenin subunit region.

Band selection and analysis: The gluten proteins in lanes with propanol extracts were divided into three sub-groups: HMW-GS, ω-gliadins and LMW-GS+gliadins (α-/γ-gliadins). The remaining HMW-GS bands in the lanes with SDS extracts were also selected (Figure 2.6). The volumes (the sum of the intensities of pixels within a band/area) of the selected areas were read by the software (Figure 2.7). The volumes of the remaining HMW bands within the residual SDS extracts were added to the volumes of the HMW band in the propanol extraction lanes. The percentages of each sub-group with total gluten protein were then calculated based on $\%\ of\ subgroup = \frac{Volume\ of\ subgroup}{Sum\ of\ volumes\ of\ all\ three\ subgroups} \times 100$. More details of this analysis method can be found in the Phoretix 1D advanced software (Version 2003.01) manual.

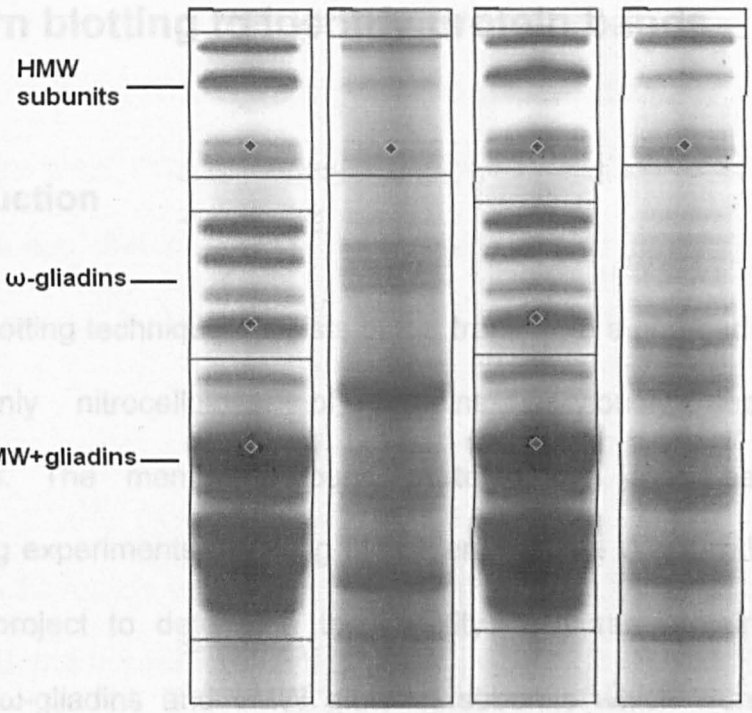

Figure 2.6 SDS-PAGE of Hereward, N350, showing the groups of bands corresponding to sample HE350R1, extraction-replicate 1, technical replicate 1. Bands left to right: fraction 2, 50% propanol extraction; fraction 2, residual SDS extractions; fraction 6, 50% propanol extraction; fraction 6, residual SDS extraction.

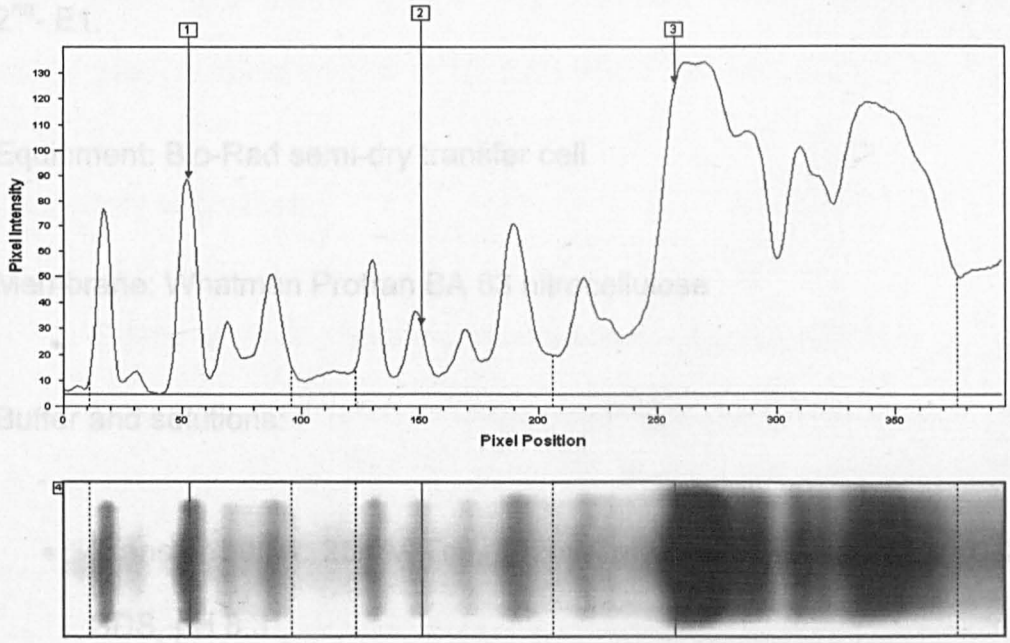

Figure 2.7 Gel bands analysis example

2.5 Western blotting to identify protein bands

2.5.1 Introduction

The western blotting technique consists of the transfer to a charged membrane, most commonly nitrocellulose, of proteins previously separated by electrophoresis. The membrane-bound proteins can then be used for immunostaining experiments, allowing their identification. Western blotting was used in this project to determine the identity of gluten protein bands, in particular the ω-gliadins and LMW glutenin subunits which were compress together on SDS-PAGE and are therefore difficult to discriminate on gels.

2.5.2 Materials

Samples: HE350R1-2nd-E1, HE350R1-2nd-E1, HE350R1-2nd-E1, and IS350R1-2nd- E1.

Equipment: Bio-Rad semi-dry transfer cell

Membrane: Whatman Protran BA 83 nitrocellulose

Buffer and solutions:

- Transfer buffer: 25mM Tris, 192mM glycine, 20% methanol, 0.02% (w/v) SDS, pH 8.3
- Tris-buffered saline (TBS): 20mM Tris, 500mM NaCl, pH 7.5
- Tween-Tris buffered saline (TTBS): TBS+0.05% (v/v) Tween 20

- Blocking agent: 5% (w/v) skimmed powdered milk solution
- Antibody buffer: TTBS+1% bovine serum albumin (BSA)
- Temperate stain: Ponceau S solution, made by Sigma-Aldrich
- Stain solution: premixed BCIP®/NBT, made by Sigma-Aldrich
- Colour enhance buffer: 0.1M Tris, 0.1M NaCl, 0.05M MgCl2, pH 9.5

Primary antibodies:

- IFRN[6] 0610 mouse monoclonal antibody, which recognises gliadins and LMW-GS, but not HMW-GS (Brett et al., 1999), diluted 1:100 in 1% BSA PBST
- INRA[7] ω-2 rabbit polyclonal antibody, which recognises ω-2 type gliadins, diluted 1:100 in 1% BSA PBST (Denery-Papini et al., 2000)
- INRA ω-5 rabbit polyclonal antibody, which recognises ω-5 type gliadins, diluted 1:100 in 1% BSA PBST(Denery-Papini et al., 2000)
- INRA ω-5 mouse monoclonal antibody, which recognises ω-5 type gliadins, diluted 1:100 in 1% BSA PBST (unpublished)

Secondary antibodies:

Goat anti-rabbit alkaline phosphatase conjugates (SIGMA)

Goat anti-mouse alkaline phosphatase conjugates (SIGMA)

[6] IFRN: Institute of Food Research, UK
[7] INRA: Institut National de la Recherche Agronomique, France

2.5.3 Method

The combined 1-propanol extracts and the SDS/DTT extract of each sample (HE350R1-2nd-E1, HE350R1-2nd-E1, HE350R1-2nd-E1, and IS350R1-2nd-E1,) were separated in adjacent lanes in the same gel (method described in last section). The separated proteins were transferred onto nitrocellulose membrane following the equipment manufacturer's instructions. Ponceau S solution was used to reversibly stain protein bands so that lanes could be marked by pencil and easily identified after immunostaining. The Ponceau S staining was then removed by washing sequentially with water and TBS, the membranes were blocked with blocking solution for 1h at room temperature and then incubated in primary antibody solution for 1h. The membranes were washed twice (5 min each) in TTBS and incubated in secondary antibody solution for 1 h. They were then washed three times (5 min each) in TTBS, followed by a 5 min rinse in TBS, then developed using BCIP®/NBT solution. The method was taken from Fido et al.,1995 (Fido et al., 1995). The membranes were dried then compared with images of stained SDS-PAGE gels in order to identify specific protein bands.

2.6 Determination of molecular size distribution of gluten proteins by SE-HPLC

Size-exclusion high performance liquid chromatography (SE-HPLC) is a chromatographic technique that separates proteins on the basis of their molecular sizes. Sample preparation normally involves dissolving of proteins in SDS buffer (with or without reducing agents), sonication (which results in some shearing and therefore dissolves larger polymers), purification, and dilution. The samples are then loaded onto an HPLC system and separated through a size exclusion column. The SE-HPLC column contains particles with pores which retain proteins for a length of time that depends on the size of the protein: smaller molecules are trapped and therefore slowed in their flow, while larger molecules pass through without delay. The absorbance of a sample which passed through the column is recorded at several time points to build the protein molecular size distribution profile of the sample. Depending on the pore size in the column it can be used to fractionate proteins with varying ranges of molecular weights, including polymeric and monomeric gluten proteins.

In this project, SE-HPLC analysis was performed by the Cereal and Milling Department of Campden BRI on total protein extracts from wheat pearling fractions and milling fractions of four selected cultivars: Hereward, Cordiale, Malacca and Istabraq, using the well-established Profilblé® method developed jointly by ARVALIS and l'Institut National de la Recherche Agronomique (INRA), Details of similar methods can be also found in literature (Morel and Bar-L'Helgouac'h, 2000, Morel et al., 2000).

2.7 Fluorescence microscopy

Immunofluorescence is a technique which uses fluorescent dyes labelled antibodies to bind target proteins. This allows the distribution of the target proteins to be observed with fluorescence microscopy and analysed using appropriate software.

2.7.1 Sample preparation

Materials and equipments

Plant material: Developing seeds of each of the seven cultivars, two N levels, and three bio-blocks (section 2.1.2). Seeds were collected at 15 dpa and 21 dpa

Buffer: 0.1M sodium cacodylate, pH 7.4

Fixative: 4% paraformaldehyde + 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.4

Ethanol: series from 10%, to 100% in 10 % steps (dry ethanol)

LR White resin medium grade (TAAB L012)

Resin series: ethanol 100%: LR White 4:1, ethanol 100%: LR White 3:2, ethanol 100%: LR White 2:3, ethanol 100%: LR White 1:4, 100% LR White

Capsules for embedding: polypropelene (TAAB Laboratories Equipment Ltd)

Embedding oven

Reichert-Jung Ultracut ultramicrotome

Poly-L-lysine hydrobromide coated multi-well slides

Method

Transverse sections (approximately 1mm thick) were cut out from developing grains then fixed in fixative on a rotator at room temperature for 5 h. Sections were then washed three times (20 min each) in 0.1M sodium cacodylate buffer and dehydrated in a graded ethanol series from 10% to 100% (30 min each grade, except 3x20 min at 100%). Subsequently, the sections were infiltrated in a series of increasing concentrations of LR White resin medium grade (1h each concentration), then in 100% LR White resin for four days. Finally, the sections were embedded within capsules with fresh resin and baked in an oven at 55°C for 16h, in a nitrogen flow for the first half hour.

The embedded samples were cut into 1 µm semi-thin sections which were collected on distilled water drops on polylysine coated multi-well slides, and then dried on a hot plate at 40°C.

2.7.2 Immunolocalisation using light microscopy and image analysis

Materials and equipment

Buffers: Phosphate buffered saline buffer (PBS), Sigma A4417; PBST (PBS+0.1% Tween 20, pH7.4)

Blocking agent: 3% bovine serum albumin (BSA), diluted in PBST

Primary antibodies:

- IFRN 0610 mouse monoclonal antibody, which recognises gliadins and LMW-GS, but not HMW-GS (Brett et al., 1999), diluted 1:100 in 1% BSA PBST
- INRA R2-HMW rabbit polyclonal antibody, specific for HMW-GS (Denery-Papini et al., 1996) diluted 1:100 in 1% BSA PBST

Secondary antibodies:

- Alexa Fluor 488 goat anti-rabbit IgG antibody, diluted 1:500 in 1% BSA in PBST; produced by Invitrogen
- Alexa Fluor 568 goat anti-rabbit IgG antibody, diluted 1:500 in 1% BSA in PBST; produced by Invitrogen

Microscope: Zeiss Axiophot epifluorescence microscope, installed with Prior ProScan II platform, Retiga Exi CCD digital camera (produced by Qimaging) and MetaMorph Software 7.7 (produced by Molecular Devices)

Immunofluorescence

Sections were rinsed with PBST, and blocked in blocking agent (3% (w/v) BSA) for 40 min. Afterwards they were incubated in R2-HMG rabbit polyclonal antibody, diluted in 1% BSA PBST (1:100) for 1h, followed by a 5 min wash with PBST and1h incubation in IFRN 0610 mouse monoclonal antibody diluted in 1% (w/v) BSA PBST (1:100). The excess primary antibodies were then removed by 3x5 min rinse with PBST. The sections were then incubated for 1 h in the dark with a combination of Alexa Fluor 488 goat anti-rabbit IgG antibodies and Alexa Fluor 568 goat anti-rabbit IgG antibodies, which were both diluted 1:500 in 1% (w/v) BSA PBST. Sections were rinsed 2x5 min in PBST, 3x5 min in PBS and 3x5 min in sterile distilled water. The slides were stored in the dark at 4°C overnight and examined on the following day.

Light microscope scanning and image analysis

Immunostained sections were scanned using a Zeiss Axiophot epifluorescence microscope system (20x objective). Different filters were used to visualise fluorochromes conjugated to secondary antibodies reacting with primary antibodies specific for HMW-GS (green colour) or LMW-GS+gladins (red colour). The image acquiring settings were: monochrome, 800ms exposure time, binning 1x1, manual focus.

The images were processed in MetaMorph 7.7 following an original procedure designed for this analysis (Figure 2.8):

1. Background and shading correction: statistical correction—average, which calculates an average intensity value from a selected region

(whole section in this case), then subtracts the value from the whole image.

2. Isolate of regions of interests: six rectangular transects (three from lobe region toward lobe centre, three from dorsal region toward ventral region) were selected and isolated. The size of the regions was 487.5x200 (μm^2), 650x200 (μm^2) or 812.5x200 (μm^2), depending on size of the section.
3. Distance (from aleurone layer) map: created by drawing the border line at the level of the aleurone layer on an isolated region, then using Arithmetic function to produce a copy with the border line only (Arithmetic: constant value 1, bit depth 1, operation Maximum using Binary Operations: Euclidean distance. In this image, the intensity of a pixel equals its t distance from the border line (1 pixel=0.325177, under 20x magnification).
4. Object mask: the region image was first threshholded: (Inclusive, lowest value varied from 100-300 depending on image quality). Next, a binary image was produced from it and all objects in the binary image were identified using the "create regions around objects" function. "Integrated Morphometry" (IMA) was then used to clear the image by filtering objects which were too small (area<1), too dark (average intensity<200), or unwanted (such as cell walls) (shape factor <0.2). The filter settings varied with different quality pictures. In the end, a cleared object mask was produced using IMA.
5. Mask split: the object mask was applied on the Euclidean distance map and then equally divided into pieces based on the distance to the aleurone layer using average intensity filter. (Three pieces for 487.5x200 (μm^2) region, four for 650x200 (μm^2) and five for 812.5x200 (μm^2)). The

masks were named by the order of distance from the border with 1 being the closest and 5 the furthest.

6. Data reading: the divided masks were applied to the original region image using IMA. The total area and total intensity of each area (divided mask) within the region were recorded. This revealed a relationship between those properties and the distance from the aleurone layer.

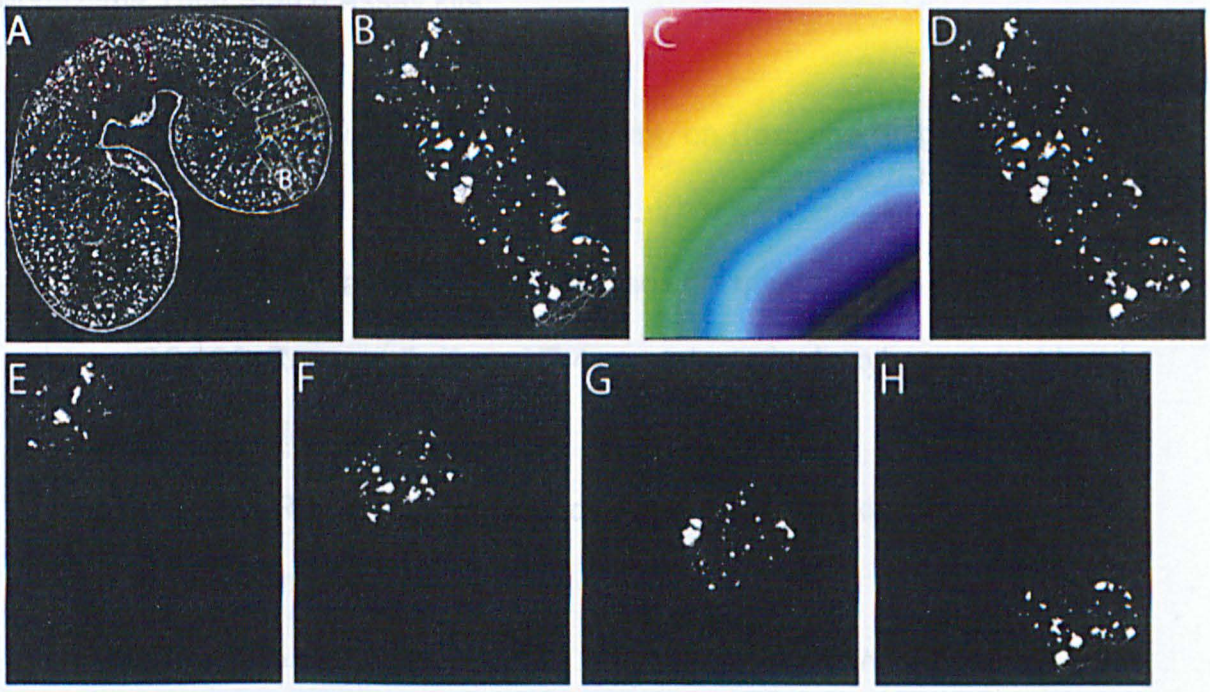

Figure 2.8 MetaMorph image analysis of HE100_15DPA_sample2, the image was acquired for HMW-GS 2.8.2 immunolocalisation. (A) Whole section with selected regions, (B) a region isolated from lobe area, (C) Euclidean distance (from aleurone layer) map of the selected region, (D) Cleared object mask of the selected region, (E)-(H), divided masks based on distance from the aleurone layer, E was the furthest area and H the closest.

2.8 Total starch content

Materials and equipment

Samples: Wheat pearling fractions of cultivars Hereward, Cordiale, Malacca and Istabraq) prepared as in section 2.2.2)

Megazyme Total Starch Assay kits:

- Thermostable α-amylase (1600 U/mL on Ceralpha reagent at pH 5.0 and 40 °C)
- Amyloglucosidase (3300 U/mL on soluble starch at pH4.5 and 40 °C)
- GOPOD Reagent Buffer: pH7.4, contains p-hydroxybenzoic acid and sodium azide (0.04w/v)
- GOPOD Reagent Enzymes: contains glucose oxidase, peroxidase and 4-aminoantipryine
- D-Glucose standard solution: 1.0mg/mL in 0.2% (w/v) benzoic acid
- Standardised maize starch control: 82.8% starch

Other reagents:

- Sodium acetate buffer: 100mM, pH5.0, plus 5mM $CaCl_2$
- Potassium hydroxide solution: 2M

Method

The principle of this method is using α-amylase to hydrolyse starch to maltodextrin, which is then quantitatively hydrolysed into D-glucose by

amyloglucosidase. Then D-glucose is then isoxidised to D-gluconate with the release of an equal amount (mole) of hydrogen peroxide, which can be quantified in a colour reaction (involves 4-aminoantipryine, p-hydroxybenzoic acid and peroxidase) which gives a quinoneimine dye that can be measured by spectroscopy (wavelength 510nm). The starch content of the sample can then be calculated by comparing this absorbance with the absorbance of a glucose standard solution, which is quantified using the same method. The details of the protocol can be found in Megazyme Total Starch Assay kit manual (http://secure.megazyme.com/downloads/en/data/K-TSTA.pdf example a). Bulked samples of three biological replicates rather than separate replicates were used due to limited amount of fractions. The experiment was split into two blocks, and two technical replicates analysed tested within each block.

2.9 Amylose/amylopectin ratio

Materials and equipments

Samples: the same wheat pearling fractions as used in last section

Megazyme Amylose/Amylopectin Assay kits including:

- Freeze dried concanavalin A (Con A)
- Amyloglucosidase (3300 U/mL on soluble starch at pH4.5 and 40 °C), plus fungal α-amylase (500 U/mL on Ceralpha reagent at pH 5.0 and 40 °C)
- GOPOD Reagent Buffer: pH7.4, contains p-hydroxybenzoic acid and sodium azide (0.04w/v)
- GOPOD Reagent Enzymes: contains glucose oxidase, peroxidase and 4-aminoantipryine
- D-Glucose standard solution: 1.0mg/ml in 0.2% (w/v) benzoic acid
- Starch reference sample: 66% amylose

Other reagents:

- Sodium acetate buffer: 100mM, pH 4.5, plus 0.2mg/ml sodium azide
- Concentrated Con A solvent: 600mM, pH 6.4 sodium acetate buffer, plus 3.4mM calcium chloride, 3.4mM magnesium Chloride, and 3.5mM manganese(II) chloride
- Dimethyl sulphoxide (DMSO): analytical reagent grade (BDH Analar)

Method

This method uses DMSO to disperse the starch in the sample which is then precipitated in ethanol, allowing separation from lipids. The precipitated starch is dissolved in an acetate/salt solution and an aliquot is used to determine total starch content. Amylopectin is then specifically precipitated from the remaining starch solution by the addition of Con A, which complexes branched polysaccharides to form a precipitate; the remaining starch in the supernatant is then measured as described in section 2.8. The concentration of amylose in the starch sample is determined as the ratio of starch in the supernatant of the Con A precipitated sample, to that of the total starch sample. The details of the protocol can be found in Megazyme Amylose/Amylopectin Assay kits manual (http://www.megazyme.com/downloads/en/data/K-AMYL.pdf). Bulked samples of three biological replicates were used due to limited amount of fractions. The experiment was split into two blocks, and two technical replicates were analysed within each block.

2.10 Gelatinisation characteristics: DSC

Introduction

Differential scanning calorimetry (DSC) is widely used to observe the physical transformation of a sample during a heating/cooling process. In starch research, DSC is used to investigate the gelatinisation and retrogradation properties. In a DSC experiment, the heat flow is recorded during a controlled heating/cooling process. Any changes (peak/valley) in the heat flow indicate changes in the physical structure of the sample, such as phase transitions. Those changes can be recorded in several ways, such as the transformation onset temperature or the energy required for the transformation (gelatinisation enthalpy in starch gelatinisation).

Materials and equipment

Samples: Wheat pearling fractions of cultivars Hereward and Istabraq

Equipment: Pyris 1 Differential Scanning Calorimeter and aluminium standard sample pans, produced by PerkinElmer

Method

The wheat pearling fractions were mixed with water in a ratio of 1:2 (g flour: g water) and left at room temperature for 1h. A 40±5 mg (accurate to 0.0001g) aliquot was then weighted and sealed in a standard sample pan, which was also weighted to 0.0001g accuracy. The pan with the sample was loaded into the holder with a sealed empty pan (weighted to the same accuracy). Both pans

were heated from 25°C to 95°C simultaneously at a heating speed of 10°C/min. The heat flow of the sample was measured and recorded using the heat flow of the empty pan as a baseline reference. The onset temperature, peak temperature, end temperature, and gelatinisation enthalpy (J/g) of the peak were calculated using the DSC system software. The sample was then dried in an oven at 130°C for 90 min and weighted in order to calculate the gelatinisation enthalpy of the dry solids, which is more accurate as the moisture content of the sample varies during the heating process. Two technical replicates of each sample were analysed.

2.11 Determination of damaged starch

Materials and equipments

Samples: Wheat pearling fractions of Hereward (as used in last section)

Megazyme Starch Damage Assay kits, includes

- Fungal α-amylase (1000 U/mL on Ceralpha reagent at pH 6.0 and 40 °C)
- Amyloglucosidase (200 U/mL on soluble starch at pH4.5 and 40 °C), Ammonium sulphate suspension
- GOPOD Reagent Buffer: pH7.4, contains p-hydroxybenzoic acid and sodium azide (0.04w/v)
- GOPOD Reagent Enzymes: contains glucose oxidase, peroxidase and 4-aminoantipryine
- D-Glucose standard solution: 1.5mg/mL in 0.2% (w/v) benzoic acid
- Wheat flour standard

Principle

The method uses carefully controlled treatment with fungal α-amylase to digest damaged starch granules with minimum breakdown of undamaged granules, the damaged starch granules being more susceptible to α-amylase. Dilute sulphuric acid is used to stop the enzyme reaction. The products of the reaction, maltosaccharides plus α-limit dextrins are then degraded to glucose by amyloglucosidase and measured using the same colourimetric method used in

the total starch kit described in section 2.8. Starch damage is expressed as a percentage of flour weight.

The damaged starch test was carried out by the Cereal and Milling Department of Campden BRI.

2.12 Starch particle size distribution

Materials and equipments

Samples: Wheat pearling fractions of Hereward (methods described in section 2.2.2)

QICPIC high speed image analysis system with RODOS dry disperser (up to 450fps with 1024x1024 square pixels, 1ns exposure time), made by Sympatec.

Principle

The analysis used a dynamic image analysis system. The flour were dispersed and flowed through the system at controlled speed while a high-speed camera took numerous images. The the images were then processed by the instrument software to measure and count the particles. More details can be found at http://sympatec.com/EN/ImageAnalysis/ImageAnalysis.html.

The size particle distribution analysis was carried out by the Cereal and Milling Department of Campden BRI.

Chapter 3: Physical properties of grain and pearling behaviours

3.1 The physical properties of grains

3.1.1 Results

The SKCS test data were analysed with the weighted ANOVA[8] method using GenStat (14th edition). Each data point was a mean of 300 seeds. The S.D. (standard deviation) of data points was used as "weight" (a mathematical function to give certain elements more influence on the result, used in here to include the system errors which was originally excluded in the data set) in the analysis. The ANOVA results are presented in table 3.1 and means are summarised in appendix 8 and compared in Figure 3.1

Table 3.1 Factors affect physical properties of wheat

Physical properties	Factors	P value	l.s.d.	d.f.
Weight	Cultivar	<0.001	2.35	26
	N-fertilisation	0.002	2.35	26
	Cultivar × N-fertilisation	0.02	2.35	26
Size/diameter	Cultivar	0.002	0.08	26
	N-fertilisation	0.599	0.08	26
	Cultivar × N-fertilisation	0.028	0.08	26
Hardness Index	Cultivar	<0.001	0.23	26
	N-fertilisation	<0.001	0.23	26
	Cultivar × N-fertilisation	<0.001	0.23	26
Moisture	Cultivar	<0.001	0.90	26
	N-fertilisation	0.099	0.90	26
	Cultivar × N-fertilisation	<0.001	0.90	26

[8] ANOVA: analysis of variance, a statistical test, for analysing the effect of experimental factors on tested data.
[9] l.s.d: Least significant differences of means (5% level)

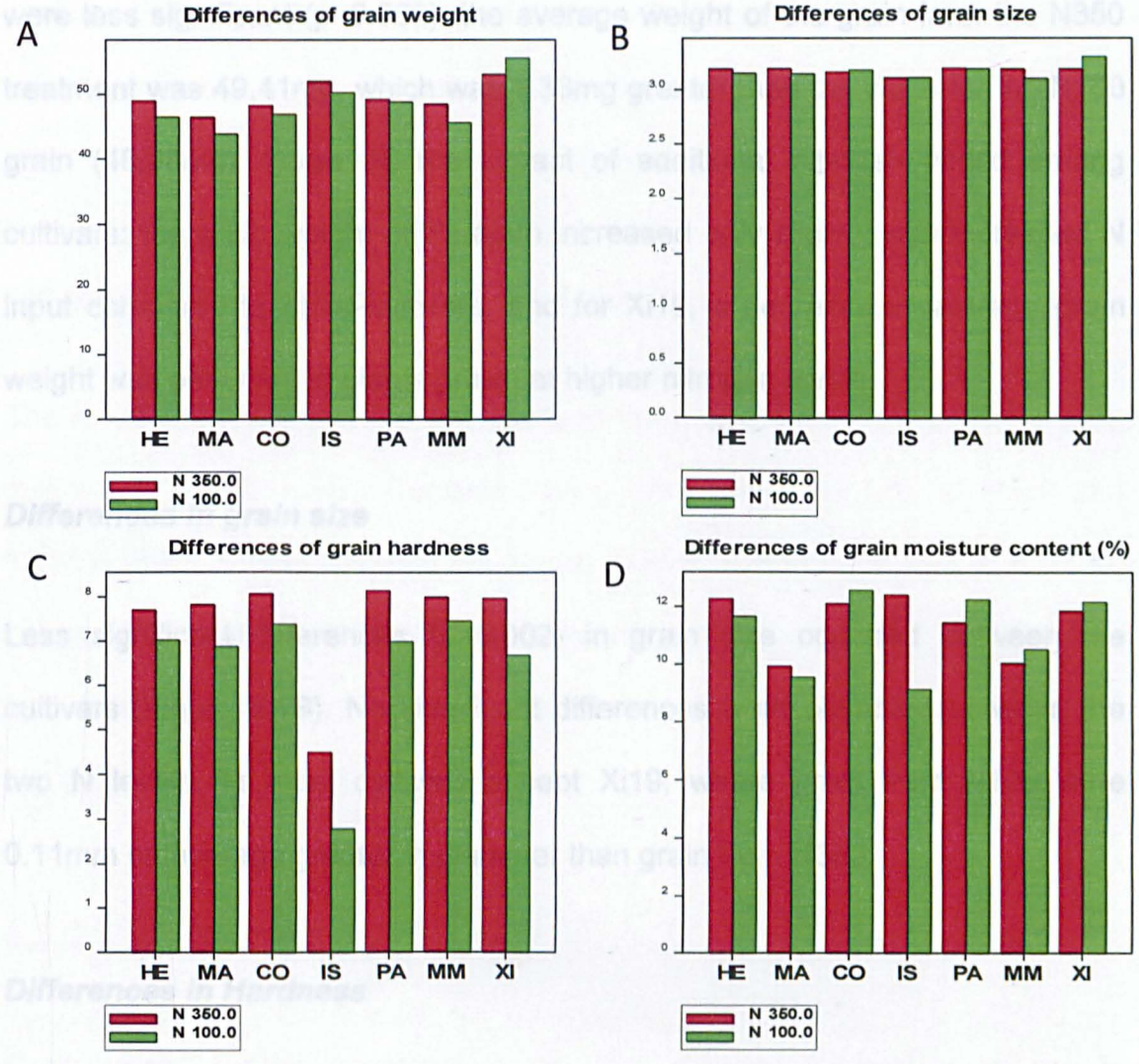

Figure 3.1 Differences in grain physical properties: A) Fresh grain weight (mg), 5% l.s.d 2.35; B) grain size, represented in diameter (mm), 5% l.s.d 0.08; C) Hardness Index, square root transformations were applied, 5% l.s.d 0.23; D) moisture content (%), 5% l.s.d 0.90

Differences of grain weight

Figure 3.1A showed significant differences in grain weight ($p<0.001$[9]) between cultivars. Xi19 had highest grain weight among the seven cultivars, followed by Istabraq, while Malacca had the lowest. Differences between the nitrogen levels

[9] p-value: the probability of tested factor is not significant. $p<0.001$ is commonly accepted as significant differences exist. $p<0.05$ is also may accepted as significance indicator, however, may considered as less significant.

were less significant (p=0.002): the average weight of the grain from the N350 treatment was 49.41mg, which was 1.33mg greater than the value for the N100 grain (48.08mg). However, the impact of additional nitrogen varied among cultivars: the grain weight of Paragon increased only slightly with increased N input compared to other cultivars, and for Xi19, a decrease in average grain weight was observed in plants grown at higher nitrogen levels.

Differences in grain size

Less significant differences (p=0.002) in grain size occurred between the cultivars (Figure 3.1B). No significant differences were observed between the two N levels for most cultivars except Xi19, where grain from N100 were 0.11mm on average greater in diameter than grain from N350.

Differences in Hardness

Significant differences (p<0.001) in grain hardness were found among the cultivars. Paragon was the hardest when grown at N350) while Malacca was the hardest when grown at N100, with Hardness Indexes of 66.40 and 55.68, respectively. By contrast, Istabraq was the softest at all N levels. This is consistent with Istabraq being the only soft cultivar with Hardness Index values of 20.25 (N350) and 7.80 (N100). Higher nitrogen also appeared to significantly impact on grain hardness (p<0.001), with increases in Hardness Index of 13.82 points, on average, being observed between N350 grain (average Hardness Index 56.85) and N100 grain (average Hardness Index 43.03). Istabraq was the most affected by N input, with the Hardness Index being increased almost three-fold between N100 and N350 grain. Xi19, Malacca and Paragon showed

between 23% and 30% increases in their hardness when N100 and N350 grain were compared, while Hereward, Cordiale and Maskman were the least affected with only 13%-17% increases in hardness.

Differences of grain moisture content (%)

The moisture contents of the grain ranged from approximately 9 to13% among cultivars ($p < 0.001$), with Cordiale having the highest (12.12% at N350 and 12.58% at N100) and Malacca the lowest (9.94% at N350 and 9.56% at N100) values. No significant differences in moisture content associated with N levels were observed, with the exception of Istabraq which had a 3.3% lower value at N100 compared to N350.

3.1.2 Discussion

Grain weight is often presented in the form of average single kernel weight (SKCS data), test weight/bulk weight (weight of a specific volume) or thousand kernel weight (TKW). Although differences in wheat yield are mainly related to differences in grain number rather than size (Evans, 1978, Demotes-Mainard et al., 1999), grain weight still considered to be an indication of flour yield and it is therefore used worldwide for wheat grading (Carson and Edwards, 2009, Halverson and Zeleny, 1988). A strong correlation between genotype and grain weight has been reported in the literature (Yücel et al., 2009). Also, grain weight can be influenced by environmental factors, in particular, by growing conditions during grain filling (Graybosch et al., 1995), with N supply playing a significant role (Nass and Reiser, 1975, Gebeyehou et al., 1982, Warraich et al., 2002). Abedi et al. (2011) found that N deficiency at the grain filling stage resulted in a

decrease in TKW. The grains analysed in this project were treated with split nitrogen supplies in March, April and May, 2009. The N350 plots were treated at all three time points, but the N100 plots were only treated at the first two time points. No extra N was given at the grain filling stage. However the last treatment (mid-May) was very close to grain filling. Considering this and the results of Abedi et al. (2011), we may assume the differences of grain weight in some of our test lines were due to the differences in N supply at the pre-grain filling stage.

Grain size is closely related to grain weight and is an important factor in determining the test weight (Halverson and Zeleny, 1988). It also has an influence on milling and end product quality (Berman et al., 1996, Dziki and Laskowski, 2004). Some correlation between genotype and grain size has been found, however not significantly distinguishable as the differences were not consistent for different cultivars (Yücel et al., 2009, Sadowska et al., 2001); and non-significant effects of N-fertilisation level were found (Sadowska et al., 2001). Our data verified these results.

Grain moisture content is commonly considered to be determined by harvest, handling, transport, and storage conditions. Grain moisture is closely associated with wheat quality and market value (Hellevang, 1995). It also influences other characteristics of wheat with positive correlations between moisture and grain size/TKW being reported (Tabatabaeefar, 2003). A negative correlation between moisture and hardness was also reported (Mis and Grundas, 2002). The cell wall properties of grain may also influence the drying of grain as wheat cell wall polysaccharides are known to have high water

retaining ability. Therefore, differences in cell wall composition among cultivars would influence the grain moisture content.

Grain hardness is considered to be one of the most important factors determining wheat processing quality (Dziki and Laskowski, 2005, Pasha et al., 2010), since it greatly influences the energy input required for milling (Hrušková et al., 2006, Hruskova and Svec, 2009), and consequently the degree of starch damage (Belderok et al., 2000, Carson and Edwards, 2009), and is also considered as an indicator of grain protein content (Pasha et al., 2010, Belderok et al., 2000). Grain hardness may result from the porosity/density of the endosperm, and in particular of the protein matrix (Pasha et al., 2010, Dobraszczyk et al., 2002), or from the degree of adhesion between starch and the protein matrix (Pasha et al., 2010, Giroux and Morris, 1998). Wheat hardness is a genetically determined trait largely controlled by one major locus, the *Ha* locus (Dobraszczyk et al., 2002, Tranquilli et al., 1999) on chromosome 5D. Although, mainly determined by genotype, hardness it is also affected by growing conditions such as N fertilisation (Saint Pierre et al., 2008, Makowska et al., 2008), as also observed in this study.

