PROJECT REPORT No. 141

THE EFFECT OF MORPHOLOGICAL STRUCTURE ON THE DIGESTIBILITY OF BARLEY AND WHEAT ENDOSPERMS

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THE EFFECT OF MORPHOLOGICAL STRUCTURE ON THE DIGESTIBILITY OF BARLEY AND WHEAT ENDOSPERMS

by

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Overall Summary

The main aim of this investigation was to identify the relationship between the structure and composition of barley and wheat endosperms and ease of conversion during malting and distilling. The research at BRF International focused on barley endosperm structure and its malting quality whereas the effect of endosperm structure of wheat on distilling was carried out at the International Centre for Brewing and Distilling (ICBD) at Heriot-Watt University.

For both cereals, endosperm structure strongly influenced their ability to be converted during the malting and distilling processes. Structurally different areas of the grain, known as mealy and steely areas, were identified in single sections and in whole grains. These structural differences can be traced to the variety of the grain and also to the growing and environmental conditions. One of the factors which affected endosperm structure was the application of fungicide to the growing crop. This increased the mealiness of both barley and wheat grains, probably due to enhanced photosynthetic activity throughout the growth period of the plants.

In barley, malting varieties contained fewer steely grains than did the feed variety. Malt quality was inversely related to the proportion of steely grains. Endosperm structure was related to the concentration of structural components such as ß-glucan and protein. For example, steeliness was associated with higher concentrations of ß-glucan and specific proteins, particularly γ-hordein. The rate of moisture uptake and re-distribution across the endosperm was greatly dependent on its structure. In turn, moisture distribution affected the

distribution activity of key hydrolytic enzymes such as ß-glucanase. The extent of endosperm modification during malting was therefore, affected by the actual location of steely areas within the endosperm. Thus steely patches in the proximal endosperm restricted the distribution of moisture into the central endosperm and reduced the effectiveness of hydrolytic enzymes. A method was developed to measure the proportion of steely and mealy areas in a given sample. This method depends upon the extent of light transflectance through the endosperm.

Wheat can be further sub-divided into soft and hard types according to endosperm structure, which is varietally determined. The small starch granules and matrix proteins of hard wheats produce a much more rigid structure than seen in soft wheat varieties. For both wheat and barley, steeliness was influenced by the growing conditions. During distilling, spirit yield is the paramount factor and soft wheat varieties normally produce the highest alcohol yield. Some hard wheat varieties were potentially useful for distilling, provided that the grain nitrogen content was low. The pentosan content of wheat was significantly higher than in barley and mealy grains of hard wheat had significantly higher levels than soft wheat. The effect of pentosans on foam during whisky production was studied and ß-glucanase and protease were more influential in reducing foam whereas xylanase reduced wort viscosity.

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Summary

Structural differences in the endosperm of barley grains were studied using light and electron microscopy methods. Two main structural types were identified and were classified as mealy and steely. The mealy areas of the endosperm have a white friable structure whereas the steely areas are tightly packed with high levels of proteins, small starch granules and cell wall materials such as ß-glucan.

Patches of steely areas may occur randomly within a mealy endosperm. Both the extent of steeliness and the distribution of steely areas within the grain can influence modification. It is therefore necessary to study the structure of the whole grain rather than individual sections. A light transflectance method was developed which can identify the mealy and steely grains in a given sample. The proportion of such grains in each variety was important in determining malt quality.

One of the factors which could affect the mealiness of the endosperm is fungicide treatment of the growing barley plant. Grain from untreated plots was significantly more steely than grain from plots which had been treated with fungicide.

The extent of mealiness and steeliness was associated with differences in the proportion of endosperm components such as hordein proteins and ß-glucan. These in turn affected the distribution of water and of the cell wall degrading enzyme, ß-glucanase, within the endosperm.

Three barley varieties, two malting (Chariot and Blenheim) and a feed (Target) containing

different proportions of mealy and steely grains were studied in relation to the concentration and distribution of protein, ß-glucan and ß-glucanase.

The distribution of moisture was mapped using the iodine vapour method (Davies, 1991) which was further developed to quantify the distribution of moisture in the endosperm as the Overall Hydration Value (OHV). The rate of moisture uptake differed slightly between samples but all reached the required moisture level of around 43% by the end of steeping. Redistribution of moisture across the endosperm was, however, greatly dependent on endosperm structure and this is represented by the OHV. Chariot, with a high OHV, was better able to distribute moisture than Blenheim which was better than Target. Both the malting varieties had a higher number of grains which were mealy and so were better able to distribute water evenly throughout the endosperm.

The influence of variety and total nitrogen (TN) content on distribution within the endosperm of both total and defined proteins was examined. Within a single corn, protein was concentrated mainly in the embryo and distal regions with the inner, mid-endosperm containing lowest levels. As the TN of the grain increased the malting varieties, Blenheim and Chariot, accumulated nitrogen mainly in the embryo whereas Target showed higher levels in the central endosperm.

Proteins were further characterised using SDS-PAGE. No differences were observed in the banding patterns within a variety when whole barley grains at different levels of total nitrogen were analysed. Differences were found in the protein profiles of the embryo proteins when compared to those in the endosperm. Immuno-gold assay techniques showed that γ-hordeins

were mainly present in the sub-aleurone and outer endosperm and were confined to steely areas, being absent from mealy areas of the endosperm. C-hordeins on the other hand were found in both mealy and steely areas and were evenly distributed throughout the central endosperm.

In general, protein modification in Chariot was better than in Target. High molecular weight D-hordeins were completely degraded during malting. Hordeins with molecular weight of 34kD and 97kD were also degraded during malting. These hordeins were absent from the friable fraction of the malt but were detected in the non-friable fraction.

The amount of ß-glucan in Target was higher than Blenheim and Chariot due to the high proportion of steely grains present in this sample. Most of this ß-glucan was concentrated in the proximal and distal areas of the endosperm. Both of these areas are very important for water uptake by the endosperm during steeping, thus variations in the concentration of specific components will affect subsequent water distribution. This in turn will determine the homogeneity of endosperm modification and consequently malt quality.

Significant differences in the distribution of cell wall degrading enzymes were observed between malting and feed varieties. With the feed variety, Target, the activity of ß-glucanase was low during most part of germination period, and remained concentrated in the proximal endosperm. With the malting variety, Chariot, however, most of the glucanase activity had developed by the third day of germination, and distributed more evenly across the endosperm.

1.0 INTRODUCTION

The principal aim of malting is to produce malt which is uniformly modified. If the malt modification is not homogeneous, the undegraded areas in the endosperm give poor extracts and high wort viscosities, which can cause processing difficulties during filtration, resulting in formation of gels and hazes in the final product (Bamforth and Barclay, 1992). Stringent specifications are therefore set for malting barley (Table I) but even when such requirements for the raw material quality have been attained, difficulties in processing can still be encountered due to uneven modification of barley endosperm. The main cause of this problem is thought to be differences in the structure of the endosperm which influence the rate and extent of water redistribution from the embryo. This in turn changes the synthesis, distribution and activity of enzymes in the areas of the endosperm where the moisture redistribution rate is slow.

During grain development synthesis of storage proteins, hordeins, and small starch granules overlap. Both climatic conditions and the genetics of the grain alter the levels of endosperm components, resulting in changes in the endosperm morphology (Palmer, 1989). Such changes will alter the structure of the cells, the ratio of small to large starch granules, the density of the protein matrix and cell wall cross linking and could, ultimately, lead to uneven modification during malting.

Differences in endosperm structure can be classified according to the degree of packing of starch (both large and small granules), proteins and cell wall polymers. Mealy (M) areas of the endosperm are loosely packed, with open spaces between starch granules, whereas the

steely (S) areas are densely packed, containing a high level of cell wall polymers, proteins and small starch granules.

The degradation of grain cell walls and the protein matrix surrounding the starch granules, the source of brewers, extract will differ according to their steeliness. For example, high levels of ß-glucans and their cross-linking to proteins will affect water redistribution. Whilst there have been several suggestions on how water enters the grain (Briggs *et al.*, 1981; Chapon, 1960; Pollock, 1962) it is important that the endosperm becomes evenly hydrated so that hydrolytic enzymes produced from the aleurone together with those latent in the endosperm, can uniformly degrade the structural components of the endosperm in any bulk sample. The normal measure of water in the grain during malting is the total moisture content at cast, which in commercial malting is in the region of 43-46% (Kirsop, 1966, 1975; Kirsop *et al.,* 1967). Even at this moisture level, some parts of the endosperm can be relatively dry compared to others, making subsequent enzyme activity during germination limited to areas where hydration has occurred with ensuing uneven modification (Axcell *et al.*, 1983).

It is generally agreed that the protein content of barley plays an important role in endosperm modification through its influence on the rate of water and enzyme distribution. A controlled modification of the protein reserves is necessary, to supply amino acids for yeast growth and flavour production during fermentation, to provide the proteins required for beer foam but, at the same time, to reduce the proteins which interfere in the final stages of brewing, such as those which form hazes. It is therefore important to study the distribution of specific proteins in the endosperm.

The major storage proteins in barley (hordeins), are classified as prolamins due to their high proline content. These have been further divided into sulphur rich, sulphur poor and high molecular weight (Kreis *et al.*, 1983; Kreis and Shewry, 1989) as shown in Table II. The proportion of hordeins increases with the grain total nitrogen and can account for up to half the nitrogen of the grain (Shewry and Miflin, 1985). The breakdown of hordeins during malting has been linked to malt quality by various authors. Baxter (1981) has shown that a well modified malt contains less than half the amount of hordein present in the original barley with a pronounced decrease in the B-hordein fraction. Similarly, it has been suggested that gel proteins containing both B and D hordein fractions can interfere with wort filtration and analysis (Moonen *et al.,* 1987; Smith and Lister, 1983). The distribution of hordein in the endosperm and its degradation during malting is, therefore, an important aspect. In this investigation, we have studied hordein modification by SDS-PAGE and immuno-histological methods using scanning electron microscopy and x-ray micro analysis.

It is well known that undegraded ß-glucans can present a number of potential problems to the brewer (Bamforth, 1994; Gjertesen, 1966; Jorgensen *et al.*, 1985) but their distribution in the grain is not well documented, particularly with regard to mealy and steely areas. The amount of high molecular weight, water soluble ß-glucan decreases sharply during malting, due to the action of endo-hydrolases (Bamforth and Martin, 1983). In high ß-glucan barley, the water insoluble ß-glucans also decrease, mainly between days four and five of germination (Bamforth and Martin, 1981a). A positive correlation has therefore been postulated between the total ß-glucan content of the grain and rate of modification (Aastrup and Erdal, 1980; Martin and Bamforth, 1980; Smart, 1978). The factors affecting the amount and physical properties of ß-glucans present at the end of malting include the amount of total ß-glucan, the

activity of ß-glucan solubilase (Bamforth and Martin, 1981b) and the time course of development of ß-glucanases (Bamforth and Martin, 1981a), and these were investigated in the present work.

Two malting varieties (Chariot and Blenheim), containing different proportions of mealy and steely grains, and a feed variety (Target) with a high proportion of steely grains, were chosen. Differences in the concentration and distribution of water, nitrogen, protein, ß-glucan and ß-glucanases were studied in relation to the differences in the proportion of mealy and steely grains in each variety.

2.0 MATERIALS AND METHODS

2.1 Materials

Barley cultivars, Blenheim and Chariot, were obtained from the National Institute of Agricultural Botany (NIAB), Cambridge, UK from their National List Trials and cultivar Target was supplied by Usbourne Grain Ltd, Andover, UK. All chemicals used were obtained from Sigma Chemicals UK (unless otherwise stated) and were of the highest purity available.

2.2 METHODS

2.2.1 Micromalting

This was carried out at 16°C using either 350g or 2kg of barley with one of the two interrupted steeping schedules of wet(w) or air-rest(a) periods: (1) 8w/16a/24w or (2) 7w/17a/7w/17a/1w. Germination was for five days and green malts were dried in an air-flow oven for 8h at 45°C followed by 16h at 65°C.

2.2.2 Pearling

Barley was dehusked using a small scale pearling machine driven by a electric motor. The main chamber of the machine was lined with an abrasive metal mesh and grains were pearled against the abrasive surface with the help of wire brush for 5-8 minutes depending on the variety.

2.2.3 Light transflectance method

Pearled grains were placed on a glass plate and a Koch Light fibre optic light source (model

KLS 85) diffused through a Whatman-105 Lens paper was used to illuminate the grains from below. Alternatively, a light box was used.

2.2.4 Scanning electron microscopy

Transverse sections of barley grains were gold coated and examined using a Phillips 505 scanning electron microscope (SEM) operating at 25kV. Analysis of wet barley samples was carried out using the Oxford CT1000 cryo unit. Samples were placed on a stub and plunged frozen in liquid nitrogen / nitrogen slush at -180°C and transferred under vacuum to the cryo-stage in the SEM. The sample was than etched by decreasing the stage temperature to -130°C. When the sample was sufficiently etched, it was coated with either gold or carbon.

2.2.5 X-ray microanalysis

Elemental analysis was carried out using EDAX PV9900 x-ray microanalysis system which attached to the SEM.

2.2.6 Moisture content

This was measured according to IOB Recommended Method of analysis (1991).

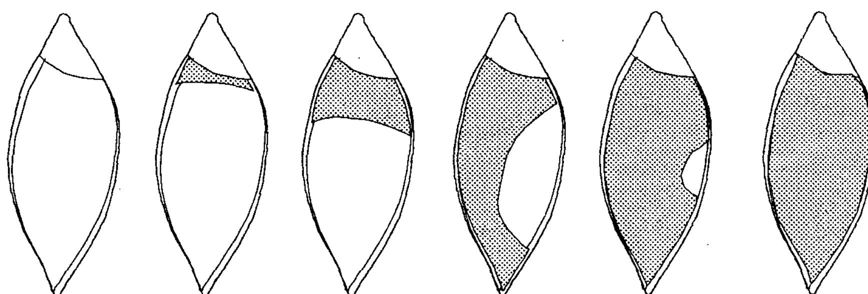
2.2.7 Iodine vapour staining

The method described by Davies (1991) was used to study the distribution of water in the endosperm during steeping.

2.2.8 Iodine vapour scoring

Grains from each variety were exposed to iodine vapour, according to method 2.2.7, and the

area of the endosperm which became stained was scored according to the following procedure:



0	0-1/4	1/4-1/2	1/2-3/4	3/4-1	1	Water redistribution
	0.125	0.375	0.625	0.875	1	Score

The grains were divided into two main populations: mealy (M) and steely (S). The steely grains also included patches of mealy endosperm (M/S), but these areas were very small and were therefore classified as steely for water distribution studies.

For each of the mealy and steely populations an Iodine Vapour Score (IVS) was calculated:

IVS= Σ (Number of grains in each group x score).

Thus, for a given sample (n=100) the Overall Hydration Value (OHV) after 16h was calculated from:

OHV = [(M/100) x IVS(M)] + [(S/100) x IVS(S)]

where M = Number of mealy grains; S= Number of steely grains.

2.2.9 Nitrogen analysis

Total nitrogen in whole grains and sections of grain were analysed by either the Kjeldahl or the Dumas methods (Leco Instruments (UK) Ltd, UK) according to the IOB Recommended

2.2.10 Total protein extraction

Total proteins were extracted from ground samples of barley endosperm in 0.7% (w/v) Tris, containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol (ME) and then precipitated with 10% (w/v) trichloroacetic acid (TCA) and analysed by SDS-PAGE or BCA protein assay.

2.2.11 Hordein extraction

Hordeins were extracted sequentially for 1h using the following : (a) Hordein I :- 50% (v/v) propan-1-ol at 60°C and (b) Hordein II :- 50% (v/v) propan-1-ol + 2% (v/v) (ME) at 60°C. After each extraction the samples were centrifuged at 10000g for 10 minutes, dried in a rotary evaporator and resuspended in SDS-PAGE sample buffer. Protease inhibitors PMSF (200μM), EDTA (100μM), Leupeptin (1μM) and Pepstatin (1μM) were added during extraction to stop any protease activity during extraction

2.2.12 SDS-PAGE analysis

Discontinuous gel electrophoresis was performed under reducing conditions by the method of Laemmli (1970) using the instrument supplied by Hoefer Scientific Instruments, USA.

2.2.13 Immuno-gold labelling of barley and malt hordeins

Barley and malt grains were cut in half and blocked in phosphate-buffered saline (PBS) containing Tween-20 (PBST): 140mM NaCl, 1.5mM KH_2PO_4, 8mM Na_2HPO_4, 2.7mM KCl, pH 7.4, and 0.05%(v/v) Tween-20) and 1%(w/v) skimmed milk powder. The grains were then washed three times in PBST for 15min and fixed in 205 buffer (2%(v/v)

paraformaldehyde and 0.05%(v/v) glutaraldehyde). Grains were incubated with primary antibody (anti-hordein IgG), diluted 1:200 with blocking buffer, for 30min, washed five times for 15min in PBS and incubated with secondary antibody (Goat anti-mouse IgG attached to 10nm gold particles, from BioCell Research Laboratories, UK), diluted 1:1000 in PBS, for 30min. Grains were then washed five times for 15min in PBS before silver enhancing using the BioCell Silver Enhancing kit and visualized using the cryo-scanning electron microscope.

2.2.14 ß-glucan determination using Calcofluor Flow Injection Analyzer

A Tecator 5700 ß-glucan analyser was calibrated using purified barley ß-glucan standards which were provided with the instrument. These standards were prepared from ß-glucans of a relatively low molecular weight.