3.2 Grain physical properties and pearling

Figure 3.2 reports the results of a pearling efficiency trial. This shows a logarithmic-like relationship between pearling time and degree of grain removed. The results reflect the physical principles of the pearling mill: the seeds are trapped between the stone mill wheel and a wheel-shaped sieve during milling. As the mill wheel rotates at high speed, frictional forces are applied on the seeds when they came in contact with the mill wheel causing abrasion of the contact surface of the seeds to release fine flour. Such forces also move the seeds around the mill creating new contact surfaces. The flour passes through the sieve while the pearled cores remain within the mill for further pearling. Since the size of the seeds become gradually smaller during pearling, the contact surface between seeds and mill wheel gradually decreases, causing a reduction in the pearling efficiency.

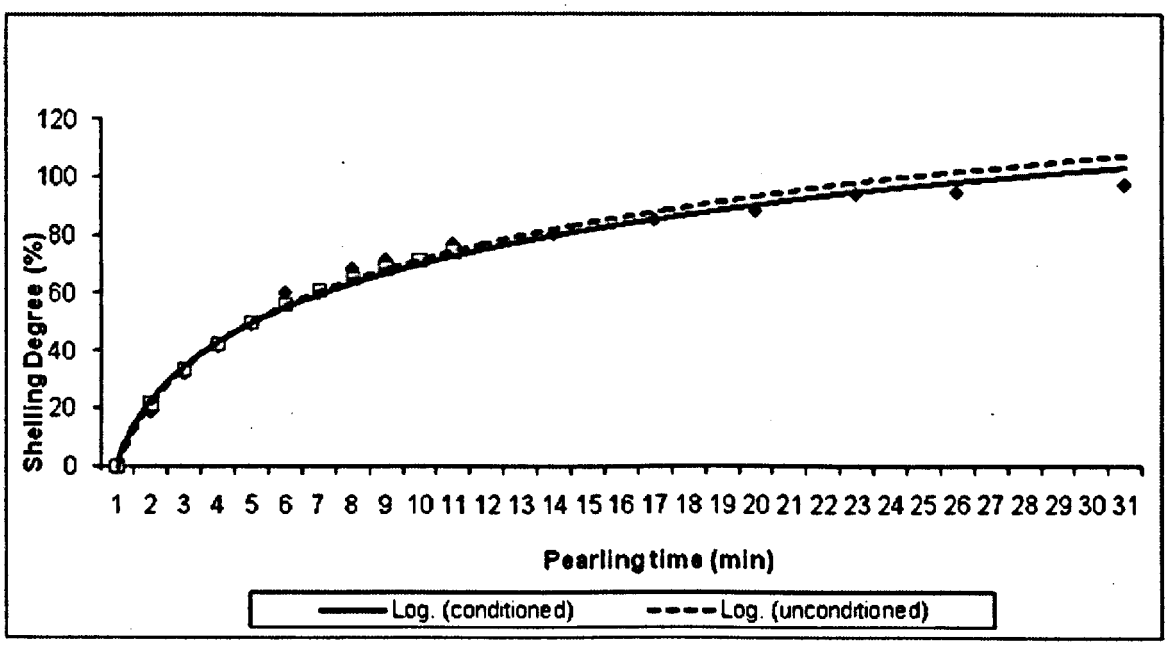

Figure 3.2 Pearling efficiency test of PA350, unconditioned seeds moisture content 13.37%, conditioned seeds moisture content 15.58%

3.2.1 Effects of moisture content on pearling efficiency

In theory, the moisture content may affect pearling as high moisture may alter grain volume and texture and, therefore, influence the pearling process. To test this hypothesis, pearling trials were carried out using conditioned and unconditioned seeds of Paragon (N350). The results (Figure 3.2) showed no significant differences in pearling efficiency between conditioned and unconditioned seeds (unconditioned seeds moisture content 13.37%, conditioned seeds moisture content 15.58%), including during the first seven minutes which is the time interval that was used for our experiments. Although a much higher moisture content could have an effect, it would be difficult to maintain and could also cause changes in the structure/composition of the grain. Therefore, the moisture content was considered to be unimportant and no conditioning procedure of the grain was carried out.

3.2.2 Effects of grain size and weight

Although the size and shape of the grain may affect the extent of the contact surface during pearling, no significant differences in pearling behaviour related to grain size or shape were found among the cultivars. This may be because any small differences were masked by more important factors, such has hardness or within the high intrinsic error of the pearling procedure. The mill used in these studies was a small scale laboratory mill, not an analytical instrument. Also, grain samples were examined before pearling to remove seeds with irregular size/shape. Therefore, the influence of grain weight was minimal.

3.2.3 Effect of grain hardness and hardness gradient

Grain hardness was considered to be the most important factor affecting the pearling process. The cubic interpolative curves built for predicting the times required for removing fractions of defined weight (described in section 2.3.1) provided supporting evidence of this assumption. Figure 3.3 showed the differences in cubic interpolative curves between two hard wheats (Hereward and Malacca) and soft wheat (Istabraq). The trends for Hereward and Malacca were logarithmic-like, which indicated that the pearling speed gradually decreased as pearling progressed. By contrast, Istabraq differed in the early stage (before 1 min) of the pearling process (marked in red circles in Figure 3.3), with the pearling speed increasing. Hypothetically, if no other variables are involved, the pearling efficiency curves should always be logarithmic-like because the grain size will gradually decrease. The difference observed with Istabraq therefore suggests that there is another variable in the system which results in an increase in speed as pearling proceeds. This variable is hardness, with Istabraq being the only soft textured cultivar that was studied. Considering all the above and the working principle of the pearling mill, it is sensible to suggest that there is a decreasing gradient of hardness from the outer layer of the grain towards the centre which would result in increases in pearling speed during the pearling process. Moreover, the different behaviour observed for Istabraq suggests that gradients in hardness have stronger effects in soft cultivars. This may also indicate that soft wheats have stronger gradients in hardness than hard wheats.

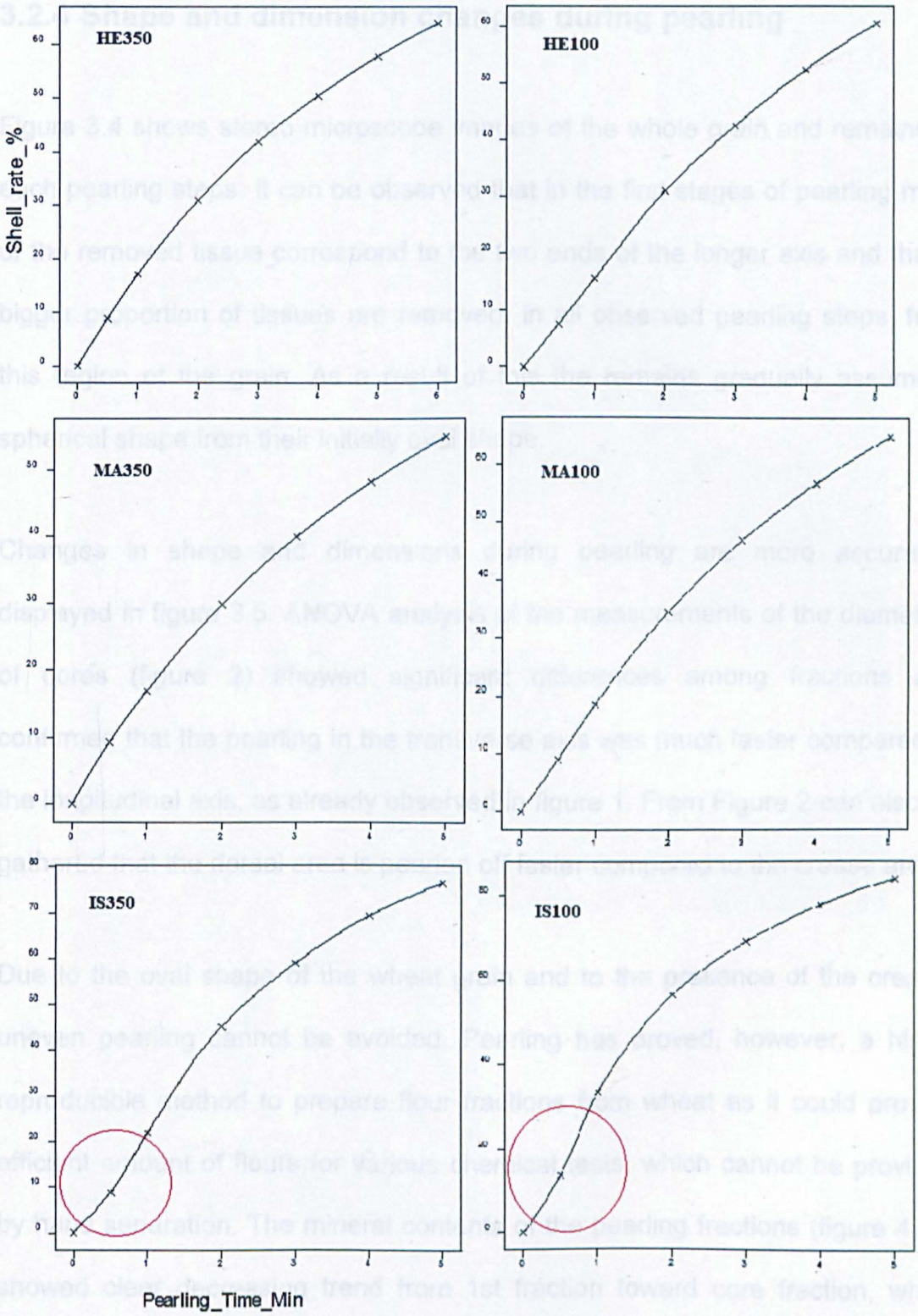

Figure 3.3 Cubic interpolative curves of HE350, HE100, IS350, and IS100, produced by GenStat. Marked in red circles: differences in shape caused by hardness gradients

3.2.4 Shape and dimension changes during pearling

Figure 3.4 shows stereo microscope images of the whole grain and remains of each pearling steps. It can be observed that in the first stages of pearling most of the removed tissue correspond to the two ends of the longer axis and that a bigger proportion of tissues are removed, in all observed pearling steps, from this region of the grain. As a result of this the remains gradually assume a spherical shape from their initially oval shape.

Changes in shape and dimensions during pearling are more accurately displayed in figure 3.5. ANOVA analysis of the measurements of the diameters of cores (figure 2) showed significant differences among fractions and confirmed that the pearling in the transverse axis was much faster compared to the longitudinal axis, as already observed in figure 1. From Figure 2 can also be gathered that the dorsal area is pearled off faster compared to the crease area.

Due to the oval shape of the wheat grain and to the presence of the crease, uneven pearling cannot be avoided. Pearling has proved, however, a highly reproducible method to prepare flour fractions from wheat as it could provide efficient amount of flours for various chemical tests, which cannot be provided by hand separation. The mineral contents of the pearling fractions (figure 4.21) showed clear decreasing trend from 1st fraction toward core fraction, which gave a good indication of the purities of pearling fractions (more details can be found at section 4.3.3).

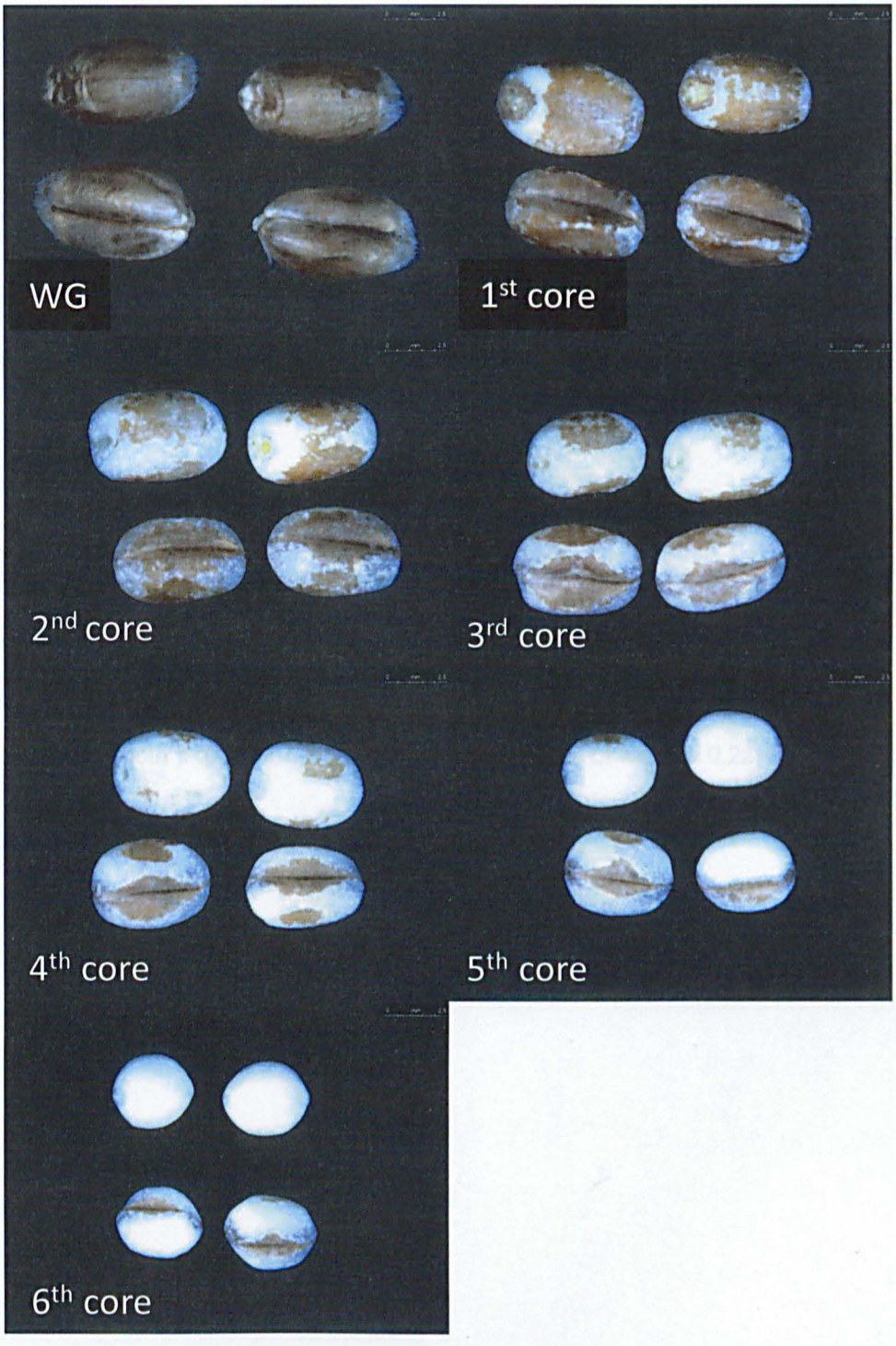

Figure 3.4 image collection of whole grains (WG) and remaining cores after each pearling step of HE350. Images were taken using Leica M205 FA Stereomicroscope (Leica, Wetzlar, Germany), exposure time 99 ms, auto white balance.

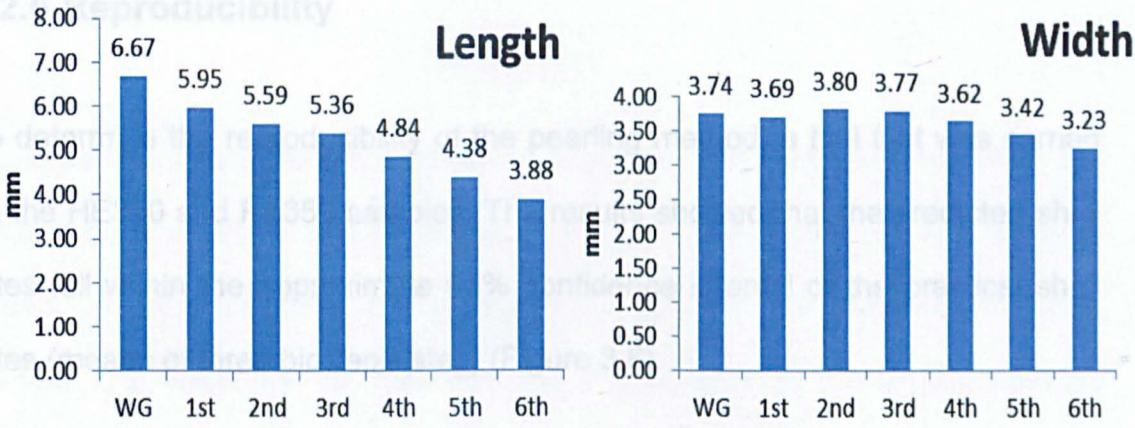

Figure 3.5 the measurements of diameters of whole grains (WG) and remaining cores after each pearling step of HE350. Data were generated using Seed Analyser Marvin (GTA Sensorik GmbH, Germany). Each data were average of ten measurements. Significant differences among fractions were found in both diameters (both $p<0.001$), l.s.d. of length is 0.24, l.s.d. of width is 0.22.

3.2.5 Reproducibility

To determine the reproducibility of the pearling method, a trial test was carried on the HE350 and PA350 samples. The results showed that the predicted shell rates fell within the approximate 95% confidence interval of the practical shell rates (means of three bio-replicates) (Figure 3.6).

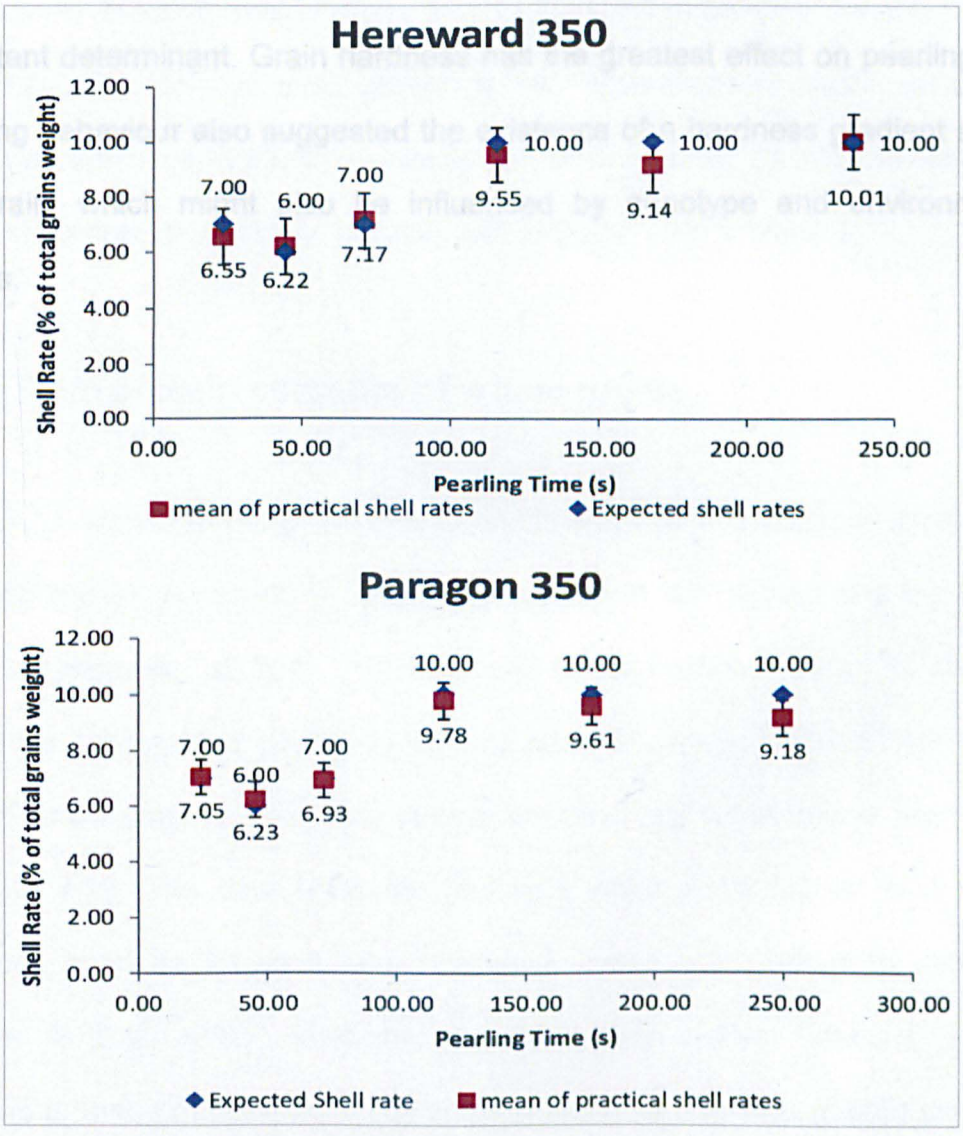

Figure 3.6 Reproducibility tests of HE350 and PA350, error bars on practical shell rates indicated the approximate 95% confidence intervals

3.3 Conclusions

My experiments showed that genotype was correlated with differences in the weight, size, moisture and hardness of the grain. N fertilisation had a major impact on grain hardness and also affected grain weight, size and moisture content to a lesser extent. However, the effects of N on the latter properties were not consistent among the cultivars suggesting that genotype was a more important determinant. Grain hardness has the greatest effect on pearling. The pearling behaviour also suggested the existence of a hardness gradient across the grain, which might also be influenced by genotype and environmental factors.

Chapter 4: Grain protein composition and distribution

4.1 Total protein content

The dataset included total protein contents (TPC, % in dry-flour weight) of eight fractions of seven cultivars, grown at two N-fertilisation levels with three biological replicates. No technical replicates were required as internal standards are used to ensure accuracy. In total, 336 samples were analysed.

4.1.1 Total protein contents of whole grains

Table 4.1A shows whole grain TPC of all lines at two N application levels (100 and 350 kg/ha). Hereward and Istabraq presented the highest and the lowest TPC, respectively, at both high and low N application levels. All cultivars showed a positive response to additional nitrogen fertilizer. The differences in TPC in whole grain for the same cultivar between two N treatments are ranked in Table 4.1B. The data show that cultivars which have higher total protein contents, such as Paragon and Hereward, responded better to additional nitrogen fertilizer when compared the low protein cultivar Istabraq, ANOVA analysis of these data (table 4.1C) showed significant effects of both genotype and N-fertilisation on TPC (both $p<0.001$). Significant interactions between the two factors were also found ($p=0.003$).

Table 4.1 Total protein contents (% of dry-flour weight) of whole grains, and influence factors

A: Whole grain TPC (average of three biological replicates)							
N350 lines	HE350	PA350	MM350	CO350	MA350	XI350	IS350
	14.36	13.97	13.51	13.40	12.94	12.94	11.80
N100 lines	HE100	CO100	MM100	MA100	PA100	XI100	IS100
	9.35	9.18	9.12	8.95	8.89	8.55	8.21
B: Difference of whole grain TPC between N350 and N100							
	PA	HE	MM	XI	CO	MA	IS
	5.07	5.02	4.39	4.39	4.22	3.99	3.59

C: ANOVA analysis result			
factors	**P value**	**l.s.d.**	**d.f.**
Cultivar	<0.001	0.3517	26
N-fertilisation	<0.001	0.1880	26
Cultivar x N-fertilisation	0.003	0.4973	26

4.1.2 Gradients of total protein content across grain

The data for TPC of pearling fractions were analysed with ANOVA using GenStat (14th edition). The effects of three main factors: cultivar, N level and fraction were tested together with all two-way and three-way interactions (table 4.2). Means are showed in table 4.3.

Table 4.2 Factors affect TPC of pearling fractions

Factors	**P value**	**l.s.d.**	**d.f.**
Cultivar	<0.001	0.1610	194
N-fertilisation	<0.001	0.0860	194
Fraction	<0.001	0.1610	194
Cultivar × Fraction	<0.001	0.4259	194
N-fertilisation × Fraction	<0.001	0.2277	194
Cultivar × N-fertilisation	<0.001	0.2277	194
Cultivar × N-fertilisation × Fraction	<0.001	0.6023	194

Table 4.3 Total protein content (% of dry flour weight) data summary

Variety	N fertilisation level	Fractions						
		1st	2nd	3rd	4th	5th	6th	core
CO	100	10.49	12.08	11.57	10.49	9.18	8.32	7.92
	350	14.99	18.81	18.13	16.42	14.48	12.83	10.94
HE	100	10.49	13.22	12.60	11.12	9.75	8.78	7.98
	350	16.07	20.92	21.03	18.87	16.47	14.25	10.94
IS	100	9.06	10.37	9.80	9.29	8.66	8.04	7.35
	350	14.08	16.19	15.16	13.79	12.43	11.29	10.03
MA	100	9.58	11.80	11.40	10.49	9.18	8.21	7.58
	350	14.02	18.70	18.13	16.07	14.14	12.26	10.32
MM	100	9.46	12.37	12.20	10.94	9.46	8.49	7.64
	350	14.82	19.89	19.21	17.21	14.93	12.88	10.49
PA	100	10.66	13.11	12.31	10.60	9.01	7.98	7.30
	350	15.68	20.86	20.58	18.47	15.79	13.97	10.37
XI	100	10.03	12.31	11.57	9.98	8.61	7.75	7.41
	350	15.16	19.15	18.18	15.68	13.45	11.69	10.37

Figure 4.1 shows that there is a significant TPC gradient across the grain in all cultivars and both N levels ($p<0.001$). In all lines, TPC peaked in the 2nd fraction and then gradually decreased toward the core (the only exception is HE350, in which TPC peaked in the 3rd fraction).

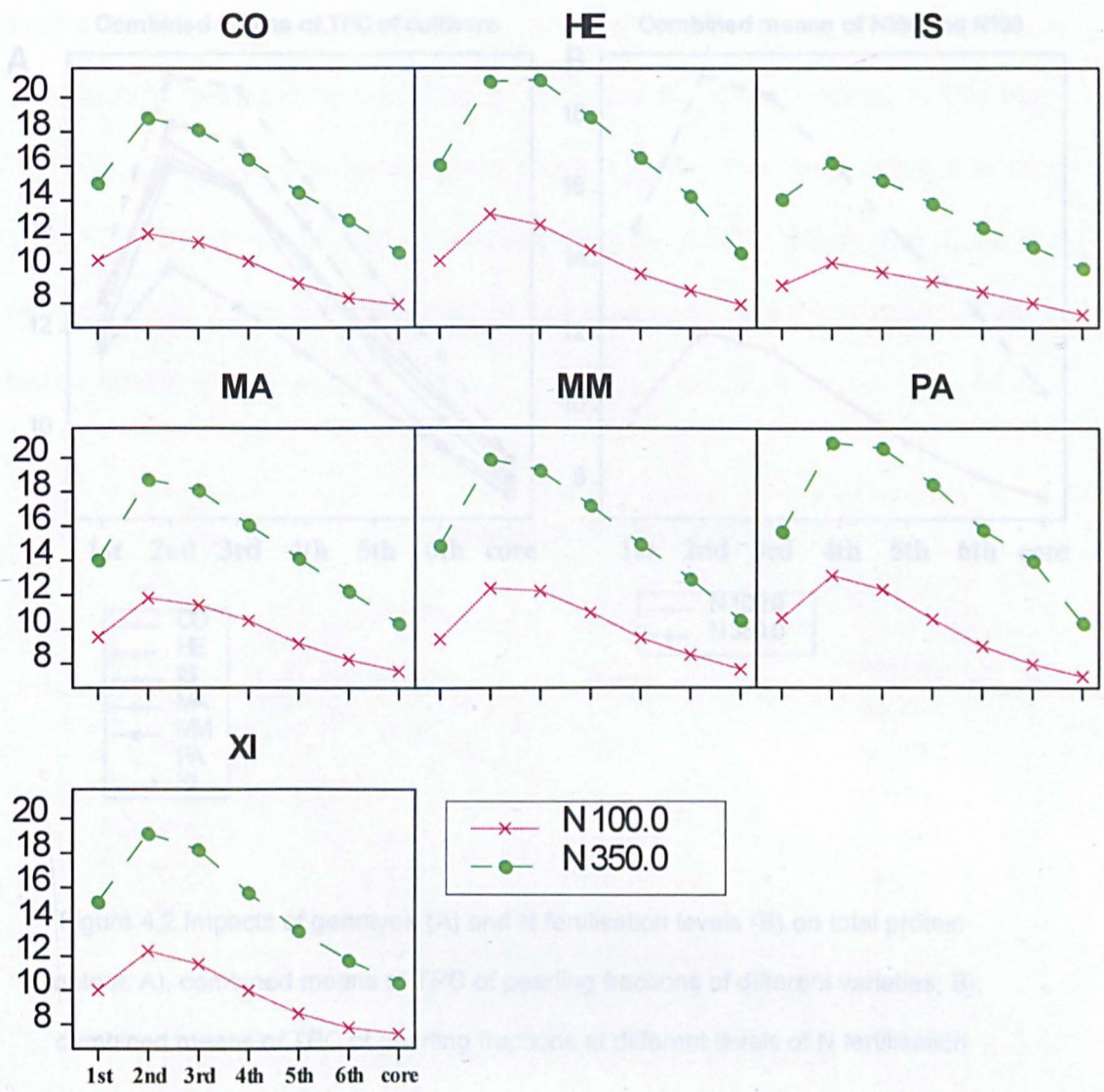

Figure 4.1 Gradients in total protein content (% of dry flour weight) in pearling fractions

The effect of genotype is shown in figure 4.2A. Genotype affected the TPC across the grain ($p<0.001$), with fractions of Hereward and Paragon having the highest TPCs and Istabraq having the lowest. Genotype also affected the gradients of TPC as shown by the significant interaction ($p<0.001$) between cultivars and fractions. This results in differences in the slopes and/or shapes of the gradients. Hereward and Paragon showed highest peaks and steepest slopes from the peak to the core. By contrast, Istabraq had the shallowest gradient.

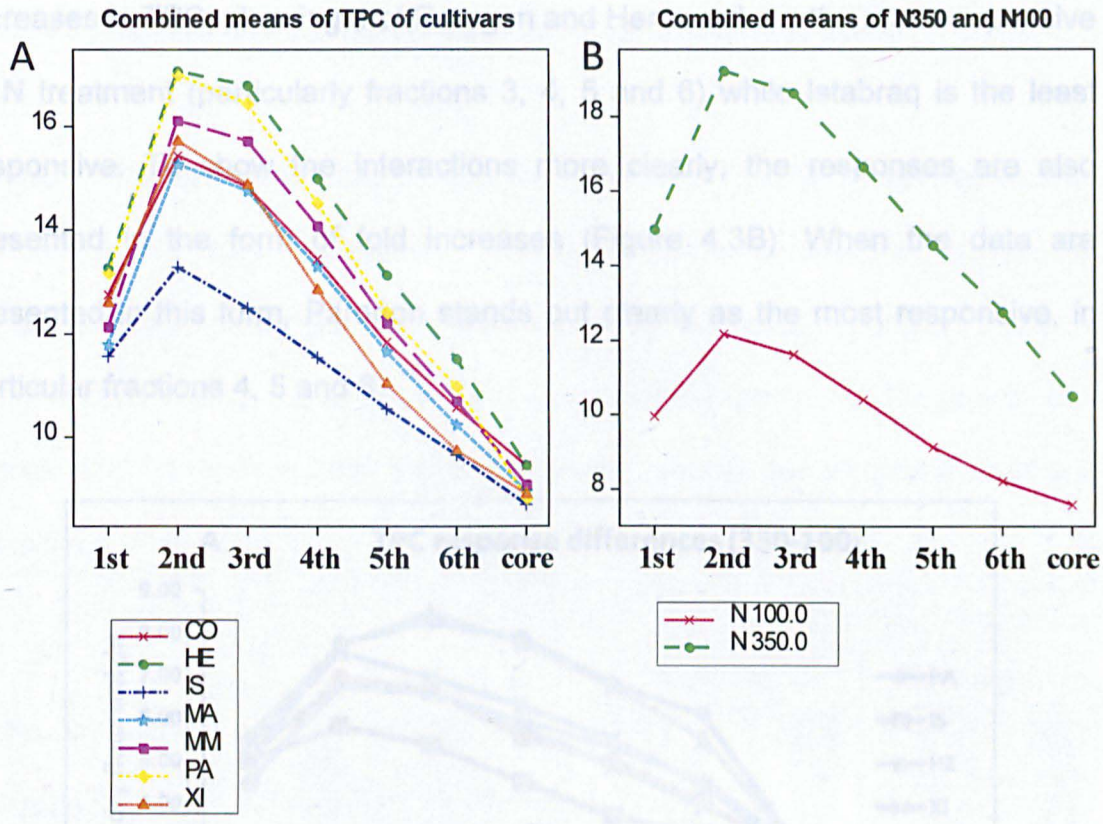

Figure 4.2 Impacts of genotype (A) and N fertilisation levels (B) on total protein content: A), combined means of TPC of pearling fractions of different varieties; B), combined means of TPC of pearling fractions at different levels of N fertilisation

Figure 4.1 showed a significant effect of additional nitrogen fertilizer ($p<0.001$) on the TPC of all fractions. However, the responses were not the same for different fractions. Figure 4.2B shows a shallower gradient for TPC in the combined means of the N100 samples compared to N350, with the increase being highest for fractions 2 and 3 (+7.04% and +6.99%, respectively) and the lowest for the core fraction (+ 2.91%).

Significant interactions between cultivar and N level and three way interactions between cultivar, N-level and fractions were also found (both $p<0.001$). Figure 4.3 illustrates the differential responses to nitrogen fertilizer of the seven cultivars. In figure 4.3A, the response is quantified in terms of absolute

increases in TPC, showing that Paragon and Hereward are the most responsive to N treatment (particularly fractions 3, 4, 5 and 6) while Istabraq is the least responsive. To show the interactions more clearly, the responses are also presented in the form of fold increases (Figure 4.3B). When the data are presented in this form, Paragon stands out clearly as the most responsive, in particular fractions 4, 5 and 6.

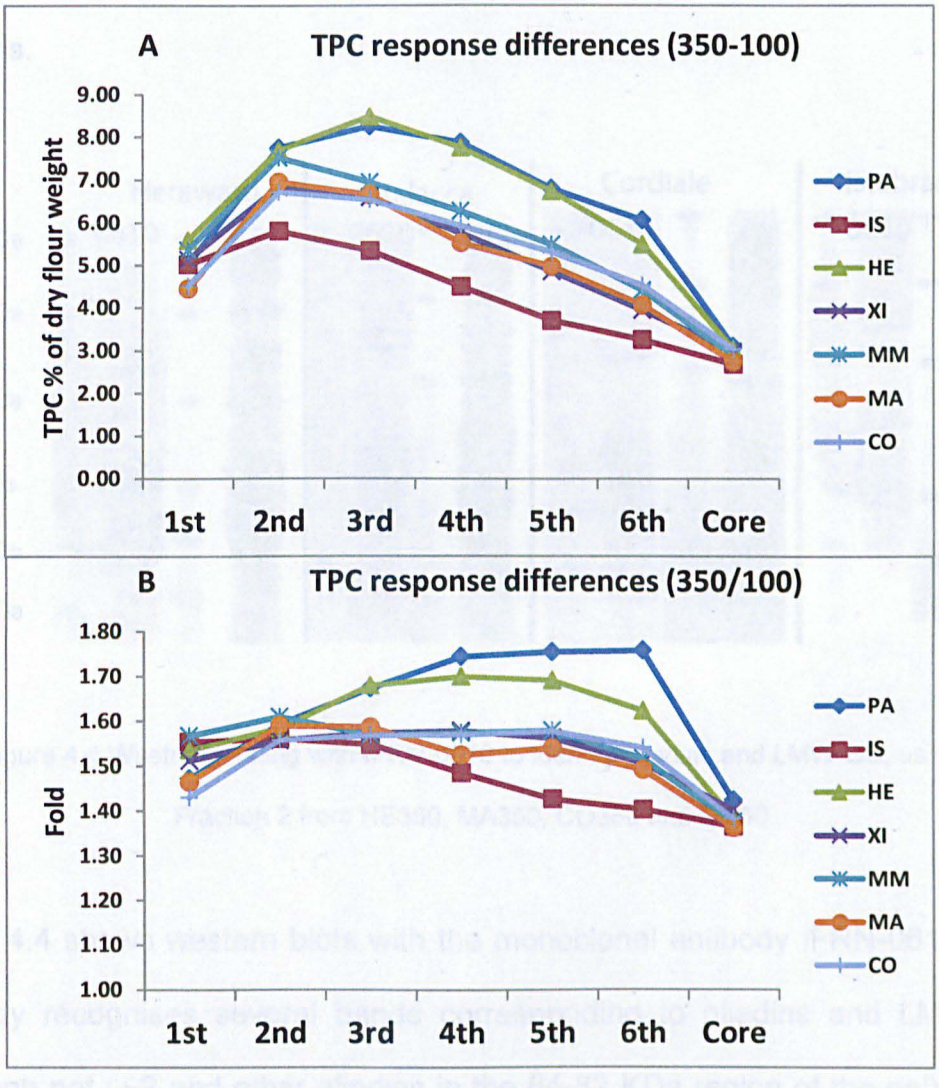

Figure 4.3 Differences between the TCP of pearling fractions from the N350 and N100 samples:

A) Differences in TPC, value= TPC of N350 fraction − TPC of N100 fraction;

B) TPC increase in fold, value = TPC of N350 fraction/TPC of N100 fraction

4.2 Western blotting to identify protein bands

Western blotting with antibodies specific for different gluten protein types was carried out to study qualitative protein gradients across the grain. The experiment used pearling fraction 2 from the N350 samples of cultivars because these fractions had the highest TPC (or second highest for Hereward, see Figure 4.1). Therefore, the antibody binding should be strongest in these fractions.