The ß-glucans in barley and malt samples were dissolved before determination. This was achieved by incubating 100mg of barley or 250mg malt flour with 9.9ml Termamyl α-amylase (Novo Nordisk, Denmark) in a boiling water bath for 1h. The extract was quickly cooled to room temperature, 10ml of 75mM sulphuric acid was added and placed in a boiling water bath for 10min. The samples were cooled to room temperature and centrifuged at 10000g for 10min. This second extraction permitted partial degradation of ß-glucans into soluble fractions. It was important not to degrade the ß-glucans too far since they will not complex with calcofluor. Therefore, it was crucial that acid extraction was carried out for exactly 10min.

2.2.15 ß-glucan solubilase determination

The level of ß-glucan solubilase was measured in barley and malt using the method described

by Moore *et al.*, 1996. The substrate was prepared from barley (*var.* Target) which was dehusked using 9M H_2SO_4 (Bamforth *et al*, 1979). The flour was refluxed for 1h with 2 parts 70%(v/v) ethanol. After cooling the solids were washed twice with cold ethanol and allowed to dry before remilling. The flour (100g) was rinsed with 50ml heat treated alpha-amylase (Yin and MacGregor, 1989) and 1.25 litre buffer (50ml 1M $NaCl_2$, 5ml 1 $CaCl_2$, 25ml 1M sodium acetate made up to 500ml pH 5.7) and incubated for 10 min at 65°C. 50mM EDTA (2ml) was added to stop the reaction. The substrate was washed three times with water at 65°C, left to dry and then remilled. The assay was performed using this substrate and 500mM potassium phosphate buffer pH 6.4 [to 1ml] were mixed and incubated at 65°C for 10min; cold buffer (0.5ml) was then added before centrifuging in a microfuge for 10min. Controls (to account for the freely soluble portion of ß-glucan) employed boiled enzyme, and all assays were performed in duplicate. ß-Glucan released in the assay was measured using the method of McCleary and Glennie-Holmes (1985).

2.2.16 ß-glucanase determination

Levels of the enzyme ß-glucanase were measured using a dye linked barley azo-ß-glucan substrate as described by McCleary and Shameer (1987).

2.2.17 Determination of potential enzyme activity from isolated aleurone

Chariot and Target grains were dehusked using a small-scale pearling machine, de-embryonated and the distal end then excised. The seeds were surfaced sterilized in 0.1% (v/v) sodium hypochlorite for 20min, rinsed in 1mM HCl, further rinsed in water and finally transferred to bottles containing sterile water. The grains were incubated for 36h before separating the aleurone layers from the starchy endosperm under sterile conditions. Isolated

aleurones from 10 grains were then incubated in 0.5mM sodium acetate and 10mM calcium chloride (pH 5.0 with acetic acid), containing 0.05%(w/v) ampicillin and 0.05%(w/v) chloramphenicol. Gibberellic acid (GA; 10^{-6}M) was added to half the samples. The supernatant was collected from aleurones incubated for 48h and 96h and the aleurones themselves were homogenised in a pestle and mortar with a small amount of sand and 2ml of aleurone buffer (100mM sodium chloride, 10mM calcium chloride and 50mM sodium acetate (pH 5.7 with acetic acid)). The activation of the aleurone and the release of enzymes into the buffer was then assessed in terms of α-amylase activity using ß-limit dextrin as substrate, based on the method of Smith *et al.*, (1978).

3.0 RESULTS AND DISCUSSION

3.1 Malting performance

During malting, the aim is to achieve uniform degradation of all areas of the endosperm. Malt analysis for the three varieties used in this study ranked Chariot better than Blenheim which in turn was better than Target (Table III). This variation in performance can be compared with differences in water uptake and its redistribution with underlying variation in endosperm structure.

3.2 Endosperm morphology

Structural examination of the endosperms, from both malting and feed varieties of barley, by light (Figure 1a) and scanning electron microscopy (Figure 1b) has shown that steely areas of barley were densely packed with cell wall polymers, such as ß-glucans, proteins and starch granules. These differences in endosperm structure occur randomly and there are many different patterns which emerge from different sections of the grain. Consequently, the identification, selection and quantification of steeliness could be problematic. For example, Figure 2 shows that steely areas could be missed completely depending on the way in which the section is taken. Therefore, structural differences have been further identified in whole corns of dehusked barley using a light transflectance method.

The light transflectance method, originally used to classify wheat grains according to their endosperm structure (Chandra, Thornton, and Palmer, unpublished observation), has been further developed to study the structure of the barley and to identify areas which may be hard to modify. Unlike wheat, however, threshed barley retains its husk and this obstructs the path of light. Dehusking with 50%(v/v) sulphuric acid was found to be unsuitable since this altered

the morphology of the outer layers of the endosperm and blocked light at the grain surface. Pearling, by abrading the grain against a wire mesh, proved a suitable and more rapid alternative and was used to prepare grains for light transflectance studies. The white, mealy areas of the grain absorb light and appear dark and opaque whereas steely areas appear translucent (Figure 3). Certain steely areas were found to include pockets of mealy endosperm, and were further sub-divided into completely steely (S) and partly steely (M/S).

Table IV shows the percentage M, S and M/S grains of each variety identified using the light transflectance method. These samples were obtained from the National Institute of Agricultural Botany (NIAB) variety trials and were either untreated or treated with fungicide. The untreated samples show the performance of a variety when disease has been allowed to develop naturally whereas the treated samples which are normally used for commercial production shows the full potential of a variety.

Application of fungicide to the growing plant was one of the factors which was found to influence endosperm structure. Fungicide treatment increased the mealiness of the malting varieties, Blenheim and Chariot but, in the feed variety Target, fungicide-treated plants still produced a large proportion of grains which were mainly steely but also partly mealy/steely. The retention of healthy leaves in fungicide-treated plants will improve grain filling, by extending the time available for transport of photosynthetic products into the ear, but does not totally overcome varietal tendencies to produce mealy or steely grains.

3.3 Water uptake

During steeping the rate of water uptake was similar for both Chariot and Target (Figure 4). although in the initial period of steeping Target had a slightly lower moisture content than Chariot. During the second steep water uptake in Target was initially slow, but water continued to be absorbed so that the final moisture content was similar to that of Chariot. This slower rate of uptake during the first part of second steep may be due to slower redistribution of moisture in Target.

3.4 Water distribution

The iodine vapour method (Davies, 1991) was used to study moisture redistribution in both malting and feed grade varieties. This technique was modified to take into account the effect of structural differences in the samples. Table V shows how the relative proportions of mealy and steely areas affect hydration. The iodine vapour score (IVS) shows that, in general, malting varieties hydrated better than did the feed variety and also that mealy grains hydrated better than the steely grains. There was an apparent anomaly, in that mealy areas in Target hydrated as well as or better than mealy areas in the malting varieties. In Target, however, this was greatly offset by a much higher proportion of poorly hydrating steely areas. Consequently, the percentage of mealiness and steeliness must be taken into account when calculating an overall hydration value (OHV) for each variety. Thus Chariot and Blenheim attained better overall hydration than did Target.

3.5 Protein distribution

Differences in grain morphology which are thought to affect water distribution may be due to variations in the proportion and distribution of protein and ß-glucans. It is generally

thought that high nitrogen barleys are steely, however, high nitrogen does not necessarily correlate with endosperm steeliness. Some malting varieties with high protein contents can contain a greater proportion of mealy grains whilst a feed variety, at a similar nitrogen levels can produce grains which are mainly steely. The distribution of nitrogen and hence protein was therefore measured for each variety to show where the nitrogen was deposited within the grain as total nitrogen (TN) increased. The combustion method was selected as the best way to measure the concentration of nitrogen (protein) in selected parts of the grain. Other methods, such as the BCA (Bicinchoninic acid) assay, were used to measure proteins but were found to be unsuitable for measuring total proteins from different parts of the grain due to limitations of the assay and the extraction procedures necessary.

Figure 5 shows the distribution of nitrogen in transverse sections of grain, illustrating the distribution (a) lengthwise from the embryo to the distal end along the length of the corn and (b) radially from the aleurone through to the central endosperm. For a particular TN level the nitrogen was mainly concentrated in two areas : the embryo and the distal region. Within the endosperm, the outer layers contained more protein than the inner mid-endosperm.

A comparison between varieties containing different proportion of protein has shown that, for malting varieties, the extra protein is associated with the embryo. For example, Chariot at a TN of 1.81% has 4%(w/w) in the embryo, whereas Target at 2.03% TN contains only 3.1%(w/w) in the embryo. This concentration of protein in the embryo results in lower proportion of protein in the Chariot endosperm in comparison to Target, which has more of the extra protein in the central endosperm where it is more likely to be stored as hordein. The extra protein in the Chariot embryo may help promote healthy roots and shoot formation. In

Target, on the other hand, the extra hordein in the endosperm will interfere with water and enzyme distribution and thus reduce the homogeneity of modification.

Proteins were further characterized using the SDS-PAGE method as shown in Figures 6-9. The banding pattern was essentially the same in grains from a single variety with different TN levels (Figure 6) and also in different sections (Figures 7 and 8) of the grain. Although, the banding pattern was the same, the amount of protein in each band varied. The main differences in banding were found between the embryo and the endosperm.

3.6 Protein degradation

Total protein extracts of malt showed that certain high molecular weight proteins were degraded during the malting process (Figure 9). Therefore, hordein degradation during malting was studied by SDS-PAGE as shown in Figures 10 and 11. A comparison was made between barley, whole malt and friable / non-friable malt fractions from a friabilimeter. Hordeins were extracted in the presence of protease inhibitors [Phenylmethylsulfonyl fluoride (PMSF), Ethylenediaminetetra acetic acid (EDTA), Leupeptin, and Pepstatin] to see if protein breakdown occurred during extraction. No changes were seen in the protein profile on addition of the protease inhibitors, thus confirming that the degradation of hordeins occurred during malting and was not an artifact of extraction. Hordeins were extracted as two fractions. The first fraction, Hordein I, was extracted in aqueous alcohol which would extract mostly the low molecular proteins. The second fraction Hordein II, contained a reducing agent in addition to alcohol to promote the breakdown of the disulphide groups present in high molecular weight hordeins. Hordeins with molecular weights of 34kD and 97kD (γ- and D-hordeins, respectively) were found in barley and non-friable fractions but were completely

degraded in the malt and friable fraction. These hordeins are therefore important since their degradation is associated with malt friability.

An immuno-gold method was developed to locate hordein in the grain endosperm. Figures 12a to 12c show the binding of antibodies (Table VI) specific to barley hordeins. Figures 13a and 13b show examples of the distribution of γ and C hordeins in the Target barley endosperm. The γ-hordeins were present in the outer endosperm and sub-aleurone areas whereas the C-hordeins were evenly distributed throughout the central endosperm (Table VII). C-hordeins were found in both mealy and steely areas, while γ-hordeins were absent in the mealy endosperm and concentrated in the steely areas (Table VIII).

Chariot malt showed better overall hordein modification than Target (Table IX). Generally, the hordein modification in the proximal endosperm for both the varieties was good but the central and distal areas of Target were less modified than in Chariot.

3.7 ß-Glucan distribution

Figure 14 shows that there can be substantial differences in ß-glucan between mealy and steely grain of some varieties. Steely grains not surprisingly contained higher levels of ß-glucan. In Chariot the total levels were generally low with the ß-glucan concentration of both mealy and steely grains being similar. In Blenheim the ß-glucan levels were higher in steely grains when compared to Chariot. Target, on the other hand, had ß-glucan levels in mealy grains similar to Chariot but the steely grains contain nearly twice the amount of the mealy grains. As the latter comprise 70% of the grains (Table IV) this means that the overall ß-glucan content of the feed variety was significantly higher than for the malting varieties.

Figure 15 shows the distribution of ß-glucan within grains. It is apparent that both proximal and distal areas of the endosperm of the feed variety Target contained more ß-glucan than the comparable areas in Chariot. Similar ß-glucan levels were, however, found in the central endosperm of both varieties.

3.8 ß-Glucan degradation

During malting the ß-glucans were well degraded in both the lager and ale malts of malting varieties (Figure 16). However, a large amount of ß-glucan remained in the feed variety even after treatment with gibberellic acid. A possible reason for this was the higher concentration of ß-glucan present in the proximal endosperm of this barley. Most of the degradation of ß-glucans occurs during germination although, since ß-glucan solubilase is present in raw barley the process of cell wall attack is presumed to start as soon as the barley is steeped (Bamforth and Martin, 1981a). The levels of ß-glucan solubilase in Blenheim and Chariot barleys were much higher than in Target, although levels in all three malts were similar (Table IX). Therefore, during malting Blenheim and Chariot barley are better able to solubilise ß-glucan than is Target.

3.9 ß-Glucanase distribution

It is recognised that the normal pattern of modification (as determined by Calcofluor staining on horizontal longitudinal sections), is for cell wall breakdown to occur first in the cells adjacent to the scutellum, then to continue under the aleurone layer near the scutellum, and finally towards the distal end of the grain (Aastrup & Erdal, 1980). Alpha-amylase activity also follows a similar pattern (Gibbons, 1981; MacGregor and Matsuo, 1982). Any effects of mealy and steely areas of the endosperm, however, may not be apparent in a study using

horizontal half sections. In the current project, therefore, experiments were designed to examine the total ß-glucanase levels in pre-defined sections of whole corns.

Results in Figure 17 show that ß-glucanase activity remained very low in the feed variety during the first four days of germination and seemed to accumulate in the proximal end of the grain. The greatest increase was seen between days 4 and 5. In comparison, Figure 18 shows that in Chariot, ß-glucanase activity developed earlier, at day 3, and a greater proportion of the total activity was found in the central endosperm. The length of time the enzyme was effective is thus greater in this malting variety, resulting in better ß-glucan degradation. These data also showed that Chariot had higher levels of enzyme in the proximal endosperm earlier in germination. Since this is a primary area for water ingress, improved degradation of ß-glucans could provide a better access for the hydrolytic enzymes to distribute evenly throughout the endosperm.

The aleurone response experiments showed that production of enzymes by aleurone layers was similar for both varieties. The effects on the distribution of ß-glucanase may therefore be attributed to the changes in the morphological structure of the endosperm *per se* rather than differences in physiology.

4.0 CONCLUSIONS

Barley endosperm may be either mealy or steely in structure. Malting varieties such as Chariot and Blenheim contained significantly higher proportion of mealy grains than did the feed variety Target. Such large variations in their endosperm structures has resulted in a lower malting quality of Target in comparison to the malting varieties.

The ratio of mealy and steely grains within one variety can be affected by the application of fungicide to the growing crop.

Steely areas can be randomly distributed within the endosperm and are associated with higher concentrations of ß-glucan and protein. Steeliness appears to be a major factor restricting the even distribution of water across the endosperm during steeping. This is supported by the observation that the mealy grains in feed varieties are capable of hydrating as well as those in malting varieties. However, the high proportion of steely grains in the feed variety resulted in much poorer endosperm hydration overall.

Uneven distribution of water across the endosperm can itself affect the distribution and activity of key enzymes. The location of steely areas is therefore crucial. For example, high levels of ß-glucan associated with steely areas in the proximal endosperm of Target are thought to have restricted the distribution and activity of ß-glucanases, restricting the enzyme to the proximal end of the endosperm and preventing it from reaching the central endosperm in time to have any real effect in malting.

Steely areas of the endosperm are also associated with a specific protein, γ-hordein, which was not found in the mealy areas. Complete degradation of both 34kD (γ) and 97kD (D) hordein would appear to be necessary in order to obtain a friable malt. Observations also suggest that a feed variety such as Target is more likely to store extra nitrogen within the endosperm as hordeins rather than as soluble proteins within the embryo, thus adding to the difficulty in modifying higher nitrogen samples.

The morphological structure of the endosperm, therefore, impacts on malt quality by affecting water distribution. This will affect the release and extent of activity of various enzymes, thus changing the pattern of protein and cell wall breakdown. The proportion and location of steely areas within grains are thus crucial factors impinging on malt quality. This project has developed a method by which mealy and steely grains can be identified using light transflectance. This technique should provide a valuable tool for any future studies involving cereal morphology. Details are given in Appendix 1.

5.0 Appendix I

It is clear from this work that endosperm structure has a important role in determining the quality of barley for production of high quality malt and the quality of wheat for use in distilling. The structural differences are clearly visible as mealy and steely areas in a whole corn by using the light transflectance method (Figure 2). Currently the system is used by visually discriminating grains into mealy and steely groups, however, it is not possible to assess the area covered by mealy and steely parts of the grain endosperm. At BRF International we have further developed this method for use in an instrument called "Light Transflectance Meter". A prototype is shown in Figure 19. This can be used to quantify the mealiness or steeliness of a sample. The prototype can only analyse single grains but this could be further developed to accommodate 50 to 100 grains simultaneously. The endosperm steeliness could then be related to extracts in barley malt and spirit yield from wheat. This method currently being developed and is to be trialled in the malting and distilling industry against current methods.

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List of Figures

Figure 1. Characterization of mealy and steely areas in barley endosperm

(a) Light microscopy analysis

(b) Scanning electron micrographs

M= Mealy; S= Steely and M/S= Mixture of both mealy and steely endosperm.

Figure 2. Problems associated with the study of single sections of grain. Shows how structural differences in the endosperm could be missed when studying single cut areas of the grain.

Figure 3. Light transflected barley grains. Differences in endosperm morphology was identified as mealy and steely areas. The mealy areas (M) absorb light and appear dark and the steely areas (S) transflect light and appear translucent.

Figure 4. Rate of water uptake. Rate of water uptake in Chariot and Target barleys during malting using the steep schedule 8:16:24.

Figure 5. Distribution of nitrogen in different parts of endosperm of malting and feed varieties.