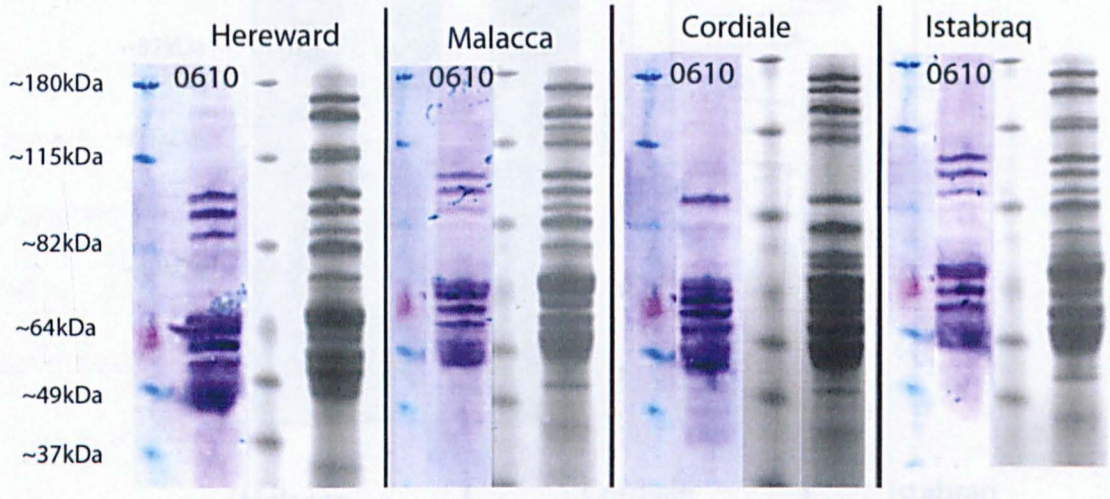

Figure 4.4 Western blotting with IFRN-0610 to identify gliadins and LMW-GS, using Fraction 2 from HE350, MA350, CO350 and IS350

Figure 4.4 shows western blots with the monoclonal antibody IFRN-0610. This antibody recognises several bands corresponding to gliadins and LMW-GS, (although not ω-2 and other gliadins in the 64-82 KDa region of the gel) (Brett et al., 1999). In Hereward, Malacca and Istabraq, the antibody binds to three bands in the ω-5 region, but only one band in Cordiale. Figure 4.5 shows western blots carried out with polyclonal antibodies specific for ω-2 or ω-5 gliadins (Denery-Papini et al., 2000). The labelling patterns are again similar for

Hereward, Malacca and Istabraq, and different for Cordial in which only two bands were clearly labelled compared with four in the other cultivars. The ω-2 antibody also labelled Cordial differently from the other three varieties (only two bands in the 82-115 kDa region rather than three).

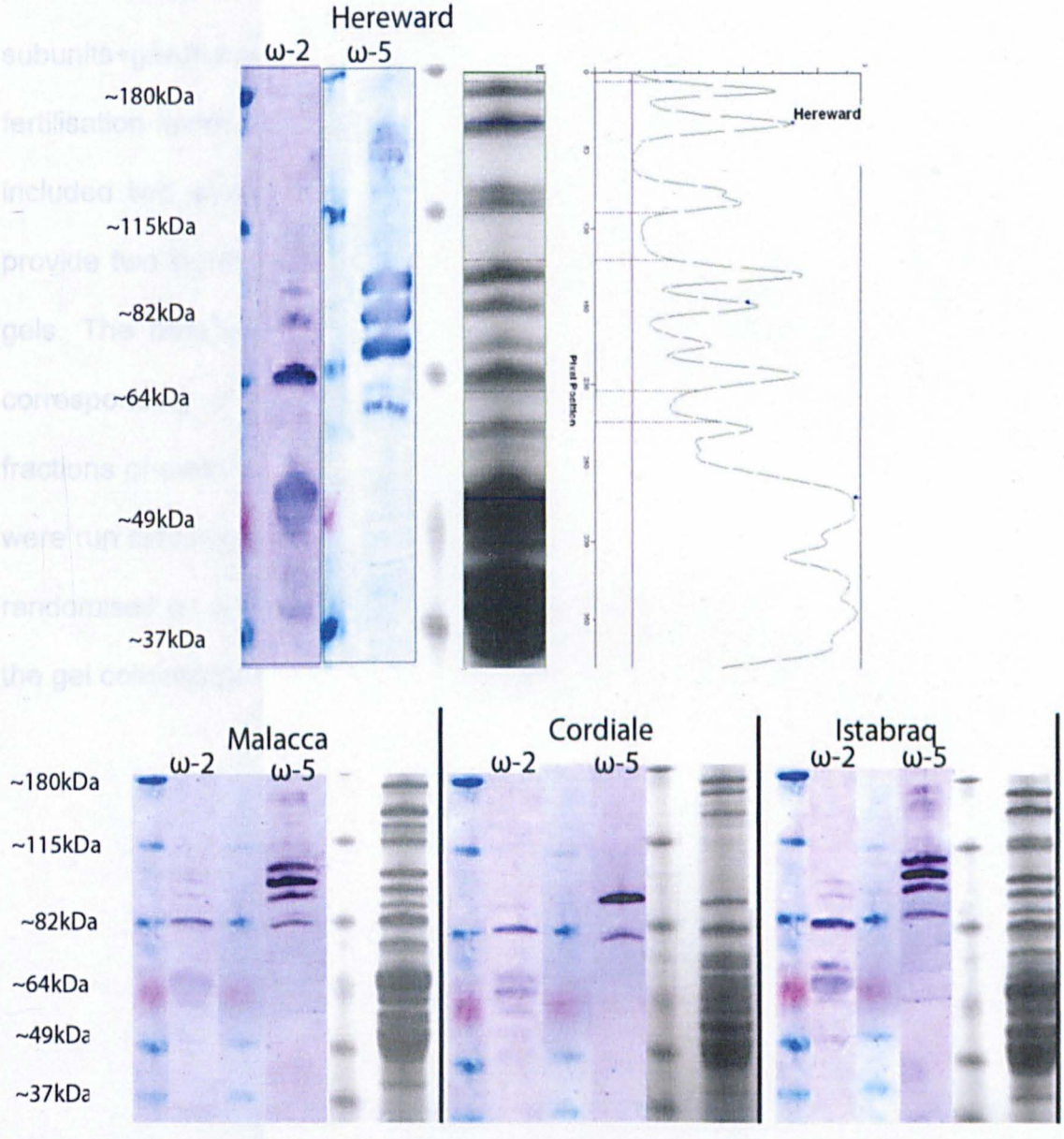

Figure 4.5 Western blot images to identify ω-2 and ω-5 gliadins, samples are Fraction 2 from HE350, MA350, CO350 and IS350

4.3 Quantification of gluten protein profiles by scanning of SDS-PAGE gels

Scanning was used to determine the percentages, expressed on a total gluten protein basis, of HMW glutenin subunits, ω-gliadins and LMW glutenin subunits+gliadins (α- and γ-) in eight fractions of four cultivars, at two N-fertilisation levels and with three biological replicates. Each biological replicate included two extraction replicates; each extraction replicate was run twice to provide two technical replicates. In total, 768 samples were separated on 192 gels. The data set has 23 missing values[10] (Healy and Westmacott, 1956) corresponding to unclear or distorted bands. Protein extracts of the eight fractions of each line (same cultivar, N level, bio-rep, extract-rep, and tech-rep) were run simultaneously on two gels. The loading positions of the extracts were randomised on each gel. Figure 4.6 shows a set of gels randomly selected from the gel collections.

[10] Missing values: in ANOVA test, lower than 5% missing values in total observations is considered has no significant effect on the accuracy of the test.

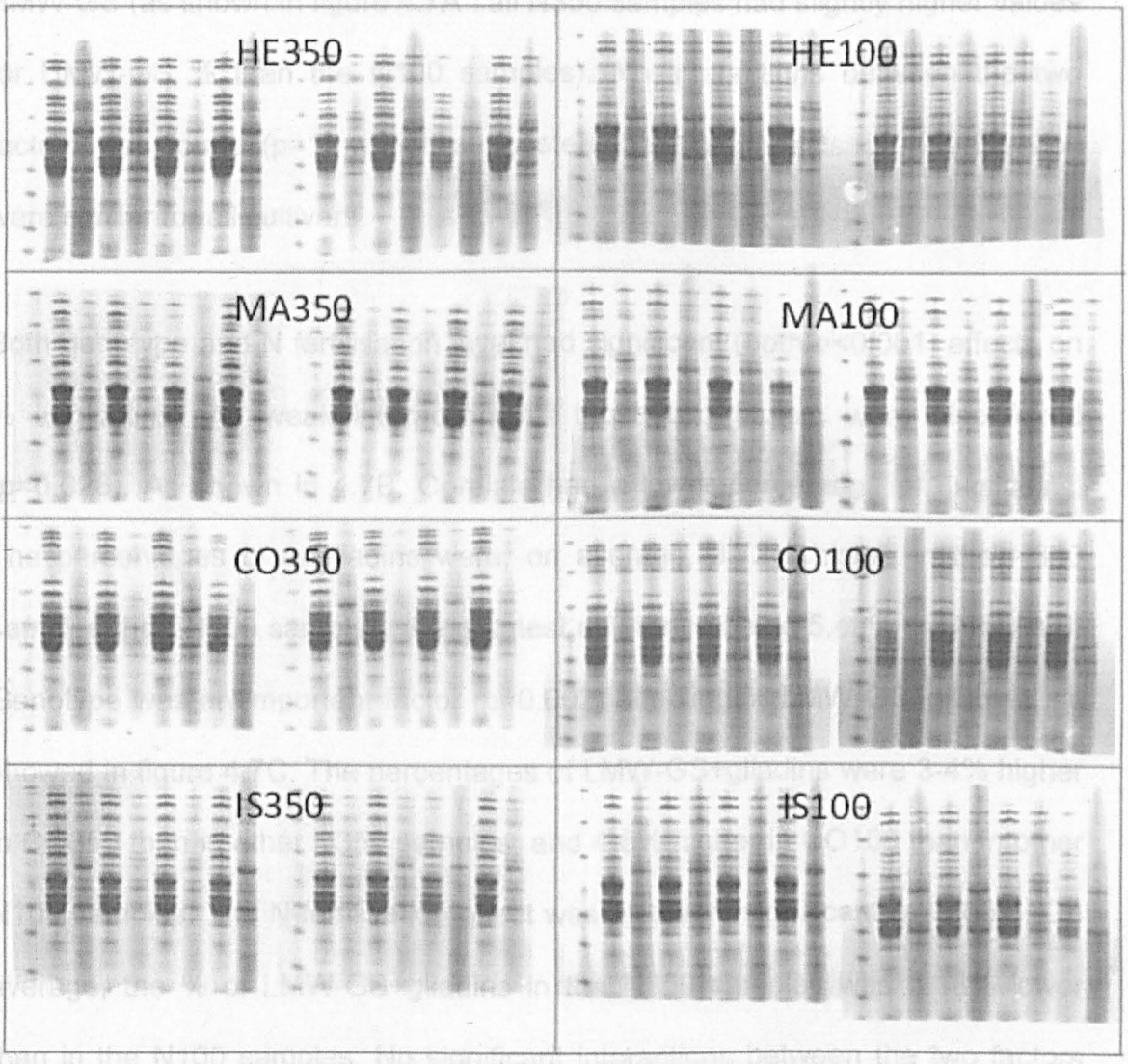

Figure 4.6 Examples of gels of test cultivars: each paired gels consist the protein extractions extracted by 1-propanol and the residues of a pearling fractions set (fraction 1st-6th+core+wholegrain). The extractions and residues were always run next to each other; the loading positions of the extracts were randomised on each gel. The gels showed here were randomly selected from the gel collections.

4.3.1 Gluten protein profile of whole grains

Data from gel scanning and results from ANOVA analysis are summarised in figure 4.7 and table 4.4, respectively., No significant genotype effects (p=0.079) and only slightly significant N-fertilisation effects (p=0.035) were found for

HMW-GS (as shown in figure 4.7A : all N350 samples had slightly higher values for HMW-GS % than the N100 samples). No interactions between the two factors was found (p=1), which suggested that the impacts of N-fertilisation were similar for all cultivars.

Both genotype and N fertilisation level had significant (both p<0.001) effects on % ω-gliadins and weak interactions of those two factors were also found (p=0.026). As shown in 4.7B, Cordiale had a lower percentage of ω-gliadins. The percentages of ω-gliadins were, on average, 3.72% higher in the N350 samples than N100 samples, the greatest difference being 5.49% in Hereward. Genotype was an important factor (p=0.002) affecting % LMW-GS+gliadins, as showed in figure 4.7C. The percentages of LMW-GS+gliadins were 3-4% higher in CO350 than in other N350 samples, and 4-6% higher in CO100 than in other N100 samples. The N-fertilisation effect was also very significant (p<0.001). On average, the % of LMW-GS+gliadins in the N350 samples was 5.18% lower than in the N100 samples. No significant interactions between the two factors were found (p=0.573), which suggests that the impacts of N fertilisation did not differ among the cultivars.

Table 4.4 Factors affect gluten profiles of whole grains

Protein sub-groups	Factors	P value	l.s.d.	d.f.
HMW-GS	Cultivar	0.079	-------	-------
	N-fertilisation	0.035	1.331	14
	Cultivar x N-fertilisation	1.000	-------	-------
ω-gliadins	Cultivar	<0.001	0.997	14
	N-fertilisation	<0.001	0.705	14
	Cultivar x N-fertilisation	0.026	1.410	14
LMW+gliadins	Cultivar	0.002	2.363	14
	N-fertilisation	<0.001	1.671	14
	Cultivar x N-fertilisation	0.573	-------	-------

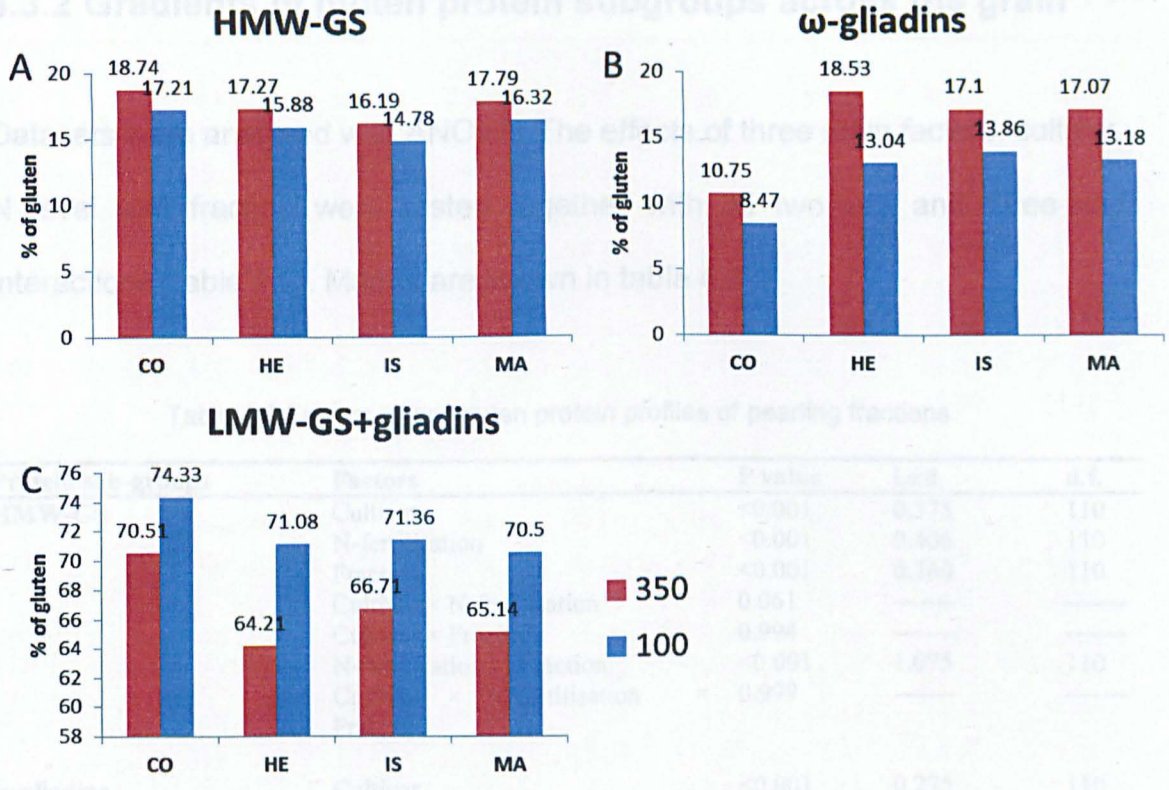

Figure 4.7 Gluten profiles of whole grains, data represent means of replicates.

4.3.2 Gradients of gluten protein subgroups across the grain

Datasets were analysed with ANOVA. The effects of three main factors: cultivar, N level and fraction were tested together with all two-way and three-way interactions (table 4.5). Means are shown in table 4.6

Table 4.5 Factors affect gluten protein profiles of pearling fractions

Protein sub-groups	Factors	P value	l.s.d.	d.f.
HMW-GS	Cultivar	<0.001	0.575	110
	N-fertilisation	<0.001	0.406	110
	Fraction	<0.001	0.760	110
	Cultivar × N-fertilisation	0.061	-------	-------
	Cultivar × Fraction	0.994	-------	-------
	N-fertilisation × Fraction	<0.001	1.075	110
	Cultivar × N-fertilisation × Fraction	0.999	-------	-------
ω-gliadins	Cultivar	<0.001	0.275	110
	N-fertilisation	<0.001	0.195	110
	Fraction	<0.001	0.364	110
	Cultivar × N-fertilisation	<0.001	0.390	110
	Cultivar × Fraction	<0.001	0.729	110
	N-fertilisation × Fraction	<0.001	0.515	110
	Cultivar × N-fertilisation × Fraction	0.750	-------	-------
LMW+gliadins	Cultivar	<0.001	0.701	110
	N-fertilisation	<0.001	0.496	110
	Fraction	<0.001	0.928	110
	Cultivar × N-fertilisation	<0.001	0.992	110
	Cultivar × Fraction	0.727	-------	-------
	N-fertilisation × Fraction	<0.001	1.312	110
	Cultivar × N-fertilisation × Fraction	0.993	-------	-------

Table 4.6 Summery of means of gluten protein subgroup% in gluten

				HMW-GS				
	N-level	1st	2nd	3rd	4th	5th	6th	Core
CO	100	7.18	14.93	16.39	17.81	19.42	19.76	20.17
	350	7.93	18.83	19.99	20.45	20.90	20.77	19.42
HE	100	4.40	12.81	14.59	15.52	16.76	17.75	18.01
	350	6.00	17.34	18.36	19.20	19.10	19.14	18.38
IS	100	4.86	12.52	14.47	15.43	15.95	16.38	17.71
	350	6.24	15.25	16.45	18.03	17.43	17.90	18.13
MA	100	4.53	12.82	14.07	15.42	15.82	16.85	17.96
	350	7.31	16.71	18.60	18.92	19.17	19.66	18.88
				ω-gliadins				
	N-level	1st	2nd	3rd	4th	5th	6th	Core
CO	100	8.33	7.69	8.20	8.49	8.57	8.65	9.82
	350	9.86	11.02	11.15	10.81	10.53	10.62	10.57
HE	100	12.87	13.25	13.48	13.60	13.20	13.06	13.28
	350	18.82	19.69	20.06	19.79	18.87	18.49	16.80
IS	100	15.24	14.91	14.40	14.68	14.22	14.10	13.94
	350	17.67	18.36	17.87	17.46	17.23	16.72	16.21
MA	100	11.66	12.35	13.94	13.53	13.45	12.86	13.64
	350	16.63	17.46	18.08	17.77	17.13	17.01	16.34
				LMW-GS+gliadins				
	N-level	1st	2nd	3rd	4th	5th	6th	Core
CO	100	84.50	77.39	75.41	73.70	72.01	71.59	70.01
	350	82.21	70.15	68.86	68.74	68.57	68.61	70.01
HE	100	82.73	73.93	71.93	70.88	70.04	69.20	68.71
	350	75.18	62.97	61.58	61.01	62.03	62.37	64.82
IS	100	79.91	72.57	71.13	69.89	69.83	69.52	68.35
	350	76.08	66.39	65.68	64.51	65.34	65.38	65.66
MA	100	83.80	74.83	71.99	71.05	70.73	70.29	68.39
	350	76.07	65.83	63.32	63.31	63.70	63.34	64.78

HMW-GS

Figure 4.8 shows clear gradients in % HMW-GS across the grain (fraction factor, $p<0.001$). Low proportions of HMW-GS were present in fraction 1. This was probably due to contamination of the bran+aleurone fraction with the starchy endosperm, since HMW glutenin subunits are not expressed in the aleurone or in the outer layers. The proportions of HMW-GS increased sharply in the 2nd fraction and then slowly in subsequent fractions toward the core.

Although the relative amounts of HMW-GS in each fraction differed between cultivars (cultivars factor $p<0.001$), the shape of the curves describing these trends were similar (interaction--Cultivar × Fraction, $p=0.994$). As shown in figure 4.9A, Cordiale had the highest % HMW-GS, while Istabraq had the lowest. Figure 4.9B shows that additional nitrogen fertiliser had positive effects on % HMW-GS in all fractions (2.3% increase on average) in all cultivars (-N-fertilisation factor, $p<0.001$), although the extent of the increase varied between fractions, with the 2nd and 3rd fractions showing the highest increases (+ 7.04% and +6.99%, respectively). The fraction corresponding to the core, on the other hand, showed the smallest increase in % HMW-GS (+2.91%). No interactions between cultivar × N fertilisation were found ($p=0.061$), suggesting that the four cultivars respond in a similar way to additional nitrogen fertiliser. No three-way interactions of cultivar × N fertilisation × fraction was found ($p=0.999$), and it is reflected in the fact that N fertilisation has a similar effect on the shape of the curve describing the % HMW-GS in all four cultivars (see fig 4.8).

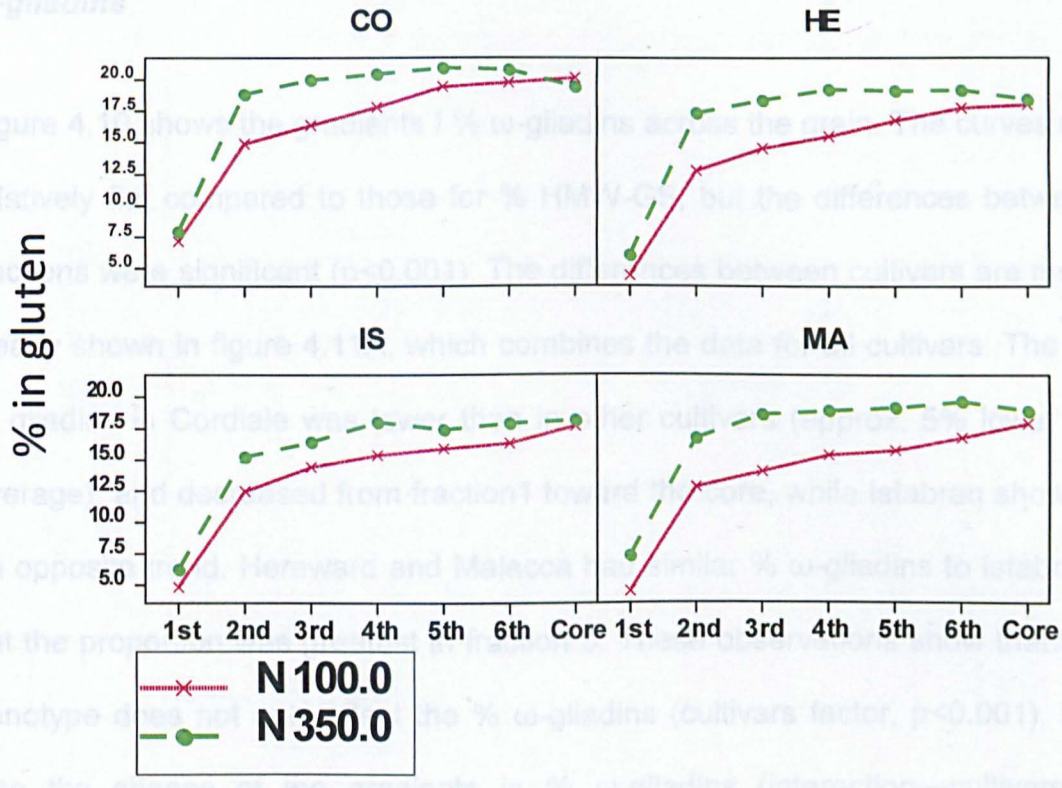

Figure 4.8 Gradients in % HMW-GS across the grain

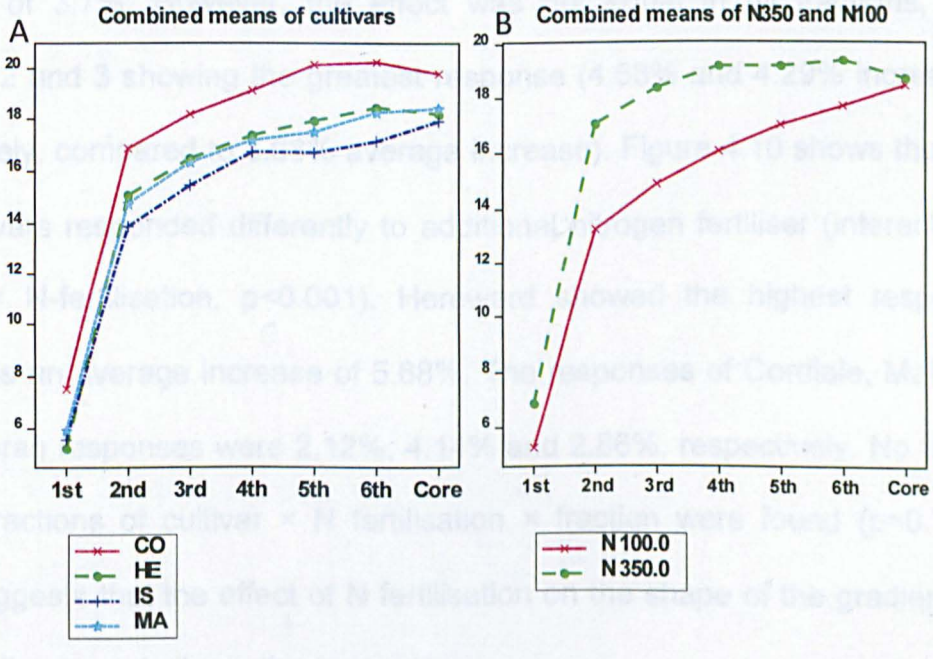

Figure 4.9 Effects of factors on % HMW-GS (% of gluten):

A) Cultivar factor, means were average of two N-levels;

B) N-fertilisation level factor, means were average of four cultivars

ω-gliadins

Figure 4.10 shows the gradients I % ω-gliadins across the grain. The curves are relatively flat compared to those for % HMW-GS, but the differences between fractions were significant ($p<0.001$). The differences between cultivars are more clearly shown in figure 4.11A, which combines the data for all cultivars. The ω-% gliadins in Cordiale was lower than in other cultivars (approx. 5% lower on average), and decreased from fraction1 toward the core, while Istabraq showed an opposite trend. Hereward and Malacca had similar % ω-gliadins to Istabraq, but the proportion was greatest in fraction 3. These observations show that the genotype does not only affect the % ω-gliadins (cultivars factor, $p<0.001$), but also the shapes of the gradients in % ω-gliadins (interaction—cultivars × fractions, $p<0.001$). Higher N fertilisation level had a positive effect on the % % ω-gliadins in all fractions (N-fertilisation factor, $p<0.001$), with an average increase of 3.7%. However, the effect was not equal in all fractions, with fractions 2 and 3 showing the greatest response (4.58% and 4.29% increases, respectively, compared to 3.68% average increase). Figure 4.10 shows that the four cultivars responded differently to additional nitrogen fertiliser (interaction--cultivar × N-fertilisation, $p<0.001$). Hereward showed the highest response which was an average increase of 5.68%. The responses of Cordiale, Malacca and Istabraq responses were 2.12%, 4.14% and 2.86%, respectively. No three-way interactions of cultivar × N fertilisation × fraction were found ($p=0.750$), which suggests that the effect of N fertilisation on the shape of the gradients in % ω-gliadins was similar in the four cultivars.

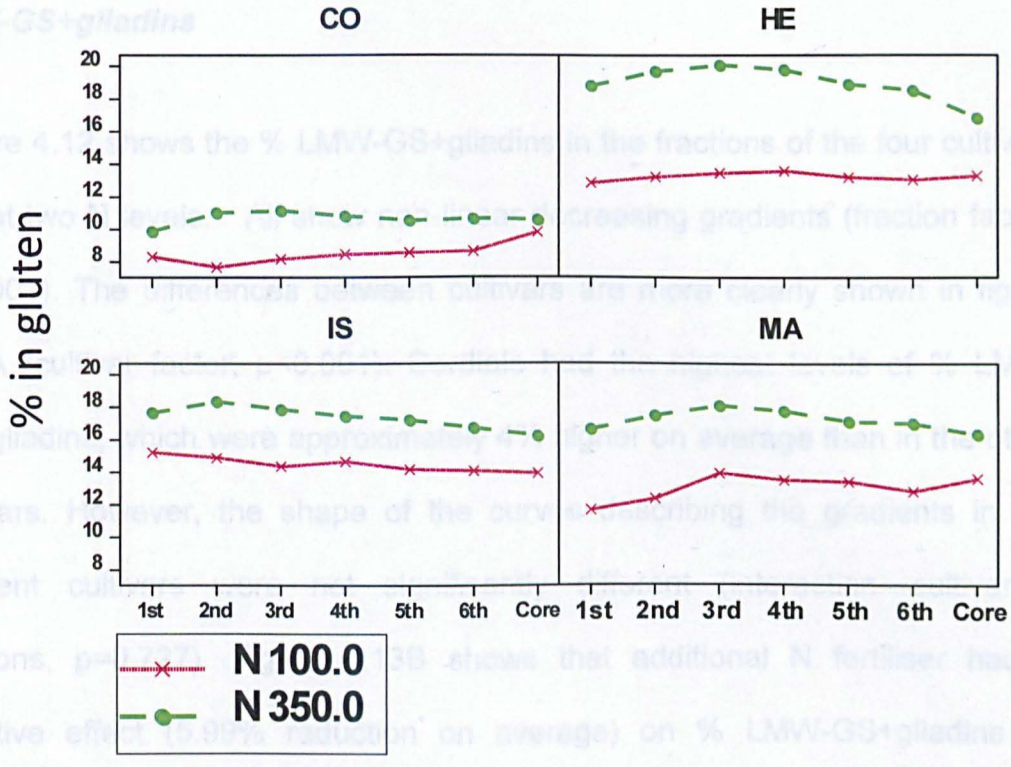

Figure 4.10 Gradients in % ω-gliadins (% of gluten) gradients across the grain

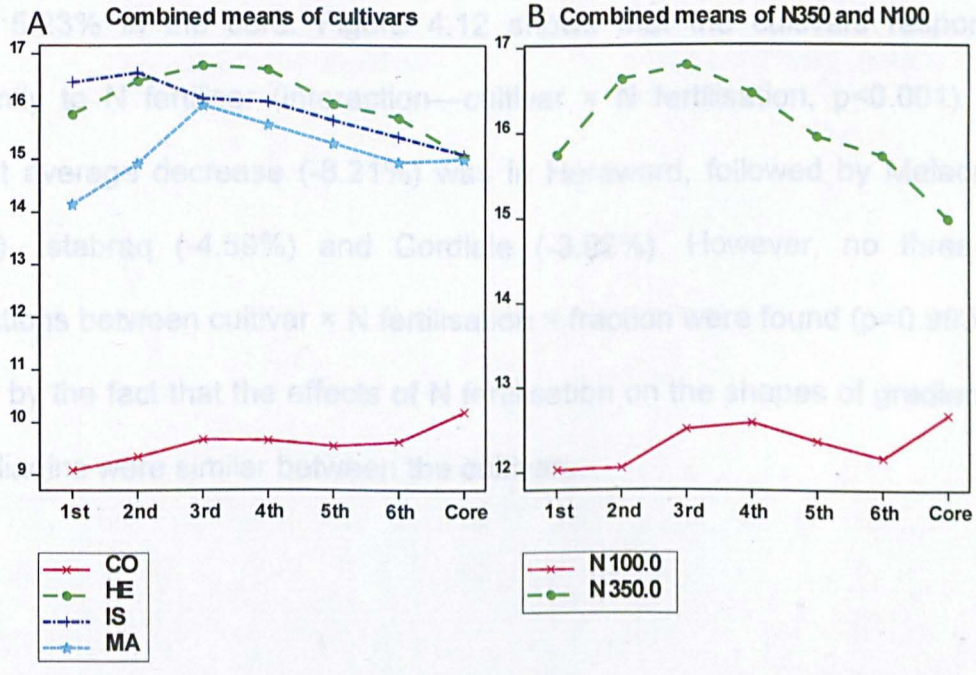

Figure 4.11 Factors impacting on % ω-gliadins (of gluten):

A), Cultivar factor, means were average of two N-levels

B), N-fertilisation level factor, means were average of four cultivars

LMW-GS+gliadins

Figure 4.12 shows the % LMW-GS+gliadins in the fractions of the four cultivars and at two N levels. All show non-linear decreasing gradients (fraction factor, $p<0.001$). The differences between cultivars are more clearly shown in figure 4.13A (cultivar factor, $p<0.001$). Cordiale had the highest levels of % LMW-GS+gliadins, which were approximately 4% higher on average than in the other cultivars. However, the shape of the curves describing the gradients in the different cultivars were not significantly different (interaction—cultivar × fractions, $p=0.727$). Figure 4.13B shows that additional N fertiliser had a negative effect (5.99% reduction on average) on % LMW-GS+gliadins (N fertilisation factor, $p<0.001$). Also, the decrease was not even across the grain (interaction—N-fertilisation × fraction, $p<0.001$). The decrease was by about 5.34% in the 1st fraction, peaked at 8.34% in the 2nd fraction to then gradually fell to 5.23% in the core. Figure 4.12 shows that the cultivars responded differently to N fertiliser (interaction—cultivar × N fertilisation, $p<0.001$). The highest average decrease (-8.21%) was in Hereward, followed by Malacca (-7.25%), Istabraq (-4.59%) and Cordiale (-3.92%). However, no three-way interactions between cultivar × N fertilisation × fraction were found ($p=0.993$), as shown by the fact that the effects of N fertilisation on the shapes of gradients in % ω-gliadins were similar between the cultivars.

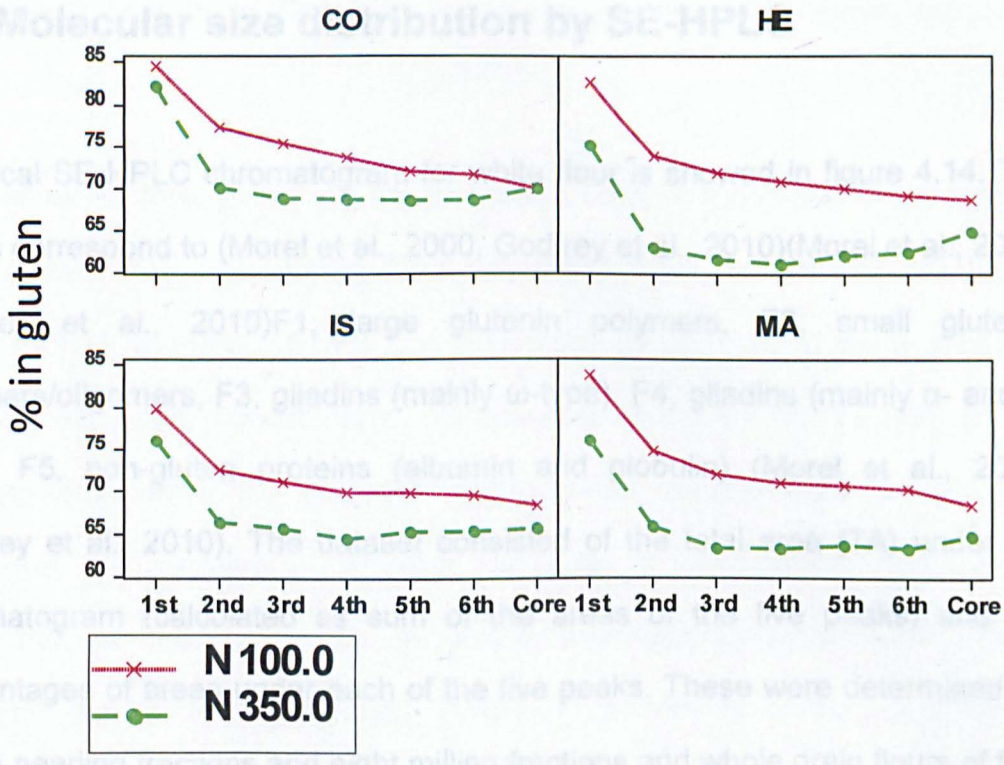

Figure 4.12 Gradients in % LMW-GS+gliadins (% of gluten) gradients across the grain

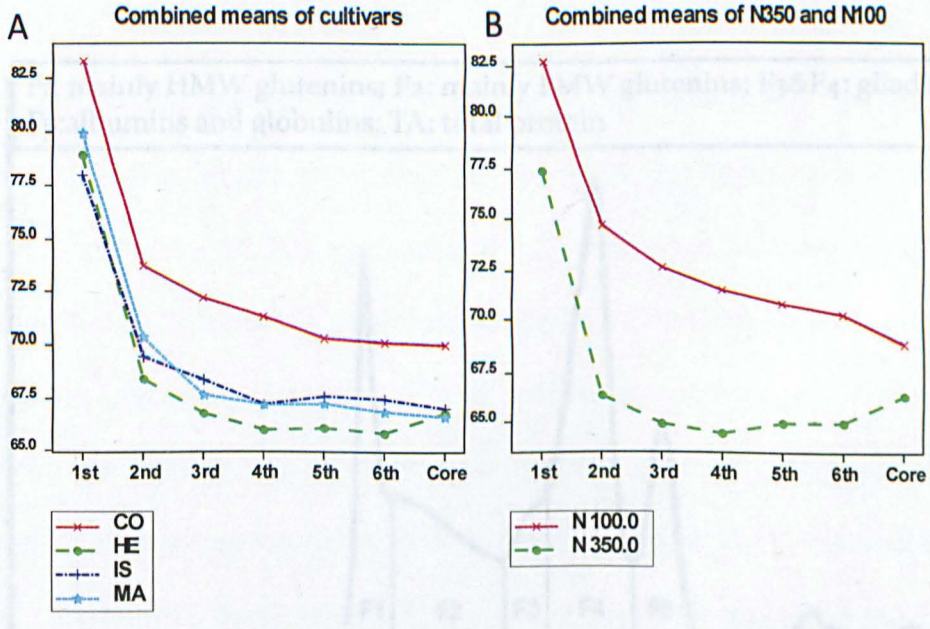

Figure 4.13 factors affecting % LMW-GS+gliadins (% of gluten):

A) Cultivar factor, means were average of two N-levels;

B) N fertilisation level factor, means were average of four cultivars

4.4 Molecular size distribution by SE-HPLC

A typical SE-HPLC chromatogram for white flour is showed in figure 4.14. The peaks correspond to (Morel et al., 2000, Godfrey et al., 2010)(Morel et al., 2000, Godfrey et al., 2010)F1, large glutenin polymers, F2, small glutenin polymers/oligomers, F3, gliadins (mainly ω-type), F4, gliadins (mainly α- and γ-type), F5, non-gluten proteins (albumin and globulin) (Morel et al., 2000, Godfrey et al., 2010). The dataset consisted of the total area (TA) under the chromatogram (calculated as sum of the areas of the five peaks) and the percentages of areas under each of the five peaks. These were determined for seven pearling fractions and eight milling fractions and whole grain flours of four cultivars at two N fertilisation levels. Only one biological replicate of each line was analysed resulting in a total of 128 samples. The short of biological replicates was due to the high cost of the analysis.

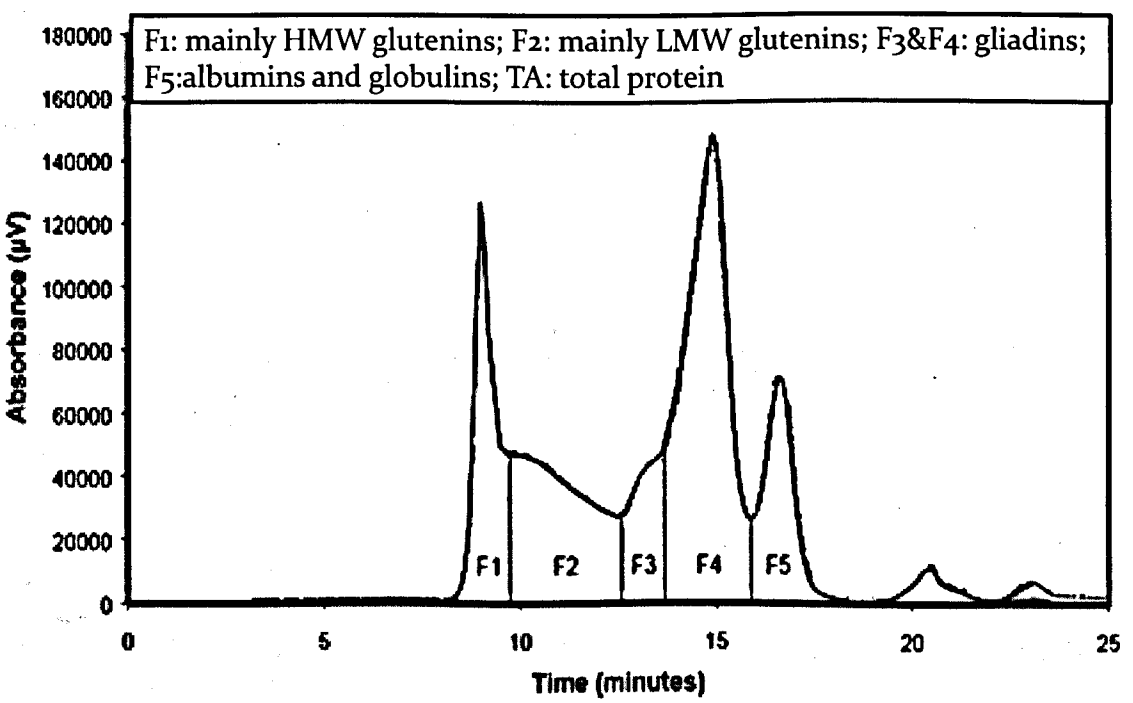

Figure 4.14 typical SE-HPLC chromatogram of a wheat flour protein extract (Millar, 2003)

The values for total area (TA), the % area of each of the gluten protein peaks (F1-F4) and three key parameters calculated from the dataset (% gluten (F1%+F2%+F3%+F4%), F1/F2 and F3+F4/F1 (Millar, 2003)) were tested in ANOVA using the highest order interaction (cultivar×N-level×fraction) as the residual term, which is a statistical method used when no data for biological replicates are available (S. Powers, personal communication, April, 2012). Using this method, the results of two-way interactions may be less reliable, therefore they are not presented. However, the effects of the main factors (cultivar, N level and fraction) are valid because the test includes extra hidden replicates[11] within the experimental structure.

4.4.1 Results of analyses whole grains

The data for whole grains (figure 4.15) were not tested by ANOVA. The differences discussed in this paragraph are therefore the differences most likely to occur in a complete ANOVA test using a fully replicated data set. Differences between the two N fertilisation levels can be seen in the data for total area (TA) and % gluten (figure 4.15A and 4.15B). However, differences within other parameters are less obvious. Genotype effects can be observed for all parameters. Istabraq had lowest %F1 and %F2 and the highest %F3, %F4 and F3+F4/F1 ratio. It also had lowest F1/F2 ratio at the N350 level and the second lowest F1/F2 ratio at the N100 level. Malacca had highest % gluten at the N350

[11] hidden replicates: when a test are not replicated or doesn't contain biological replicates, each level of each factor can serve as a "hidden replicate" for the levels of the other factor (S. Powers, personal communication, April, 2012).

level but the lowest % gluten at the N100 level. Hereward had the highest TA at the N350 level and relatively high % gluten, %F1 and %F2, and relatively low F3+F4/F1 ratio. Cordiale had highest F1/F2 ratio at both N levels.

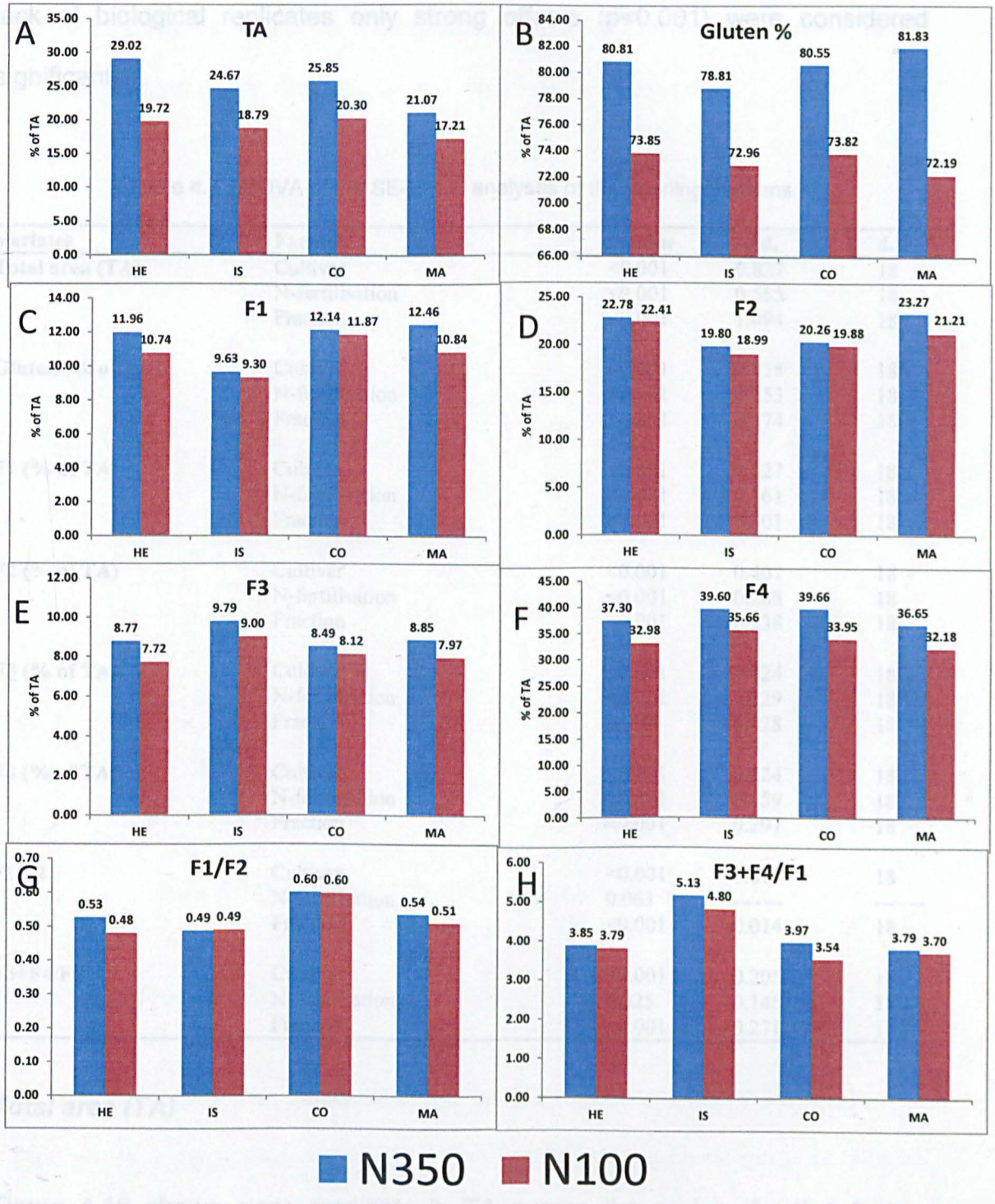

Figure 4.15 SE-HPLC data for whole grains

4.4.2 Analyses of pearling fractions

Table 4.7.are summarises the results of the ANOVA test of the SE-HPLC data for the pearling fractions. A table of means is given in Appendix 1. Due to the lack of biological replicates only strong effects ($p<0.001$) were considered significant.

Table 4.7 ANOVA of the SE-HPLC analyses of the pearling fractions

Variates	Factors	P value	l.s.d.	d.f.
Total area (TA)	Cultivar	<0.001	0.827	18
	N-fertilisation	<0.001	0.585	18
	Fraction	<0.001	1.094	18
Gluten (% of TA)	Cultivar	<0.001	0.358	18
	N-fertilisation	<0.001	0.253	18
	Fraction	<0.001	0.474	18
F1 (% of TA)	Cultivar	<0.001	0.227	18
	N-fertilisation	<0.001	0.161	18
	Fraction	<0.001	0.301	18
F2 (% of TA)	Cultivar	<0.001	0.407	18
	N-fertilisation	<0.001	0.288	18
	Fraction	<0.001	0.538	18
F3 (% of TA)	Cultivar	<0.001	0.324	18
	N-fertilisation	<0.001	0.229	18
	Fraction	0.009	0.428	18
F4 (% of TA)	Cultivar	<0.001	0.224	18
	N-fertilisation	<0.001	0.159	18
	Fraction	<0.001	0.297	18
F1/F2	Cultivar	<0.001	0.011	18
	N-fertilisation	0.063	-------	-------
	Fraction	<0.001	0.014	18
F3+F4/F1	Cultivar	<0.001	0.205	18
	N-fertilisation	0.025	0.145	18
	Fraction	<0.001	0.271	18

Total area (TA)

Figure 4.16 shows clear gradients in TA across the grains (fraction factor, $p<0.001$). The shape of the trends was very similar to the gradients in TPC (figure 4.1). This is not surprising since the TA determined by SE-HPLC has

been shown to reflect the total protein content of the flour (Millar, 2003, Morel and Bar-L'Helgouac'h, 2000). Both the cultivar and N level were significant (both $p<0.001$). The absolute values of TA (instrument arbitrary units) of all of the N350 fractions were higher than those of N100 fractions, in agreement with the TPC results discussed above. The gradient was more pronounced in Hereward and less pronounced in Istabraq at both N levels, (Figure 4.16).

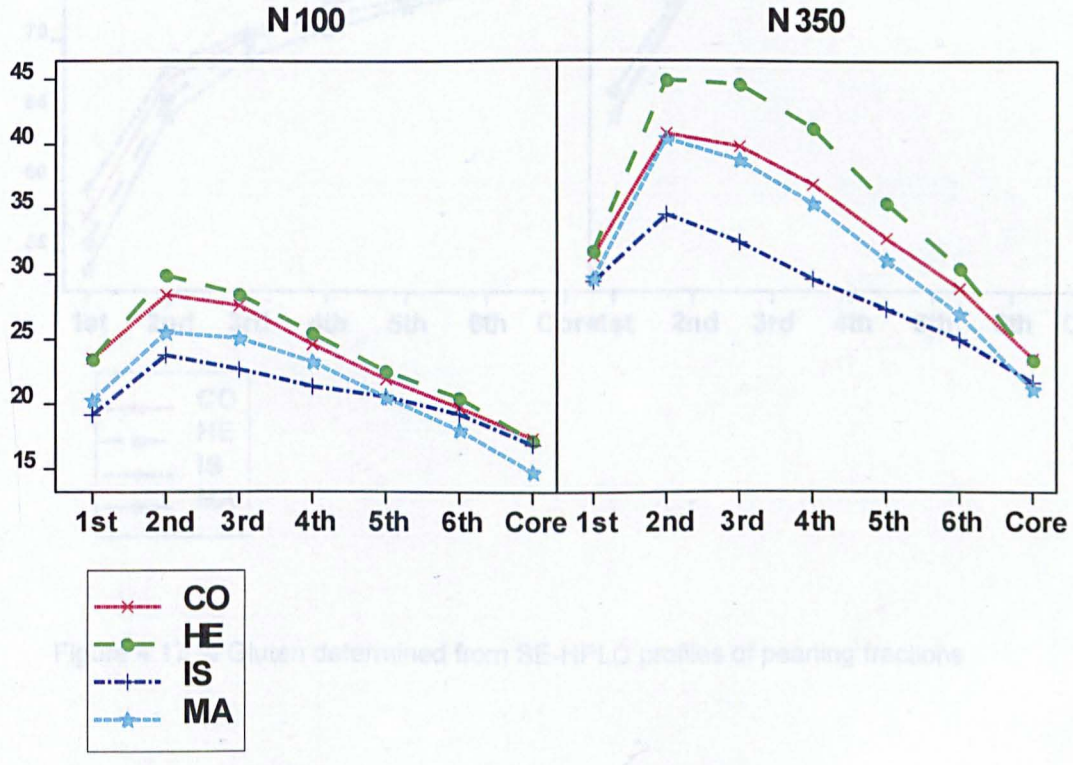

Figure 4.16 Total areas of SE-HPLC profiles of pearling fractions

Gluten% (of total protein)

The % gluten protein was calculated as the sum of F1%, F2%, F3%, and F4%, based on the classification of the SE-HPLC peaks showed in figure 4.14. Figure 4.17 shows the gradients (fraction factor, $p<0.001$) in gluten percentage across the grain. All lines show a non-linear relationships with the values being higher in the N350 samples than in the N100 samples, indicating a positive effect of

additional nitrogen fertiliser on % gluten (7.4% increases on average) (N-level factor, $p<0.001$). Although the cultivar factor was found to be significant ($p<0.001$), the differences between cultivars are not seen in figure 4.17.

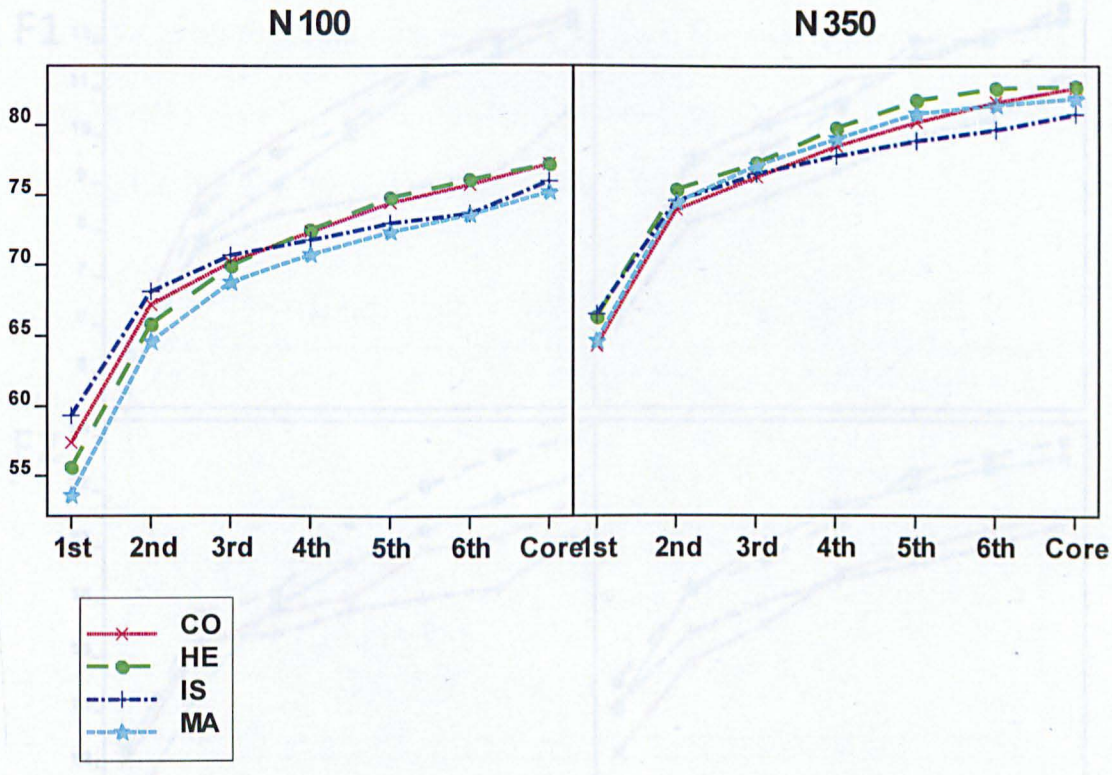

Figure 4.17 % Gluten determined from SE-HPLC profiles of pearling fractions

Large polymer peak %F1 and small polymer peak %F2

Both large and small polymer classes showed similar gradients across the grain (fraction factor, $p<0.001$) to % gluten (figure 4.18). The cultivar effects ($p<0.001$) on the gradients were clearer than in the case of the gradient in % gluten. The fractions of Istabraq had lower values for %F1 (figure 4.18A) than the other cultivars. In general, Hereward and Malacca showed higher values for %F2 (figure 4.18B) compared with Cordiale and Istabraq; however this was not consistent in all fractions. ANOVA indicated that N fertilisation had significant

positive effects on both peaks (both $p<0.001$), however, this is not obvious in figure 4.18.

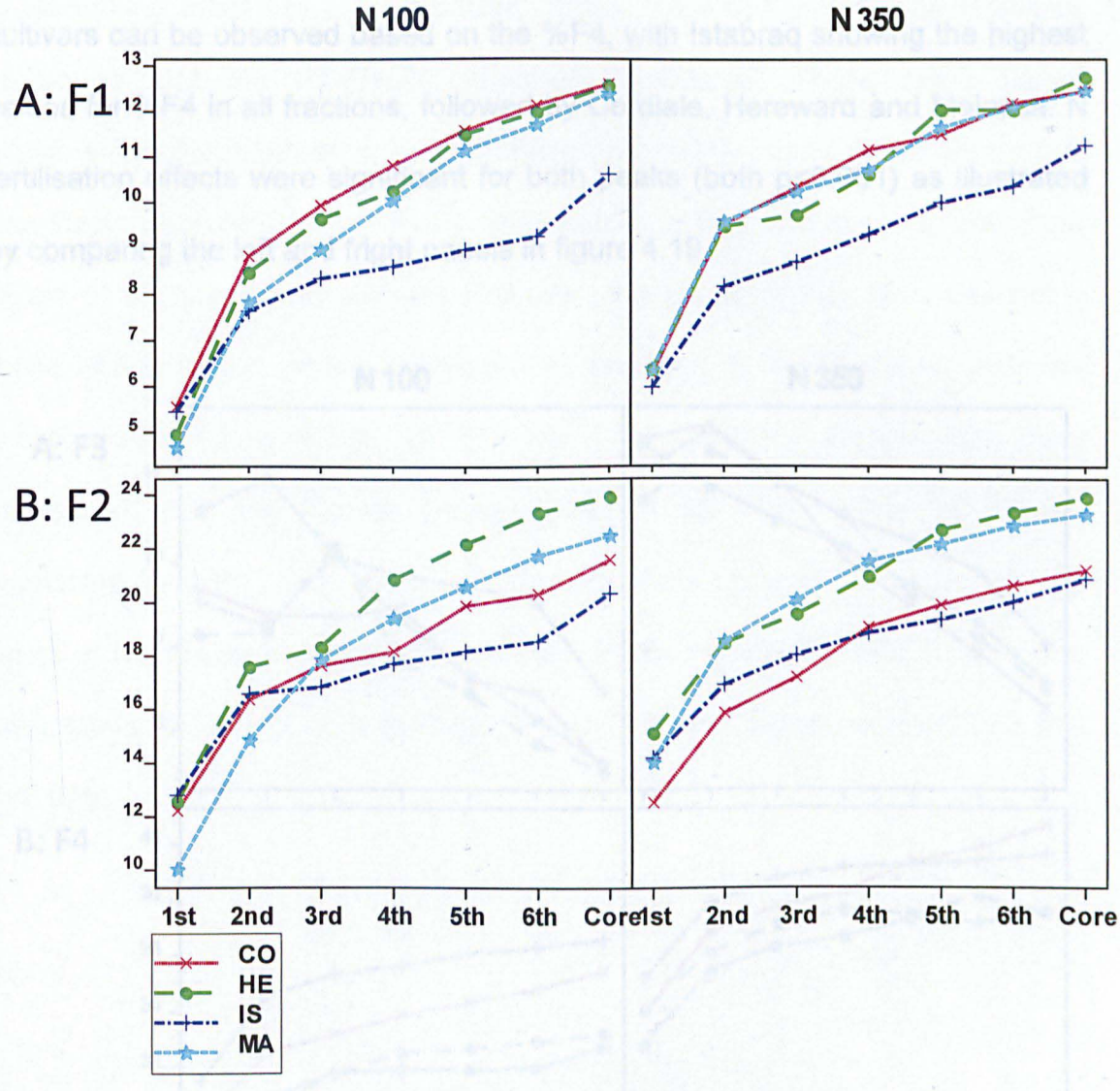

Figure 4.18 %F1 and %F2 determined by SE-HPLC of pearling fractions
A) larger polymer peak F1, B) smaller polymer peak F2

ω-gliadin enriched peak F3 and α- and γ- gliadin enriched peak F4

Figure 4.19 shows significant gradients across the grain for the two main gliadin fractions (factor-fraction, $p<0.001$). The F3 peak, enriched in ω-gliadins, showed a decreasing trend from the outer layer of the endosperm toward the

centre, while the F4 peak, enriched in α- and γ- gliadins, follows an opposite trend. Differences among cultivars, although statistically significant ($p<0.001$), were not clearly seen for %F3 in Figure 4.19, while a clearer separation of cultivars can be observed based on the %F4, with Istabraq showing the highest values for %F4 in all fractions, followed by Cordiale, Hereward and Malacca. N fertilisation effects were significant for both peaks (both $p<0.001$) as illustrated by comparing the left and fright panels in figure 4.19.

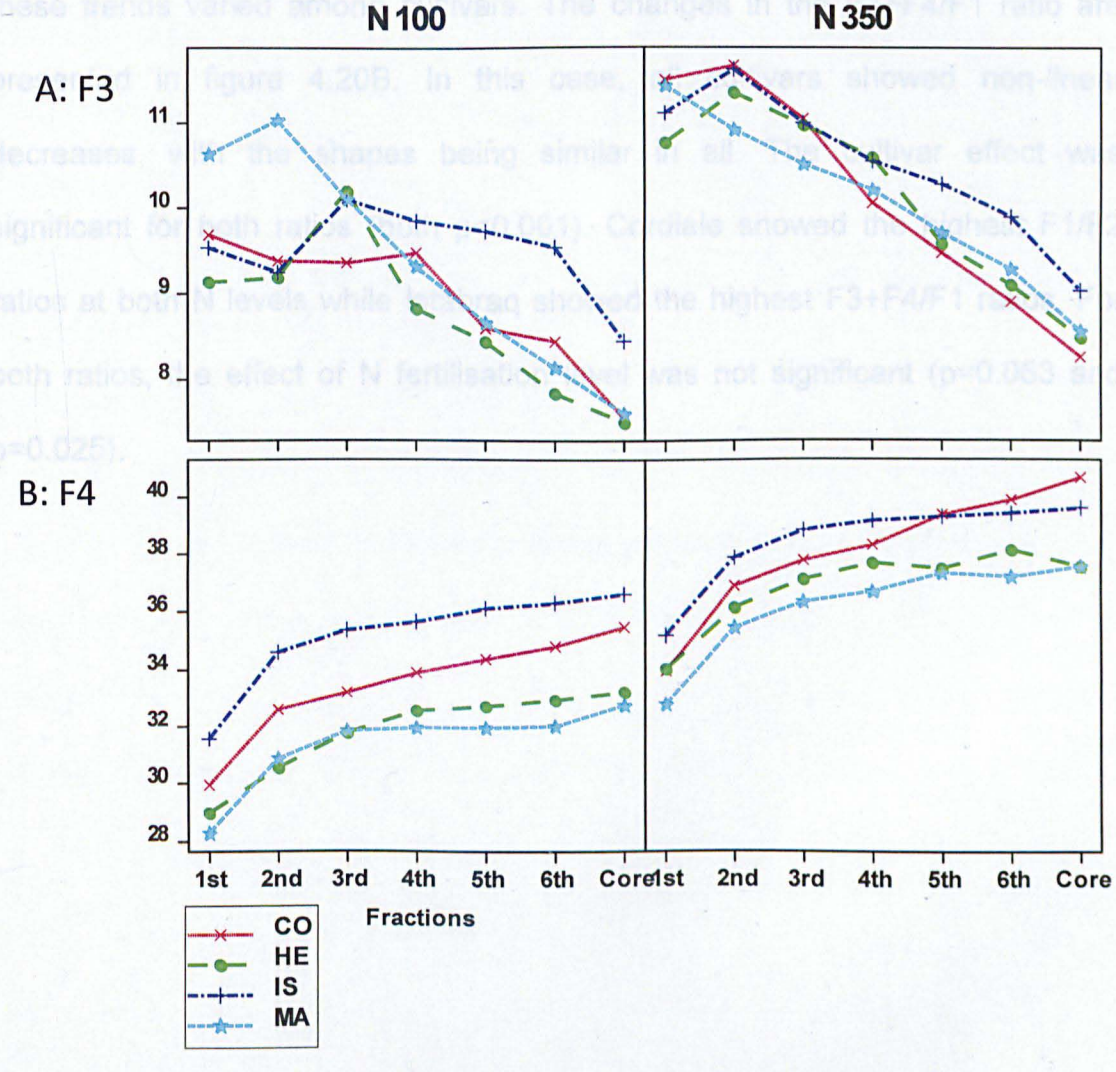

Figure 4.19 % gliadins determined by SE-HPLC of pearling fractions

A) ω-gliadin enrich peak F3, B) α- and γ- gliadin enrich peak F4

Quality parameters: F1/F2 ratio and F3+F4/F1 ratio

The F1/F2 ratio and F3+F4/F1 ratio have been shown to relate to the breadmaking quality of a flour (Millar, 2003), the F1/F2 ratio being positively correlated and the F3+F4/F1 negatively correlated. These ratios varied significantly across the grain (fraction factor, $p<0.001$). Figure 4.20A shows that non-linear increases in the F1/F2 ratio occurred from the outer to the inner layers of the grain in al cultivars However, the shape of the curve describing these trends varied among cultivars. The changes in the F3+F4/F1 ratio are presented in figure 4.20B. In this case, all cultivars showed non-linear decreases, with the shapes being similar in all. The cultivar effect was significant for both ratios (both $p<0.001$). Cordiale showed the highest F1/F2 ratios at both N levels while Istabraq showed the highest F3+F4/F1 ratios. For both ratios, the effect of N fertilisation level was not significant ($p=0.063$ and $p=0.025$).

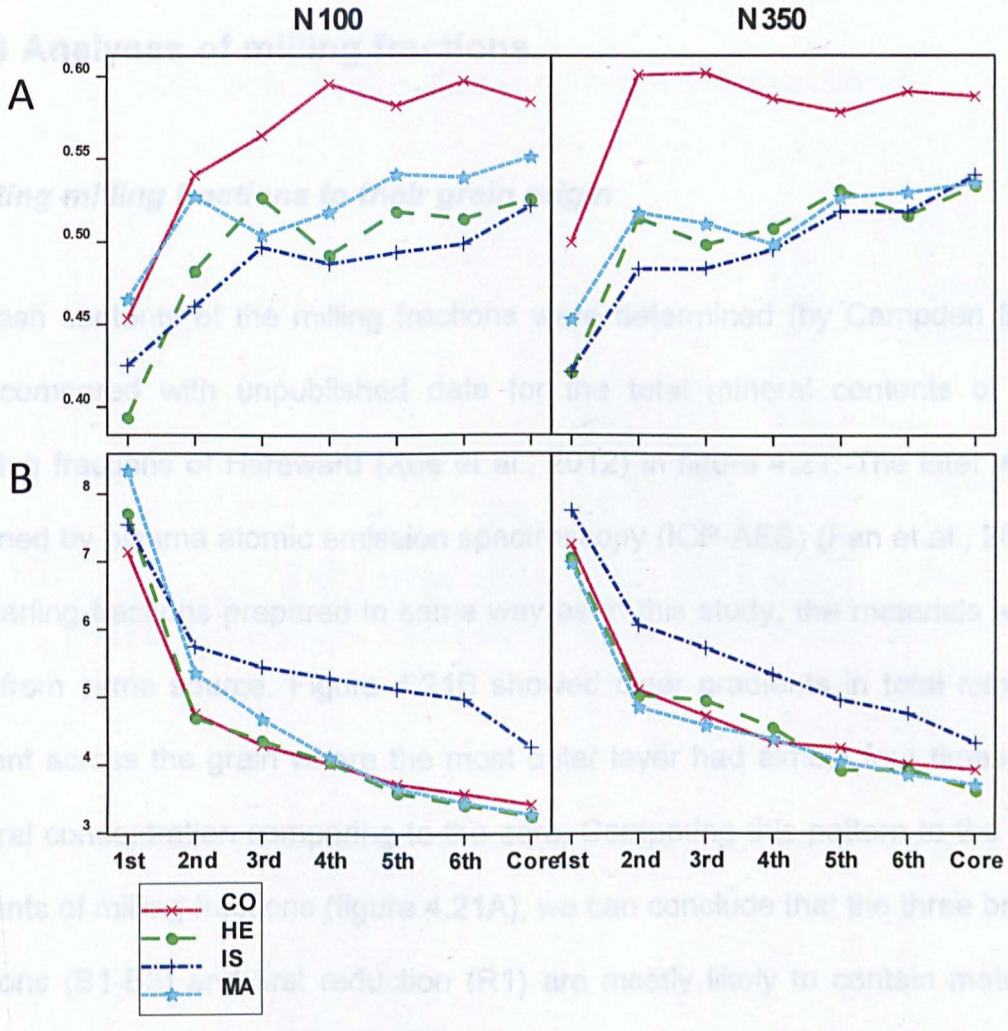

Figure 4.20 Ratios of fractions separated by SE-HPLC: A) F1/F2 ratio, B) F3+F4/F1 ratio

4.4.3 Analyses of milling fractions

Relating milling fractions to their grain origin

The ash contents of the milling fractions were determined (by Campden BRI) and compared with unpublished data for the total mineral contents of the pearling fractions of Hereward (Xue et al., 2012) in figure 4.21. The later were obtained by plasma atomic emission spectroscopy (ICP-AES) (Fan et al., 2008) of pearling fractions prepared in same way as in this study, the materials were also from same source. Figure 4.21B showed clear gradients in total mineral content across the grain where the most outer layer had almost four times the mineral concentration comparing to the core. Comparing this pattern to the ash contents of milling fractions (figure 4.21A), we can conclude that the three break fractions (B1-B3) and first reduction (R1) are mostly likely to contain material from centre of the endosperm. The bran, offal and R3 fractions are likely to be mostly consisted with material from the outer layers of the grain including the bran, aleurone and subaleurone, and the R2 fractions may comprise mixtures of material from both regions. Based on this observation, the milling fractions were arranged in an order of offal flour, bran flour, R3, R2, R1, B3, B2, and B1 to correspond pearling fractions 1, 2, 3, 4, 5, and the core.

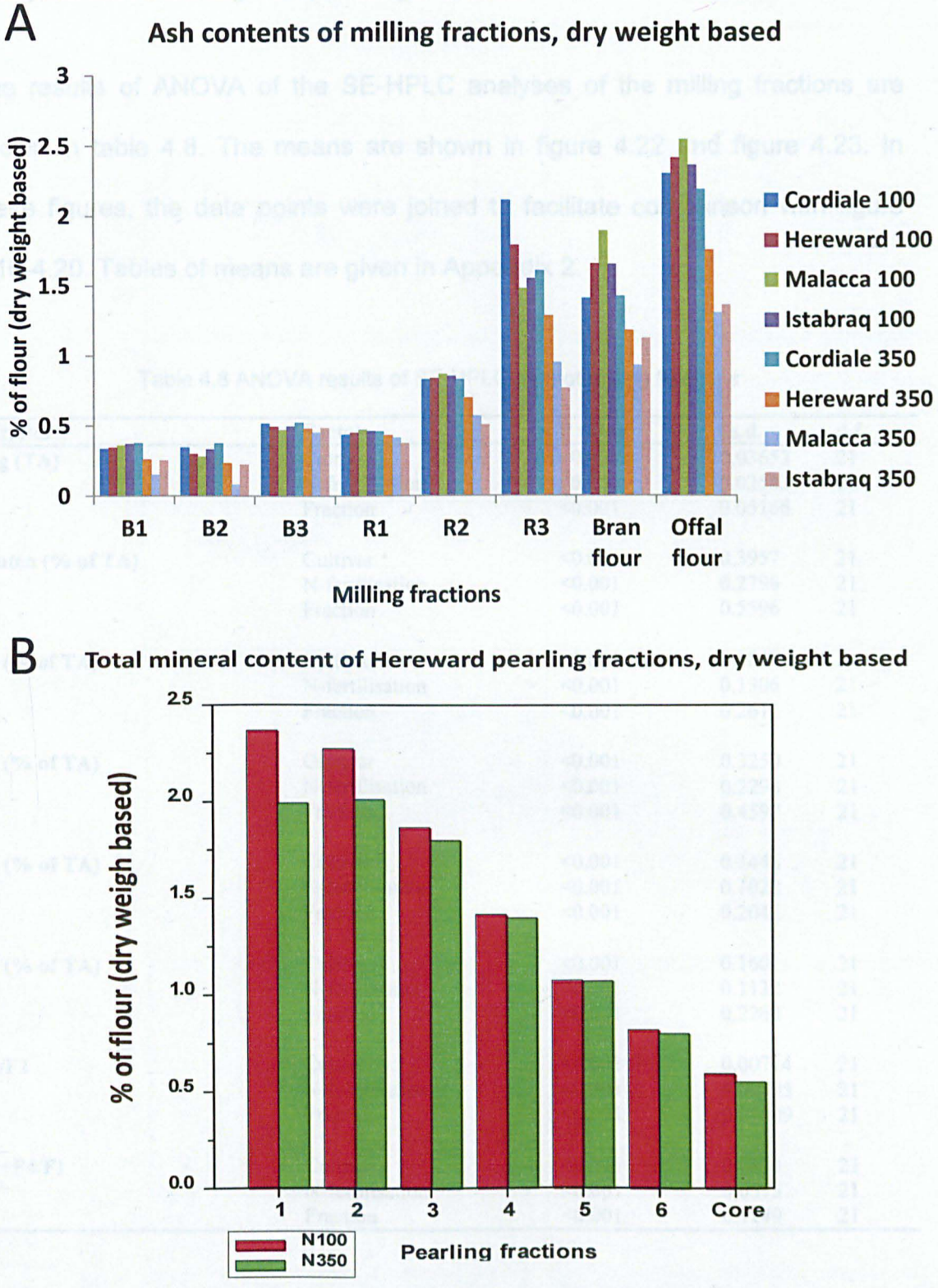

Figure 4.21 Comparison of the mineral contents of the milling fractions and pearling fractions: A) ash contents of milling fractions of four cultivars, B) total mineral content of Hereward pearling fractions (Xue et al., 2012)

Comparison of milling and pearling fractions

The results of ANOVA of the SE-HPLC analyses of the milling fractions are shown in table 4.8. The means are shown in figure 4.22 and figure 4.23. In these figures, the data points were joined to facilitate comparison with figure 4.16-4.20. Tables of means are given in Appendix 2.