Figure 6. Effect of Total nitrogen on protein composition

Lane number: S- Molecular weight markers

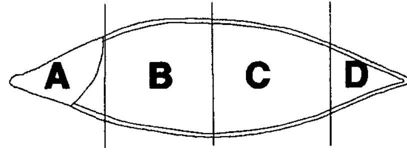
1- Blenheim barley at TN 1.54

2- Blenheim barley at TN 1.60

3- Blenheim barley at TN 1.91

4- Blenheim barley at TN 1.93

Figure 7. SDS-PAGE of transverse sections of Blenheim barley



Lane number: 1- Section A from Blenheim barley (TN 1.4)

2- Section B from Blenheim barley (TN 1.4)

3- Section C from Blenheim barley (TN 1.4)

4- Section D from Blenheim barley (TN 1.4)

S- Molecular weight markers

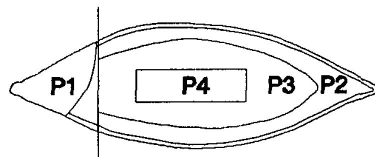
1- Section A from Blenheim barley (TN 1.9)

2- Section B from Blenheim barley (TN 1.9)

3- Section C from Blenheim barley (TN 1.9)

4- Section D from Blenheim barley (TN 1.9)

Figure 8. SDS-PAGE of pearled sections of Blenheim barley



Lane number: S-Molecular weight markers

1- Section P1 from Blenheim barley (TN 1.4)

2- Section P2 from Blenheim barley (TN 1.4)

3- Section P3 from Blenheim barley (TN 1.4)

4- Section P4 from Blenheim barley (TN 1.4)

S-Molecular weight markers

1- Section P1 from Blenheim barley (TN 1.9)

2- Section P2 from Blenheim barley (TN 1.9)

3- Section P3 from Blenheim barley (TN 1.9)

4- Section P4 from Blenheim barley (TN 1.9)

Figure 9. Degradation of proteins during malting. SDS-PAGE (10% acrylamide gel) of 2% (w/v) SDS + 5% (v/v) 2-mercaptoethanol extract of Blenheim and Sprite barleys and malts.

Lane number: S- Molecular weight markers

1- Blenheim barley

2- Blenheim malt

S- Molecular weight markers

3- Sprite barley

4- Sprite malt

Figure 10 Degradation of Hordein I during malting. Degradation of low molecular weight hordein I fraction extracted in propanol with and without protease inhibitors from: B: Barley; M:Total malt F: Friable fraction and NF: Non-friable fraction from friabilimeter.

Figure 11 Degradation of Hordein II. Degradation of high molecular weight hordein II fraction extracted in Propanol + ME with and without protease inhibitors from: B: Barley; M:Total malt F: Friable fraction and NF: Non-friable fraction from friabilimeter.

Figures 12a-c Distribution of proteins in barley endosperm. Key: LS - Large starch granules; SS - Small starch granule; P - Protein; CW - Cell wall. Scanning electron micrographs of hordein distribution in barley endosperm (a) General distribution; (b) Shows specific binding to matrix protein without binding to starch (c) Silver enhanced gold particles which can be visualized using scanning electron microscopy to study the binding of antibody to protein.

Figure 13 a-b Distribution of protein in barley endosperm. Key: P - Protein; Ad1 - Digital image of grain; AgL - Digital map of silver enhanced immuno-gold labelled proteins. Digital mapping of silver in grain endosperm using x-ray microanalysis. (a) Distribution of C-hordeins (b) Distribution of γ-hordeins.

Figure 14 ß-glucan content of mealy and steely barley grains. Total ß-glucan content of mealy and steely grains of cultivars Chariot, Blenheim and Target. The mealy and steely grains were selected from each variety using the Light Transflectance method.

Figure 15 ß-glucan distribution in barley endosperm of cultivars with differences in their morphological structure. Barley ß-glucan distribution in the endosperm of

cultivars Chariot and Target. The levels in the proximal endosperm, the central endosperm and the distal area are shown. Each area represents an average of 50 endosperms.

Figure 16 ß-glucan degradation during malting. A comparison of ß-glucan degradation during malting. Levels shown are in barley, lager malt (malted without gibberellic acid), ale malt (malted with gibberellic acid).

Figure 17 ß-glucanase activity during germination in different areas of Target barley. Distribution of ß-glucanase in different areas of Target barley endosperm during germination. The areas are: embryo, proximal endosperm, central endosperm and distal endosperm.

Figure 18 ß-glucanase activity during germination in different areas of Chariot barley. Distribution of ß-glucanase in different areas of Chariot barley endosperm during germination. The areas are: embryo, proximal endosperm, central endosperm and distal area.

Figure 19 Instruments used in the evaluation of mealy and steely grains.
(a) Pearling Machine (b) Light Transflectance Meter.

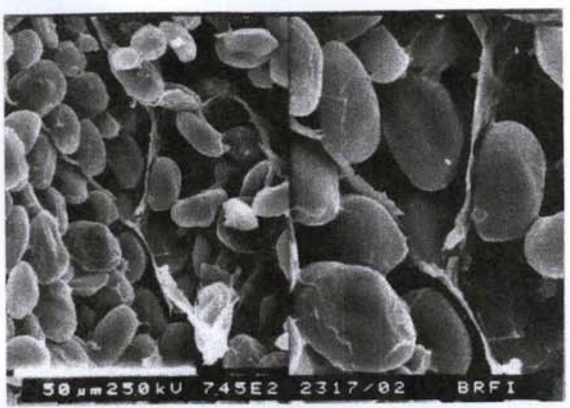
Figure 1. Characterisation of mealy and steely areas in barley endosperm.

M

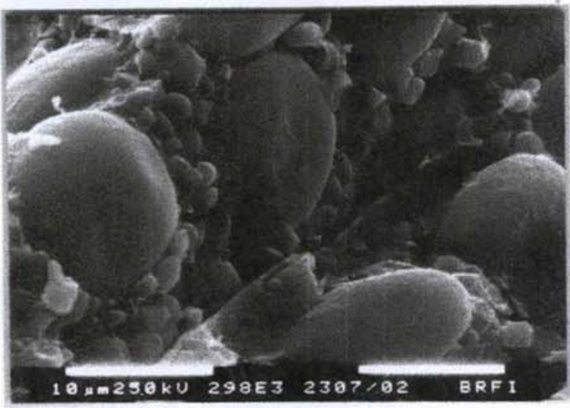
S

M/S

(a)



M



S

(b)

Figure 2. Problems associated with the study of single sections of grain.

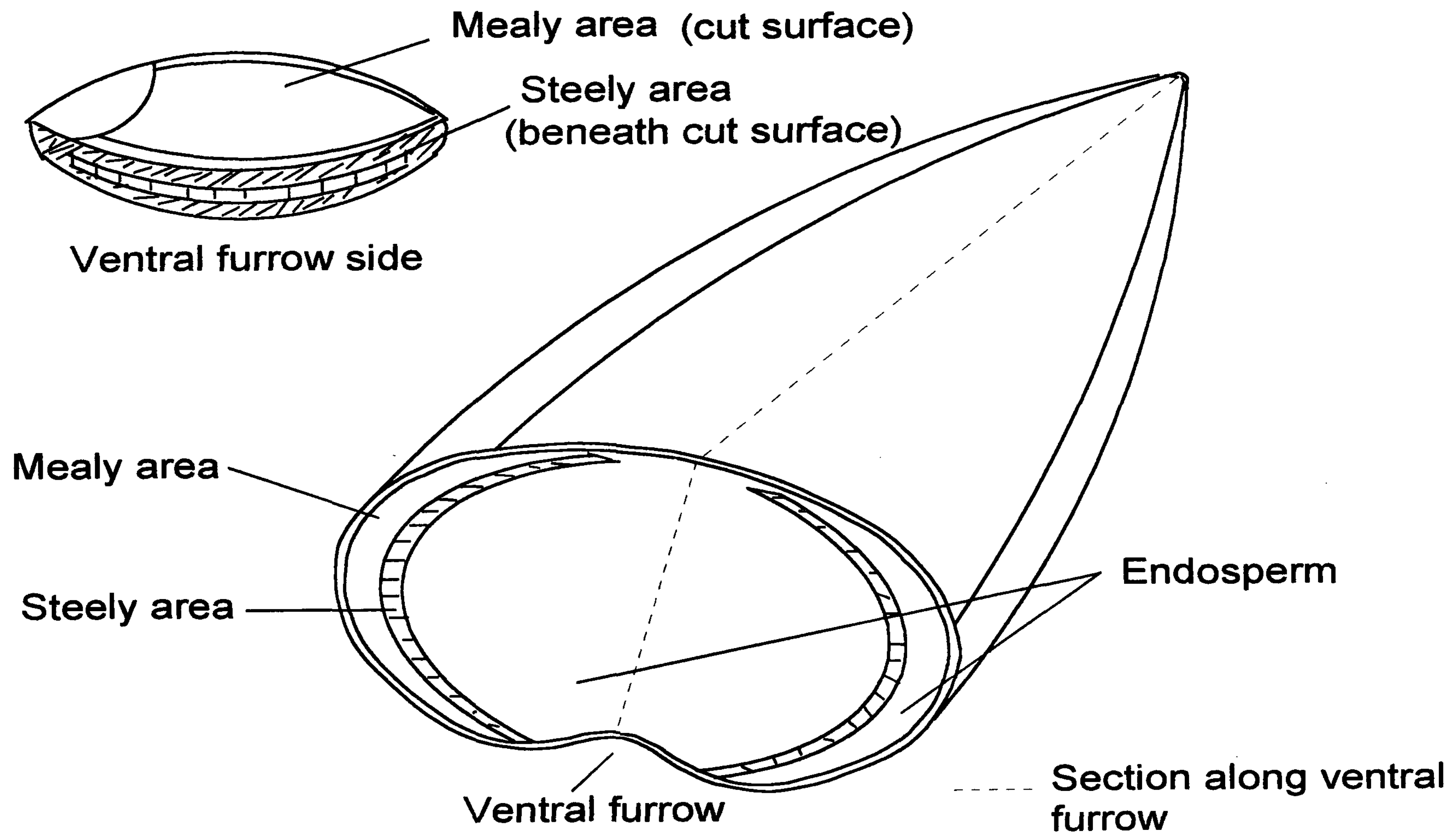


Figure 3. Light transflected barley grains.

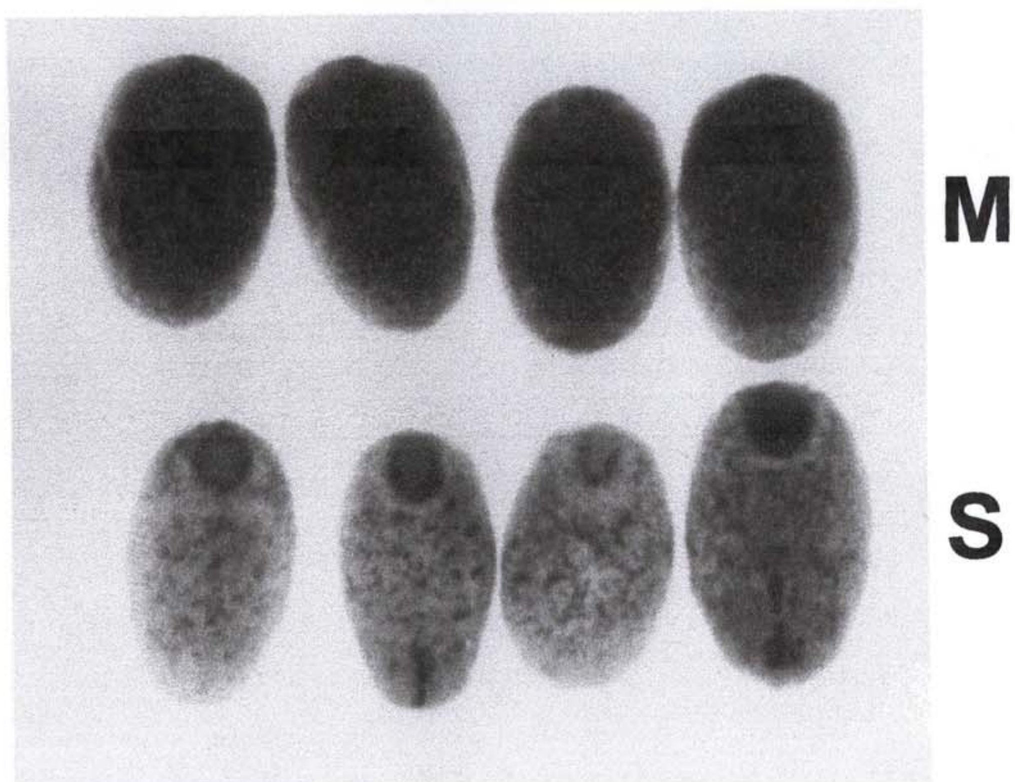


Figure 4. Rate of water uptake.

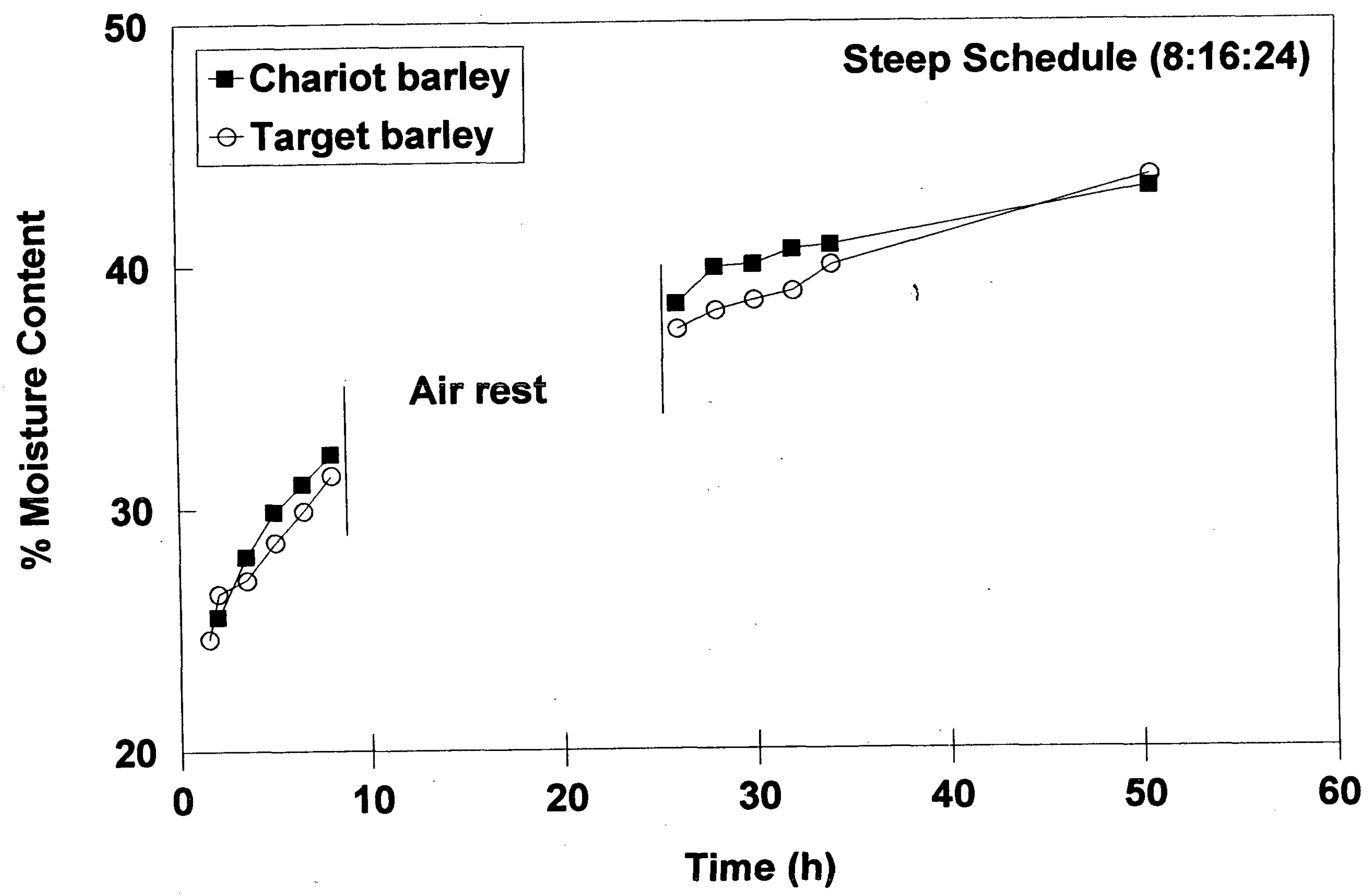
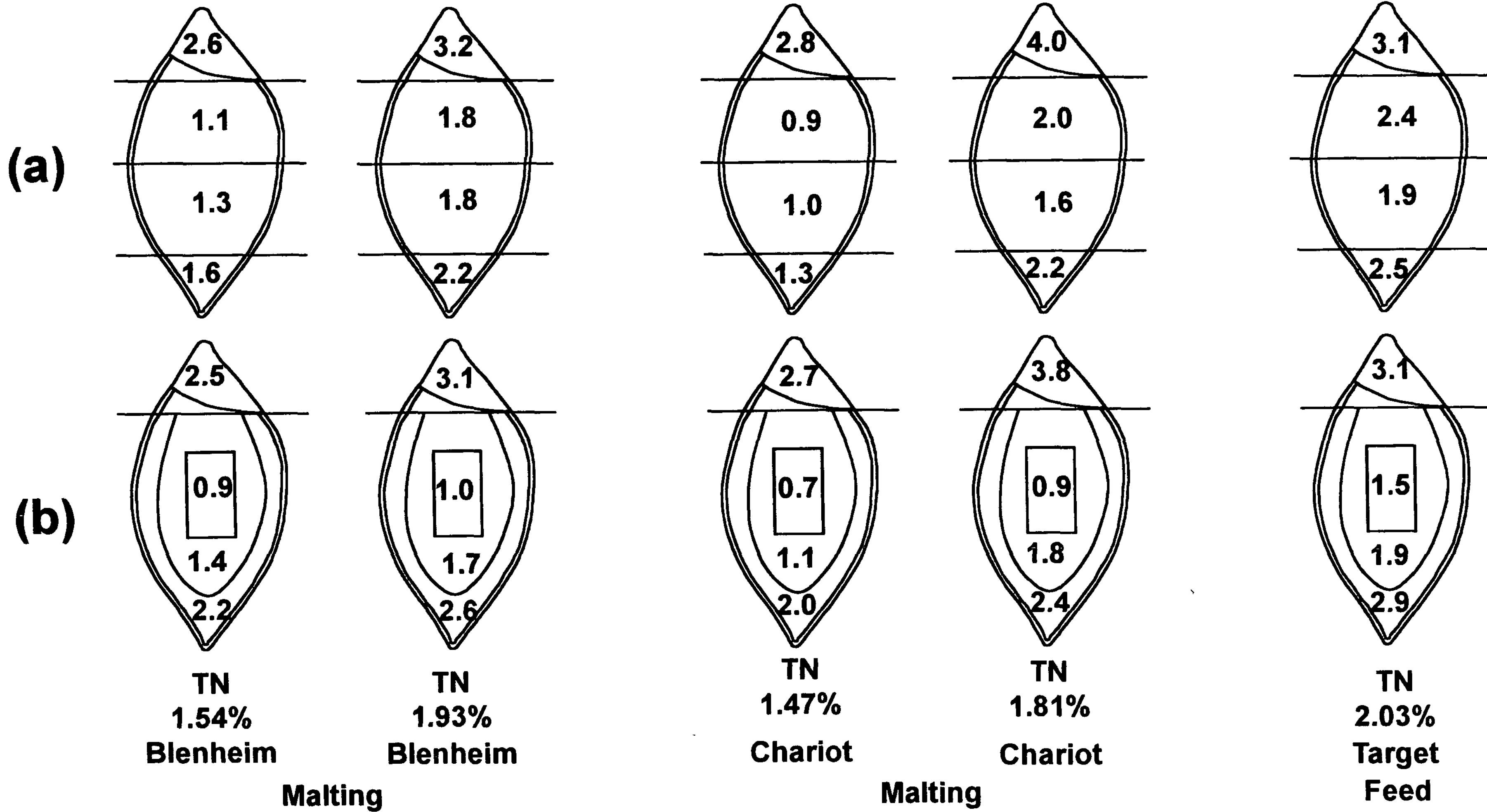