Table 4.8 ANOVA results of SE-HPLC data of milling fractions

Variates	Factors	P value	l.s.d.	d.f.
Log (TA)	Cultivar	<0.001	0.03653	21
	N-fertilisation	<0.001	0.02583	21
	Fraction	<0.001	0.05166	21
Gluten (% of TA)	Cultivar	<0.001	0.3957	21
	N-fertilisation	<0.001	0.2798	21
	Fraction	<0.001	0.5596	21
F1 (% of TA)	Cultivar	<0.001	0.1847	21
	N-fertilisation	<0.001	0.1306	21
	Fraction	<0.001	0.2611	21
F2 (% of TA)	Cultivar	<0.001	0.3250	21
	N-fertilisation	<0.001	0.2298	21
	Fraction	<0.001	0.4597	21
F3 (% of TA)	Cultivar	<0.001	0.1446	21
	N-fertilisation	<0.001	0.1022	21
	Fraction	<0.001	0.2045	21
F4 (% of TA)	Cultivar	<0.001	0.1601	21
	N-fertilisation	<0.001	0.1132	21
	Fraction	<0.001	0.2264	21
F1/F2	Cultivar	<0.001	0.00714	21
	N-fertilisation	<0.001	0.00505	21
	Fraction	<0.001	0.01009	21
F3+F4/F1	Cultivar	<0.001	0.0813	21
	N-fertilisation	<0.001	0.0575	21
	Fraction	<0.001	0.1149	21

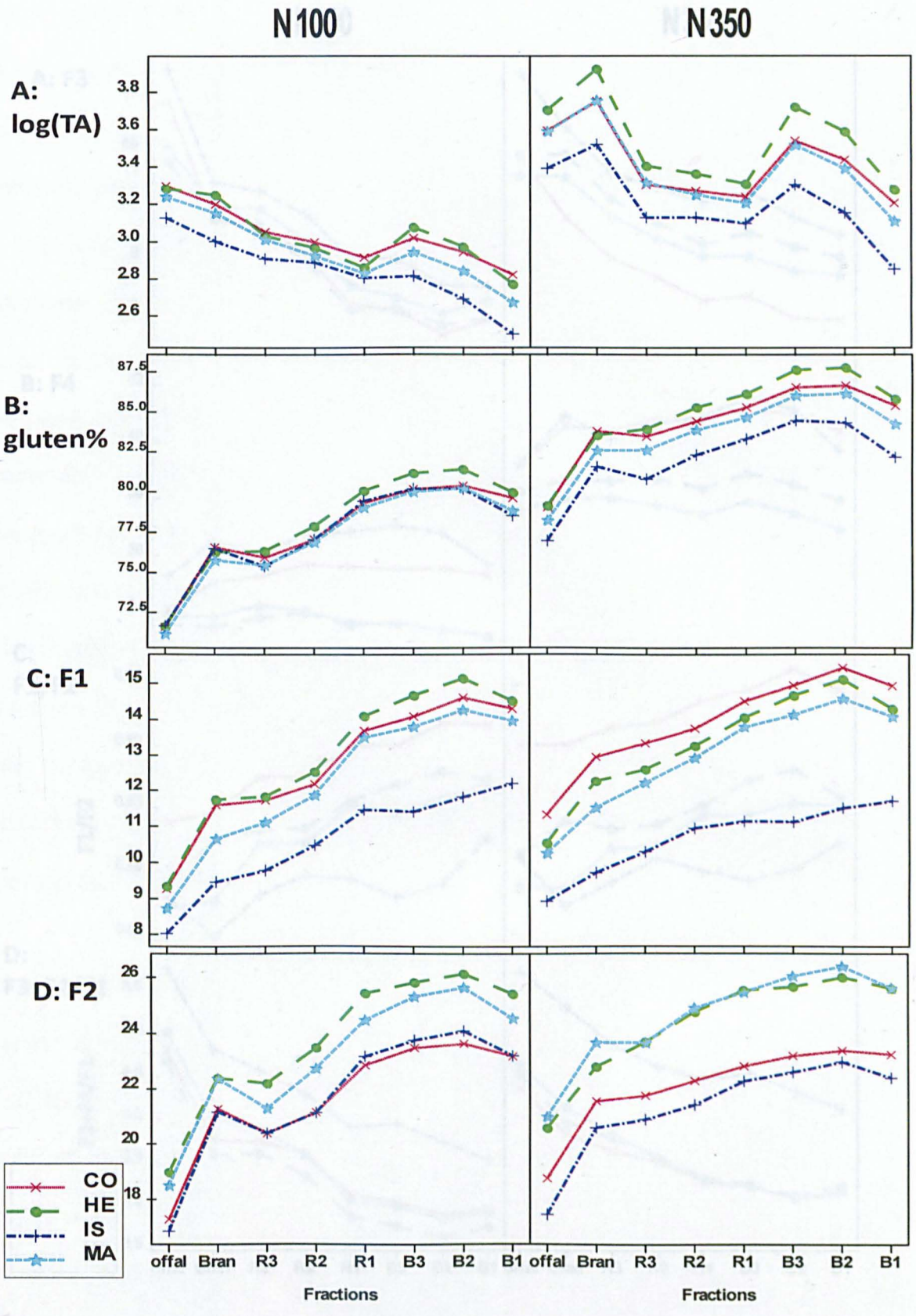

Figure 4.22 SE-HPLC analyses of the milling fractions, part 1: A) log (TA); B) Gluten% (of total protein); C) larger polymer peak F1%, D) smaller polymer peak F2%

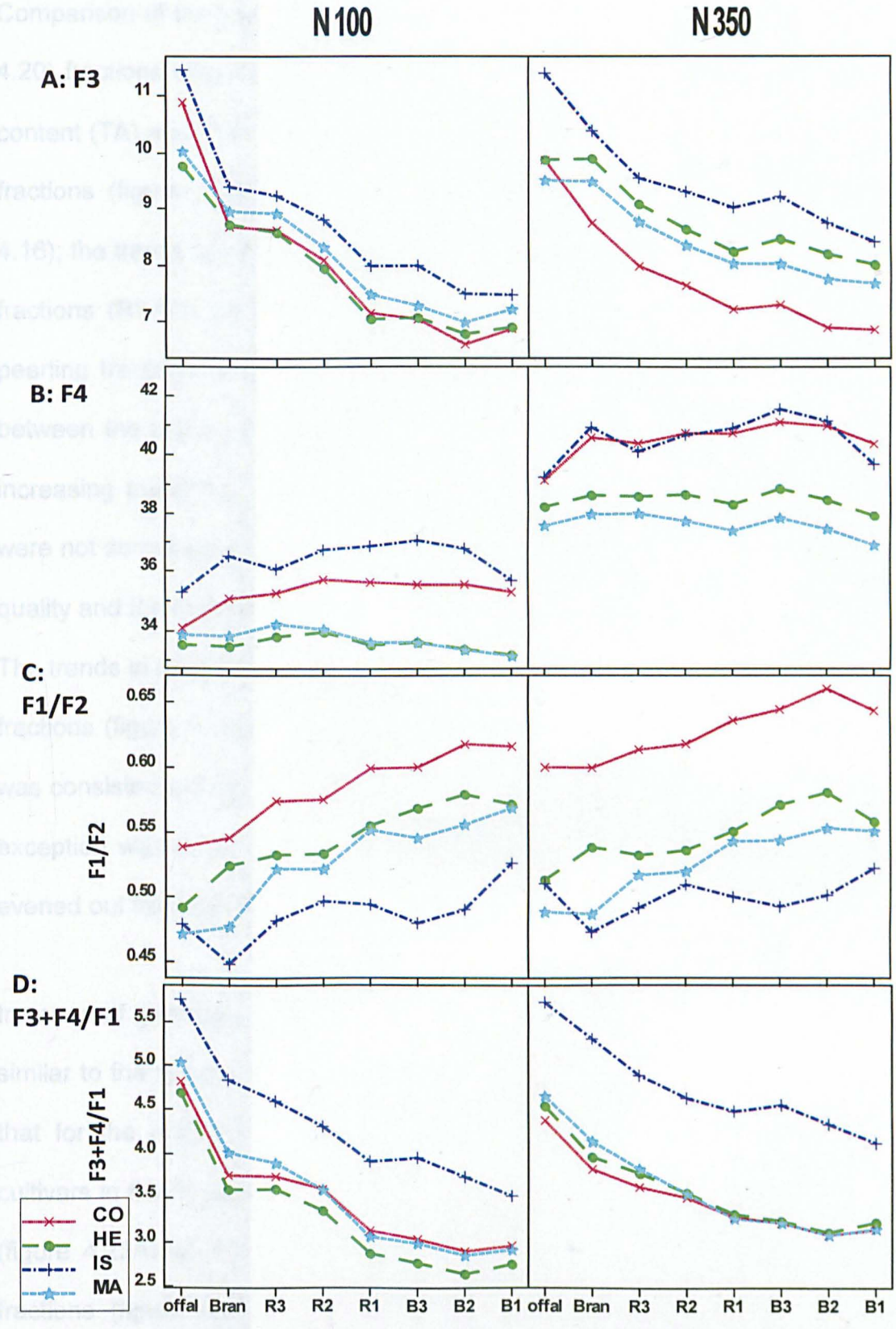

Figure 4 23 SE-HPLC analyses of the milling fractions, part 2: A) ω-gliadin enrich peak F3%; B) α- and γ- gliadin enrich peak F4%; C) F1/F2 ratio; D) F3+F4/F1 ratio

Comparison of the analyses of the milling (figure 4.22-4.23) and pearling (4.16-4.20) fractions shows similar patterns for most variates except for total protein content (TA) and F1/F2 ratio. For TA, although the general trends for the milling fractions (figure 4.22A) differed from those for the pearling fractions (figure 4.16); the trends for the milling break fractions (B3-B1) and the milling reduction fractions (R3-R1) did follow the same decreasing pattern as the endosperm pearling fractions (fraction 4^{th} to Core). The reason for the differences in TA between the break and reduction fractions is unclear. For the F1/F2 ratio, the increasing trend from the offal and bran fractions toward the break fractions were not surprising since the F1/F2 ratio is positively correlated to breadmaking quality and the quality differences between milling fractions are well established. The trends in the F1/F2 ratios of the milling fractions (figure 4.23C) and pearling fractions (figure 4.20A), were similar, except for the 1^{st} pearling fraction which was consisted of bran and was therefore expected to have a low F1/F2 ratio. An exception was for Cordiale at N350, where the trend in the pearling fractions evened out from the 2^{nd} fraction. The cause of this exception is unknown.

In terms of genotype influences, the results for the milling fractions were very similar to the results for the pearling fractions. The only minor differences were that for the milling fractions, the differences among the trends of selected cultivars in the % gluten at N350 (figure 4.22B) and in the %F3 at both N levels (figure 4.23A) were more obvious compared to the same trends for pearling fractions (figure 4.17 and 4.19A). For the N fertilisation factor, the milling fractions responded similarly to the pearling fractions and increased nitrogen fertiliser had positive effects on total protein (TA), % gluten and %F4. However, there were no clear effects on %F1, %F2, %F3, and F3+F4/F1 ratio despite been statistically significantly. For the F1/F2 ratio, a positive response to

nitrogen fertiliser was noted for cultivar Cordiale. However, such responses could not be identified for the other three cultivars. The similar effects of genotype and nitrogen fertiliser were expected since the milling and pearling fractions were from same field trial. The minor differences might be caused by the fact that both sets of analyses lacked biological replicates.

4.5 Fluorescence microscopy study

4.5.1 Spatial patterns of gluten distribution

Figure 4.24, 4.25, 4.26 and 4.27 show the immunolocalisation of proteins in transverse sections of wheat grains. The sections were double labelled to show the distributions of HMW-GS (green signal) and LMW-GS and gliadins (red signal). Four regions of the grain, consisting of the subaleurone in the lobe region, the central endosperm in the lobes, the subaleurone in the dorsal region and the cell layers just below this (opposite the endosperm cavity) were studied in detail. To confirm the nitrogen fertilisation effect, total protein contents of the mature seeds of Hereward and Istabraq lines grown in glasshouse (section 2.1.2) were tested using Dumas method (section 2.3). The results (table 4.9) indicated that, the additional nitrogen fertiliser application did transfer to higher protein contents in these lines.

Table 4.9 Total protein contents of mature seeds of Hereward and Istabraq lines grown in glasshouse

Cultivar	N level (kg/ha)	Total protein content (% of dry flour weight, means of three biological replicates)	Standard Deviation
Hereward	350	20.95	0.55
	100	17.55	2.24
Istabraq	350	17.15	0.78
	100	11.18	1.25

Distribution of HMW-GS

In general, the HMW-GS were enriched in endosperm layers two to three layers from the aleurone. The protein bodies labelled by the anti-HMW antibody in the

outer endosperm layers were larger than the ones in the centres of the lobes, also labelled by this same antibody.

Differences also existed between protein bodies in the lobe and dorsal regions as the former were larger and more heavily labelled by the anti-HMW antibody. These differences were more obvious in sections of Hereward (figure 4.24). In general, at 21 dpa in Hereward the protein bodies appear to be larger and more heavily labelled by the anti-HMW antibody than in Istabraq (figure 4.26 and 4.27). No visual differences were found between the two N levels, or between two development stages.

Distribution of LMW-GS and gliadins

The LMW-GS and gliadins (red signals) recognised by the 0610 monoclonal antibody appeared to be more abundant in protein bodies in the dorsal area rather than in the lobe area (figure 4.24, 4.25, 4.26, 4.27A). In the lobe region the labelling with 0610 was observed mostly in the subaleurone layer. Several images, such as figures 4.24F, 4.25A 4.25F and 4.26A, showed that strong red signals were found in outer endosperm layers, especially at the dorsal edge regions. On the other hand, figures 4.24C, 4.24E, 4.25E and 4.26E showed relatively strong distributions of LMW-GS and gliadins in the lobe centre and dorsal centre areas. The effects of cultivar, N level and development stage were not clearly observed due to weak signals and smaller protein bodies compared to those recognized by anti-HMW-R2 antibodies. They will be studied in detail using image digitalization analysis in next section.

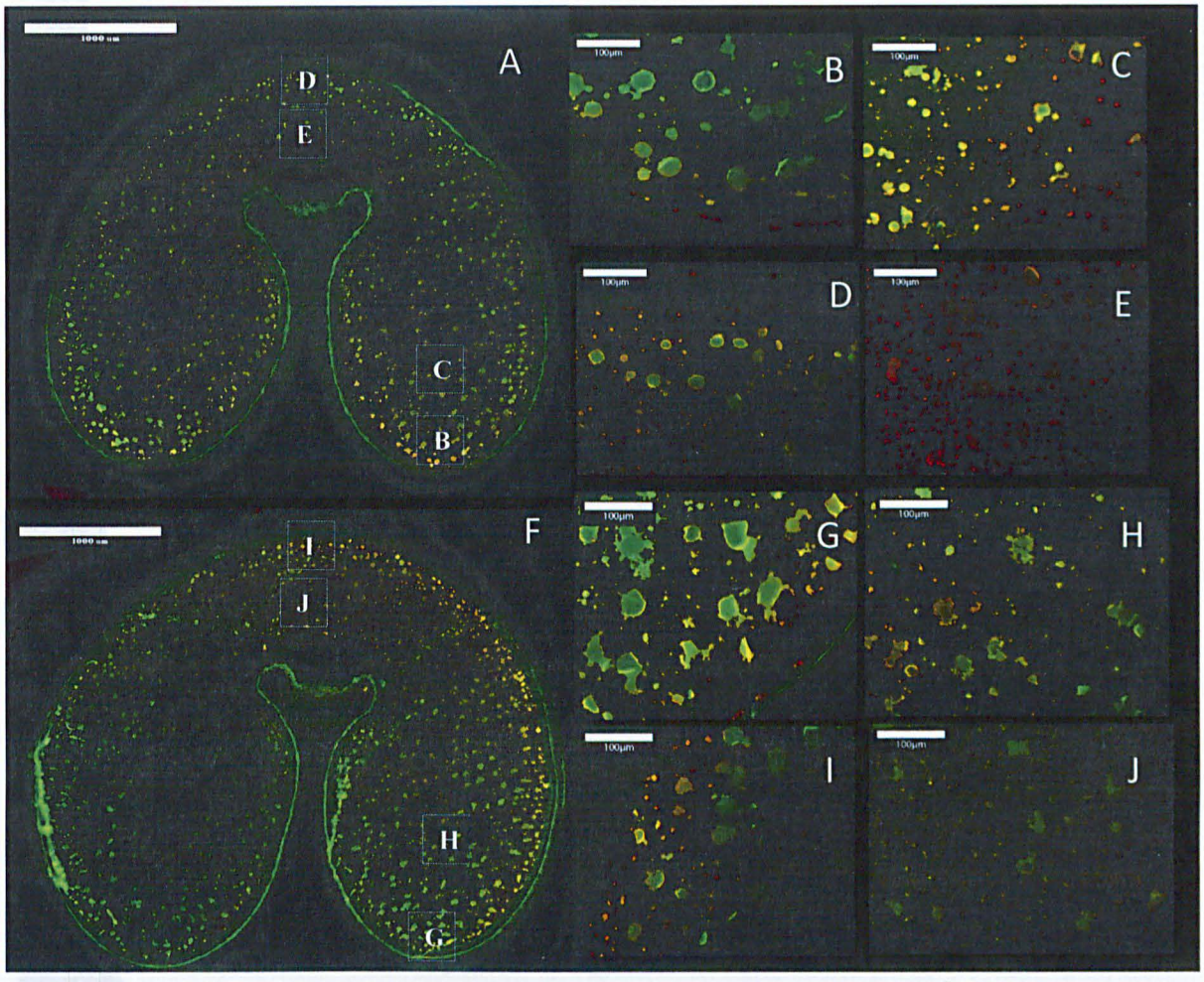

Figure 4.24 Transverse sections of **HE350** grains showing the distribution of HMW-GS (green signal) and LMW-GS and gliadins (red signal): A) HE350 15 d.p.a. scale bar=1000µm. areas labelled B-E corresponded to detailed images B-E (scale bar=100 µm); B) lobe edge; C) lobe centre; D) dorsal edge; E) dorsal centre; F) HE350 21 d.p.a. scale bar=1000µm. areas labelled G-J corresponded to detailed images G-J (scale bar=100 µm); G) lobe edge; H) lobe centre; I) dorsal edge; J) dorsal centre

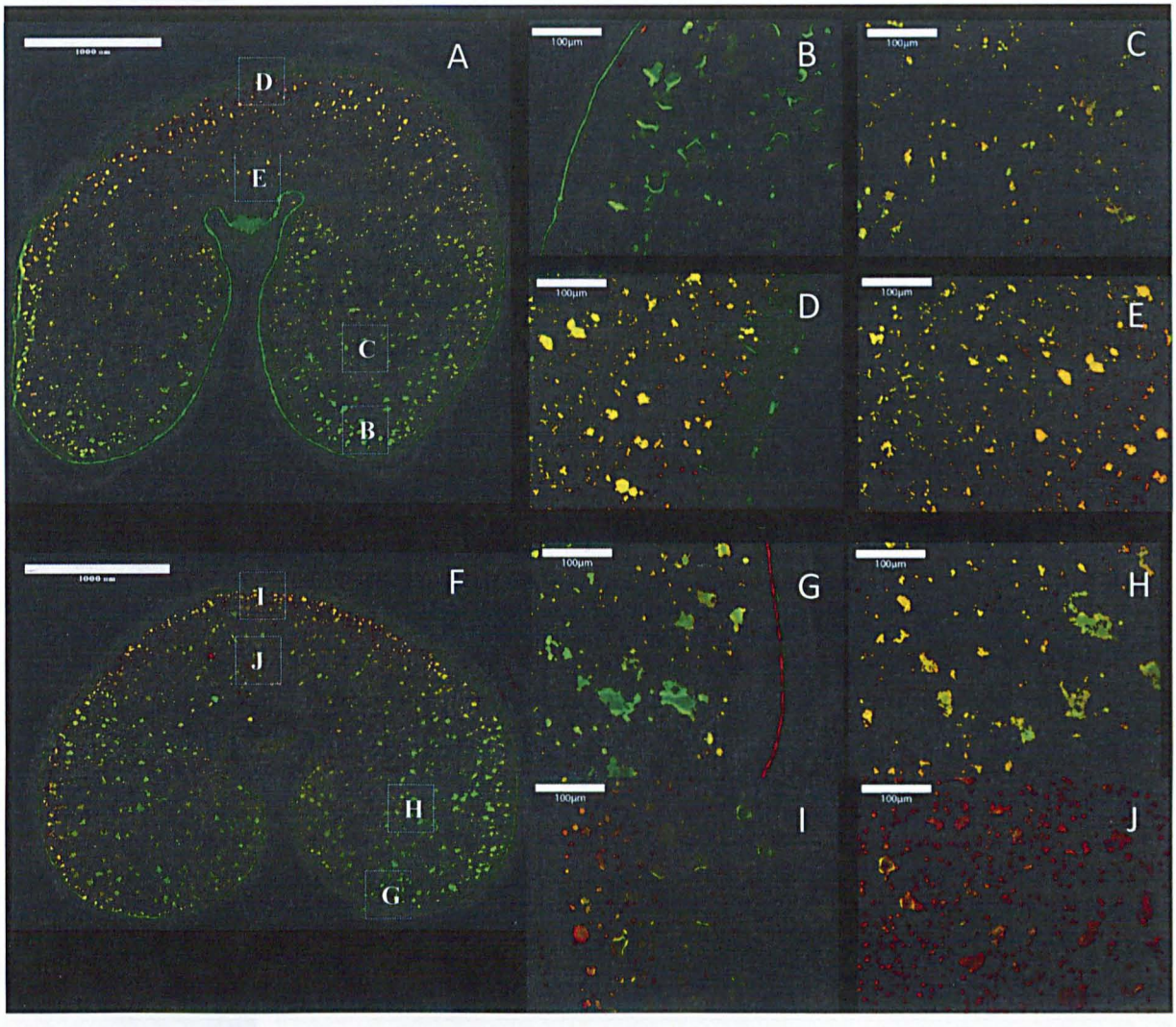

Figure 4.25 Transverse sections of **HE100** grains showing the distribution of HMW-GS (green signal) and LMW-GS and gliadins (red signal): A) HE100 15 d.p.a. scale bar=1000µm. areas labelled B-E corresponded to detailed images B-E (scale bar=100 µm); B) lobe edge; C) lobe centre; D) dorsal edge; E) dorsal centre; F) HE100 21 d.p.a. scale bar=1000µm. areas labelled G-J corresponded to detailed images G-J (scale bar=100 µm); G) lobe edge; H) lobe centre; I) dorsal edge; J) dorsal centre;

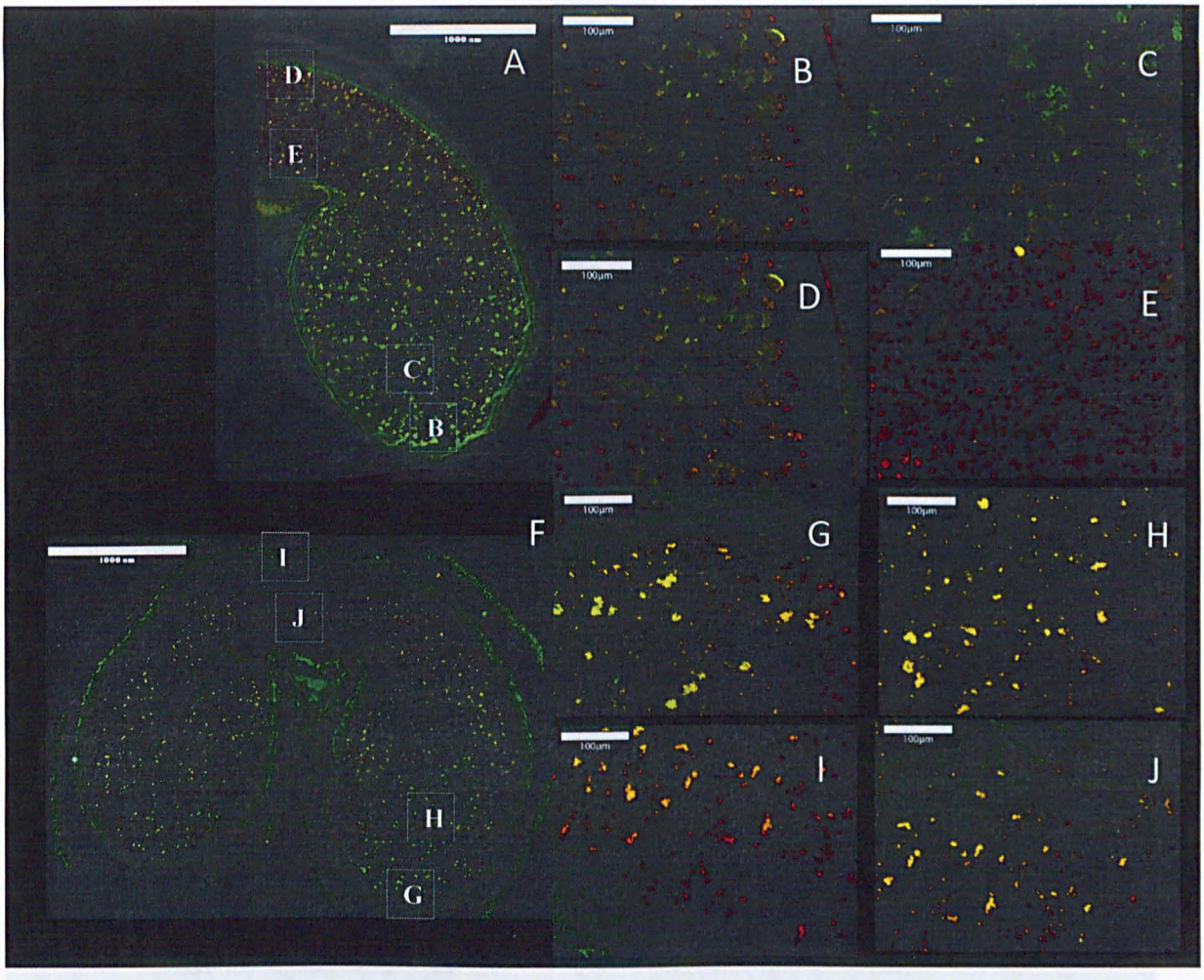

Figure 4.26 Transverse sections of **IS350** grains showing the distribution of HMW-GS (green signal) and LMW-GS and gliadins (red signal): A) IS350 15 d.p.a. scale bar=1000µm. areas labelled B-E corresponded to detailed images B-E (scale bar=100 µm); B) lobe edge; C) lobe centre; D) dorsal edge; E) dorsal centre; F) IS350 21 d.p.a. scale bar=1000µm. areas labelled G-J corresponded to detailed images G-J (scale bar=100 µm); G) lobe edge; H) lobe centre; I) dorsal edge; J) dorsal centre;

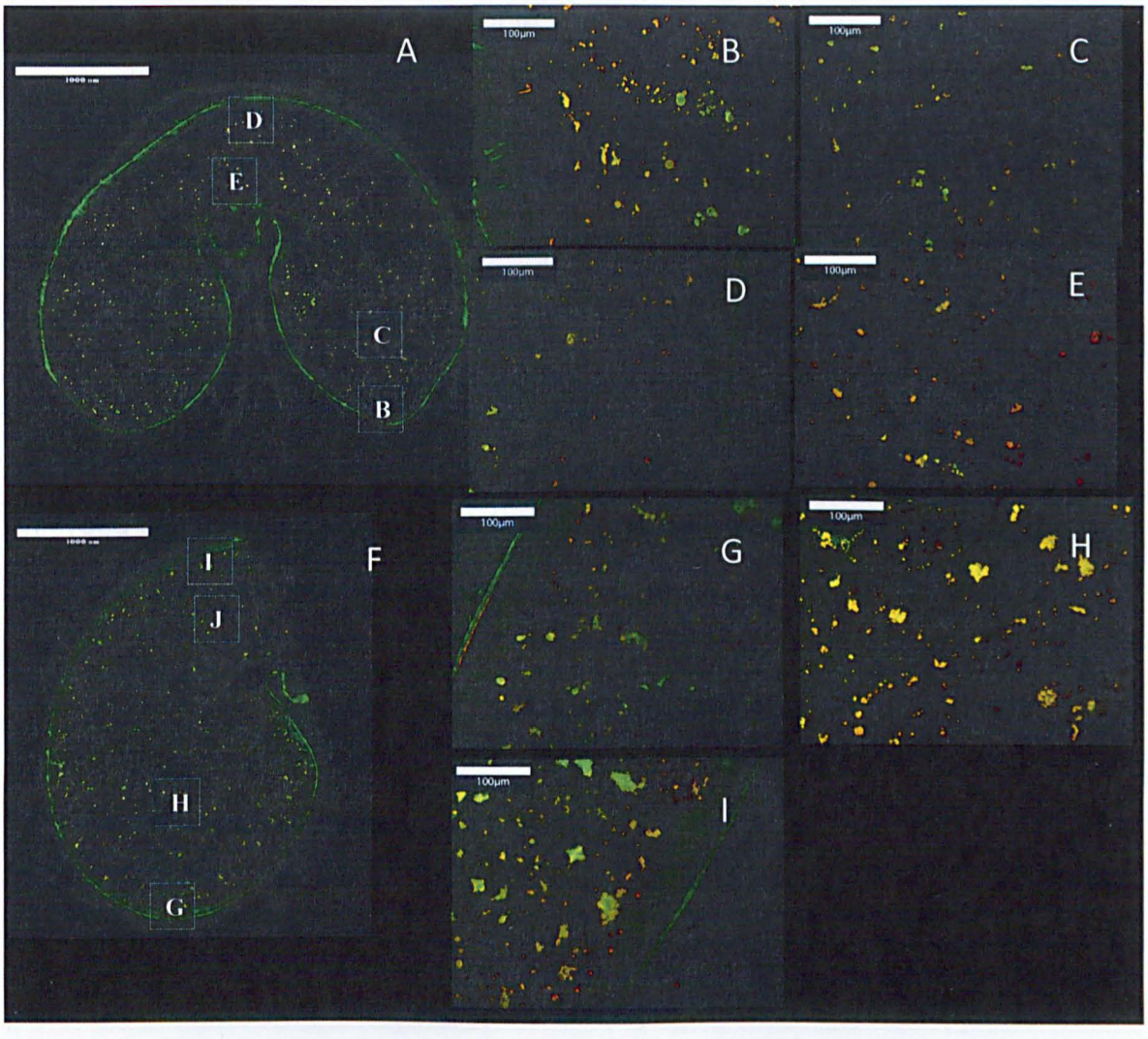

Figure 4.27 Transverse sections of IS100 grains showing the distribution of HMW-GS (green signal) and LMW-GS and gliadins (red signal): A) IS100 15 d.p.a. scale bar=1000µm. areas labelled B-E corresponded to detailed images B-E (scale bar=100 µm); B) lobe edge; C) lobe centre; D) dorsal edge; E) dorsal centre; F) IS100 21 d.p.a. scale bar=1000µm. areas labelled G-I corresponded to detailed images G-J (scale bar=100 µm); G) lobe edge; H) lobe centre; I) dorsal edge

4.5.2 Digitalisation of images

The dataset of images showing double immunofluorescence labelling (method described in Chapter 2, section 2.8) was analysed using ANOVA. The lobe regions and dorsal region were analysed separately. Three transects were

studied from each region (an example can be found at figure 2.8A, chapter 2). Each transect was divided transversely into four (three for dorsal regions) sections, which were marked from 1 to 4 (3 for the dorsal regions), section 1 being the closest and section 3 the furthest from the aleurone layer of the grain. The total area (of the protein bodies) and total intensity were both log (natural) transformed to achieve normal statistic distributions. In total, 15 out of 16 images were analysed (one image of IS100, 21d.p.a. was not able to be analysed due to sample damage) to give 672 data pairs (including 42 missing pairs). All ANOVA results are given in appendix 4.

The results showed that most factors and interactions at the seed level (seed stratum) were not flagged as important. This can be explained by large variation (see m.s. columns of appendix 4) existed between individual seeds due to biological variation since the seeds were harvested from random spikes of the plant. However, at the seed level, the variety factor for HMW-GS in the lobe region was significant for both protein body size ($p=0.019$) and intensity ($p=0.022$), despite the large variation between seeds. This shows a clear difference between Hereward and Istabraq in HMW-GS distribution in the lobe region.

When transects and positions are taken into account (at Seed.Transects stratum), the variation becomes much smaller because the biological variation between transects within a seed is much smaller. Therefore, the interactions of influential factors can be tested. At this level, when an interaction between position[12] and a factor or multiple factors is flagged as significant, it indicates

[12] The term of "position" used in this section reflect the geographic position of the data point on the axis from aleurone layer toward centre endosperm at lobe region, or the axis from aleurone layer toward crease at dorsal region

that the factor/factors has/have influences on the pattern of protein body size/intensity across the grain, but not the general level of protein size/intensity.

Distribution of HMW-GS

ANOVA (see appendix 4, table 1) showed significant interactions of Position×Variety×N in analyses of log (area) in the lobe regions (p=0.017), although for log (total_Intensity) the same interaction was not flagged as important (p=0.059). Considering the p value was very close to the significance limit of 0.05 and the fact that those two values were paired data, the Position×Variety×N interaction of log (total_Intensity) should also be considered significant. The distribution patterns of the HMW-GS in the two cultivars and at the two nitrogen levels are presented in figure 4.28. Both protein body size (Figure 4.28 A) and intensity of labelling (Figure 4.28 B) show decreasing gradients from the outer layers of the lobe toward the centre. Hereward shows steeper gradients for protein body size and labelling intensity compared to Istabraq. Although the N fertilisation effect was included in the significant interaction --Position×Variety×N, it had no clear impact on the shapes of gradients considering the relatively large l.s.d. values (l.s.d.=0.6465 for protein size, l.s.d.=0.9295 for protein intensity, figure 4.28). Because of the large l.s.d., the difference between the two trends in protein intensity of Istabraq at different N levels is also considered insignificant. The fact that the data set of IS350 only contains one replicate may also contribute to this difference. None of the interactions involving development stage (DPA) was found to be significant.

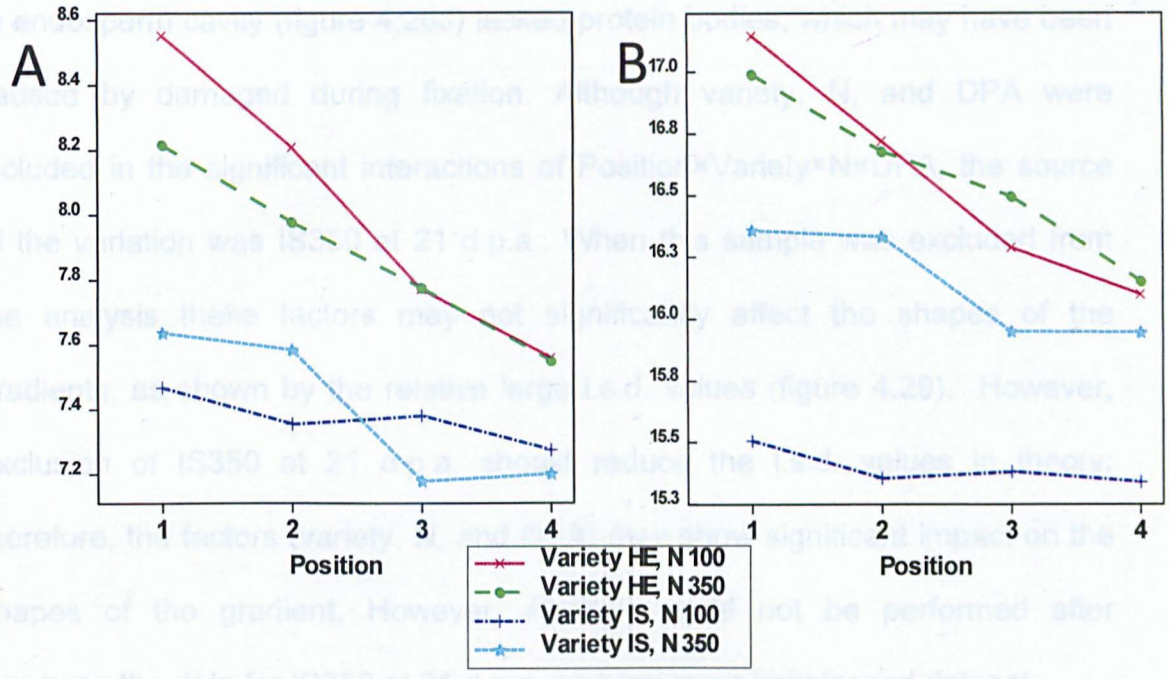

Figure 4.28 Distribution of HMW-GS in the lobe regions of grain. Data for two development stages (15 and 21 d.p.a.) were integrated since neither factor DPA nor DPA related interactions were found to be significant (see appendix 4, table 1). A) Distribution of sizes of protein bodies recognised by anti-HMW-R2, l.s.d.=0.6465 at d.f. 10 when comparing across cultivars/N-levels, l.s.d.=0.27 at d.f. 111 when comparing within same cultivar and N-level. B) Distribution of total intensity of protein bodies recognised by anti-HMW-R2, l.s.d.=0.9295 at d.f. 10 when comparing across cultivars/N-levels, l.s.d.=0.2906 at d.f. 111 when comparing within same cultivar and N-level.