Figure 5. Distribution of nitrogen in different parts of endosperm of malting and feed varieties.



Units : % wt/wt (dry wt.)

Each section represents data averaged from 15 - 20 grains

Figure 6. Effect of total nitrogen on protein composition

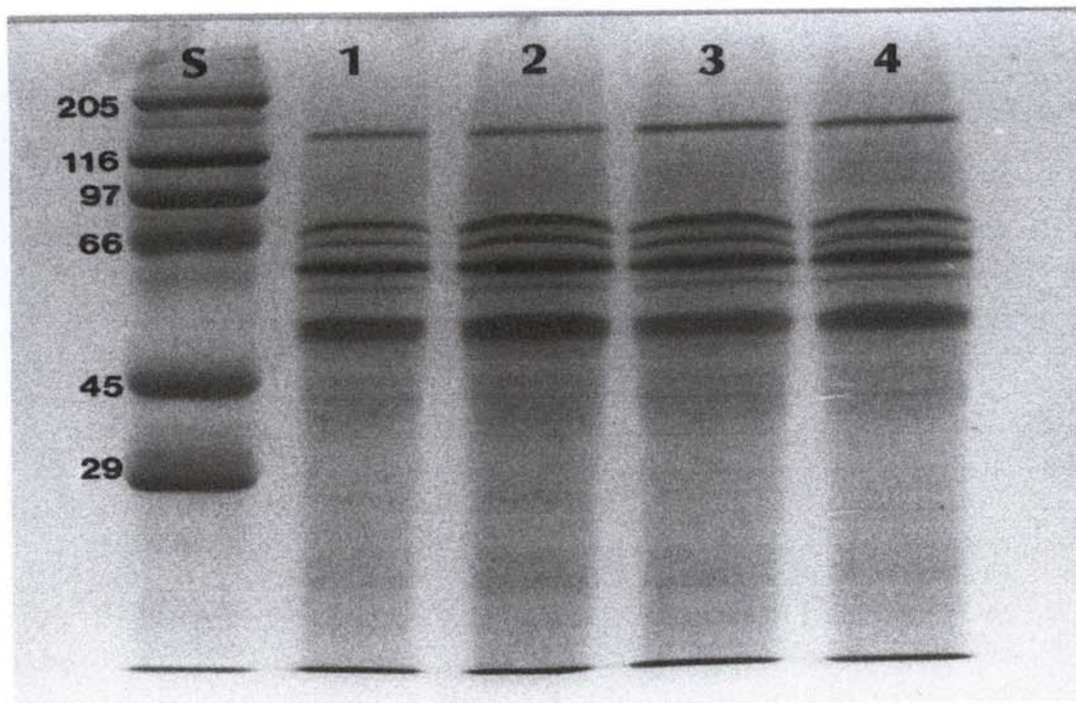


Figure 7. SDS-PAGE of transverse sections of Blenheim barley.

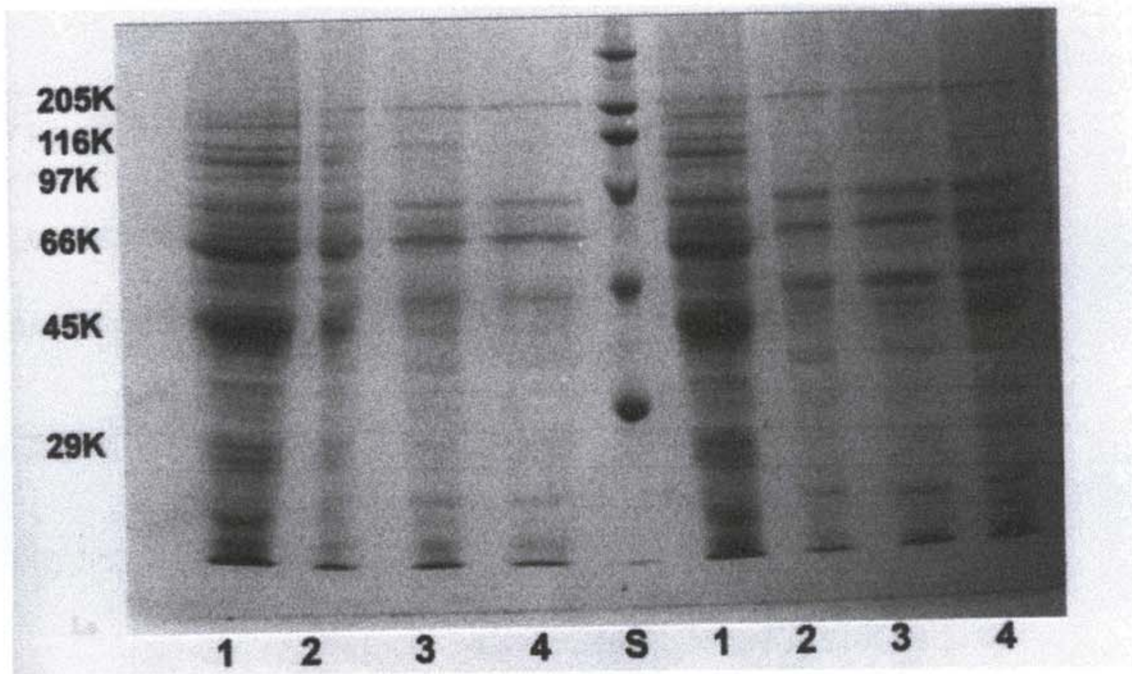


Figure 8. SDS-PAGE of pearled sections of Blenheim barley.

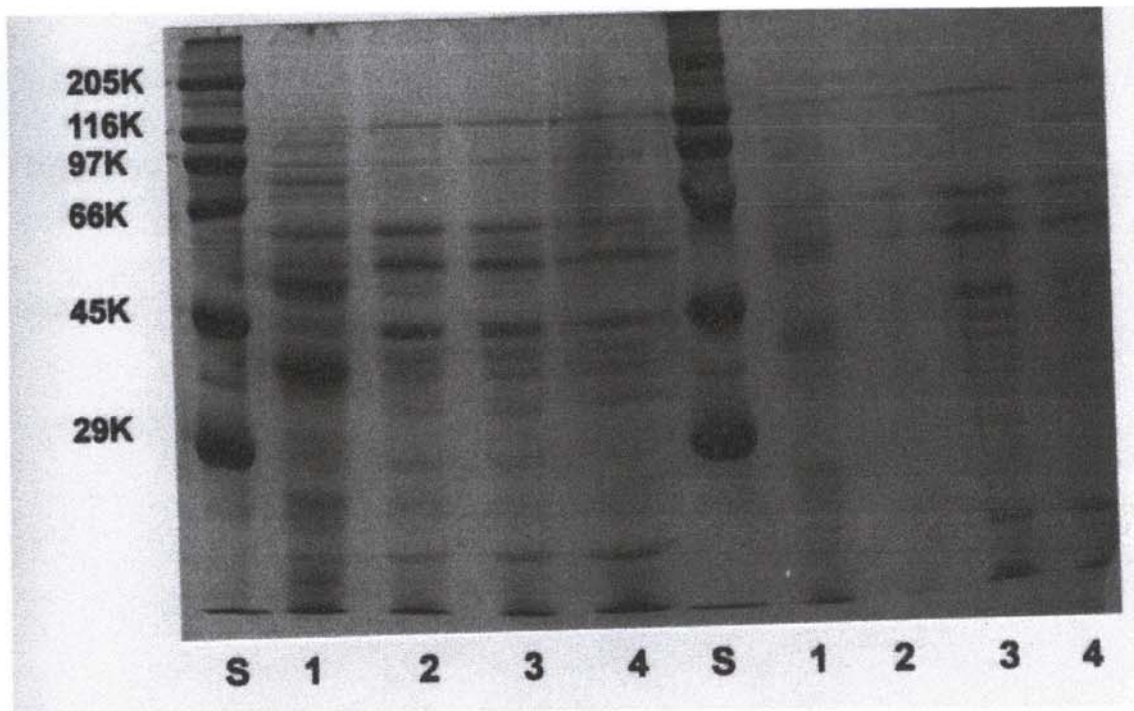


Figure 9. Degradation of proteins during malting.

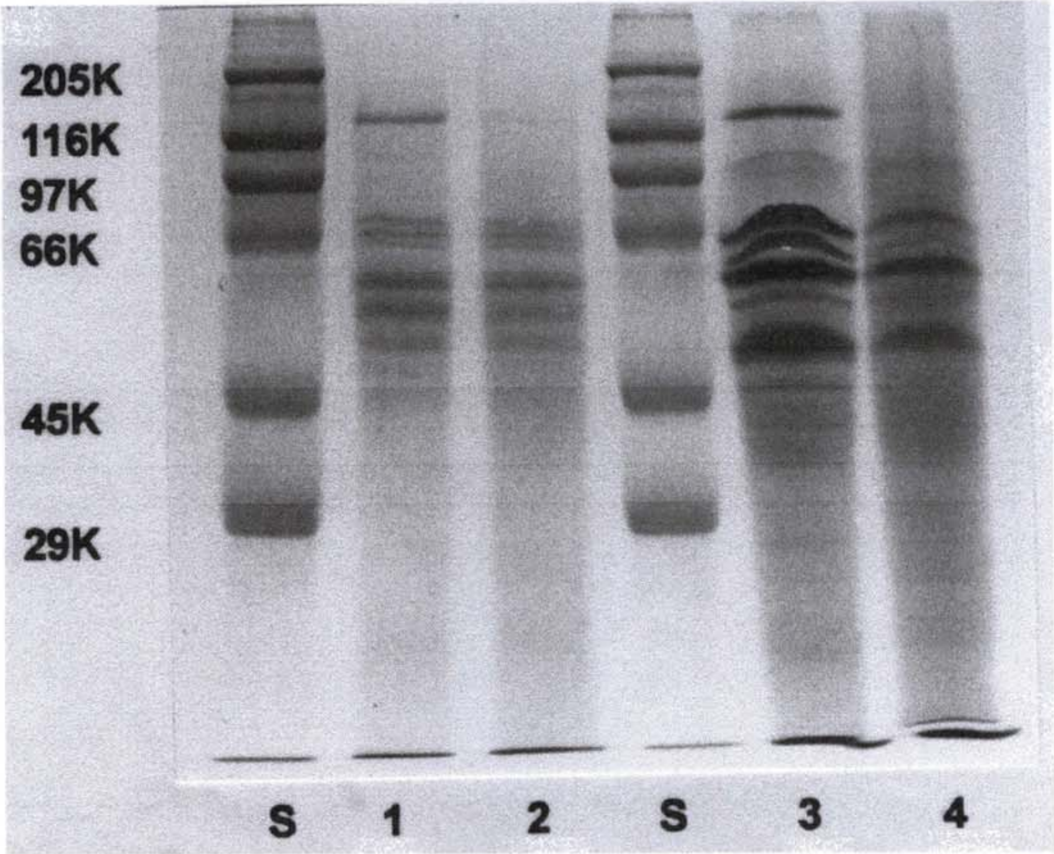


Figure 10. Degradation of Hordein I during malting.

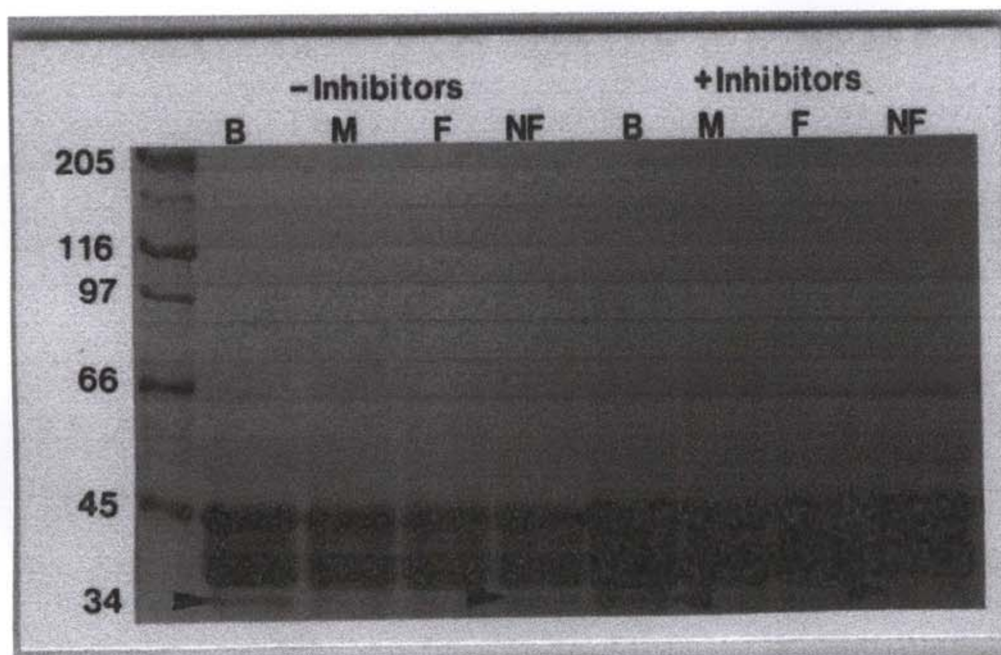
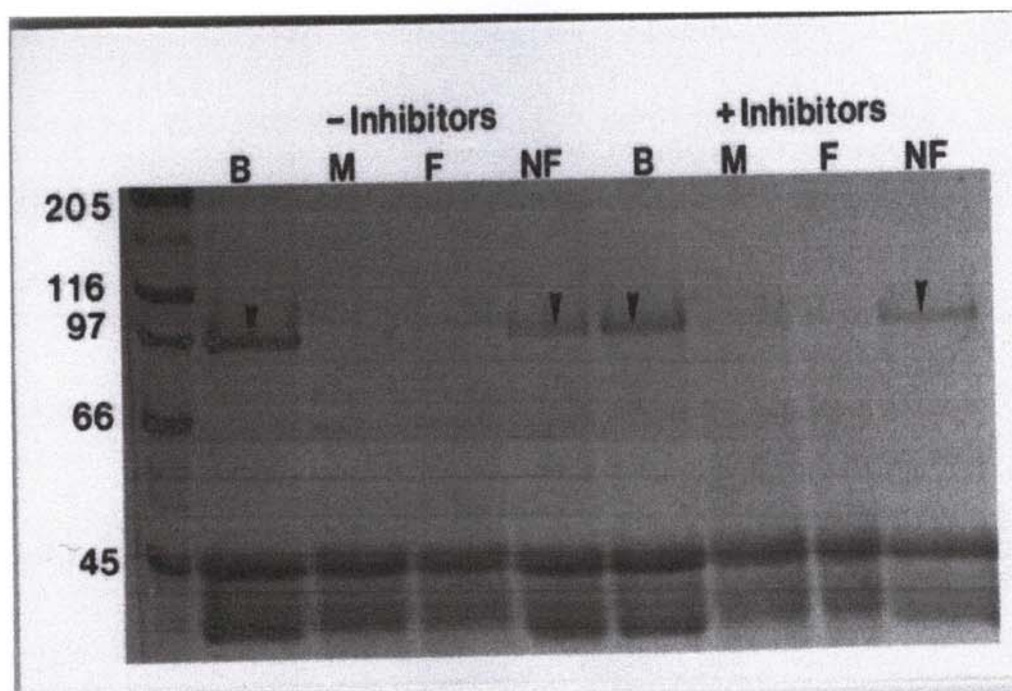
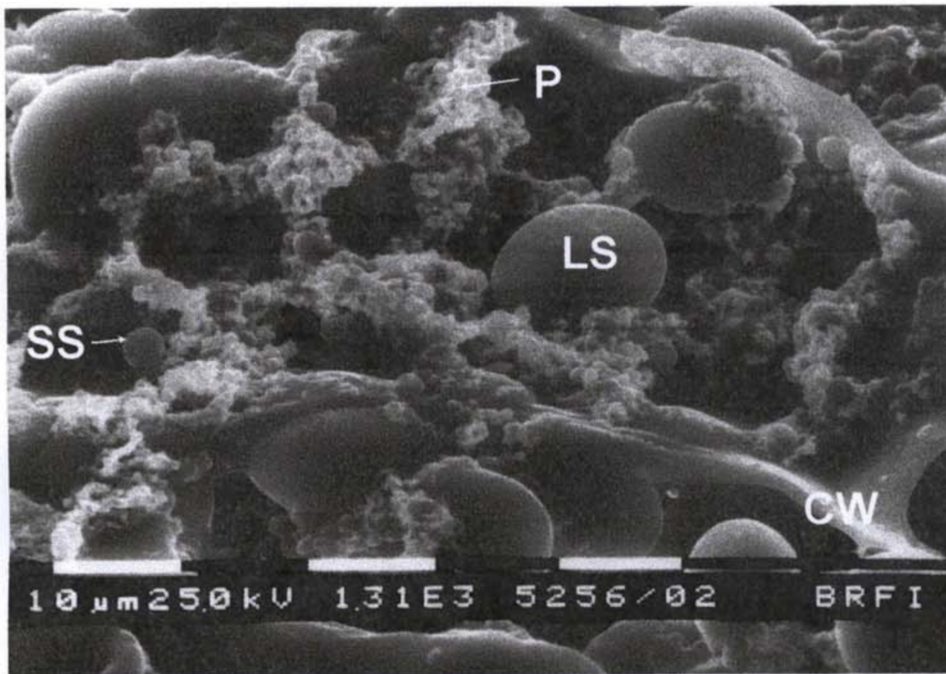


Figure 11. Degradation of Hordein II during malting.