For the dorsal regions, significant interactions of Position×Variety×N×DPA were found for both log (area) and log (total intensity) ($p=0.021$ and 0.034, respectively). Figure 4.29 shows decreasing gradients of log (area) and log (total intensity) from the outer endosperm layers toward internal layers. Interestingly, IS350 at 21 d.p.a. had exceptionally low levels of log (area) and log (total intensity) at positions 1 and 3 and shows an opposite trend when compared to the data for 15 DPA. Figure 4.26 shows that this result is due to the fact that the cells closest to aleurone layer (figure 4.26l) and the area close

to endosperm cavity (figure 4.26J) lacked protein bodies, which may have been caused by damaged during fixation. Although variety, N, and DPA were included in the significant interactions of Position×Variety×N×DPA, the source of the variation was IS350 at 21 d.p.a.. When this sample was excluded from the analysis these factors may not significantly affect the shapes of the gradients, as shown by the relative large l.s.d. values (figure 4.29). However, exclusion of IS350 at 21 d.p.a. should reduce the l.s.d. values in theory; therefore, the factors (variety, N, and DPA) may show significant impact on the shapes of the gradient. However, ANOVA could not be performed after removing the data for IS350 at 21 d.p.a. as it led to an imbalanced dataset.

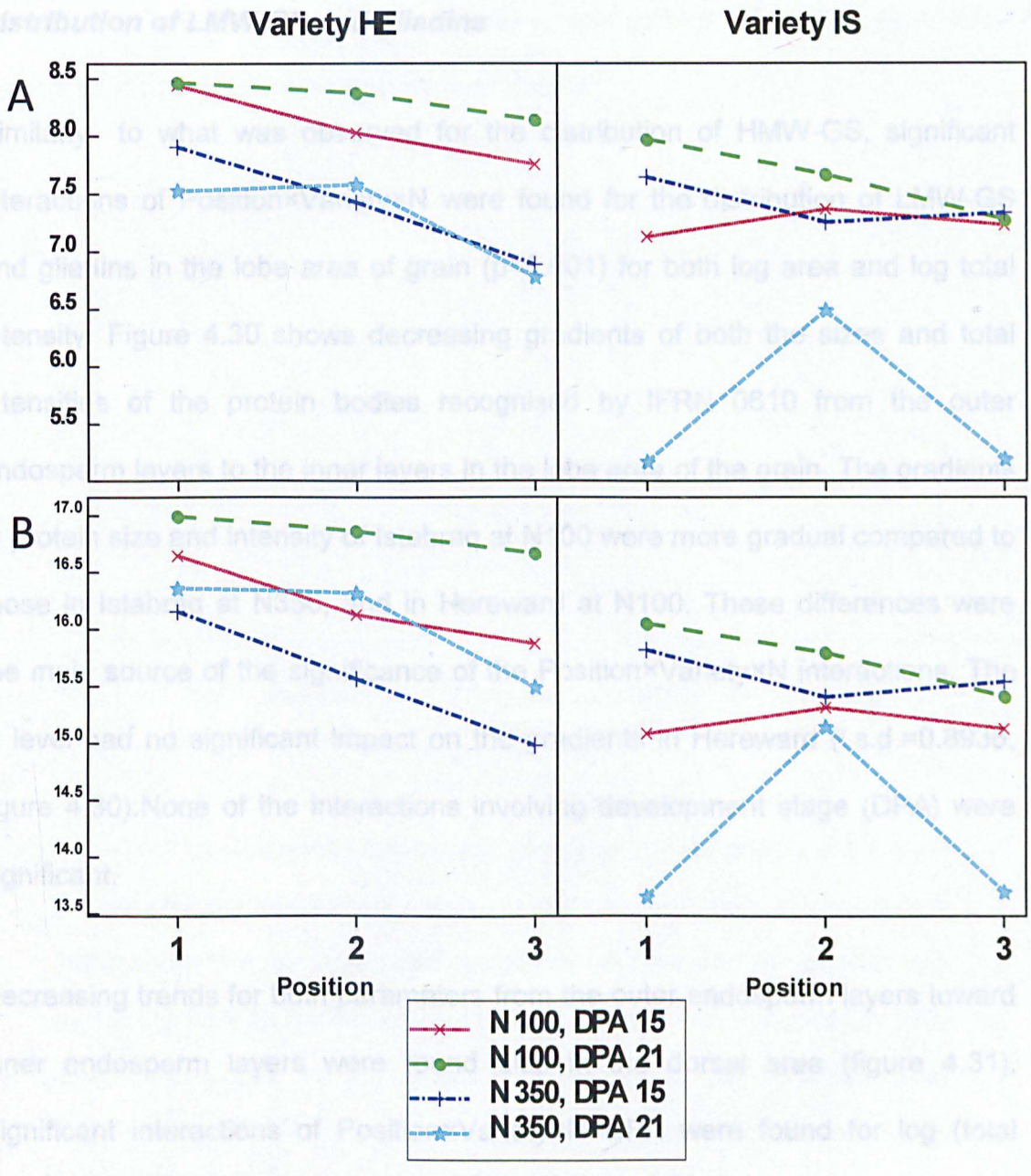

Figure 4.29 Distribution of HMW-GS in the dorsal regions of grain. A) Distribution of sizes of protein bodies recognised by anti-HMW-R2, l.s.d.=1.8652 at d.f. 8 when comparing across cultivars/N-levels/development stage, l.s.d.=0.5076 at d.f. 74 when comparing within same level of cultivar, N-level and development stage. B) Distribution of total intensity of protein bodies recognised by anti-HMW-R2, l.s.d.=0.5892 at d.f. 8 when comparing across cultivars/N-levels/development stage, l.s.d.=2.1389 at d.f. 74 when comparing within same level of cultivar, N-level and development stage.

Distribution of LMW-GS and gliadins

Similarly to what was observed for the distribution of HMW-GS, significant interactions of Position×Variety×N were found for the distribution of LMW-GS and gliadins in the lobe area of grain ($p<0.001$) for both log area and log total intensity. Figure 4.30 shows decreasing gradients of both the sizes and total intensities of the protein bodies recognised by IFRN 0610 from the outer endosperm layers to the inner layers in the lobe area of the grain. The gradients in protein size and intensity of Istabraq at N100 were more gradual compared to those in Istabraq at N350, and in Hereward at N100. These differences were the main source of the significance of the Position×Variety×N interactions. The N level had no significant impact on the gradients in Hereward (l.s.d.=0.8936, figure 4.30).None of the interactions involving development stage (DPA) were significant.

Decreasing trends for both parameters from the outer endosperm layers toward inner endosperm layers were found also in the dorsal area (figure 4.31). Significant interactions of Position×Variety×N×DPA were found for log (total intensity) ($p=0.019$). The p value of same interaction for log (area) was very close to the significant level ($p=0.053$). Considering that the log (area) and log (total intensity) were paired data, this interaction should also be considered significant. Similarly to the data for HMW-GS, significantly lower values for protein body size and intensity were measured close to the aleurone and endosperm cavity for IS350 at 21 d.p.a.. As discussed above, this was due to loss of protein in these areas. Also similar to the HMW-GS data, the significant interactions of Position×Variety×N×DPA mainly resulted from variation in IS350 at 21 d.p.a.. When the data for IS350 at 21 d.p.a. were excluded, these

factors may become insignificant. However, this cannot be verified as ANOVA cannot be carried out on an unbalanced dataset.

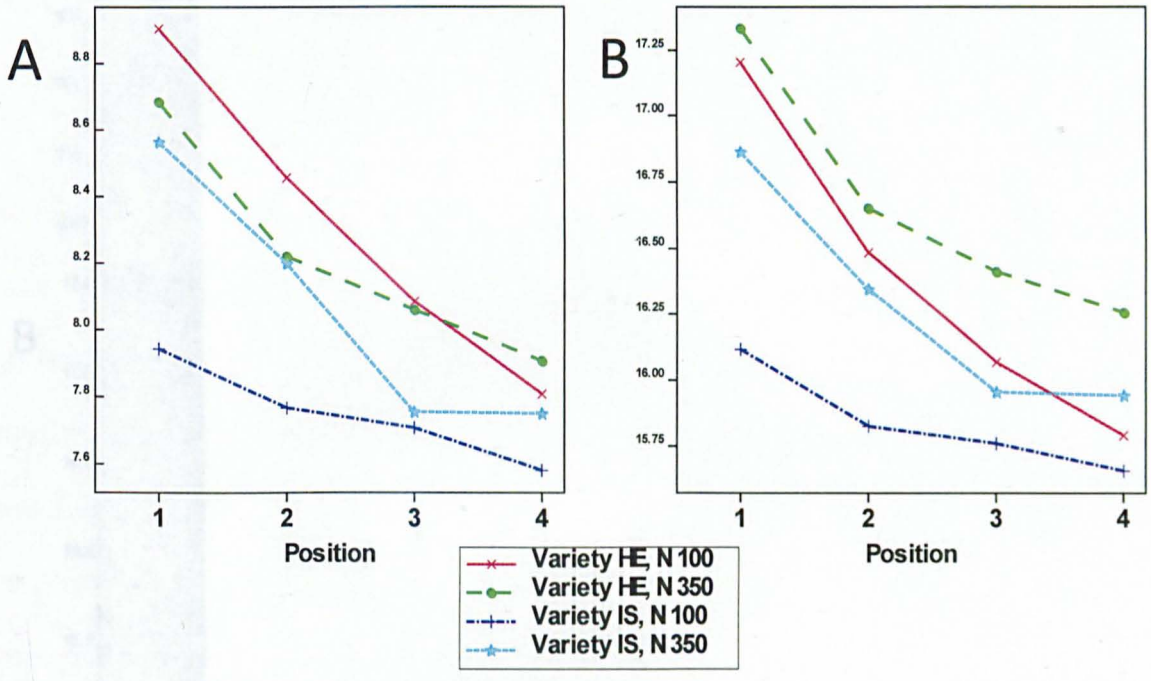

Figure 4.30 Distribution of LMW-GS and gliadins of the lobe regions of the grain. The data for two development stages (15 and 21 d.p.a.) were integrated since neither factor DPA nor DPA related interactions were found to be significant (see appendix 4, table 3). A) Distribution of sizes of protein bodies recognised by IFRN 0610, l.s.d.=0.5439 at d.f. 9 when comparing across cultivars/N-levels, l.s.d.=0.2109 at d.f. 111 when compared within the same cultivar and N level. B) Distribution of total intensity of protein bodies recognised by IFRN 0610, l.s.d.=0.8936 at d.f. 7 when compared across cultivars/N levels, l.s.d.=0.2063 at d.f. 111 when comparing within same cultivar and N level.

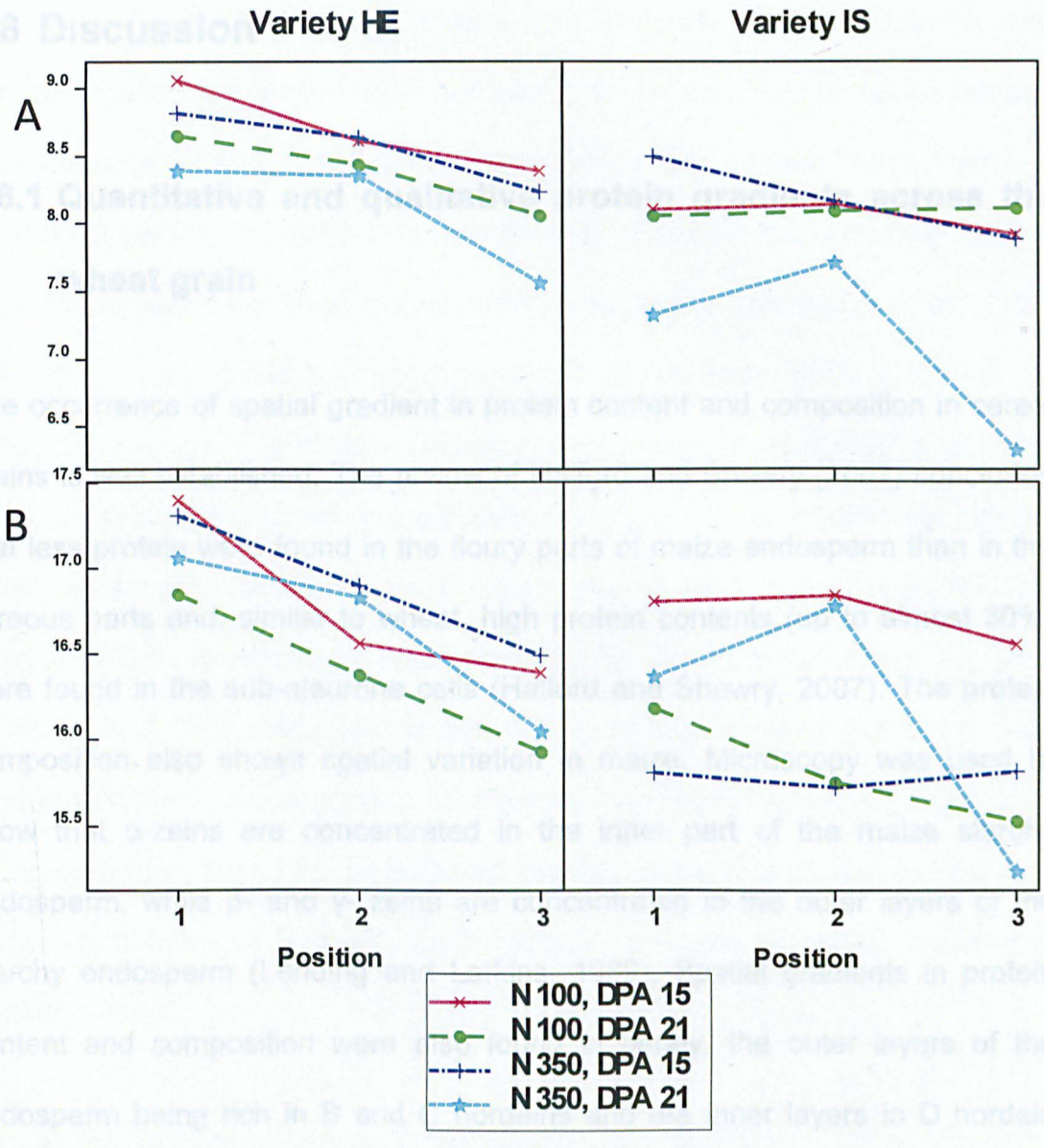

Figure 4.31 Distribution of LMW-GS and gliadins in the dorsal regions of the grain. A) Distributions of sizes of protein bodies recognised by IFRN 0610, l.s.d.=1.3140 at d.f. 7 when compared across cultivars/N levels/development stages l.s.d.=0.3298 at d.f. 74 when compared within same level of cultivar, N level and development stage. B) Distribution of total intensity of protein bodies recognised by IFRN 0610, l.s.d.= 1.2879 at d.f. 8 when compared across cultivars/N levels/development stages, l.s.d.=0.4009 at d.f. 74 when compared within same level of cultivar, N-level and development stage.

4.6 Discussion

4.6.1 Quantitative and qualitative protein gradients across the wheat grain

The occurrence of spatial gradient in protein content and composition in cereal grains is well established. The review of Halford and Shewry (2007) concluded that less protein were found in the floury parts of maize endosperm than in the vitreous parts and, similar to wheat, high protein contents (up to almost 30%) were found in the sub-aleurone cells (Halford and Shewry, 2007). The protein composition also shows spatial variation in maize. Microscopy was used to show that α-zeins are concentrated in the inner part of the maize starchy endosperm, while β- and γ- zeins are concentrated in the outer layers of the starchy endosperm (Lending and Larkins, 1989). Spatial gradients in protein content and composition were also found in barley, the outer layers of the endosperm being rich in B and C hordeins and the inner layers in D hordein (Millet et al., 1991, Davies et al., 1993). For wheat, a high protein content in the subaleurone cells has been reported (Evers, 1970), but detailed information on the spatial distribution of protein content and composition has not been reported until a recently study (Tosi et al., 2011). The author of this thesis was a co-author of this paper.

Differences among wheat endosperm cells may be expected due to the development process of grain. The cells in the layer closest to the aleurone (subaleurone) are the youngest and the cells in centre part of endosperm are the oldest since the aleurone cells continue to divide periclinally (Shewry and

Halford, 2002) until about two weeks after anthesis. The subaleurone cells contain smaller and fewer starch granules than cells in the central endosperm (Bechtel et al., 2009), but they have a higher protein content (Kent, 1966). In addition, a gradient in total protein content may also exist in all endosperm cells, from the subaleurone layer toward centre endosperm (Normand et al., 1965, Tosi et al., 2011). My results (section 4.1.1) provide solid evidence for quantitative gradient in protein content across the grain. However, it has also been suggested that approximately amounts of protein (by weight) are present in all endosperm cells. Therefore, the gradient in protein content could resulted from dilution by starch, which also shows significant gradients across the grain (Evers, 1970). However, Ugalde and Jenner (1990) showed that starch was deposited evenly on dorsal side of the wheat endosperm, except for a decrease in the outermost layer. They concluded that the gradient in protein content was not due to differences in starch content (Ugalde and Jenner, 1990a, Ugalde and Jenner, 1990b).

The protein gradient is not only quantitative, also qualitative. There is indirect evidence from studies of wheat mill streams which showed significant differences in HMW-GS and LMW-GS content among white flour milling fractions (Yahata et al., 2006, Wang et al., 2007). This indicates that the wheat endosperm is heterogeneous in terms of gluten protein composition. My results from the analysis of pearling fractions (figure 4.17, 4.8, 4.10, and 4.12) and immunofluorescence images (section 4.5.1) confirm the existence of qualitative protein gradients. Similar immunofluorescence results have been reported previously from our laboratory (Tosi et al., 2009, Tosi et al., 2011). Combining the SDS-PAGE and SE-HPLC data with TPC data (figure 4.32), the gradients in

total gluten protein percentage in flour and percentages of major gluten protein types in flour could be observed.

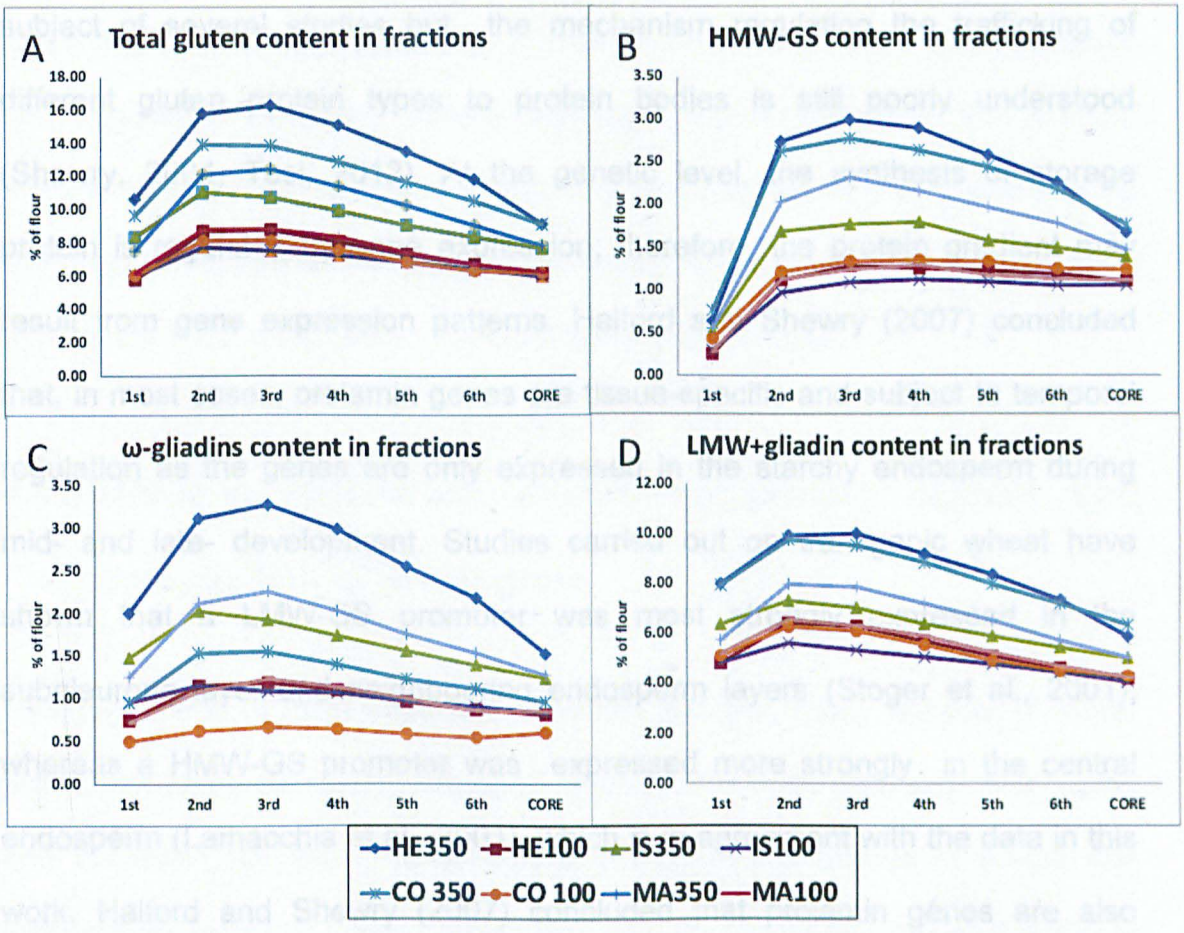

Figure 4.32 Contents of total gluten protein and protein groups in pearling fractions. Data were calculated based on TPC data of pearling fractions (table 4.3), SE-HPLC data of pearling fractions (appendix 1) and gluten profile data of pearling fractions (table 4.6). A) Total gluten % of pearling fractions, calculation based on gluten content in total protein (from SE-HPLC data) × TPC. B)-D) sub-gluten protein group contents of pearling fractions, =total gluten % of flour × sub-group % of gluten protein (from gluten profile data).

4.6.2 Origin of protein qualitative and quantitative gradients

The synthesis and deposition of cereal seed storage proteins have been the subject of several studies but the mechanism regulating the trafficking of different gluten protein types to protein bodies is still poorly understood (Shewry, 2004, Tosi, 2012). At the genetic level, the synthesis of storage protein is regulated by gene expression; therefore, the protein gradient may result from gene expression patterns. Halford and Shewry (2007) concluded that, in most cases, prolamin genes are tissue-specific and subject to temporal regulation as the genes are only expressed in the starchy endosperm during mid- and late- development. Studies carried out on transgenic wheat have shown that a LMW-GS promoter was most strongly expressed in the subaleurone layer and neighbouring endosperm layers (Stoger et al., 2001), whereas a HMW-GS promoter was expressed more strongly in the central endosperm (Lamacchia et al., 2001), which is in agreement with the data in this work. Halford and Shewry (2007) concluded that prolamin genes are also regulated by nutritional availability, specifically, the availability of nitrogen and sulphur (Halford and Shewry, 2007). Various studies have shown that N-fertilisation influences the protein content of the grain as well as the protein composition (Wieser and Seilmeier, 1998, DuPont et al., 2006). Despite all the above information, many things about storage protein synthesis are still unknown, such as how gene expression is triggered by development and environmental signals, as well as what causes the temporal and spatial patterns of expression within endosperm (Halford and Shewry, 2007). Considering all above, we can only assume that, the spatial patterns of protein content and composition are related to the tissue specificity of gene expression, and/or the availability of nutrients during development. Hypothetically, the level and pattern

of expression of prolamin genes may not only be regulated by the tissue specificity, but also by the location of the cells within the tissue. Also, the patterns of nutrition transport might may in spatial differences in nutrient availability, which may regulate gene expression level (Shewry 2012, personal communication). However, similarly to storage protein synthesis and deposition, the transportation of nutrients is not fully understood. Currently there is no evidence in support any of these hypotheses. More detailed gene expression studies are required for better understanding of this subject.

4.6.3 Nitrogen fertilisation effects on protein content and composition of wheat grain

Nitrogen is one of most important nutrients, affecting many aspects of wheat production and grain quality. Nitrogen fertiliser effects on yield are complicated and influenced by many other factors, therefore it is difficult to predict the outcome in terms of yield. However, the positive effect of post-anthesis application of nitrogen on grain protein content and gluten content is well documented (Johansson et al., 2001, Pechanek et al., 1997, Borkowska et al., 1999, Godfrey et al., 2010). But some reports have shown either no effect or negative effects of additional nitrogen fertiliser on the gluten index (GI) (Mis and Grundas, 2001, Ames et al., 2003). However, the true relationship between the GI and wheat quality has been questioned (Bonfil and Posner, 2012, Borkowska et al., 1999). Nitrogen fertilisation also affects the composition of gluten proteins. Our results indicated that additional nitrogen fertiliser led to higher proportions of HMW-GS and ω-gliadins and lower proportions of LMW-GS and other gliadins, which agrees with previous studies (Daniel and Triboi, 2000,

Wieser and Seilmeier, 1998, DuPont et al., 2006). Godfrey (2008) found similar positive effects of nitrogen fertiliser on the proportion of gliadins (F3% and F4%) by SE-HPLC which agrees with our results. However, Godfrey (2010) also reported a decreased proportion of large glutenin polymers (F1%) with increased N application. My analyses showed that the proportion of large polymers was positively affected by N application, although the differences were small. In order to understand this contradiction, it is essential to understand differences between gluten protein subunits and SE-HPLC fractions. Previous studies combined SE-HPLC and SDS-PAGE to reveal the composition of SE-HPLC fractions. This showed that SE-HPLC fractions do not contain single types of proteins but are mixtures of different proteins. For example, the large polymer peak F1 and small polymer peak F2 are both enriched in HMW-GS and LMW-GS, but in different proportions (Larroque et al., 1997, Tosi et al., 2005). My gluten profile results showed HMW-GS proportion in gluten and LMW-GS+other gliadins were influenced by N applications conversely, therefore it is understandable that the N-fertilisation effects on two SE-HPLC polymer peaks (F1 and F2) were not obvious because the contradict N application effects on HMW-GS proportion in gluten and LMW-GS proportion in gluten.

More interestingly, our results showed differential effects of nitrogen application on protein content and composition within the grain. The protein contents of endosperm layers close to aleurone were increased more by additional N fertiliser than those in the centre of endosperm (figure 4.3A), which led to a greater increase in TPC, and to higher proportions of HMW-GS and ω-gliadin in gluten. This may relate to nutrient transport as discussed above. My results also indicated that the pattern of responses to N fertilisation across the grain varied among cultivars. Figure 4.3B showed that, in terms of fold increase in TPC, the

good quality bread making wheats Paragon and Hereward showed greater responses in the layers toward the centre of the endosperm, whereas Istabraq showed higher responses in the layers close to aleurone. Differential responses of cultivars also occurred in storage protein composition. Figures 4.10 and 4.11, show that increase N level led to higher proportions of ω-gliadins and lower proportions of LMW+α- and γ-gliadins within all of the cultivars. Also, Hereward showed greater responses than other three cultivars. Effects of cultivar×N interactions on wheat grain protein content have been frequently reported. Luo et al. (2000) and Ames et al. (2003) found significant interactions of genotype and treatment (nitrogen and sulphur) for flour protein % (Luo et al., 2000, Ames et al., 2003). Luo et al. (2000) also suggested that genotype is the only significant cause of difference in the quantities of HMW-GS and LMW-GS (Luo et al., 2000). However, they did not describe how the protein bands were divided into HMW-GS and LMW-GS groups, therefore, this result cannot be compared to ours. Other environmental factors such as temperature, year and sulphur supply also played important roles in determine wheat protein content and/or composition (DuPont et al., 2006, Barraclough et al., 2010, Flaete et al., 2005). Regrettably, the effects of these factors were not tested in this project. Therefore, we cannot determine whether these factors have roles in determining the spatial patterns of TPC and gluten composition.

4.6.4 Wheat storage protein and end-use properties

As described in Chapter 1, the quantity and quality of wheat storage protein have been widely studied and shown to be significant in relation to wheat end-use properties, especially breadmaking quality. Carson and Edwards (2011) reviewed that wheat flour protein content requirements can ranged from 7% to

13.5% for varies types of products (Carson and Edwards, 2009). For cake making, about 8% flour protein content is suitable (Cornell and Hoveling, 1998). For breadmaking, at least 12% of grain protein, which could produce flour with about 11% protein content, is required (Halverson and Zeleny, 1988). Even higher protein content is desirable since it leads to higher loaf volume (Shewry et al., 2009a). The quality of wheat protein or more specifically, the composition of wheat gluten proteins is as important as the quantity of protein for wheat products, especially for bread making. It is well established that the molecular size distribution of gluten proteins influences breadmaking quality, especially the proportion of large polymeric proteins (Millar, 2003, Field et al., 1983). More detailed research has revealed that the different groups of wheat gluten proteins play different roles in dough formation. The gliadins provide viscous flow and extensibility to the dough, and the glutenins determined the elasticity (Van Der Borght et al., 2005). The LMW-GS determine the extensibility and contribute to dough strength, where the HMW-GS are considered to be major contributors to dough elasticity (Shewry et al., 2009a). Therefore, understanding the spatial variation in the quantity and quality of wheat storage proteins are not only important for further research to understand storage protein synthesis, trafficking and deposition in the grain; but may also be exploited for the development of novel food processes to improve product variety and quality. For example, the relationship between milling fractions and pearling fractions showed in this study could lead to improvement of common milling process.

4.7 Conclusions

My results revealed differences in the spatial distribution patterns of wheat grain protein content and composition from outer starchy endosperm layers toward centre endosperm. Protein content was highest in the layers closest to the aleurone layer and then gradually decreased toward the central endosperm. Genotype and nitrogen fertiliser had effects on total protein content and on the spatial patterns of total protein content. The layers closest to the aleurone layer were most responsive to additional nitrogen. The same layers also showed most distinct differences when compared across cultivars.

The SE-HPLC analyses showed that the total content of gluten (the sum of F1, F2, F3, and F4 peaks) increased from the outer layers toward the central endosperm. Similar patterns were observed for the proportions of large polymers (F1), small polymers (F3), and monomers enriched in α- and γ- gliadin (F4). The proportion of monomers enriched in ω-gliadin (F3) showed the opposite trend. Genotype had strong effects on some of these parameters but not on all. Istabraq had a lower level of %F1 and the highest F3+F4/F1 ratio, and Cordiale stood out as having highest F1/F2 ratio. Higher N levels had positive effects on % gluten and %F4; however, the effects were not obvious for other parameters despite been statistically significant. I also compared the SE-HPLC results of milling fractions and pearling fractions, and found some associations between the two sets of fractions, which may indicate the structural origins of milling fractions.

Within the gluten proteins, the SDS-PAGE results showed that the proportion of HMW-GS gradually increased toward the centre of the endosperm. By contrast,

the proportions of LMW-GS+α- and γ- gliadins decreased in the same direction. The proportion of ω-gliadin was relatively stable across the grain. The composition of gluten proteins was strongly influenced by genotype. For example, Cordiale had a low level of ω-gliadin (% in gluten) but relatively high levels of LMW-GS+α- and γ- gliadins (% in gluten). N level had significant effects on gluten composition. Additional nitrogen increased the proportions of HMW-GS and ω-gliadin, and consequently reduced the proportions of LMW-GS+α- and γ- gliadins. Similar to total protein content, the layers closest to aleurone responded greatest to the additional nitrogen.

The microscopy study provided visual evidence for spatial gradients in HMW-GS and other gluten proteins. The micrographs showed that, at the seed level, both HMW-GS and other gluten proteins were expressed more highly in the layers close to the aleurone layer than the centre of endosperm. The protein bodies in the outer layers were also larger. There were also differences in gluten protein concentration in different area of the grain. The LMW-GS and gliadins tended to be concentrated more in the dorsal area than in the lobe area (in transverse sections). Similar tendency was observed for HMW-GS; however the differences were not as distinct.

Chapter 5: Starch content and properties

5.1 Total starch content

The data set included total starch content (TSC, % of flour weight) of four cultivars and two N fertilization levels. Bulked flours of three biological replicates were used for analysis due to the limited amount of flour of each fraction. The analysis was run in two blocks, with two technical replicates being tested within each block. In total, 256 samples were tested including one missing value.

5.1.1 Results of total starch content of whole grains

The dataset of whole grains was not tested by ANOVA due to the lack of replicates. Therefore, the differences discussed in this paragraph are those most likely to occur in a complete ANOVA test using a fully replicated data set. Figure 5.1A shows the ranking of the cultivars on the basis of their whole grain TSC. Surprisingly, the highest ranked wheats in both N-levels appeared to be the hard breadmaking wheats and not the soft feed wheat Istabraq. Figure 5.1B showed differences between the two N levels for each cultivar. Hereward showed the greatest reduction in TSC with additional nitrogen fertiliser, while Cordiale showed a small increase in TSC with higher N fertilisation.

Table 5.1 Total starch contents (%) of whole grains

A: Whole grain TSC				
N350 lines	MA350	IS350	CO350	HE350
	66.75	64.37	62.64	61.85
N100 lines	HE100	MA100	IS100	CO100
	76.83	76.62	68.14	61.8
B: Difference of whole grain TSC between N350 and N100				
	HE	MA	IS	CO
	-14.98	-9.87	-3.77	0.84

5.1.2 TSC gradients across grain

The data sets for %TSC of the pearling fractions were analysed by ANOVA using highest order interaction (cultivar×N-level×fraction) as residual term due to the lack of biological replicates (described at section 4.4.1). All effects of main factors (cultivar, N-level and fraction) were tested using hidden replicates within the experiment structure. Two-way interactions were not analysed as they were less reliable due to lack of replicates. The results of ANOVA and means of data are shown in table 5.2.

Table 5.2 TSC ANOVA results and means summary

A: Influential factors			
Factors	P value	l.s.d.	d.f.
Cultivar	<0.001	2.577	18
N-fertilisation	<0.001	1.822	18
Fraction	<0.001	3.408	18

B: Means of total starch content (% in flour weight)

	1st	2nd	3rd	4th	5th	6th	core
CO100	20.06	46.03	58.48	65.94	72.23	73.51	71.50
CO350	17.06	38.26	47.89	50.20	54.94	73.21	70.25
HE100	21.30	47.57	54.95	60.21	62.82	76.18	75.27
HE350	19.31	41.45	49.18	53.94	71.05	78.08	76.79
IS100	29.23	52.59	63.10	66.90	69.74	73.40	69.01
IS350	22.44	47.75	54.80	63.04	64.00	67.49	63.82
MA100	20.70	53.82	66.39	72.06	76.76	71.53	77.23
MA350	19.23	47.64	56.71	66.89	78.80	70.74	71.56

Figure 5.1 showed clear gradients in %TSC across the grain. A gradual increase toward the central endosperm was observed in all lines, and is reflected in the significant influence ($p<0.001$) showed by the ANOVA results for the fraction factor. The cultivar factor was also significant ($p<0.001$), suggesting a role of genotype in the %TSC of fractions across the grain. Figure 5.2A showed that, until the 6th fraction, the increase in Malacca was greatest followed by Istabraq. The increases in Hereward and Cordiale were similar. In terms of differences in shape, Malacca showed a slightly steeper increase in %TSC from the 1st fraction to the 5th fraction than other cultivars, and in particular Istabraq, , then a decrease in fraction 6, suggesting that different varieties may have different gradients in %TSC. However it cannot be verified since two-way interactions were not tested. The N level factor was also found to have a significant impact on the %TSC of the fractions ($p<0.001$). As can be observed in Figure 5.2B, all N100 fractions have higher %TSC than N350 fractions. The shapes of the trends appeared to be different indicating that different fractions may response differently to nitrogen fertiliser. However, this cannot be confirmed as no two-way interactions were tested.

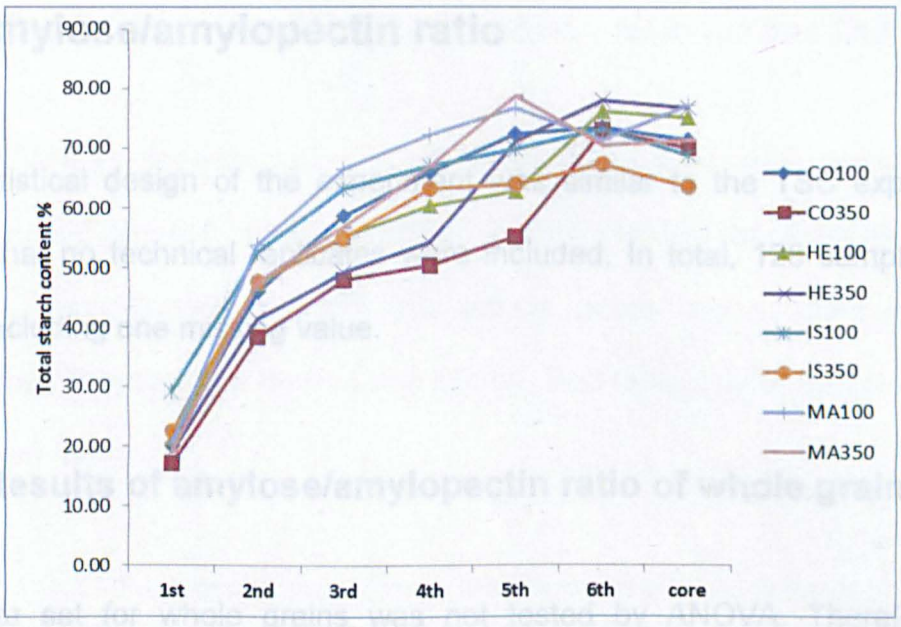

Figure 5.1, total starch content (% in flour weight) across grains

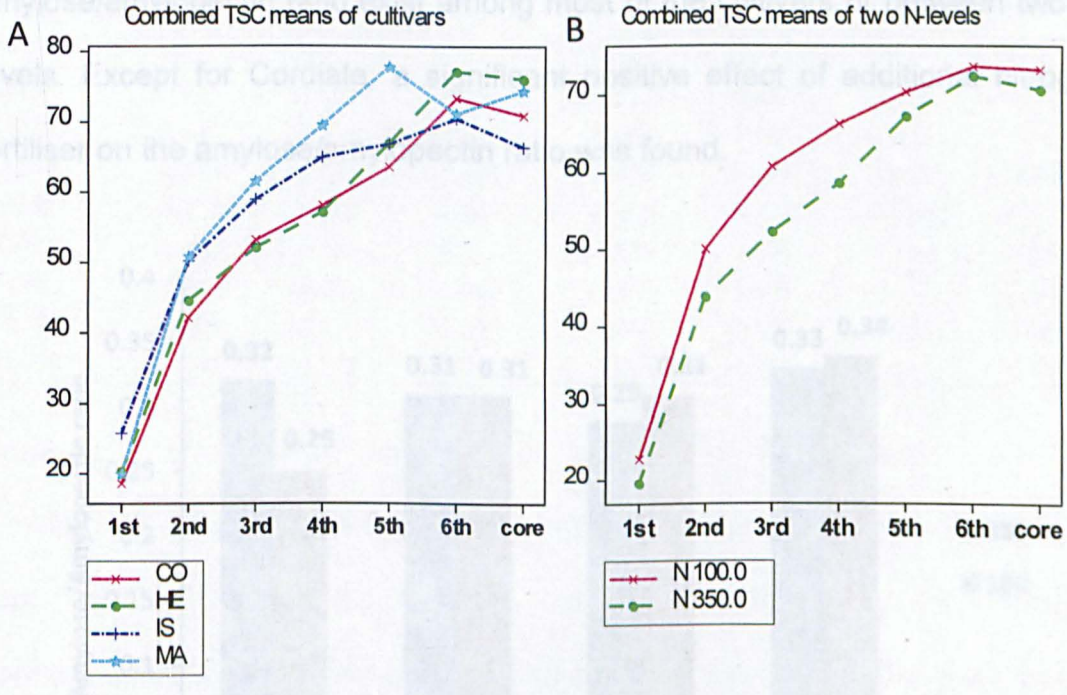

Figure 5.2 Impacts of genotype (A) and N fertilization levels (B) on total starch content (% in flour weight): A), integrated data for two N levels to show effects of cultivars; B), integrated data of four cultivars to show effects of N-level

5.2 Amylose/amylopectin ratio

The statistical design of the experiment was similar to the TSC experiment, except that no technical replicates were included. In total, 128 samples were tested including one missing value.

5.2.1 Results of amylose/amylopectin ratio of whole grains

The data set for whole grains was not tested by ANOVA. Therefore, the differences discussed below showed the tendency most likely to occur in a fully replicated ANOVA test. Figure 5.3 shows that no obvious differences in the amylose/amylopectin ratio exist among most of the cultivars or between two N-levels. Except for Cordiale, a significant positive effect of additional nitrogen fertiliser on the amylose/amylopectin ratio was found.

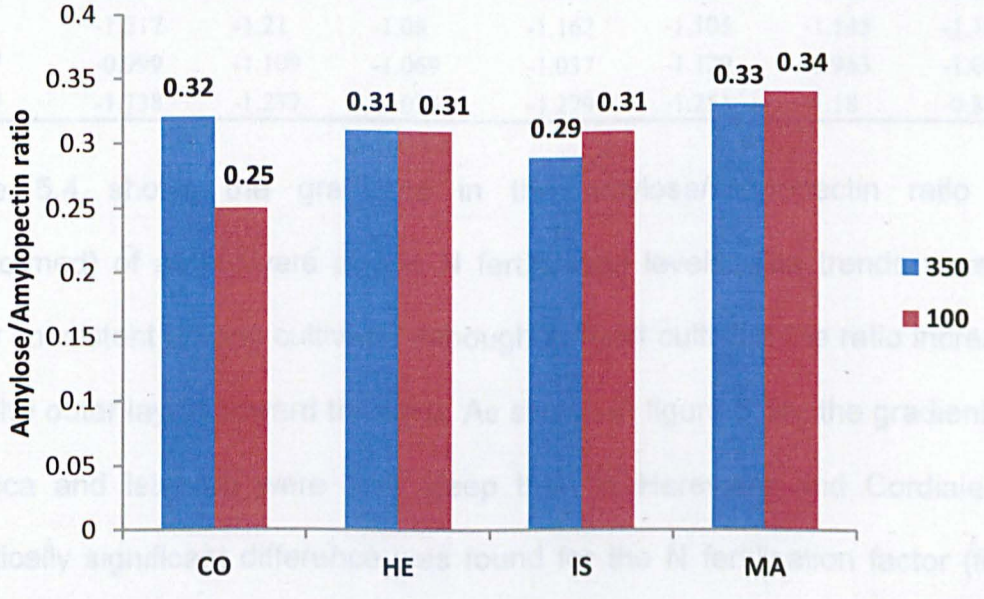

Figure 5.3 Amylose/amylopectin ratios of whole grains

5.2.2 Gradients in amylose/amylopectin ratio across the grain

The amylose/amylopectin ratios in the pearling fractions were analysed with ANOVA using highest order interaction (cultivar×N-level×fraction) as residual term; all main factors, but not interactions, were tested. Data were log transformed to achieve a normal distribution. See table 5.3 for test results and means summary.

Table 5.3 Amylose/amylopectin ratio ANOVA results and means summary

A: Influential factors

Factors	P value	l.s.d.	d.f.
Cultivar	<0.001	0.0851	73
N-fertilisation	0.601	------	---
Fraction	<0.001	0.1126	73

B: Means of log (amylose/amylopectin ratio)

	1st	2nd	3rd	4th	5th	6th	core
CO100	-2.12	-1.736	-1.436	-1.237	-0.883	-1.408	-1.26
CO350	-2.447	-1.999	-1.362	-1.367	-1.189	-1.233	-1.105
HE100	-2.064	-1.572	-1.407	-1.425	-1.139	-1.072	-1.14
HE350	-1.727	-1.502	-1.467	-1.138	-1.031	-0.926	-1.043
IS100	-0.763	-1.335	-1.257	-1.339	-1.206	-1.109	-1.081
IS350	-1.217	-1.21	-1.08	-1.162	-1.105	-1.145	-1.142
MA100	-0.999	-1.109	-1.069	-1.037	-1.129	-0.963	-1.008
MA350	-1.738	-1.277	-1.029	-1.279	-1.251	-1.18	-0.838

Figure 5.4 shows the gradients in the amylose/amylopectin ratio (log transformed) of all cultivars at two N fertilisation levels. The trends were not totally consistent among cultivars, although in most cultivars the ratio increased from the outer layers toward the core. As shown in figure 5.5A, the gradients for Malacca and Istabraq were less steep than in Hereward and Cordiale. No statistically significant difference was found for the N fertilisation factor (figure 5.5B). Figures 5.4 and 5.5 also show that the changes in the amylose/amylopectin ratio (log transformed) were not as stable as those observed for TSC. Since the analytical method used includes multiple

enzymatic reactions which were extremely sensitive to reaction times and environmental factors such as temperature, it is likely that part of this variability is due to experimental error The large variability in the results observed when the same type of analysis was carried out at Campden BRI on several technical replicates of the same pearling fraction of Hereward supported this suggestion. On the other hand, the large differences in TSC between pearling fractions (from 17% to 77% of flour weight) could also affect the determination of the amylose/amylopectin ratio.

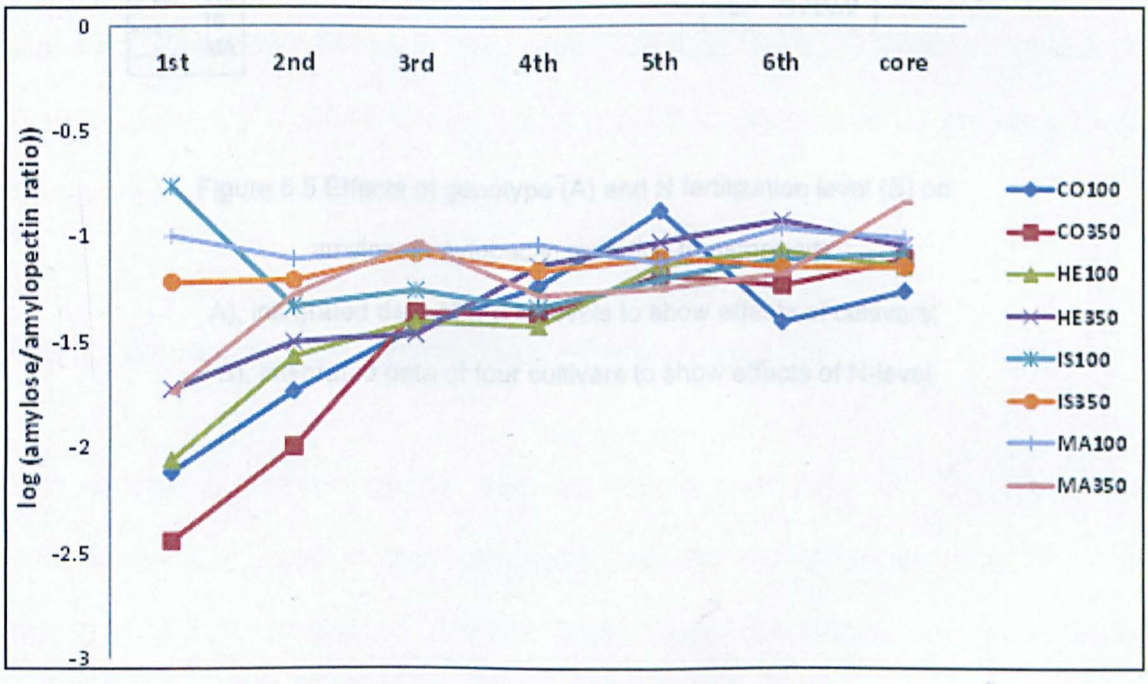

Figure 5.4 Gradients in amylose/amylopectin ratio (log transformed) across the grain

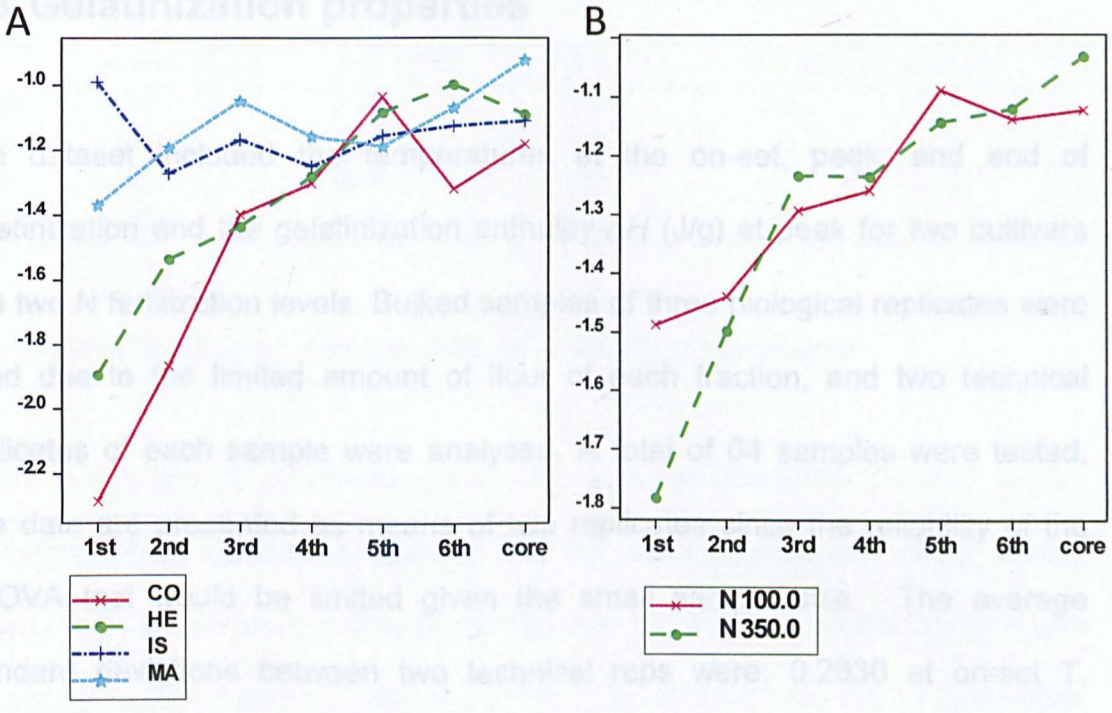

Figure 5.5 Effects of genotype (A) and N fertilization level (B) on amylose/amylopectin ratio (log transformed):

A), integrated data of two N-levels to show effects of cultivars;

B), integrated data of four cultivars to show effects of N-level

5.3 Gelatinization properties

The dataset included the temperatures at the on-set, peak, and end of gelatinization and the gelatinization enthalpy-ΔH (J/g) at peak for two cultivars and two N fertilization levels. Bulked samples of three biological replicates were used due to the limited amount of flour of each fraction, and two technical replicates of each sample were analysed. A total of 64 samples were tested. The data are presented as means of two replicates since the reliability of the ANOVA test would be limited given the small sample size. The average standard deviations between two technical reps were: 0.2630 at on-set T, 0.1989 at peak T, 0.2541 at end T and 0.0409 for gelatinization enthalpy (ΔH). See appendix 5 for the full dataset.

5.3.1 Gelatinization properties of whole grains

The dataset for whole grains was not tested with ANOVA. Therefore, the differences discussed in this paragraph showed the tendency most likely to occur in a fully replicated ANOVA test. Table 5.4 shows the parameters describing the gelatinization properties of whole grain flour. The temperatures at on-set, peak and end of gelatinisation are very similar in the HE350, IS350 and IS100 samples while in HE100 the temperatures are approximately three degrees higher. The gelatinization enthalpies varied among the tested lines with IS100 having the highest ΔH, closely followed by IS350. The Hereward samples had lower level of ΔH than the Istabraq samples, with HE350 had higher ΔH than HE100.

Table 5.4 Gelatinization properties of whole grains

	on-set T (°C)	peak T (°C)	end T (°C)	ΔH (J/g) (Dry based)
HE350	59.95	65.35	70.95	1.30
HE100	63.45	68.50	74.45	1.08
IS350	59.85	65.70	71.25	1.51
IS100	60.20	65.90	71.40	1.64

5.3.2 Gelatinization properties of pearling fractions

Figure 5.6 shows that on-set, peak and end temperatures showed similar gradients. Most showed a peak in the 2nd fraction, followed by a steady decrease in subsequent fractions. IS100 had the highest on-set temperature compared to the other lines, with the 2nd fraction of IS100 having a higher peak temperature compared to the same fractions of the other lines. Figure 5.6D shows differences in enthalpy-ΔH both between cultivars and N levels. Istabraq showed higher values for ΔH than Hereward with differences in the shapes of the gradients in the two lines. While the values for the 1st and the core fractions are quite similar in the two cultivars at both N levels, the same is not true for the fractions 2nd to 6th with the differences between N levels within the same cultivar being smaller than the differences between cultivars. Istabraq at N100 level had higher levels of ΔH than IS350 except for the1st fraction. However, an effect of N level effect was not apparent for Hereward.

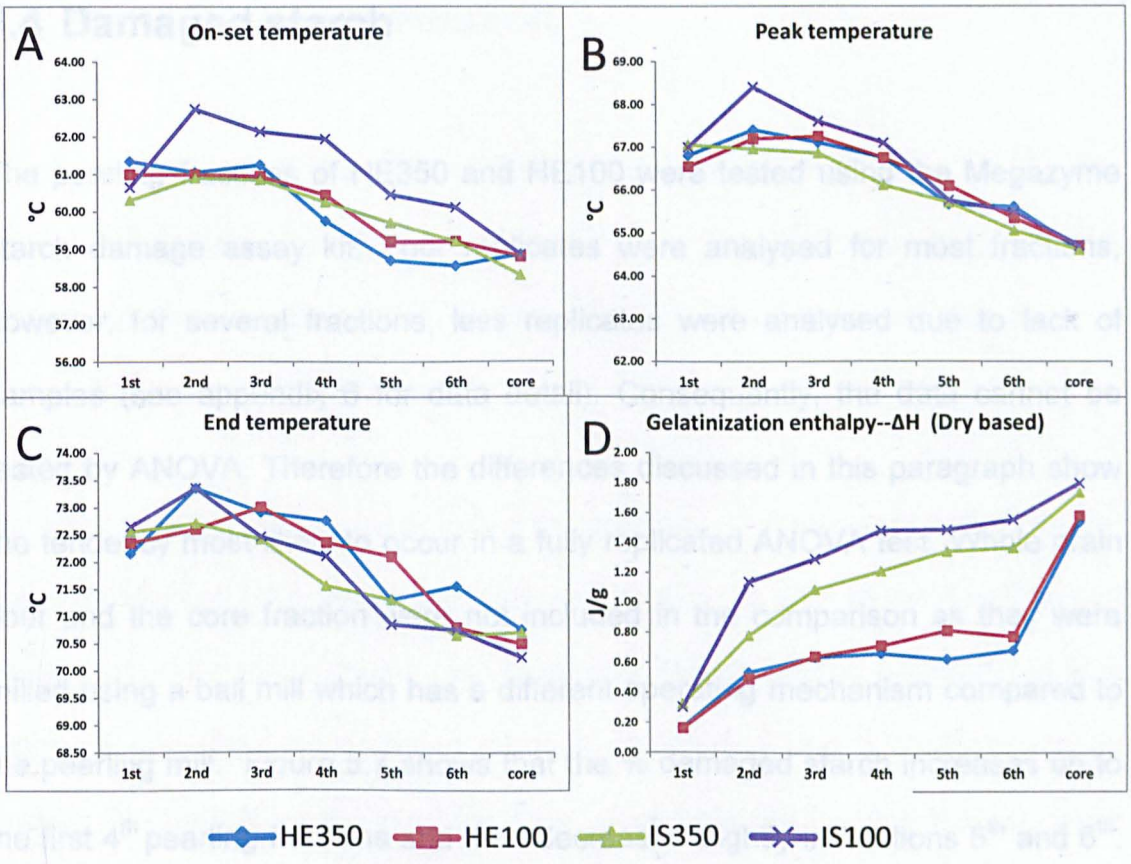

Figure 5.6 Gelatinization properties (DSC parameters) of pearling fractions of Hereward and Istabraq at 350 and 100 kg/ha nitrogen fertilisation levels. A) On-set temperature (beginning of gelatinization); B) Peak temperature (stage at which the endothermic reaction reaches the peak); C) End temperature (stage at which starch granules are completely gelatinized); D) gelatinization enthalpy-ΔH (J/g) (energy absorbed during gelatinization process).

5.4 Damaged starch

The pearling fractions of HE350 and HE100 were tested using the Megazyme starch damage assay kit. Four replicates were analysed for most fractions, however, for several fractions, less replicates were analysed due to lack of samples (see appendix 6 for data detail). Consequently, the data cannot be tested by ANOVA. Therefore the differences discussed in this paragraph show the tendency most likely to occur in a fully replicated ANOVA test. Whole grain flour and the core fraction were not included in the comparison as they were milled using a ball mill which has a different operating mechanism compared to the pearling mill. Figure 5.7 shows that the % damaged starch increases up to the first 4th pearling fractions and then decreases slightly in fractions 5th and 6th. For all fractions, except fraction 5, additional nitrogen fertiliser seemed to have a negative effect on damaged starch rate. However, the differences may not be significant considering the large standard deviations observed in the dataset.

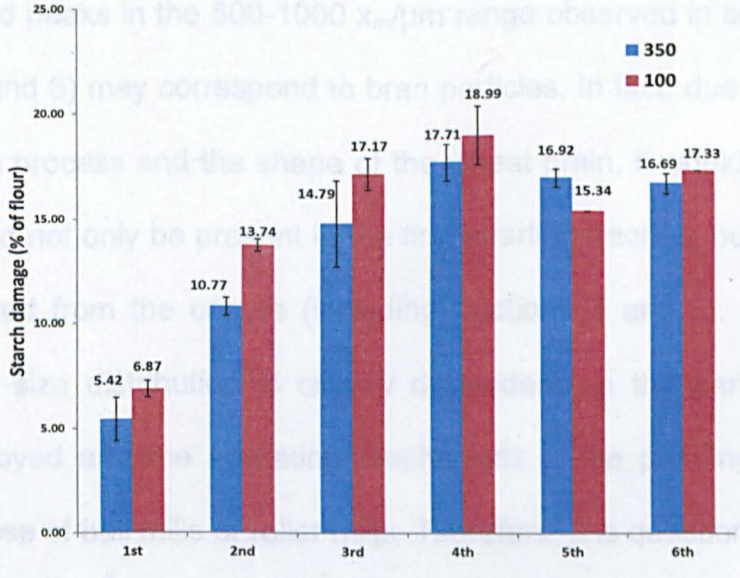

Figure 5.7 % damaged starch (of flour) of pearling fractions of Hereward at 350 and 100kg/ha nitrogen fertilisation levels, error bars show the standard deviations.

5.5 Particle size distribution

The particle size distributions of pearling fractions of HE350 and HE100 are shown in figure 5.8. See appendix 7 for full data. The 1st fraction of N 350 was lost during test due to unknown system problem. The differences discussed in this section described the tendency likely to occur in a fully replicated test.

For most pearling fractions (excluding the core and 1st fraction) at both N levels, approximately 40-50% of the particles were within the size range 40-70 x_m/μm (measured in Feret minimum--- x_m) (appendix 7) with second peaks in the size range of 800-1000 x_m/μm being found in fractions 1 and 5 at N100 and in fraction 6 at both N levels. No other differences between the two N levels were found. Noort and colleagues (2010) reported that the median particle size (measured in diameter) of the bran fractions obtained by traditional milling were 831.1μm with some particles being larger than 2000 μm (Noort et al., 2010). Similar results were obtained by (Antoine et al., 2004). It is therefore possible that the second peaks in the 800-1000 x_m/μm range observed in some fractions (fraction 1, 5 and 6) may correspond to bran particles. In fact, due to the nature of the pearling process and the shape of the wheat grain, it would be expected that bran would not only be present in the first pearling fraction, but also in later fractions derived from the crease (including fractions 5 and 6). On the other hand, particle size distribution is closely dependent on the particular milling process employed and the operating mechanism of the pearling mill is very different to those of ball mills or roller mills. Therefore, it is questionable whether results from other milling studies can used to explain the occurrence of this second peak. However, fractions 5 and 6 mostly consist of material from the

centre of endosperm and are relatively pure compared to other pearling fractions (see figure 4.21B). Therefore, the second peaks occurring in these probably had other origins. One possibility is that some of the cores may have been broken and escaped the sieve during later stages of pearling, and therefore contaminated some of the 5^{th} and 6^{th} fractions.

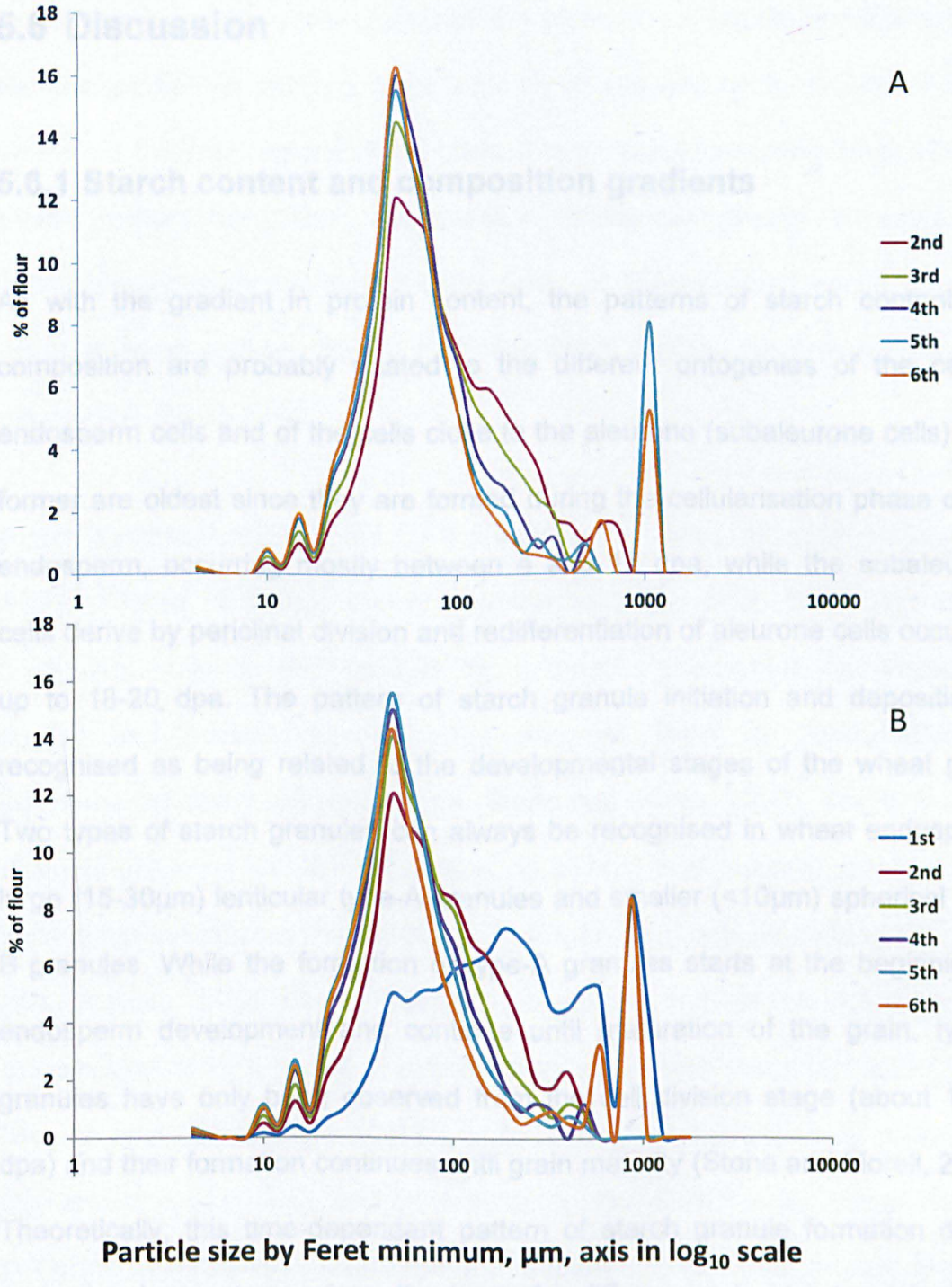

Figure 5.8 Flour particle size distributions of Hereward pearling fractions at N350 level (A) and N100 level (B). X axis values are expressed on a 10 logarithmic base scale. Whole grain flour and core fraction were not included as they were milled by ball mill which has a different operating mechanism compared to the pearling mill

5.6 Discussion

5.6.1 Starch content and composition gradients

As with the gradient in protein content, the patterns of starch content and composition are probably related to the different ontogenies of the central endosperm cells and of the cells close to the aleurone (subaleurone cells). The former are oldest since they are formed during the cellularisation phase of the endosperm, occurring mostly between 4 and 12 dpa, while the subaleurone cells derive by periclinal division and redifferentiation of aleurone cells occurring up to 18-20 dpa. The pattern of starch granule initiation and deposition is recognised as being related to the developmental stages of the wheat grain. Two types of starch granules can always be recognised in wheat endosperm: large (15-30μm) lenticular type-A granules and smaller (<10μm) spherical type-B granules. While the formation of type-A granules starts at the beginning of endosperm development and continue until maturation of the grain, type-B granules have only been observed from the cell division stage (about 10-12 dpa) and their formation continues until grain maturity (Stone and Morell, 2009). Theoretically, this time-dependant pattern of starch granule formation during grain development may form the basis for differences in starch content and particle size distribution in wheat endosperm: the older cells from the central endosperm would start accumulating starch earlier, and therefore for longer, than the subaleurone cells (figure 5.1). On the another hand, other studies have showed that starch is evenly distributed in the dorsal area of the wheat grain (Ugalde and Jenner, 1990a). However, the results of Ugalde and Jenner (1990) are not necessarily inconsistent with our results since the pearling fractions are

representative of the whole grain, not only of the dorsal region, and it is possible that the gradient in starch content is mainly determined by the pattern of starch content in the lobe regions of the grain. It is tempting to explain the gradient of starch content using this "accumulation differences" theory. However, this simple theory cannot explain the gradients of amylose/amylopectin ratio that occur in some cultivars (figure 5.4), nor can it explain the gradients in DSC parameters (figure 5.6).

Starch synthesis is regulated by the action of a series of enzymes including ADPG PPase, GBSSIa, starch synthases (SSI, SSII, SSIII and SSIV), branching enzymes (BEI and BEII), debranching enzymes (isoamylase in cereal) and possibly several other enzymes including disproportionating enzymes and starch phosphorylase (Stone and Morell, 2009). The expression of these enzymes and there activities could lead to differential starch content and/or starch structure. For example, The branching enzymes cut α-1,4 bonds and promote α-1,6 bonds therefore increasing the degree of branching. Suppression of BEII genes in wheat led to a high-amylose phenotype (>70%) (Regina et al., 2006). The GBSSIa gene is expressed solely in the endosperm (Nakamura et al., 1998), and by monitoring amylose content changes through the grain development (Morrison and Gadan, 1987) it was showed that it is expressed at relatively low level in the early stages of development to then increase from the end of the cell-division phase to maturation. The kinetics of expression of SSI and SSII has also been studied. In wheat, SSI starts to be expressed from 6 d.p.a., peaks at 8 d.p.a, remain high until 18 d.p.a, and then declines (Li et al., 1999). However, the gene control of starch synthesis is very complex and subject to regulation at multiple levels (Stone and Morell, 2009). Detailed analysis of substrate and enzyme activities during development would

be required to relate this knowledge to the effect on starch quantity and composition described here. This information could also benefit breeding programs aiming for cultivars having specific starch characteristics for producing special products such as Asian noodles.

5.6.2 Effects of nitrogen and other environmental factors

The synthesis of starch in wheat is significantly affected by environment factors, especially temperature. Temperature affects starch synthesis because it influences the development of the plant and grain in general (Wardlaw, 1994) and because more specifically it affects enzyme activity (Keeling et al., 1993). A study showed that the rate of starch deposition was slower at higher temperature, due to reduced activity of soluble starch synthase (Jenner, 1994). Heat stress may also suppress the synthesis of B-granules (Shi et al., 1994) and increase the proportions of A-granules and amylose (Panozzo and Eagles, 1998). In this study, the major differential environmental factor is N fertilisation level. Although nitrogen is not a direct substrate for starch synthesis, nitrogen supply does nonetheless influence starch synthesis by influencing plant and grain development, leaf photosynthesis, and the synthesis of enzymes required for starch synthesis. Many studies have demonstrated that nitrogen fertiliser has many effects on wheat leaf photosynthesis by influencing chlorophyll content (Evans, 1983), rubisco content (Makino, 2011, Lawlor et al., 1989), and eventually sugar synthesis (Tranaviciene et al., 2007). Nitrogen deficiency has been reported to limit the activity of starch biosynthetic enzymes in maize and rice (Thitisaksakul et al., 2012, Dian et al., 2003). My study found a negative effect of N on total % starch. However, it is not clear whether this negative effect

was caused by biological changes due to increased nitrogen fertiliser or to increased protein content under high N fertilisation. Although no significant effect of N level on amylose/amylopectin ratio was found, we did observe changes in the gelatinization properties of starch in Istabraq (a low protein high starch cultivar) associated with N fertilisation (figure 5.6), which suggested that N fertilisation changes could lead to changes in starch structure and functionality.

5.6.3 Starch content and composition in relation to processing and utilization

Amylose/amylopectin ratio

The chemical structure and functionality of amylose and amylopectin are well studied. The unique properties of amylose such as gelation and ability to bind hydrophobic molecules are at the basis of its very wide usage in the food industry and beyond as thickener, gelling agent and binder (Young, 1984). On the other hand, the use of waxy wheats (reduced amylose wheat) is very limited due to their poor agronomic performance (Graybosch, 1998). However, there are many potential uses of waxy wheat flour if blended with other flours as they have increased water absorption, higher starch damage rate (Bettge et al., 2000), reduced crumb firmness (Lee et al., 2001) and an extended shelf life (Hayakawa et al., 2004). For bread making, the most significant impact of the amylose/amylopectin ratio is on bread staling caused by starch retrogradation. The linear molecular structure of amylose undergoes retrogradation much faster than amylopectin (BeMiller and Whistler, 1996), therefore starch with a

low amylose content may have a longer shelf life. My results showed there was a clear gradient in the amylose/amylopectin ratio across the grain in Cordiale and Hereward, but not in Malacca and Istabraq. This information could potentially benefit breeding programs aimed at producing novel cultivars for specific uses.

Gelatinization properties and DSC

Starch gelatinization is a central event in food processing since moisture and heat are processing conditions commonly used in the food industry, although some food systems use relatively low moisture levels, so that most starch granules remain ungelatinized (for example, in making biscuits (BeMiller and Whistler, 1996)). In conventionally baked cakes, which are relatively high in water content, the starch gelatinization rate can range from 85% to 93% (Sakiyan et al., 2011), while in bread making, the starch is partly gelatinized during baking and the degree of gelatinization is largely depending on the moisture content and baking temperature (Yasunaga et al., 1968). Gelatinization is important as it affects many aspects of food processing and final food quality, such as loss of viscosity after the starch granules collapse, storage behaviour, and digestibility (BeMiller and Whistler, 1996). In bread making, gelatinization is importance mainly because it influences the baking time (Mondal and Datta, 2008), crumb texture (Mondal and Datta, 2008, Purlis, 2011) and staling (Yasunaga et al., 1968). The gelatinization properties are influenced by many aspects of the flour including the amylose/amylopectin ratio (Lin et al., 2008, Jane et al., 1999), amylopectin structure (Jane et al., 1999, Fredriksson et al., 1998, Kohyama et al., 2004, Kuakpetoon and Wang, 2007), interaction with lipids (Eliasson, 1994), protein/gluten content (Mohamed and

Rayas-Duarte, 2003), and starch granule size (Vermeylen et al., 2005). Without knowing all of the above properties for each fraction and how much effect they have on the gelatinization properties, it is difficult to speculate on the reasons for the gradients that were observed for DSC parameters. Regardless of this, it is important to be aware of the existence of different gelatinization properties among fractions from different part of the endosperm since these properties will affect the industrial use of flour: gelatinization enthalpy is related to processing energy input while the onset/end temperatures are related to the temperature and timing setups for food processing.

Starch damage and particle size distribution

Damaged starch can influence food processing in many ways. Damaged starch absorbs more water than native starch, and can be more easily digested by α-amylase. In bread making, it is essential to have a certain amount of damaged starch to provide sugar to support yeast activity. However, too much damaged starch can cause problems such as low loaf volume, sticky crumb and collapsed loaves (Bhandari, 2009). Starch damage is commonly recognised as a consequence of milling and the rate of damaged starch can usually be controlled at different optimum ranges depending on the product by adjusting mill parameters such as roller pressure. The typical percentage of damaged starch for bread flour is 6-11% (S. Penson, 2012, personal communication). During milling, when fractures occur through endosperm cell, starch granules break to release starch fragments and protein bodies attached to the granule surface (Barlow et al., 1973, Shewry and Morell, 2001). Milling of hard wheat tends to produces more damaged starch comparing to milling of soft wheat because of the high adhesion between starch and protein in its endosperm cells

(Barlow et al., 1973). I found a gradient of in % damaged starch in flour (figure 5.7). However, when the total starch contents were taken into account, the proportion of damaged starch in total starch did not show clear gradient across the grain (figure 5.9). The ratio of damaged/undamaged starch largely depends on the milling process. While the pearling mill uses friction force to separate the different layers of the grain, roller and ball mill use pressure to first crush the grain and then grind the fragments into flour. This may be why the pearling fractions of Hereward had higher percentages of damaged starch (approx.5-19%) than typical bread flour (6-11%).