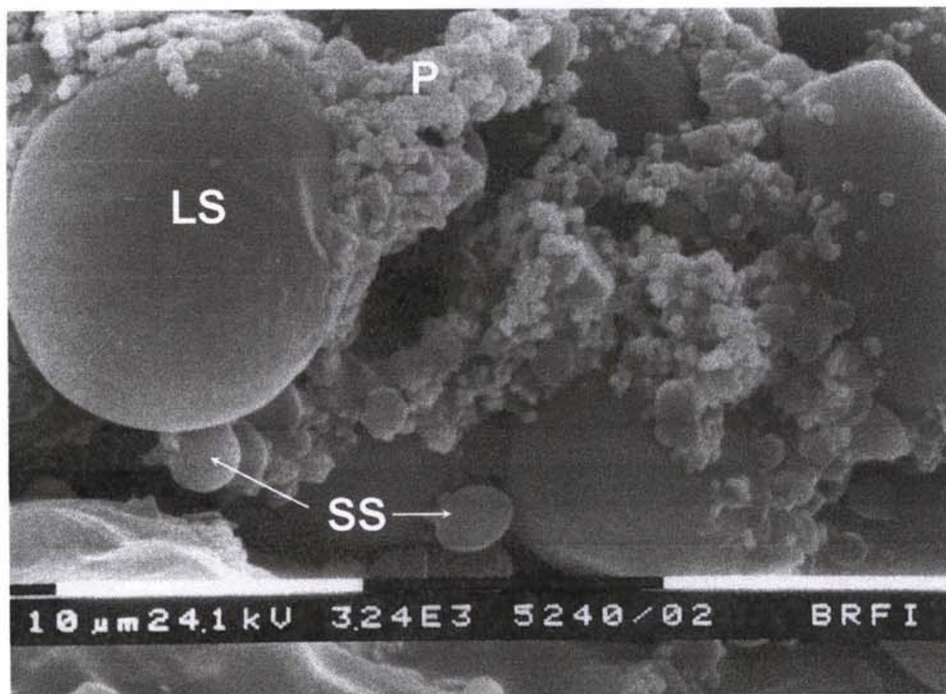


Protease inhibitors: PMSF(200µm); Leupeptin(1µm); EDTA(100µm) & Pepstatin(1µm)

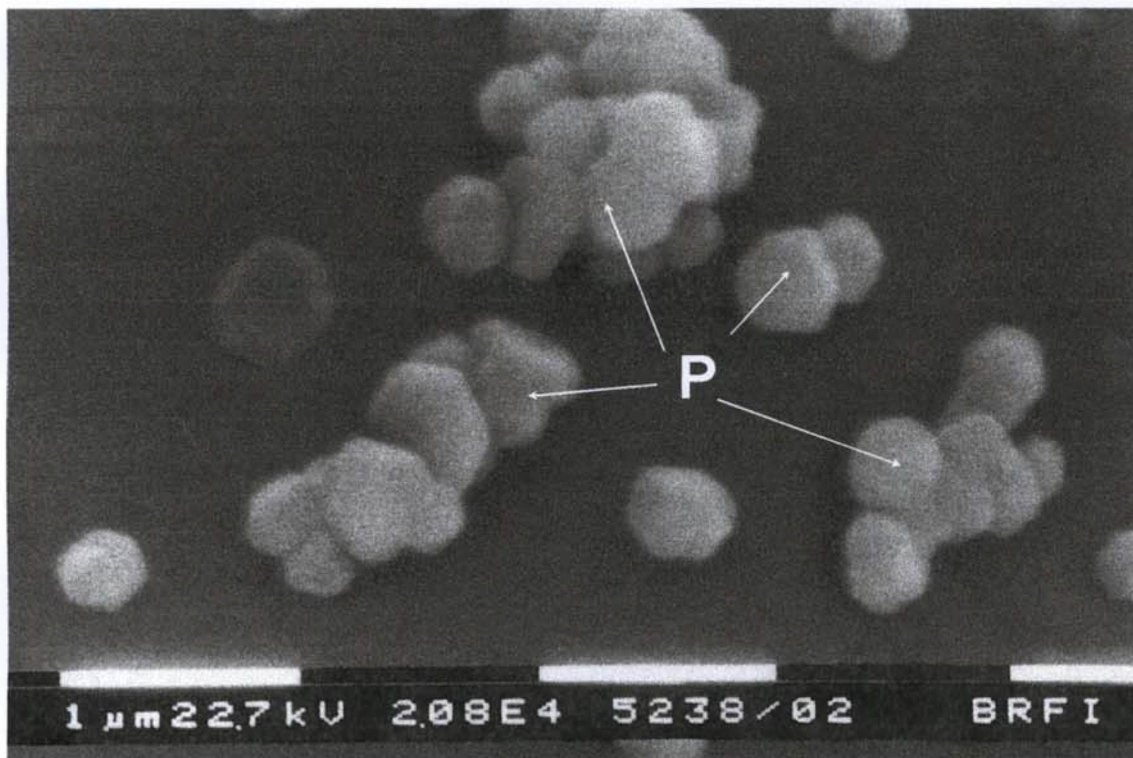
Figure 12. Distribution of proteins in barley endosperm.



(a)

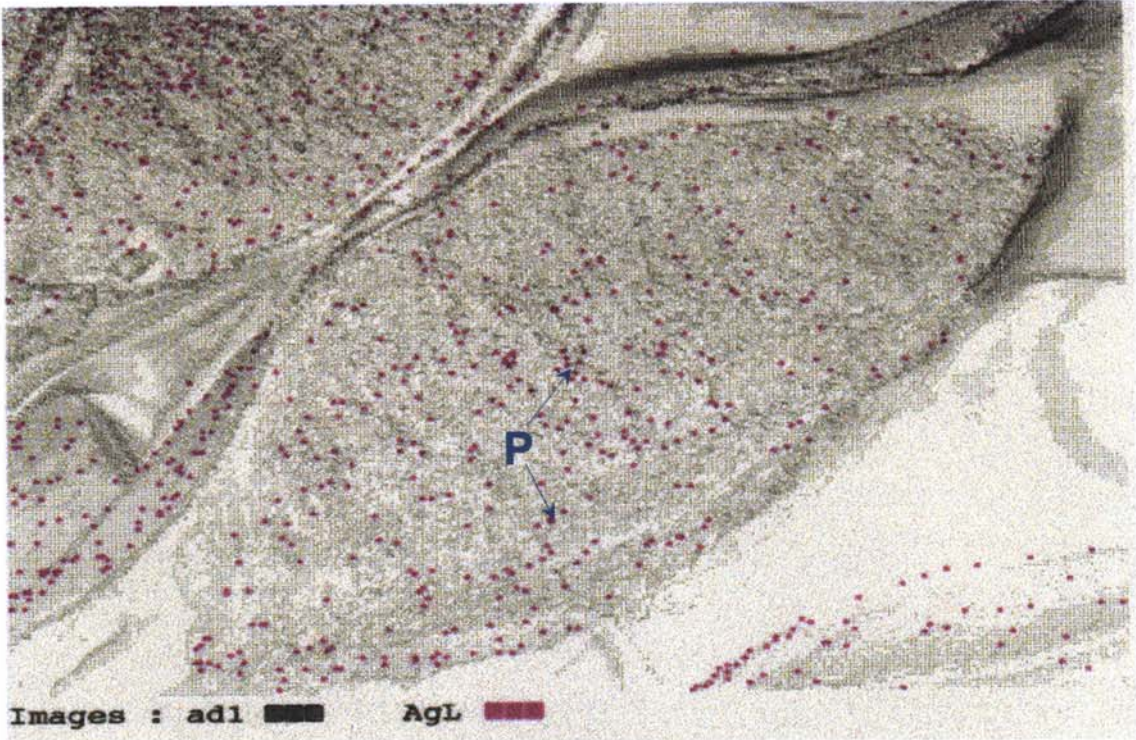


(b)

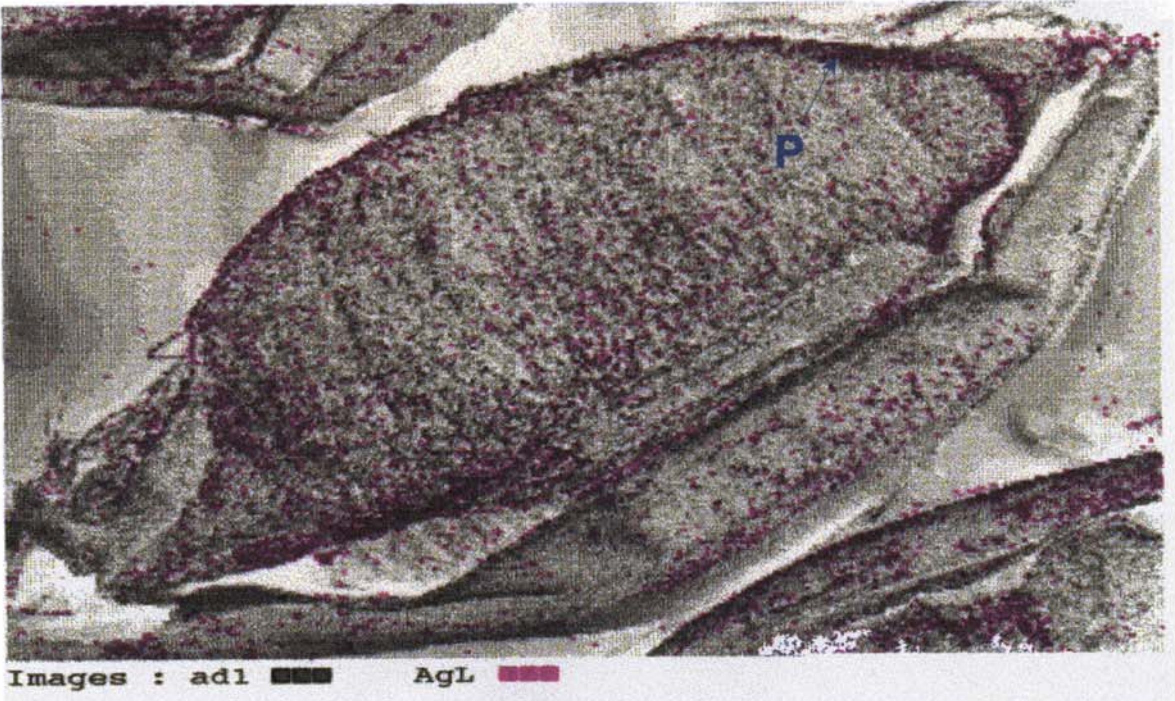


(c)

Figure 13. Distribution of proteins in barley endosperm.



(a)



(b)

Figure 14. ß-Glucan content of mealy and steely barley grains

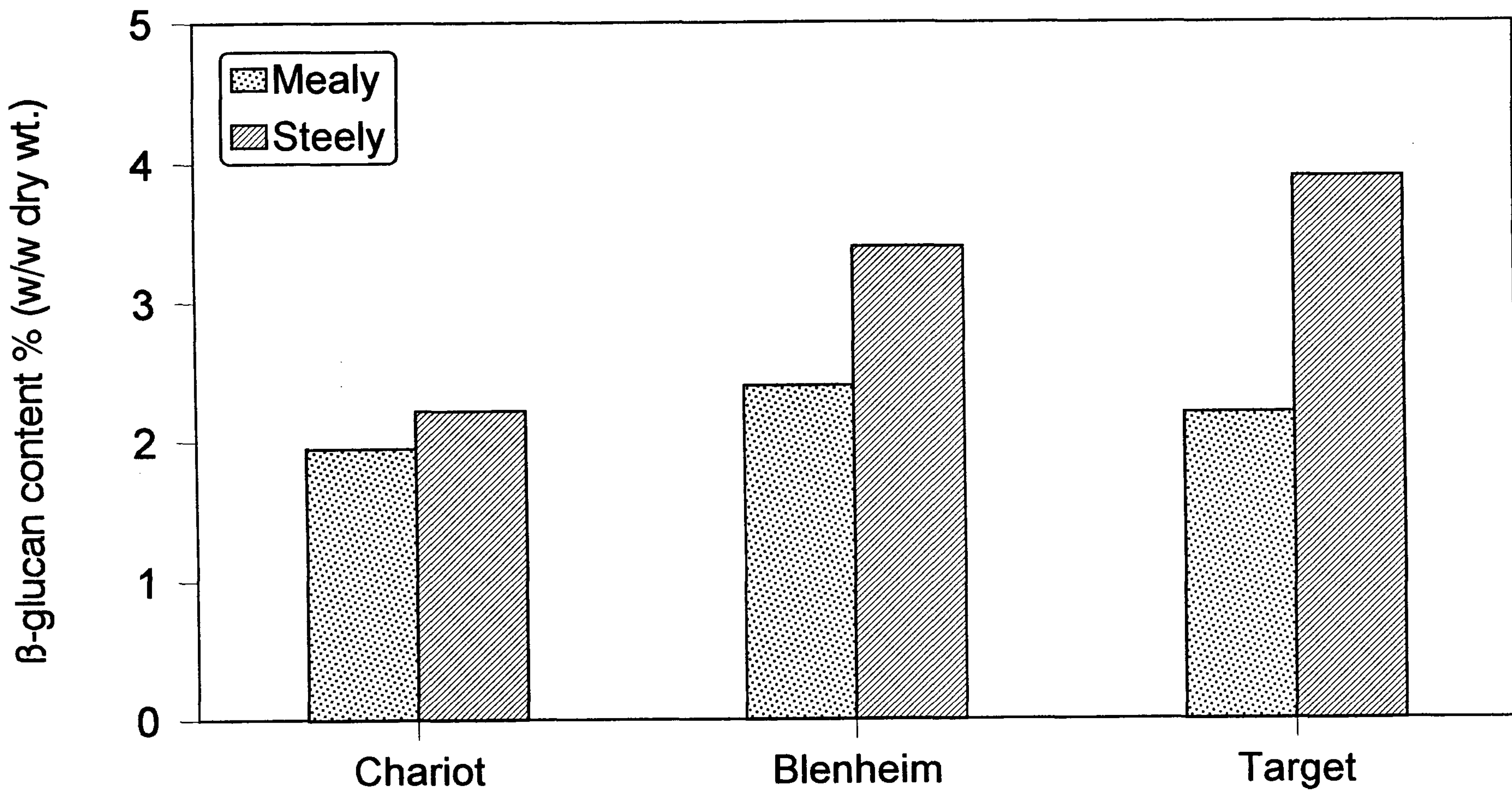
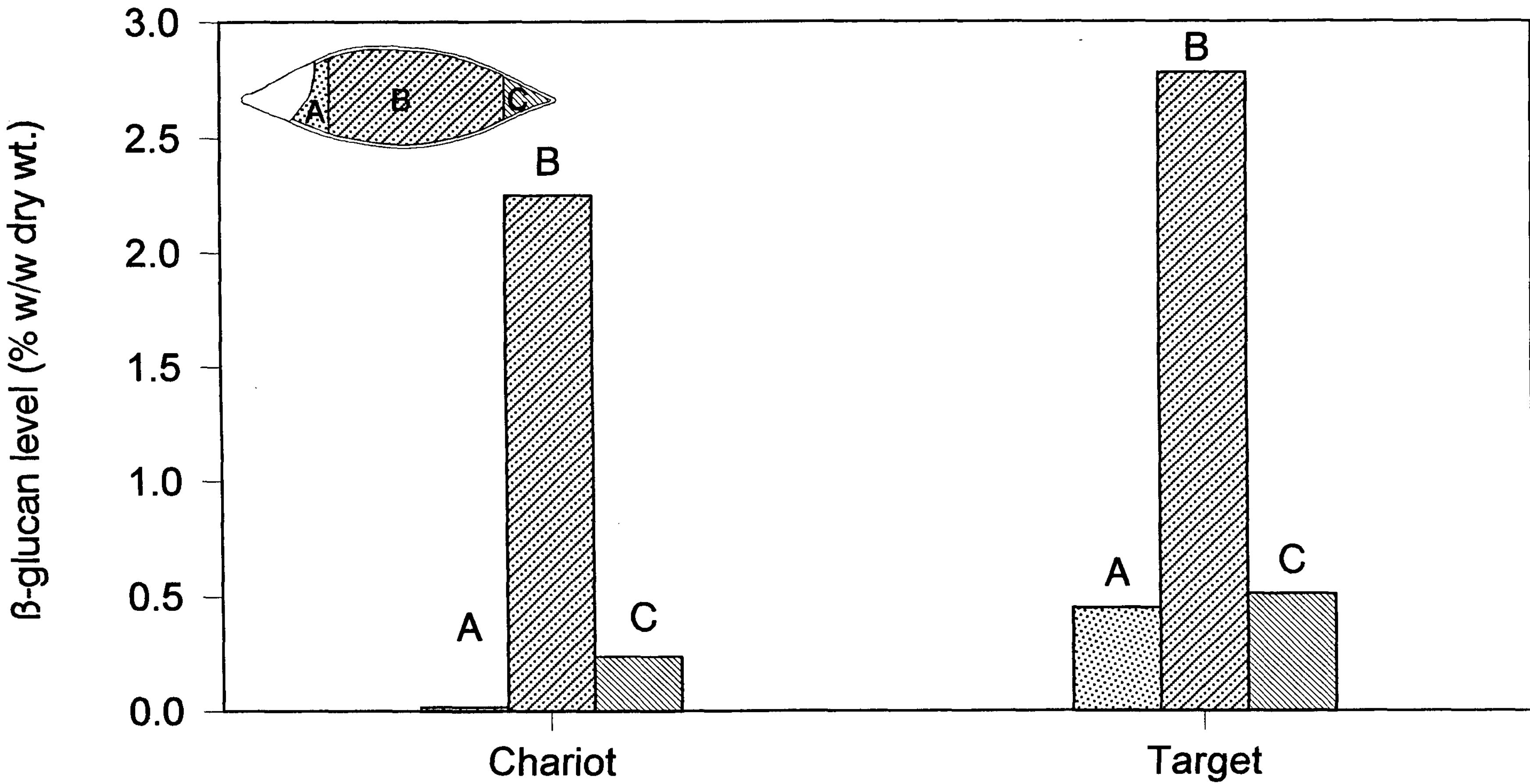


Figure 15. ß-Glucan distribution in barley endosperm of cultivars with differences in their morphological structure



Total Levels - Chariot 2.2 (% w/w), Target 3.1(%w/w)
Each area represents an average of 50 grains

Figure 16. ß-Glucan degradation during malting

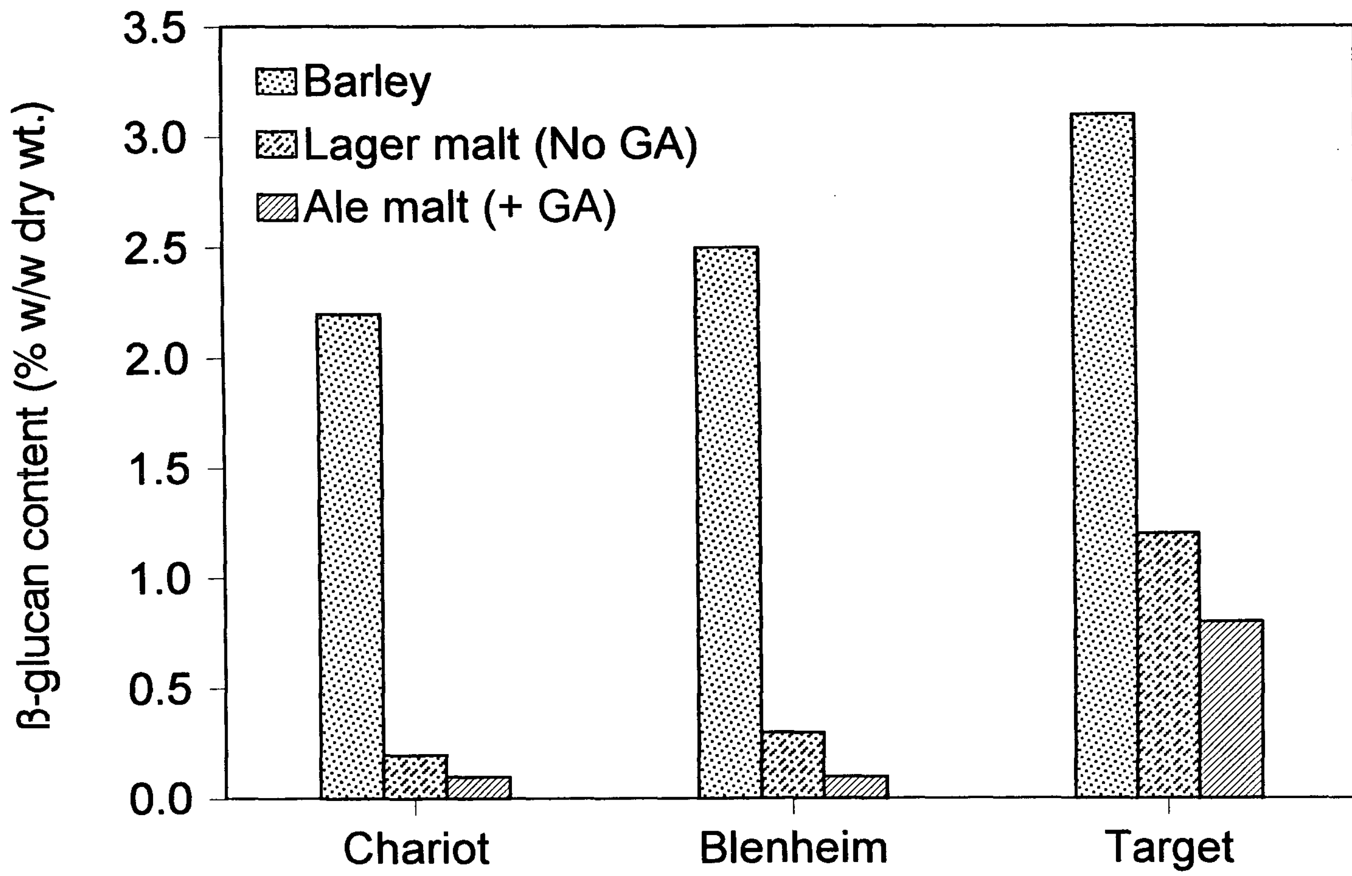


Figure 17. ß-Glucanase activity during germination in different areas of Target barley

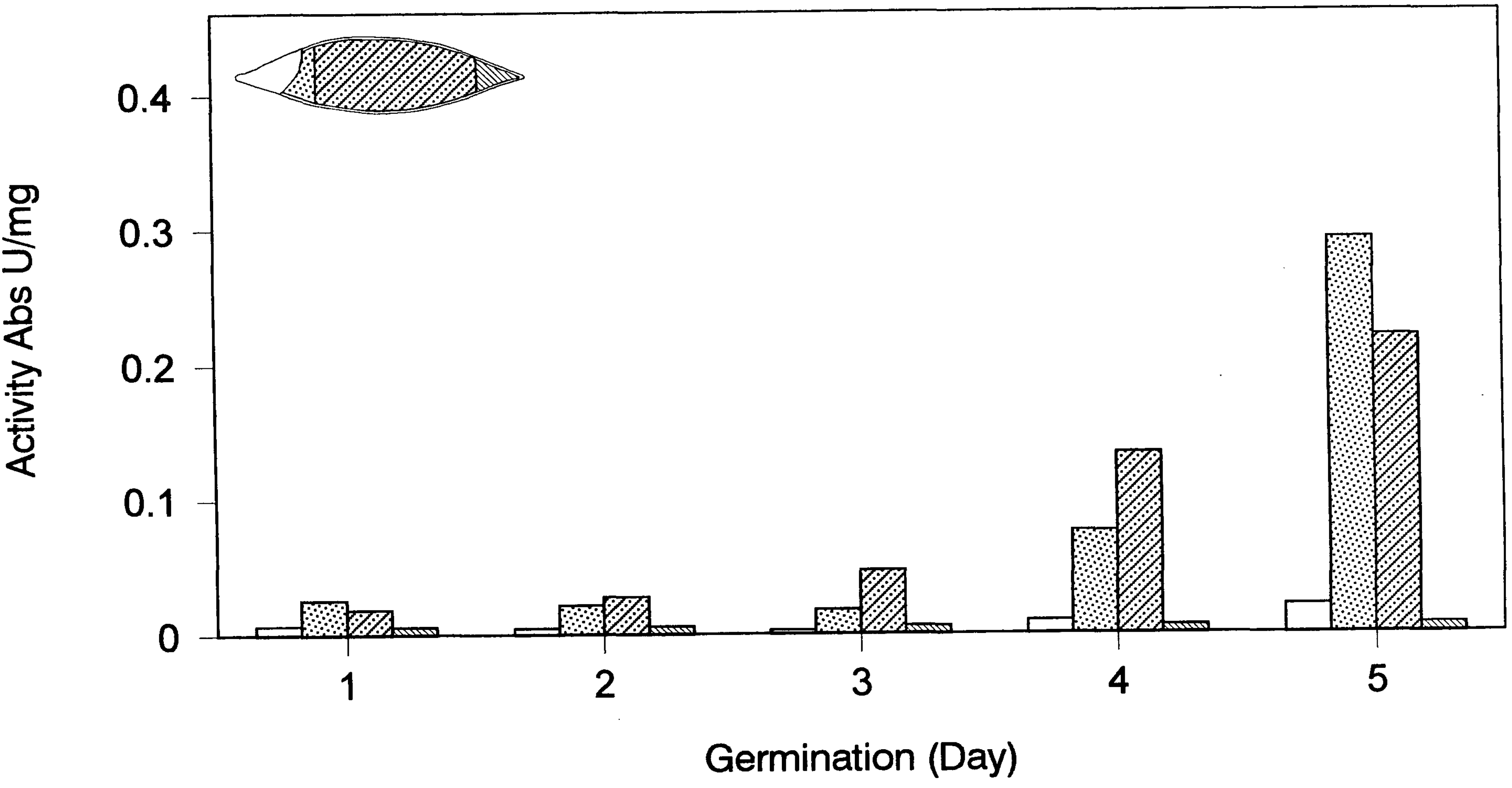
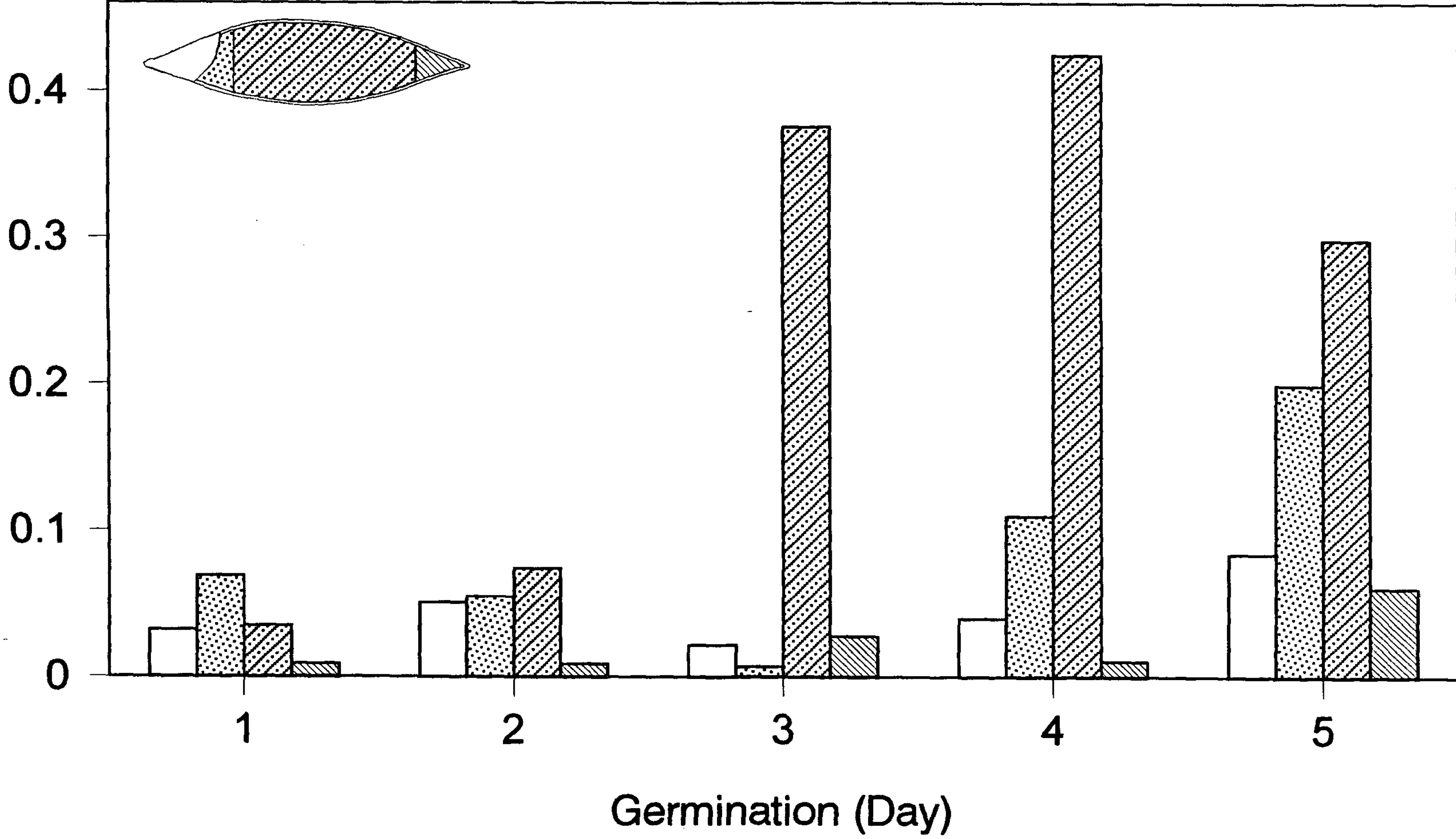


Figure 18. ß-Glucanase activity during germination in different areas of Chariot barley



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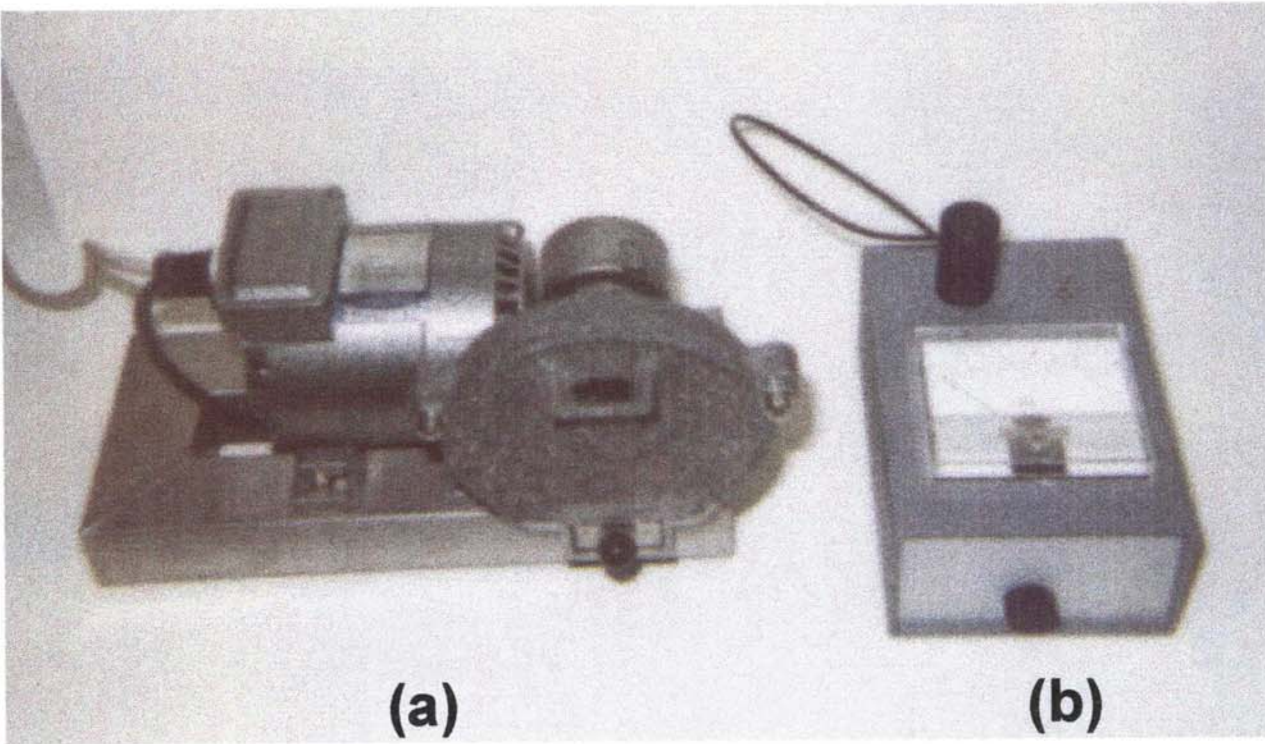

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Table I. Specification of barley suitable for malting.