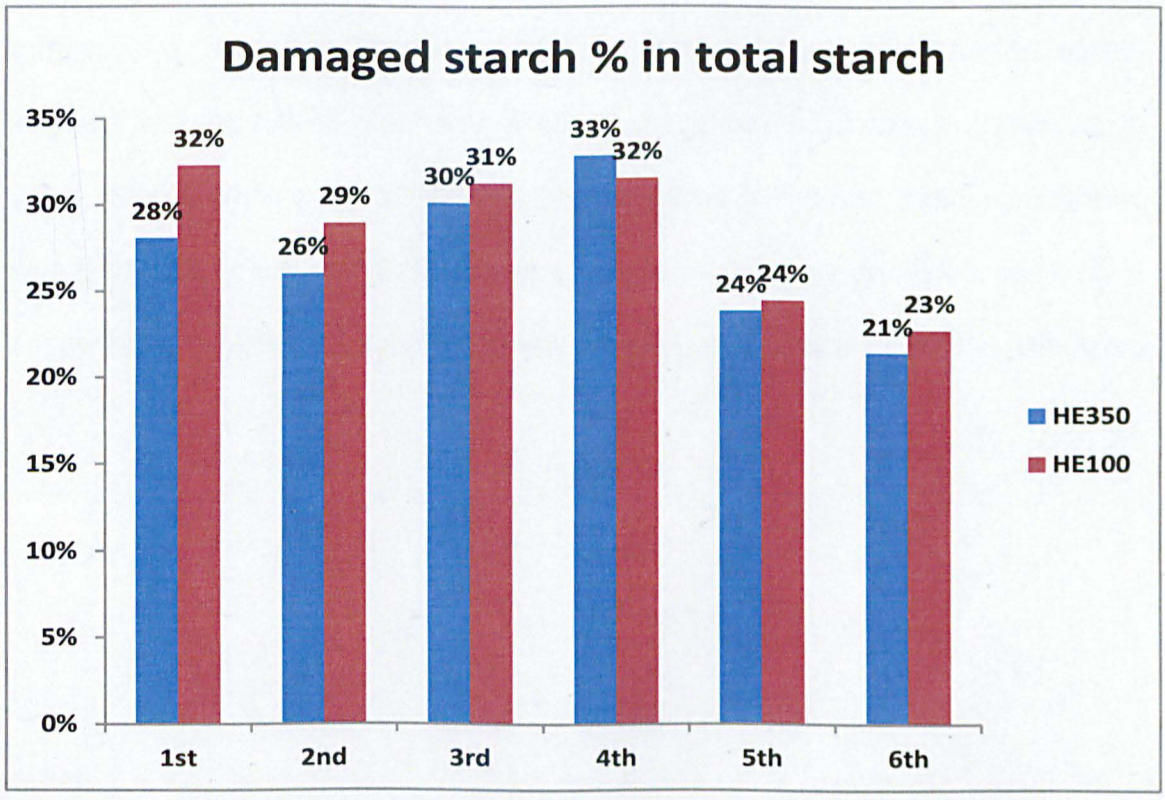

Figure 5.9 Proportion of damaged starch in total starch of Hereward pearling fractions.

The particle size distribution of flour is known to influence properties related to processing such as water absorption, solubility, and nutritional value such as

digestion rate (Al-Rabadi et al., 2009, Al-Rabadi et al., 2012). Particle size distribution is significantly related to the hardness class of wheat, with soft wheats showing a first peak at around 20 μm and a second peak at around 110 μm, while hard wheats have one main peak around 120 μm (Devaux et al., 1998). This does not agreed with our results, probably because particle size distribution is also determined by the milling method and milling parameters, which are quite different for pearling and roller mills.

5.7 Conclusions

My results shows that the total amount of starch in the endosperm of wheat is influenced by the genotype and N fertilisation and that starch is not homogeneously distributed within the endosperm, increasing from the outer layers toward the central endosperm. I also found a gradual increase in the ratio of amylose/amylopectin towards the centre of the endosperm in Hereward and Cordiale but not in Malacca and Istabraq. The causes of these gradients remain unclear. DSC showed significant gradients in gelatinization properties among the pearling fractions, including in onset temperature, peak temperature, end temperature, and gelatinization enthalpy. Determination of damaged starch showed that the pearling process produce more damaged starch compared to roller milling, while particle size analysis showed that most pearling fractions had particles in the 40-70 x_m/µm size range, with some fractions showing a second peak at 800-1000 x_m/µm, which may be due to contamination with bran.

Chapter 6: Future work and final conclusion

There are several aspects from this work would be interesting to investigate further. Firstly, due to the limitations of the pearling mill, only 50% of the grain can be pearled into pearling fractions. Therefore, the gradients of protein and starch within the inner 50% of the grain (the core) were not determined. With some modification of the pearling mill such as fitting a finer sieve and adding a speed control, it may be possible to fractionate the endosperm material closer to the crease. Secondly, in the analysis of starch, the biological samples had to be bulked to provide efficient samples for multiple tests. Therefore, the interactions of factors could not be tested. Microscopy could also be used to directly determine the spatial distribution of starch granules and the A/B-granule ratio. Pearling has the potential to allow the fractionation and reconstitution of wheat grain to produce flour with specifications required for a particular product. However, ultimately the only valid test for flour quality is by processing, making either bread or other products. It would therefore be interesting to carry out baking tests with the pearling fractions, milling fractions and recombined pearling fractions.

Overall, this project was successful as all of the research objectives were met, revealing gradients in protein content across the grain as well differential patterns of gluten subunit composition and molecular size distribution. Visual observation of spatial patterns of different gluten protein groups was achieved by microscopy. The effects of genotype and N-fertilisation were also demonstrated.

The study also showed gradients in starch content and in the ratio of amylose/amylopectin in some cultivars. Pearling may lead to greater starch damage and DSC showed that the gelatinization properties of flours may also differ depending on their origin.

Several side projects had also been carried out with colleagues at Rothamsted to determine gradients in other chemical components of wheat grain such as lipids, cell wall polysaccharides and minerals. The data generated from these projects are very promising and will contribute to a comprehensive understanding of the spatial distributions of chemical compositions in wheat.

Appendix

Appendix 1 SE-HPLC data of pearling fractions

Variety	N	Fractions	F1	F2	F3	F4	F5	AT
HE	100	1st	4.94	12.57	9.14	28.97	44.38	23.42
HE	100	2nd	8.48	17.58	9.18	30.53	34.23	29.89
HE	100	3rd	9.65	18.32	10.19	31.75	30.09	28.39
HE	100	4th	10.26	20.87	8.83	32.55	27.49	25.44
HE	100	5th	11.48	22.17	8.44	32.71	25.19	22.54
HE	100	6th	11.98	23.33	7.83	32.94	23.92	20.47
HE	100	Core	12.57	23.91	7.49	33.24	22.80	17.16
HE	100	whole	10.74	22.41	7.72	32.98	26.15	19.72
HE	350	1st	6.36	15.10	10.76	34.05	33.73	31.66
HE	350	2nd	9.48	18.44	11.34	36.14	24.60	44.92
HE	350	3rd	9.74	19.54	10.97	37.10	22.66	44.60
HE	350	4th	10.63	20.94	10.58	37.66	20.19	41.20
HE	350	5th	12.04	22.68	9.57	37.45	18.26	35.38
HE	350	6th	12.02	23.33	9.09	38.11	17.44	30.33
HE	350	Core	12.75	23.90	8.48	37.55	17.33	23.33
HE	350	whole	11.96	22.78	8.77	37.30	19.19	29.02
IS	100	1st	5.45	12.81	9.53	31.56	40.64	19.17
IS	100	2nd	7.64	16.59	9.24	34.61	31.92	23.75
IS	100	3rd	8.36	16.83	10.09	35.37	29.34	22.62
IS	100	4th	8.61	17.70	9.85	35.65	28.19	21.35
IS	100	5th	8.97	18.18	9.73	36.11	27.01	20.58
IS	100	6th	9.25	18.56	9.54	36.31	26.35	19.22
IS	100	Core	10.60	20.33	8.45	36.62	24.00	16.84
IS	100	whole	9.30	18.99	9.00	35.66	27.04	18.79
IS	350	1st	5.97	14.19	11.11	35.22	33.51	29.38
IS	350	2nd	8.18	16.90	11.62	37.89	25.42	34.54
IS	350	3rd	8.72	18.02	10.99	38.83	23.44	32.38
IS	350	4th	9.33	18.85	10.54	39.15	22.13	29.53
IS	350	5th	10.02	19.34	10.27	39.29	21.08	27.20
IS	350	6th	10.36	20.01	9.88	39.41	20.33	24.88
IS	350	Core	11.26	20.85	9.03	39.62	19.24	21.60

IS	350	whole	9.63	19.80	9.79	39.60	21.19	24.67
CO	100	1st	5.55	12.24	9.69	29.95	42.57	23.50
CO	100	2nd	8.85	16.37	9.38	32.60	32.81	28.37
CO	100	3rd	9.95	17.64	9.36	33.22	29.84	27.55
CO	100	4th	10.82	18.18	9.48	33.91	27.60	24.60
CO	100	5th	11.58	19.89	8.60	34.39	25.54	21.94
CO	100	6th	12.14	20.33	8.45	34.83	24.26	19.73
CO	100	Core	12.61	21.58	7.54	35.51	22.76	17.34
CO	100	whole	11.87	19.88	8.12	33.95	26.18	20.30
CO	350	1st	6.27	12.54	11.50	34.00	35.69	31.35
CO	350	2nd	9.53	15.86	11.67	36.91	26.03	40.81
CO	350	3rd	10.35	17.19	11.04	37.77	23.63	39.81
CO	350	4th	11.18	19.06	10.05	38.32	21.39	36.87
CO	350	5th	11.51	19.91	9.47	39.38	19.74	32.67
CO	350	6th	12.19	20.63	8.90	39.90	18.38	28.83
CO	350	Core	12.46	21.20	8.26	40.70	17.38	23.46
CO	350	whole	12.14	20.26	8.49	39.66	19.45	25.85
MA	100	1st	4.67	10.03	10.63	28.28	46.39	20.23
MA	100	2nd	7.83	14.85	11.03	30.86	35.43	25.48
MA	100	3rd	8.98	17.83	10.10	31.83	31.26	25.03
MA	100	4th	10.06	19.42	9.32	31.94	29.26	23.24
MA	100	5th	11.13	20.61	8.66	31.94	27.66	20.51
MA	100	6th	11.70	21.73	8.13	32.01	26.44	17.98
MA	100	Core	12.38	22.47	7.59	32.76	24.80	14.72
MA	100	whole	10.84	21.21	7.97	32.18	27.81	17.21
MA	350	1st	6.35	14.03	11.42	32.81	35.39	29.57
MA	350	2nd	9.58	18.53	10.91	35.46	25.52	40.40
MA	350	3rd	10.26	20.09	10.51	36.31	22.84	38.73
MA	350	4th	10.71	21.50	10.20	36.66	20.92	35.34
MA	350	5th	11.65	22.15	9.70	37.30	19.20	30.95
MA	350	6th	12.09	22.84	9.27	37.18	18.63	26.81
MA	350	Core	12.46	23.27	8.55	37.55	18.17	21.07
MA	350	whole	12.0	22.8	8.8	36.7	19.7	26.1

Appendix 2 SE-HPLC data of milling fractions and graphic summaries

Table 1, SE-HPLC data of milling fractions

Variety	N	Fractions	F1	F2	F3	F4	F5	AT
HE	100	B1	14.38	25.51	6.87	33.02	20.22	15.33
HE	100	B2	15.28	26.42	6.69	33.25	18.37	19.11
HE	100	B3	14.73	26.12	7.03	33.43	18.68	21.00
HE	100	R1	14.23	25.55	6.99	33.69	19.53	18.09
HE	100	R2	12.41	23.24	8.05	34.20	22.10	20.15
HE	100	R3	11.73	21.72	8.79	33.84	23.92	21.39
HE	100	Bran flour	11.78	22.06	8.87	33.39	23.90	25.16
HE	100	Offal flour	9.27	19.12	9.48	33.63	28.50	26.83
HE	350	B1	14.42	25.48	8.04	38.08	13.99	27.56
HE	350	B2	14.95	25.69	8.28	38.60	12.48	36.81
HE	350	B3	14.59	25.34	8.49	39.03	12.54	42.35
HE	350	R1	13.93	25.38	8.29	38.15	14.26	26.31
HE	350	R2	13.36	24.98	8.52	38.42	14.72	27.72
HE	350	R3	12.66	24.13	8.85	38.55	15.81	29.05
HE	350	Bran flour	12.20	23.08	9.75	38.72	16.25	51.63
HE	350	Offal flour	10.59	20.39	10.17	38.13	20.72	40.27
IS	100	B1	12.09	22.84	7.64	35.87	21.56	13.20
IS	100	B2	11.64	23.65	7.54	36.79	20.37	15.06
IS	100	B3	11.09	23.46	8.13	37.01	20.31	17.23
IS	100	R1	11.24	22.83	8.06	36.71	21.17	16.01
IS	100	R2	10.61	21.13	8.78	36.49	23.00	17.09
IS	100	R3	9.90	20.91	9.08	35.92	24.19	17.45
IS	100	Bran flour	9.57	21.66	9.23	36.60	22.93	20.51
IS	100	Offal flour	8.42	17.18	11.37	35.51	27.51	22.31
IS	350	B1	11.80	22.74	8.24	39.48	17.74	16.02
IS	350	B2	11.67	23.40	8.69	41.10	15.13	22.86
IS	350	B3	11.42	22.90	9.07	41.57	15.04	26.29
IS	350	R1	11.33	22.61	8.95	41.03	16.08	22.79
IS	350	R2	10.79	21.40	9.32	40.89	17.61	23.94
IS	350	R3	10.15	20.33	9.70	40.23	19.60	23.99
IS	350	Bran flour	9.60	20.05	10.56	40.81	18.97	33.14
IS	350	Offal flour	8.54	17.12	11.49	39.02	23.84	30.34

CO	100	B1	14.27	23.32	6.80	35.35	20.26	16.16
CO	100	B2	14.65	23.69	6.60	35.58	19.48	18.86
CO	100	B3	14.25	23.48	7.00	35.63	19.64	20.54
CO	100	R1	13.76	22.99	7.12	35.58	20.55	18.23
CO	100	R2	12.14	21.26	8.06	35.68	22.86	20.06
CO	100	R3	11.70	20.44	8.54	35.28	24.03	21.25
CO	100	Bran flour	11.47	21.10	8.75	35.14	23.54	25.27
CO	100	Offal flour	9.25	16.93	11.05	33.88	28.89	27.51
CO	350	B1	14.97	23.11	6.92	40.30	14.70	25.67
CO	350	B2	15.40	23.33	6.88	40.93	13.47	31.31
CO	350	B3	14.80	23.21	7.34	41.00	13.64	34.31
CO	350	R1	14.46	22.70	7.25	40.77	14.81	25.81
CO	350	R2	13.80	22.17	7.69	40.75	15.60	26.27
CO	350	R3	13.35	21.68	8.07	40.36	16.53	27.08
CO	350	Bran flour	13.07	21.70	8.66	40.51	16.06	41.29
CO	350	Offal flour	11.34	19.11	9.72	39.38	20.45	35.80
MA	100	B1	14.23	24.63	7.13	33.02	20.99	14.48
MA	100	B2	14.26	25.71	7.01	33.40	19.62	17.19
MA	100	B3	13.90	25.28	7.21	33.71	19.90	18.83
MA	100	R1	13.52	24.51	7.50	33.62	20.85	17.05
MA	100	R2	11.83	22.79	8.27	34.09	23.03	18.83
MA	100	R3	11.06	21.11	8.91	34.32	24.60	20.59
MA	100	Bran flour	10.63	22.33	8.86	33.75	24.43	22.72
MA	100	Offal flour	8.44	18.39	10.26	33.77	29.15	25.43
MA	350	B1	13.81	25.49	7.76	37.00	15.94	22.30
MA	350	B2	14.58	26.30	7.72	37.40	14.00	29.48
MA	350	B3	14.05	26.03	8.09	37.73	14.09	33.83
MA	350	R1	13.78	25.39	8.02	37.37	15.44	24.38
MA	350	R2	12.94	24.81	8.39	37.69	16.18	25.43
MA	350	R3	12.23	23.80	8.75	37.91	17.31	27.09
MA	350	Bran flour	11.51	23.66	9.56	38.06	17.20	43.91
MA	350	Offal flour	10.52	21.06	9.28	37.67	21.46	36.31

Appendix 3 Milling fraction production information

Table 1, Milling fraction production

Weight of flour (g)	Feed	B1	B2	B3	R1	R2	R3	Bran	Offal	Total product
CO100	2815	265	223	66	1322	159	36	392	299	2762
CO350	5197	333	361	127	2306	615	102	685	603	5132
HE100	2801	315	294	72	1309	137	28	373	239	2767
HE350	3671	391	306	80	1551	402	68	452	372	3622
IS100	2819	231	246	80	1145	303	59	408	311	2783
IS350	3040	290	256	79	1333	294	51	393	294	2990
MA100	2638	251	211	71	1285	154	35	363	252	2622
MA350	3593	302	300	91	1571	377	73	428	386	3528

Appendix 4 ANOVA results of bio-imaging analysis of gluten protein distribution

Table 1, HMW-GS lobe regions

Variate: LOG(Area)

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr.
Seed stratum					
Variety	1	15.3987	15.3987	9.12	0.019
N	1	0.1554	0.1554	0.09	0.77
DPA	1	0.0009	0.0009	0	0.982
Variety.N	1	0.3616	0.3616	0.21	0.657
Variety.DPA	1	0.2985	0.2985	0.18	0.687
N.DPA	1	2.6129	2.6129	1.55	0.253
Variety.N.DPA	1	1.0954	1.0954	0.65	0.447
Residual	7(1)	11.8139	1.6877	9.69	
Seed.Transects stratum		30(2)	5.2261	0.1742	1.56
Seed.Transects.Position	stratum				
Position	3	9.4991	3.1664	28.42	<.001
Position.Variety	3	1.7081	0.5694	5.11	0.002
Position.N	3	0.0696	0.0232	0.21	0.89
Position.DPA	3	0.0907	0.0302	0.27	0.846
Position.Variety.N	3	1.1765	0.3922	3.52	0.017
Position.Variety.DPA	3	0.299	0.0997	0.89	0.447
Position.N.DPA	3	0.4186	0.1395	1.25	0.294
Position.Variety.N.DPA	3	0.0405	0.0135	0.12	0.948
Residual	111(9)	12.369	0.1114		
Total	179(12)	62.3413			

Variate: LOG(Total_Intensity)

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr.
Seed stratum					
Variety	1	30.8343	30.8343	8.56	0.022
N	1	7.0254	7.0254	1.95	0.205
DPA	1	5.058	5.058	1.4	0.275
Variety.N	1	6.5074	6.5074	1.81	0.221
Variety.DPA	1	0.7497	0.7497	0.21	0.662
N.DPA	1	0.0001	0.0001	0	0.996
Variety.N.DPA	1	2.2447	2.2447	0.62	0.456
Residual	7(1)	25.2294	3.6042	19.06	
Seed.Transects stratum		30(2)	5.6723	0.1891	1.47

Seed.Transects.Position					
Position	3	10.5948	3.5316	27.36	<.001
Position.Variety	3	2.6472	0.8824	6.84	<.001
Position.N	3	0.1375	0.0458	0.36	0.785
Position.DPA	3	0.0733	0.0244	0.19	0.904
Position.Variety.N	3	0.9872	0.3291	2.55	0.059
Position.Variety.DPA	3	0.2682	0.0894	0.69	0.558
Position.N.DPA	3	0.5337	0.1779	1.38	0.253
Position.Variety.N.DPA	3	0.1221	0.0407	0.32	0.814
Residual	111(9)	14.3252	0.1291		
Total	179(12)	108.8259			

Table 2, HMW-GS dorsal regions

Variate: LOG(Area)

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr.
Seed stratum					
Variety	1	22.949	22.949	4.17	0.08
N	1	28.2842	28.2842	5.14	0.058
DPA	1	3.5504	3.5504	0.65	0.448
Variety.N	1	0.0466	0.0466	0.01	0.929
Variety.DPA	1	5.3189	5.3189	0.97	0.358
N.DPA	1	14.5197	14.5197	2.64	0.148
Variety.N.DPA	1	7.4542	7.4542	1.35	0.283
Residual	7(1)	38.5291	5.5042	34.81	
Seed.Transects stratum		30(2)	4.7436	0.1581	0.81
Seed.Transects.Position					
Position	2	6.5722	3.2861	16.88	<.001
Position.Variety	2	1.7162	0.8581	4.41	0.016
Position.N	2	0.8984	0.4492	2.31	0.107
Position.DPA	2	1.8818	0.9409	4.83	0.011
Position.Variety.N	2	0.4947	0.2473	1.27	0.287
Position.Variety.DPA	2	0.8118	0.4059	2.08	0.132
Position.N.DPA	2	2.2741	1.137	5.84	0.004
Position.Variety.N.DPA	2	1.5756	0.7878	4.05	0.021
Residual	74(6)	14.4085	0.1947		
Total	134(9)	154.6601			

Variate: LOG(Total_Intensity)

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr.
Seed stratum					
Variety	1	36.1619	36.1619	5	0.06
N	1	15.3182	15.3182	2.12	0.189
DPA	1	0.1509	0.1509	0.02	0.889
Variety.N	1	0.1495	0.1495	0.02	0.89
Variety.DPA	1	8.6948	8.6948	1.2	0.309
N.DPA	1	10.2684	10.2684	1.42	0.272
Variety.N.DPA	1	7.8062	7.8062	1.08	0.333
Residual	7(1)	50.6388	7.2341	36.23	
Seed.Transects stratum		30(2)	5.9909	0.1997	0.76
Seed.Transects.Position					
Position	2	7.4666	3.7333	14.23	<.001
Position.Variety	2	2.6835	1.3418	5.11	0.008
Position.N	2	1.1221	0.5611	2.14	0.125
Position.DPA	2	2.383	1.1915	4.54	0.014

Position.Variety.N	2	0.7194	0.3597	1.37	0.26
Position.Variety.DPA	2	1.0002	0.5001	1.91	0.156
Position.N.DPA	2	2.5241	1.262	4.81	0.011
Position.Variety.N.DPA	2	1.8619	0.9309	3.55	0.034
Residual	74(6)	19.4134	0.2623		
Total	134(9)	173.6707			

Table 3, LMW-GS and gliadins lobe regions

Variate: LOG(Area)

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr.
Seed stratum					
Variety	1	6.15808	6.15808	5.1	0.058
N	1	0.58476	0.58476	0.48	0.509
DPA	1	0.37252	0.37252	0.31	0.596
Variety.N	1	2.05151	2.05151	1.7	0.234
Variety.DPA	1	0.3466	0.3466	0.29	0.609
N.DPA	1	0.55183	0.55183	0.46	0.521
Variety.N.DPA	1	0.28469	0.28469	0.24	0.642
Residual	7(1)	8.45335	1.20762	17.88	
Seed.Transects stratum		30(2)	2.02593	0.06753	0.99
Seed.Transects.Position					
Position	3	16.12857	5.37619	79.13	<.001
Position.Variety	3	0.73452	0.24484	3.6	0.016
Position.N	3	0.2195	0.07317	1.08	0.362
Position.DPA	3	0.04103	0.01368	0.2	0.895
Position.Variety.N	3	1.44352	0.48117	7.08	<.001
Position.Variety.DPA	3	0.34663	0.11554	1.7	0.171
Position.N.DPA	3	0.37567	0.12522	1.84	0.144
Position.Variety.N.DPA	3	0.08528	0.02843	0.42	0.74
Residual	111(9)	7.5418	0.06794		
Total	179(12)	46.21056			

Variate: LOG(Total_Intensity)

Source of variation					
Seed stratum					
Variety	1	10.47955	10.47955	3.12	0.121
N	1	6.01747	6.01747	1.79	0.223
DPA	1	0.1024	0.1024	0.03	0.866
Variety.N	1	0.30751	0.30751	0.09	0.771
Variety.DPA	1	0.57661	0.57661	0.17	0.691
N.DPA	1	2.16243	2.16243	0.64	0.449
Variety.N.DPA	1	4.26017	4.26017	1.27	0.297
Residual	7(1)	23.52798	3.36114	29.78	
Seed.Transects stratum		30(2)	3.38572	0.11286	1.74
Seed.Transects.Position					
Position	3	26.42245	8.80748	135.43	<.001
Position.Variety	3	1.97575	0.65858	10.13	<.001
Position.N	3	0.17943	0.05981	0.92	0.434
Position.DPA	3	0.01566	0.00522	0.08	0.971

Position.Variety.N	3	1.36221	0.45407	6.98	<.001
Position.Variety.DPA	3	0.31356	0.10452	1.61	0.192
Position.N.DPA	3	0.18198	0.06066	0.93	0.427
Position.Variety.N.DPA	3	0.06161	0.02054	0.32	0.814
Residual	111(9)	7.21858	0.06503		
Total	179(12)	82.48506			

Table 4, LMW-GS and gliadins dorsal regions

Variate: LOG(Area)					
Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr.
Seed stratum					
Variety	1	11.85601	11.85601	4.33	0.076
N	1	7.29934	7.29934	2.66	0.147
DPA	1	3.51294	3.51294	1.28	0.295
Variety.N	1	0.15974	0.15974	0.06	0.816
Variety.DPA	1	0.43299	0.43299	0.16	0.703
N.DPA	1	3.51637	3.51637	1.28	0.295
Variety.N.DPA	1	1.98813	1.98813	0.73	0.423
Residual	7(1)	19.18037	2.74005	42.66	
Seed.Transects stratum		30(2)	1.92688	0.06423	0.78
Seed.Transects.Position					
Position	2	8.49634	4.24817	51.68	<.001
Position.Variety	2	0.47523	0.23762	2.89	0.062
Position.N	2	0.83176	0.41588	5.06	0.009
Position.DPA	2	1.77528	0.88764	10.8	<.001
Position.Variety.N	2	0.05357	0.02678	0.33	0.723
Position.Variety.DPA	2	0.68382	0.34191	4.16	0.019
Position.N.DPA	2	0.95585	0.47793	5.81	0.005
Position.Variety.N.DPA	2	0.50427	0.25214	3.07	0.053
Residual	74(6)	6.0833	0.08221		
Total	134(9)	69.15088			
Variate: LOG(Total_Intensity)					
Source of variation					
Seed stratum					
Variety	1	11.389	11.389	4.37	0.075
N	1	0.1686	0.1686	0.06	0.807
DPA	1	3.3869	3.3869	1.3	0.292
Variety.N	1	2.3586	2.3586	0.9	0.373
Variety.DPA	1	0.0149	0.0149	0.01	0.942
N.DPA	1	4.452	4.452	1.71	0.233
Variety.N.DPA	1	2.8145	2.8145	1.08	0.333
Residual	7(1)	18.2456	2.6065	32.11	
Seed.Transects stratum	30(2)	2.4354	0.0812	0.67	
Seed.Transects.Position					
Position	2	12.9538	6.4769	53.34	<.001
Position.Variety	2	1.6077	0.8039	6.62	0.002
Position.N	2	1.0591	0.5296	4.36	0.016
Position.DPA	2	2.0036	1.0018	8.25	<.001

Position.Variety.N	2	0.0598	0.0299	0.25	0.782
Position.Variety.DPA	2	0.7795	0.3898	3.21	0.046
Position.N.DPA	2	1.2158	0.6079	5.01	0.009
Position.Variety.N.DPA	2	1.0147	0.5074	4.18	0.019
Residual	74(6)	8.9853	0.1214		
Total	134(9)	71.0787			

Appendix 5 DSC results of pearling fractions of Hereward and Istabraq

Varity	N	Fractions	on-set T (°C)	peak T (°C)	end T (°C)	ΔH (J/g) (Dry based)
Istabraq	350	1st	60.30	67.05	72.55	0.32
Istabraq	350	2nd	60.90	66.95	72.70	0.78
Istabraq	350	3rd	60.85	66.85	72.40	1.09
Istabraq	350	4th	60.25	66.10	71.55	1.21
Istabraq	350	5th	59.70	65.70	71.30	1.34
Istabraq	350	6th	59.20	65.05	70.65	1.36
Istabraq	350	core	58.30	64.60	70.70	1.73
Istabraq	350	whole	59.85	65.70	71.25	1.51
Istabraq	100	1st	60.65	67.00	72.65	0.30
Istabraq	100	2nd	62.75	68.40	73.35	1.14
Istabraq	100	3rd	62.15	67.60	72.45	1.29
Istabraq	100	4th	61.95	67.10	72.10	1.48
Istabraq	100	5th	60.45	65.75	70.85	1.48
Istabraq	100	6th	60.10	65.50	70.75	1.55
Istabraq	100	core	58.85	64.70	70.25	1.80
Istabraq	100	whole	60.20	65.90	71.40	1.64
Hereward	350	1st	61.35	66.80	72.15	0.16
Hereward	350	2nd	61.05	67.40	73.35	0.53
Hereward	350	3rd	61.25	67.10	72.90	0.64
Hereward	350	4th	59.75	66.75	72.75	0.66
Hereward	350	5th	58.70	65.65	71.30	0.62
Hereward	350	6th	58.55	65.60	71.55	0.68
Hereward	350	core	58.85	64.60	70.75	1.53
Hereward	350	whole	59.95	65.35	70.95	1.30
Hereward	100	1st	61.00	66.55	72.35	0.16
Hereward	100	2nd	61.00	67.20	72.60	0.49
Hereward	100	3rd	60.95	67.25	73.00	0.65
Hereward	100	4th	60.45	66.75	72.35	0.72
Hereward	100	5th	59.20	66.10	72.10	0.82
Hereward	100	6th	59.20	65.35	70.80	0.77
Hereward	100	core	58.80	64.70	70.50	1.58
Hereward	100	whole	63.45	68.50	74.45	1.08

Appendix 6 starch damage of pearling fractions of Hereward

Nitrogen level	Fraction	Replicate				Mean	S.D.
		1	2	3	4		
HE350	1st	5.60	6.72	4.22	5.13	5.42	1.04
	2nd	10.64	11.25	------	10.42	10.77	0.43
	3rd	14.94	17.00	11.99	15.23	14.79	2.07
	4th	17.86	18.26	16.44	18.30	17.71	0.87
	5th	17.39	16.81	16.56	------	16.92	0.43
	6th	17.01	16.15	16.92	------	16.69	0.47
	Core	4.49	4.27	------	4.19	4.32	0.15
	Whole	4.47	4.43	------	4.40	4.43	0.04
HE100	1st	6.54	6.76	------	7.30	6.87	0.39
	2nd	13.68	13.61	13.49	14.17	13.74	0.30
	3rd	16.98	16.23	17.44	18.04	17.17	0.76
	4th	17.25	18.50	20.25	19.95	18.99	1.38
	5th	15.34	------	------	------	15.34	0.00
	6th	17.33	------	------	------	17.33	0.00
	Core	4.07	------	------	------	4.07	0.00
	Whole	4.38	------	------	4.47	4.43	0.06

Appendix 7 practical size distribution of pearling fractions of Hereward

N350

	1st	2nd	3rd	4th	5th	6th	Core	Whole
Particle size by Feret minimum, µm					**% of flour**			
4.23	*	0.1	0.15	0.21	0.2	0.22	0.04	0.07
5.86	*	0	0	0	0	0	0	0
6.72	*	0	0	0	0	0	0	0
8.17	*	0	0	0	0	0	0	0
9.92	*	0.39	0.56	0.76	0.74	0.81	0.14	0.22
12.05	*	0.18	0.25	0.34	0.33	0.37	0.07	0.11
14.63	*	1.01	1.4	1.84	1.83	1.99	0.39	0.62
17.77	*	0.42	0.56	0.72	0.72	0.8	0.17	0.26
21.59	*	1.68	2.33	3	3.07	3.37	0.76	1.17
26.22	*	2.5	3.36	4.28	4.4	4.91	1.15	1.79
31.85	*	4.18	5.39	6.55	6.71	7.39	1.76	2.64
38.68	*	6.94	8.79	10.22	10.23	10.86	2.67	4.1
46.98	*	12	14.35	15.86	15.43	16.23	4.73	7.25
57.06	*	11.52	13.4	14.47	13.5	13.64	5.16	7.47
69.3	*	10.93	11.65	11.97	10.89	11.05	6.5	8.57
84.17	*	8.52	8.51	8.33	7.33	7.63	5.96	7
102.24	*	7.15	6.82	6.66	5.13	5.08	5.27	6.22
124.17	*	6.03	5.47	4.15	3.27	2.91	4.37	5.47
150.82	*	5.89	4.71	3.17	2.37	2.04	4.8	5.67
183.18	*	5.13	3.85	2.74	1.94	1.37	4.7	6.38
222.49	*	4.33	3.18	1.78	0.71	0.75	5.92	7.01
270.23	*	3.43	2.45	0.85	1.11	0.61	8.19	6.04
328.22	*	1.85	1.95	1.18	0.51	0.46	8.4	5.87
398.64	*	1.62	0.46	0	0.5	0	8.79	5.48
484.18	*	1.05	0.38	0.94	0.97	0.52	5.51	2.04
588.08	*	1.64	0	0	0	1.73	2.26	1.23
714.27	*	1.53	0	0	0	0	7.72	1.48
867.53	*	0	0	0	0	0	4.57	5.85
1053.68	*	0	0	0	8.09	5.26	0	0
1279.78	*	0	0	0	0	0	0	0
1554.39	*	0	0	0	0	0	0	0
1765.97	*	0	0	0	0	0	0	0
Between 40-70	*	**41.39**	**48.19**	**52.52**	**50.05**	**51.78**	**19.06**	**27.39**
>70	*	**48.17**	**37.78**	**29.8**	**31.93**	**28.36**	**76.46**	**65.74**

N100

	1st	2nd	3rd	4th	5th	6th	Core	Whole
Particle size by Feret minimum, µm				**% of flour**				
4.23	0.04	0.14	0.21	0.29	0.34	0.3	0.1	0.09
5.86	0	0	0	0	0	0	0	0
6.72	0	0	0	0	0	0	0	0
8.17	0	0	0	0	0	0	0	0
9.92	0.15	0.5	0.75	1.01	1.15	1.03	0.32	0.3
12.05	0.07	0.23	0.34	0.45	0.51	0.47	0.15	0.14
14.63	0.39	1.26	1.82	2.36	2.67	2.48	0.87	0.8
17.77	0.16	0.5	0.71	0.89	1.01	0.96	0.36	0.33
21.59	0.65	2.09	3.02	3.82	4.4	4.27	1.73	1.57
26.22	0.96	3.04	4.22	5.3	6.08	5.97	2.57	2.29
31.85	1.65	4.72	6.21	7.53	8.42	8.22	3.54	3.15
38.68	2.89	7.57	9.39	10.82	11.52	10.78	4.79	4.2
46.98	4.97	11.95	14.03	14.99	15.59	14.36	7.56	6.37
57.06	4.75	11.04	12.26	12.97	13.13	11.32	7.42	5.98
69.3	5.13	10.26	10.85	10.61	10.5	8.8	8.64	6.74
84.17	5.22	8.78	8.7	7.94	7.53	6.04	8.05	5.82
102.24	5.92	8.34	7.87	6.66	5.69	4.34	7.71	5.7
124.17	6.14	6.93	5.95	4.61	3.73	2.8	6.18	4.71
150.82	6.49	6.33	4.41	3.43	2.5	1.66	6.68	4.03
183.18	7.37	5.3	2.88	1.93	1.69	1.24	6.15	4.49
222.49	7.05	3.99	2.07	0.99	1.15	0.55	6.42	5.18
270.23	6.25	1.99	1.43	1.23	0.72	0.74	5.65	4.71
328.22	4.54	1.77	0.75	0.98	0.42	0.95	4.39	5.7
398.64	4.49	2.33	1.21	0	0.77	0.54	2.77	5.6
484.18	5.18	0.93	0.9	1.2	0.48	0.51	1.93	5.71
588.08	5.21	0	0	0	0	3.25	2.67	1.46
714.27	1.16	0	0	0	0	0	3.35	4.69
867.53	8.38	0	0	0	0	8.4	0	4.03
1053.68	4.8	0	0	0	0	0	0	6.21
1279.78	0	0	0	0	0	0	0	0
1554.39	0	0	0	0	0	0	0	0
1765.97	0	0	0	0	0	0	0	0
Between 40-70	**17.74**	**40.82**	**46.53**	**49.39**	**50.74**	**45.26**	**28.41**	**23.29**
>70	**78.2**	**46.69**	**36.17**	**28.97**	**24.68**	**31.02**	**61.95**	**68.04**

Appendix 8 means of physical properties of wheat grains

		HE	MA	CO	IS	PA	MM	XI
Weight/mg	N350	49.03	46.49	48.30	52.03	49.29	48.52	53.09
	N100	46.51	43.85	46.94	50.33	48.84	45.65	55.58
Size/diameter mm	N350	3.19	3.19	3.16	3.11	3.20	3.19	3.19
	N100	3.15	3.14	3.18	3.11	3.19	3.10	3.30
Hardness Index	N350	59.42	61.25	65.11	20.25	66.40	64.08	63.67
	N100	49.37	47.30	54.52	7.80	48.95	55.68	44.87
Moisture (%)	N350	12.28	9.94	12.12	12.42	11.45	10.05	11.87
	N100	11.66	9.56	12.58	9.12	12.26	10.52	12.19

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