- Variety
  - All batches should be of single cultivar
- Size
  - Sieve analysis
    - 2.8mm sieve > 60%
    - 2.5mm sieve > 85%
    - 2.0mm sieve < 3%
- Moisture
  - 16% (Price reductions up to 21%)
- Total Nitrogen
  - 1.5 - 1.7%

Table II. Barley hordeins.

Hordein	Amount	MW	Form	Type
S-rich	80-90%	36-40k	Mono	γ
			Poly	B
S-poor	10-20%	40-80k	Mono	C
HMW	10%	>90k	Poly	D

Table III. Malt analysis of samples made from barley varieties with different endosperm structure

Analysis *	Chariot	Blenheim	Target
Moisture (% w/w)	4.2	4.4	4.8
H.W.E (0.2mm) (l°/kg)	315	310	299
H.W.E (0.7mm) (l°/kg)	313	306	290
F/C Difference (l°/kg)	2	4	9
T.S.N (% w/w)	0.7	0.69	0.59
T.N (% w/w)	1.6	1.81	2.03
S.N.R (%)	44	39	29
Viscosity (mPa.s)	1.46	1.63	1.93
Friability (%)	90	85	50

*According to recommended methods of the Institute of Brewing

Table IV. Endosperm characteristics of barley grains

Cultivar	Screening (mm)				Moisture (%)	T.N. (%)	Population (%)		
	>2.8	2.5-2.8	2.2-2.5	<2.2			S	S/M	M
Chariot									
-	58.4	26.6	14.5	0.5	11.4	1.47	31	26	43
+	72.1	16.8	5.9	5.5	11.8	1.60	5	10	85
Blenheim									
-	41.6	32.3	13.1	13.0	12.4	1.64	30	19	51
+	58.1	25.7	8.7	7.5	12.5	1.81	9	15	76
Target									
+	60.1	30.0	8.4	1.5	13.1	2.03	19	51	30

+: Fungicide treatment; -: Untreated; S: Steely; S/M :Steely and Mealy; M: Mealy

Table V. Hydration of cultivar with differences in the number of mealy and steely grains.

Cultivar	Population (%)	Iodine score (IVS)	Overall hydration value (OHV)
Chariot			
(M)	85	98	93
(S)	15	67	
Blenheim			
(M)	76	75	69
(S)	24	50	
Target			
(M)	30	90	47
(S)	70	29	

Table VI. MONOCLONAL ANTIBODIES

Mab	Binding characteristics
Broad specificity	
IFRN 0661	Recognises wheat, barley, rye and oat prolamins equally well. Binds predominently to B,C and γ- hordein
Narrow specificity	
IFRN 0610	Does not differentiate between wheat, barley, rye and oat prolamins. Shown to be γ- hordein specific by immunoblotting, recognises an epitope which is susceptable to proteolysis
IFRN 0614	Binding restricted to S-poor prolamins of barley, wheat and rye. Binds predominently to C-hordein and a weaker reaction with B-hordein

Table VII. Distribution of hordeins in barley & malt endosperm measured using immuno gold labelling method

Variety & antibody	Barley 1	Barley 2A	Barley 2S	Barley 3	Barley 4	Malt 1	Malt 2A	Malt 2S	Malt 3	Malt 4
Chariot										
Broad	+++	++	+++	+++	+++	+	+	+	+	++
γ-hordein	+++	++	+++	+	+++	-	+	++	-	++
C-hordein	+	+	+	+++	+++	-	-	-	+	++
Target										
Broad	+++	+++	+++	+++	+++	+	++	+++	+++	+++
γ-hordein	+++	++	+++	++	++	+	+	+++	++	++
C-hordein	+++	+	+	+++	+	-	+	++	+++	+++

Hordein Levels:
- Not detected, + Low, ++ medium, +++ High

Table VIII. Distribution of hordeins in mealy and steely endosperm measured using immuno gold labelling method

Variety & antibody	Mealy	Steely
Chariot		
Broad	+++	+++
γ -hordein	-	+++
C-hordein	+++	+++
Target		
Broad	+++	+++
γ -hordein	+	+++
C-hordein	+++	+++

Hordein Levels:
- Not detected, + Low, ++ medium, +++ High

Table IX. ß-glucan solubilase

Cultivar	Barley (mg ß-glucan solubilised / min)	Malt
Chariot	0.7	0.9
Blenheim	1.1	1.4
Target	0.3	0.9

PART II. Studies on wheat endosperm

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SUMMARY

One of the crucial factors for selection of wheat varieties for the production of whisky is its spirit yield. A number of soft and hard wheat varieties were studied. Spirit yield was highest from the soft wheats which had been grown with the aid of fungicides.

Although only the enzymes of malted barley can be used to convert wheat into Scotch grain whisky, additions of extraneous enzymes to wheat mashes gave optimal spirit yield. However, spirit produced with the aid of commercial enzymes cannot be used to make Scotch whisky, but can be used for other drinks products.

Results from electron microscopic analysis of the grains indicated that structurally, the small starch granule / matrix protein of the endosperms of hard wheat was more rigid (plastic) than that of soft wheat, causing the endosperm to fracture along cell walls, rather than across cells as in soft wheat endosperms, during milling. The structural similarity between the endosperm of hard wheat and the steely endosperm of soft wheat was separated clearly by the Stenvert hardness test but not by NIR tests. Steeliness, as assessed by eye or image analysis, was related to protein content and is influenced by nitrogen availability. Hardness, in contrast, is a genetic characteristic. For example, Mercia which had a lower percentage of steeliness than Beaver, gave a higher hardness score.

Hard wheats such as the feed wheat Brigadier have a potential for use as distilling wheats, if the nitrogen content is low and can be purchased at competitive prices, because they gave high spirit yields with enzymes. One reason for this is the observation made in this study that

the small starch granule / matrix protein of Brigadier has some structural similarities to that of soft wheat, where the protein / starch matrix is diffuse in structure.

One important feature of the processing efficiency of soft wheat is that a loose association between endosperm matrix protein and small starch granules facilitates the release of starch which is an essential requirement for high spirit yield.

A test was developed to identify rapidly wheats with soft endosperm characteristics which give higher spirit yields than hard wheats during distilling. The sedimentation test described in this report, is simple to perform and distinguished hard from soft wheats. However, the contaminating levels of hard wheat could only be detected safely at about 50% inclusion and above. It was also observed that as the contaminating levels of hard wheat increased in a sample of soft wheat, the 1 - 2.2 mm flour fraction of the sedimentation flour increased over expected values for pure soft wheat flours. Both the sedimentation test and the percentage of coarse flour fractions could be used to standardise the distilling quality of soft wheat flours, in terms of grain admixture.

A method was also developed to test for total pentosan. Although Brigadier tested as a hard wheat, it, like the soft wheats, had lower pentosan contents than the hard wheat Mercia. Our results suggested that truly hard wheats could be distinguished on the pentosan contents of their mealy grains, which was significantly higher than those for the corresponding mealy grains of Brigadier or the soft wheat varieties studied.

The bran layers of wheat are also a particularly rich source of pentosan, and may along with

endosperm cell walls contribute to the dewatering problems of distillers, spent grains. An enzyme cocktail of ß-glucanases pentosanase, and proteases may be the most efficient way of eliminating dewatering problems in the distilling process.

The negative effects of foaming caused by wheat during whisky production prompted investigations on foaming. Hard wheats foamed more than soft wheats and the foam was more stable. The relationships between foaming potential and extracted pentosans of Riband (soft wheat) and Mercia (hard wheat) were investigated.

Cell walls of hard and soft wheat released ß-glucans, pentosans and protein when extracted at 120°C. Enzymic treatment of these extracts showed that foam stability was destroyed by ß-glucanase and protease enzymes. Surprisingly, xylanase which reduced the viscosity of the extract, had little effect on the foam stability. Although the mechanisms of foam development are not fully understood, it is possible that an enzyme cocktail containing ß-glucanases and proteases is essential for the destruction of foam caused by wheat.

1.0 INTRODUCTION

1.1 The importance of wheat to the Scotch whisky industry

There are two categories of Scotch whisky, malt whisky and blended whisky. Malt whisky is made from malted barley, yeast and water. Blended whisky is usually a mixture of between 15-40% malt whisky and 85-60% grain whisky. The greater the proportion of malt whisky the higher the quality of the blend. For example deluxe blends can contain as many as 40 different single malt whiskies whereas supermarket blends can contain as few as 10 malts making up as little as 15% of the blend.

Despite the marketing value of the single malt whisky, 95% of the 87 million cases of Scotch whisky sold in the world per annum is blended. In other words, 60-85% of all whisky sold in the world each year is grain whisky.

Grain whisky is produced from a grist containing 90% cooked wheat or maize converted by the enzymes of 10% malted barley. Currently about 500,000 tonnes of wheat and 100,000 tonnes of maize are used annually in Scotland to produce 200 million litres of grain whisky. Therefore wheat is very important to the whisky distillers.

1.2 Economics of distiller's use of wheat

The use of wheat by grain whisky distillers became prevalent in the mid 1980s as prices and EU subsidies made it financially attractive. In 1982 only 10 000 tonnes of wheat was used by all the Scottish grain whisky distillers. By 1989 this had increased to almost 500 000 tonnes with a concurrent decline in the use of maize from its previously dominant position.

This demand for wheat was reflected in increasing production in Scotland from 110 000 tonnes in 1979 to around 900 000 tonnes annually from 1990. Since this upsurge in the use of wheat, grain distillers have been developing their specification for "grain-whisky-making" wheat. However, at present the use of home-grown wheat as a primary source of raw material is under threat. French maize has a net cost of £113.79 per tonne and yields c. 391 L of alcohol per tonne giving a net cost of £0.29 per L of alcohol. UK wheat has a net cost of £104.41 per tonne and yields c. 380 L of alcohol per tonne giving a net cost of £0.27 per L of alcohol (HGCA, 1995). If alcohol was ever to become cheaper to produce from maize when compared to wheat the distillers would switch from using wheat to maize. This could have a catastrophic impact on the farmers growing wheat. To prevent this from happening new wheat varieties need to be bred which give higher alcohol yields.

1.3 Distiller's use of wheat

At intake to the distillery the wheat is tested to ensure that it meets the specification, *i.e.* that it is a soft wheat with a low nitrogen content. The wheat is then screened and either lightly milled or used whole. The wheat is cooked at 150°C for 1.5 hours and then cooled and mashed at 63°C for a further 1.5 hours with 10% (w/w original barley) high diastatic power malt (DP = c. 200). After mashing, the wort is either passed directly to the fermenters, partially filtered or fully filtered. After fermentation the wash is passed to Coffey stills for distillation over a series of plates which gives a final spirit at c. 94% alcohol with the only other significant alcohols being propanol, butanol and some pentanol. The diluted spirit is then matured in oak for a minimum of 3 years before blending. Very little unblended grain whisky is bottled in Scotland. The spent grains and other residues are then processed for animal feed (Bathgate, 1989).

1.4 Common problems associated with the use of wheat in a distillery

The use of wheat by grain distillers has caused significant problems in the distilleries. Primarily, excessive foaming during mashing and fermentation causes under utilisation of vessels. Animal feed production can also be limited because wheat residues can inhibit dewatering, thus increasing energy inputs. The current strategies to overcome these problems involve breeding wheats which do not foam. However, there is as yet no light at the end of this long technological tunnel, although there may be beneficial effects of breeding out the 1B1R translocation with rye.

At present mash tuns and fermenters are under-filled by between one quarter and one third of their volume or large vessels are constructed to accommodate the foam. Both under-filling and constructing larger vessels are uneconomic.

Pentosanases have been added to spent grains to aid dewatering. However, they cannot be added to the fermentation because the Scotch Whisky Act prohibits the use of extraneous enzymes for scotch whisky production. Enzymes can however be used in neutral spirit production for gin and vodka.

1.5 The effects of cultivar, environment and the use of fungicide on the quality of wheat

The effects of climate and cultivar were examined by Rao *et al.* (1993) over a ten year period. Most of the variation observed in the varieties studied was due to environmental factors. The higher the temperature during grain filling the higher the evaporation rate, this in turn leads to a reduction in the grain filling period and an acceleration of protein

accumulation giving rise to a higher final nitrogen content (Martin del Molino, 1991 and 1992). At the same time, the thousand corn weight of the grain is reduced by increases in the ambient temperature, primarily due to a reduction in the accumulation of starch (Jenner, 1991). This reduction in starch accumulation is compensated for by the increased protein deposition which leads to no detectable increase in the rate of grain filling (Cochrane, personal communication). Rao *et al.* (1993) and Alcoz *et al.* (1993) found that in order to optimise the control of nitrogen in wheat the timing, rate and method of nitrogen application were very important. Sylvester-Bradley (1995) recommends that the soil nitrogen needs to be assessed by analysis and the target canopy of plants set. If it is assumed that each 30 Kg N/ha will provide sufficient nitrogen for one canopy unit then a target of 5 canopy units (c. 500 shoots per square metre) would require 150 Kg N/ha. Therefore any shortfall between the soil nitrogen and the requirements of the crop needs to be made up by nitrogen application in early March (in the UK.) to promote tillering and therefore give good yields. A further application of between 50 and 60 Kg N/ha in late May will ensure canopy survival.

The application of fungicide to wheat is as important as nitrogen in order to maintain yield and produce a crop suitable for grain distilling. The variety Riband is the preferred variety of the grain distilling industry (although it is not specified and no premiums are paid for Riband) it has a soft endosperm and under good conditions gives large, mealy low nitrogen grains. There are three important growth stages (GS) when fungicide treatment has the greatest influence on grain quality and yield: GS 32, the 3rd last leaf; GS 33, the 2nd last leaf and GS 59 the emergence of the flag leaf. The objective of these sprayings is to prevent infection by rain-splashed diseases like *Septoria tritici*. Infection of the upper leaves from the lower ones is caused by rain splashing spores from the soil and the lower leaves onto the

upper more important leaves. The flag leaf plays a vital role in grain filling and infection by Septoria and other diseases can dramatically reduce yields. Riband produces an average yield response of + 2.5 tonnes/ha (c. 25% of the fungicide treated yield/ha) to an application of fungicide. However, the range of response of Riband is between - 1.75 and + 5.51 tonnes/ha. Therefore correct timing and rates of fungicide application and the weather at the time of spraying can radically affect yield and quality. The range of yield response to fungicide in other varieties is less pronounced than in Riband but still significant (Wale, 1995).

2.0 MATERIALS AND METHODS

2.1 MATERIALS

Samples of wheat were supplied over three years by the Scottish Agricultural College, Bush Estate, Edinburgh from their variety trials. In the first year four cultivars were selected based on their suitability for grain whisky distilling. Cultivar Riband made up 73 % of the wheat acreage in Scotland in 1993 and along with Beaver (8 %) and Admiral (3 %) were selected as recommended soft wheats for distilling. The hard wheat cultivar Brigadier was selected for comparison. In the second year the soft wheat Hunter and the hard wheat Mercia were added. In the final year samples were obtained from the Scottish Borders and Aberdeenshire, the soft wheats Admiral and Beaver were replaced by the new varieties Consort and Encore and the hard wheat Lynx was added. Two samples of each cultivar were provided which were grown with or without the use of fungicide, during this study the use of fungicide was indicated by + or - (**Table 1**).

The wheat samples provided by the Scottish Agricultural College, Edinburgh were produced as part of the UK crop variety trials. The following is an example of the typical treatments given during growth. The winter wheat samples were sown at a seed rate of 200 kg/ha on the 12th October 1992 at Nether Finlarg in the east of Scotland on a sandy clay loam. Four additions of fertilizer were applied: P_2O_5 and K_2O were applied at 60 kg/ha on the 14th of October 1992; the first top dressing of nitrogen was applied at 50 kg/ha on the 9th March 1993, the second top dressing was applied at 50 kg/ha on the 29th March 1993 and the third top dressing was applied at 75 kg/ha on the 28th April 1993. Three fungicide applications were made to treated samples: S. Delta (@ 1.25 L/ha) and Corbel (@ 0.5 L/ha) on the 3rd

of May 1993; Patrol (@ 0.75 L/ha) and Radar (@ 1.25 L/ha) on the 14th of June 1993 and Impact Excel (@ 2.0 L/ha) and Corbel (@ 0.75 L/ha) on the 29th of June 1993.

2.1.2 Iodine solution

The iodine solution (0.2 % I_2 and 2.0 % KI) used throughout this study was prepared by "dry" mixing iodine (0.2 g) and potassium iodide (2.0 g) and leaving the mixture to stand for 10 min before slowly adding water (100 mL). The iodine solution was freshly prepared before use and stored cool and in the dark for up to one week.

2.2 METHODS

2.2.1 Determination of the moisture content of whole grains

Whole grains were taken at random, weighed and dried at 70°C until constant weight was achieved. Drying at 70°C, instead of 105°C, is slow but gives a more reliable result as it does not "drive off" any volatile compounds from the grains.

2.2.2. Determination of total nitrogen content of grain

The total nitrogen content of the grain (0.5g) was determined by the Kjeldahl method (Institute of Brewing, recommended method of analysis, 1991).

2.2.3 The light transflectance method for distinguishing mealy grains from steely grains

Whole wheat grains were placed on the glass plate of a dissection microscope and illuminated

from below. The soft white mealy portions of the endosperm of a grain appeared dark and opaque whereas the hard grey steely portions of the endosperm appeared translucent and the transmitted light "glowed" orange. The proportions of mealy, steely and piebald (mixed mealy and steely grains) grains in each sample were determined.

2.2.4 The image analysis method for determining the steeliness of wheat grains using the Cambridge Instruments Quantimet 570 Image Analyser

The Quantimet 570 image analysis system consists a console, two monitors, an Acer 80386 micro computer with hard and floppy drives linked to a JVC ky-F30/3CCD colour video camera. The software is produced under licence from Armines by Leica Cambridge Ltd and uses QBASIC.

The wheat sample was placed on a light box and focused with a "macro lens", the grey image was displayed on one of the monitors and IMAGE SETUP menu selected with the mouse. The red camera option was selected as it gave the sharpest image. The "white level" was adjusted in conjunction with the light intensity on the microscope to give good contrast and a white level of 100. Without changing the focus or the white level the shading corrector was then selected to remove the background and thus improve the contrast. The image analyser was calibrated according to the lens being used (1 pixel = 0.983μm). The size of the fields measured was set at c.10 cm. The results were protected from frame edge errors because the image analyser only included particles if their lowest right hand corner was within the frame being measured.

The grains or areas of steeliness were measured from a "binary" image which was obtained

by the "Detection" process. "Detection" is the process which extracted the binary image from the original grey-level image. The detected image was displayed as a red overlay on the grey-level image. Once DETECT had been selected from the main menu the threshold was adjusted to pick out, or "detect", the zones of interest in the image. The detected red binary image was then checked against the grey image and the threshold readjusted to give the best correspondence between the red image and the particles being measured.

The red binary image was then edited and amended according to the judgement of the operator. Touching or overlapping grains were separated using the cut tool and the mouse. If a particle was not fully detected then the cover tool allowed it to be drawn in. After each image edit the new binary image was displayed in a different colour and was allocated a new "bit plane".

The final version of the binary image was measured by selecting MEASURE FEATURE from the main menu. A number of parameters could be measured for each grain: Area (the number of detected pixels in a grain); Feret (the orthogonal distance between a pair of parallel tangents to the feature [grain] at a given direction (one of 64 different angles, e.g. Feret 0° = width and 90° = height); Length (the longest of the feret measurements); Breadth (the shortest of the feret measurements); Roundness. Further work is in progress to automate this process.

2.2.5 Measurement of hardness by Near Infra Red (NIR) analysis

Wheat hardness was measured using wheat milled to pass a 0.5 mm screen and a Technicon Infra-Alyser 400.

2.2.6 The Stenvert wheat hardness test, Stenvert (1977)

Wheat (20g) of known moisture content was placed in the hopper of a Glen Creston Micro hammer cutter mill which was fitted with a grid containing 2 mm holes and a plastic tube calibrated to a volume of 17 mL fitted to the mill exit. The speed control was turned to 600 rpm, as measured with a tachometer, the slide was pulled out to admit the wheat to the milling chamber and a stop watch started simultaneously. The tube was turned gently until the ground wheat reached the calibration mark and the watch was stopped. The results were expressed in seconds and adjusted for moisture content in the following way: 1 second was added for each % of moisture below 15 % or 1 second was subtracted for each % moisture above 15 %. The grinding resistance was converted to hardness value in accordance with the following scale:

Grade	Grinding Resistance(Seconds)
1	< 28
2	29 - 32
3	33 - 35
4	36 - 38
5	39 - 42
6	43 - 45
7	46 - 48
>7	> 49

2.2.7 The sedimentation test, a rapid method for distinguishing between hard and soft wheat

The sedimentation test for wheat hardness was developed from the method of Palmer and Harvey (1977). Wheat (16 g) was milled for 5 seconds in a Braun coffee mill. The milled wheat was sieved and the flour with a particle size less than 250 μm was collected. To 0.4 g of this flour was added 20 mL of cold (4°C) 70% ethanol in a graduated boiling tube. After shaking for 5 seconds the tube was allowed to stand for 5 minutes. If the ethanol was

cloudy then the wheat was soft, if the ethanol was clear then the wheat was hard (see **Plate 8**).

This method was further refined by diluting an aliquot (2 mL) of the suspension of wheat flour 1 : 100 and measuring the changes in turbidity over time. This allowed "numbers" to be given to the differences between the sedimentation rates of flours of hard and soft wheats.

A by-product of this sedimentation test was found to reflect increasing amounts of hard wheat contaminating a sample of soft wheat. The fraction of flour greater than 1 mm and less than 2.2 mm was found to be proportionally related to the amount of hard wheat contaminating the soft wheat.

2.2.8 Determination of the starch content of single barley endosperms

(a) Solubilisation of the starch

Starch cannot be completely dissolved in water, therefore in order that the starch could be assayed it was first solubilised using the following procedure:

Barley endosperms were crushed and weighed into screw capped Pyrex test tubes and 1 mL of 90% DMSO/10% deionised water was added. The starch was dissolved by alternately mixing and heating in a boiling water bath and then holding the tube in the boiling water bath for 90 min. The tube was then cooled and centrifuged (1800 g / 5 min) and examined for the presence of a gel which would indicate that some of the starch had not dissolved (in which case the tube was returned to the water bath). The dissolved starch was precipitated with 9 volumes of 96% ethanol and left to stand for 15 min before the tube was centrifuged

(1800 g/5 min) again and the precipitate redissolved in 1 mL of the DMSO/water solution. This solution was held in a boiling water bath for 15 min and cooled. After three days the starch was precipitated with 96% ethanol as before and was readily soluble in water.

(b) Starch determination

The solubilised starch from individual endosperms was dissolved in water and made up to 1 mL. To 400 μL of this starch solution was added 800 μL of citrate/phosphate buffer (50mM, pH 4.6) and 80 μL of amyloglucosidase (from *Aspergillus niger*). This was incubated at 60°C for 30 min. The glucose produced was assayed using the Glucose Oxidase Peroxidase (GOD POD) kit from Biocon Ltd. A standard curve was prepared with a starch solution of 80 $mgmL^{-1}$ prepared with commercially available soluble wheat starch. The standard starch solution was checked using the phenol sulphuric method for the determination of total polysaccharide.

2.2.9 Determination of total polysaccharide

The method used was based on that of Dubois *et al.* (1956). A sample or a standard glucose solution (20 to 100 μg glucose in 1 mL) was pipetted into clean, thick walled, Pyrex test tubes. To this was added 2 mL of aqueous phenol solution (5 %) and 5 mL of concentrated sulphuric acid. The sulphuric acid was added rapidly to ensure complete mixing in the tube. The tubes were cooled and left to stand for 15 min before the absorbance of each tube was read at 490 nm in a glass cuvette, against a reagent blank. Sample concentrations could be determined by reference to concurrently tested glucose standards. A factor of 0.9 was used to compensate for the difference in the molecular weight of glucose residues as a component of a polysaccharide (RMM 162) and glucose in the free form used for calibration (RMM

180).

2.2.10 Determination of reducing sugars

Reducing sugar was determined according to the method of Nelson and Symogi as described by Robyt (1984).

2.2.11 Determination of ß-glucan content.

ß-glucan content was determined using the kit supplied by Megazyme pty, Australia.

2.2.12 Isolation of pentosans

Whole wheat flour (250g in 5 batches of 50g) was cooked at 100 °C with 300 mL of distilled water in stainless steel mashing vessels, after 1 hour the cooked wheat slurry was cooled to 65 °C and stirred in a mashing bath for 3 hours with an excess of Porcine pancreatic α-amylase until no starch reaction remained (determined by the addition of iodine). The mash was cooled to 4 °C and centrifuged (5000 *g*, 10 min, 4 °C) in a Sorvall RC-24 superspeed refrigerated centrifuge and the pellet discarded. The supernatant was made up to 70% (v/v) with respect to ethanol at 4 °C and the precipitate collected by centrifugation (5000 *g*, 10 min, 4 °C). The pellet of crude pentosans was resuspended in 400 mL of water and made up to 30% (w/v) with ammonium sulphate, the precipitated (ß-glucan) was recovered by centrifugation (2500 *g*, 10 min. 4 °C) and freeze dried. The supernatant was then made up to 70% (w/v) with ammonium sulphate and the second precipitate (pentosan) was recovered by centrifugation (3000 *g*, 10 min, 4 °C) and freeze dried. Further additions of ammonium sulphate did not produce any further precipitation.

Three varieties of wheat were processed (the soft wheats Riband, Admiral and the hard wheat Mercia), all three varieties yielded approximately 2.5g of pentosans, 1 % of the original weight of grain. The viscosity (Brookfield Digital viscometer), protein content (Bradford method) and pentosan content (Phloroglucinol method) were determined. A small sample was acid hydrolysed with 2.5 N H_2SO_4 for 3 hours at 100 °C and neutralised with $BaCO_3$. The neutralised hydrolysate was spotted on a paper chromatogram and run with a solvent mixture of ethyl acetate, pyridine and water in the ratio 10:4:6 with glucose, arabinose and xylose as standards. After developing with silver nitrate the Rf values of the sugars were measured and compared with the standards.

2.2.13 Determination of the foaming potential and foam stability of solutions of the isolated pentosans

The foaming potential and foam stability of solutions of the isolated pentosans was determined by injecting 50 mL of the solutions into a 100 mL measuring cylinder and marking the initial volume of the foam (foaming potential). The volume of the foam was also noted at intervals after injection as it collapsed (foam stability).

The effect of pure enzymes on the foaming of solutions of extracted and purified pentosans was examined. The enzymes (at 10 U mL^{-1}) used were ß-xylanase from *Trichoderma viride*, α-L-arabinofuranosidase from *Aspergillus niger* and Proteinase K from *Tritirachium album*. The solutions of extracted and purified pentosans were pre-incubated at room temperature with each enzyme separately and then sequentially in different combinations. The foaming potential and foam stability of the solutions was then determined.

2.2.14 Assay for the total pentosan content of wheat, wheat flour and worts

Finely milled wheat (200 mg) was solubilised in 15 mL of NaOH (1 M) with continuous mixing for 24 hours at room temperature. After neutralisation with HCL (1 M) the solution was made up to 30 mL.

To 1 mL of this extract was added 4 mL of 1 M H_2SO_4, this was then boiled for 1, 2 and 3 hours in duplicate. After cooling the acid hydrolysed pentosans were neutralised with 4 mL of 2 M NaOH then diluted 1 mL in 25 mL of water.

An aliquot of this hydrolysed wheat or wort pentosan or a xylose standard (0 - 100 μg/mL) (2 mL or 2 mL of a suitable dilution) was mixed with 10 mL of Phloroglucinol reagent in thick walled test tubes. The reagent was freshly prepared by mixing: A. glacial acetic acid (110 mL) and fuming HCL (2 mL) and B. Phloroglucinol (1g) in absolute alcohol (5 mL containing 17.5 mg of glucose) in the ratio of 1 : 0.0463 (A : B). All glassware was acid washed and all reagents were of the highest quality available. The Phloroglucinol/glucose reagent was unstable and was replaced after 90 minutes. The test tubes were heated in a boiling water bath for 25 minutes and cooled for exactly 5 minutes before reading the difference in absorbance at 552 and 510 nm with a Philips PU 8730 UV/VIS Scanning Spectrophotometer with xylose as a standard and reagent blanks. The results were expressed as μg xylose per mg of the milled wheat.

2.2.15 Column chromatography of the isolated purified pentosans of hard and soft wheats

Sephadex G200 columns were prepared (90 x 2 cm) and 10 mg of pentosans loaded. The

elutant was 0.02 M Tris/HCl pH6.8 containing 0.001 % sodium azide. Elution was with the use of a peristaltic pump to give a flow rate of about 0.25 mL per min. Fractions (3 mL) were collected and analysed for total carbohydrate, pentosan and reducing sugars.

2.2.16 Spirit yield determination

Grain distillers measure the true alcohol yield and the maximum potential yield for all the samples of wheat they purchase.

For each fermentation 170 g of wheat was ground at setting 10 on the Minerva Grinder. This was transferred to a 1 L steel mashing beaker and 600 mL of hot tap water added along with 1 - 2 drops of silicon anti-foam agent. The beakers were then heated to 80°C, stirring occasionally. When the starch had gelatinised, foil caps were placed over the beakers and they were cooked at 40 psi for 1.5 h. After cooking the wheat was cooled to 67°C and the beakers were placed in a water bath at 65°C. Malt slurry at 54°C was added to give 11 % Original Barley (17.6 g dried malt or 31 g of green malt). The worts were mashed for 1.5 h then cooled to 40 °C. The beaker contents were then transferred to 2 L fermentation flasks and 1 % yeast (w/w, yeast/grain) was added. The volume of the flasks was adjusted to 1.1L with cold water and then incubated at 30 °C for 65 h. After fermentation wash samples were analysed for % alcohol, yield and sugar losses, distillate samples were also analysed for congeners.

The maximum potential spirit yields were determined in a similar manner to the true alcohol yields except that the enzymes were provided by Wheatzyme , Ambazyme and Nervanase supplied by ABM(diluted 20 mL in 200 mL, 1.5 mL in 200 mL, and 16 mL in 200 mL

respectively). Penicillin was also added to the fermentation to prevent infection.

Samples of wash and spent wash were collected for pentosan, starch and nitrogen determinations, these samples were also sub-divided into liquor and suspended solids by centrifugation at 5000 *g* for 10 minutes.

2.2.17 Mashing trials

Coarsely milled wheat or wheat flour (50 g, reduced if malt was used for mashing) was cooked in stainless steel mashing vessels with 300 mL of distilled water in a boiling water bath for 90 minutes. After cooling to 65°C the viscosity of the mash was examined for its resistance to stirring by a magnetic stirrer. Mashing was then carried out by the addition of either malt slurry (20 mL containing 5 g commercial malt) or a sequence of three commercial enzymes. The milled malt was slurried with cold water 90 minutes before addition to the mash in order to closely mimic the process in a distillery, a further 20 mL of water was used to rinse out the malt slurry vessel and then added to the mash giving a final volume of 340 mL. The sequence of enzymes consisted of 10 mL of Ambazyme, Nervanase and Wheat zyme diluted 133.3:1, 12.5:1 and 10:1 respectively with water, a further 10 mL of water was added to bring the final volume of the mash to 340 mL. The effect of the sequential addition of each of the enzymes on the viscosity of the mashes was observed. After mashing for 90 minutes the mashes were filtered hot for 2 hours or until reaching dryness and the volume of wort measured. The viscosity of the wort was measured using a Brookfield Digital Viscometer and the density (extract) measured using a Parr Digital Density Meter.

2.2.18 Examination of the mashing properties of commercially produced hard and soft wheat flour

Hard and soft wheat flour was obtained from the Chancelot Mill in Edinburgh.

The mashing properties of the hard and soft wheat flour was examined using both the enzyme cocktail of Ambazyme, Nervanase and Wheatzyme and with malt enzymes from a high DP malt (DP = 202). 50 g of grist was cooked with 250 mL of water at 21 psi for 1.5 hours before being cooled and mashed at 65°C for a further 1.5 hours. When the flour was mashed with malt, 45 g of flour was cooked in 250 mL of water and after cooling 5 g of malt slurried in 50 mL of water was added. The mash thickness, filtrate volume, wort viscosity, extract, foaming potential and foam stability and pentosan content of the worts were determined.

To examine the effect of bran on the mashing properties and the nature of the extracted worts, increasing amounts of bran were added to the grist. The proportions of bran in the grist were 0, 5, 10 and 15 % of 50g. This was cooked in 250 mL of water at 21 psi for 1.5 hours and then cooled to 65°C before a cocktail of Ambazyme, Nervanase and Wheatzyme was added for a further 1.5 hours. The mash thickness, filtrate volume, wort viscosity, extract, foaming potential and foam stability and pentosan content of the worts were determined.

2.2.19 Determination of wort foaming potential and foam stability

Wort (50 mL) was rapidly injected into a 100 mL measuring cylinder and the volume of the foam marked (foaming potential), the volume of the foam was noted at intervals after

injection as it collapsed (foam stability). Solutions of isolated pentosans were prepared by dissolving the freeze dried pentosans in hot water and measuring the foaming properties rapidly while the solutions were still hot.

2.2.20 Isolation of hard and soft wheat endosperm cell walls

Wheat (500 g) was milled at setting 3 in a Miag mill. The milled wheat was passed through a series of endocott sieves and the fraction greater than 250 μm and less than 355 μm was collected. Small amounts of the flour were suspended in cold (4 °C) 70 % ethanol and repeatedly ground with fine sand in a pestle and mortar to remove the starch. The resulting slurry was washed over a fine sieve (60 μm) with cold 70 % ethanol to remove the free starch. The grinding and washing steps were repeated until no more starch was released. The cell walls were then further purified by differential sedimentation in 70 % ethanol in a tall cylinder. The cell walls remained in suspension and could be decanted off after 10 minutes of sedimentation. The sediment was returned to the grinding stage for further purification. The cell walls were stored cold (4 °C) under 70 % ethanol and not freeze dried. The nitrogen, moisture and pentosan contents of the cell walls were then determined.

2.2.21 The effect of commercial enzyme preparations on the foaming of extracts of isolated cell walls

Extracts of were prepared by cooking the isolated cell walls under conditions similar to those in a grain distillery, *i.e.* 121°C for 1.5 hours. As with worts, the foaming potential and foam stability of the extracts was determined by rapidly injecting 50 mL into a 100 mL measuring cylinder and marking the initial volume of the foam (foaming potential). The volume of the foam was also noted at intervals after injection as it collapsed (foam stability).

The nature of the extract and the foaming potential were further examined by the addition of commercial preparations of enzymes. The enzymes (at 100 UmL^{-1}) used were: Bioxylanase from *Trichoderma reesei*; Bioglucanase ME, a complex of 1-4 and 1-3:1-4 ß-glucanase; Bioglucanase B, pure lichenase from *Bacillus subtilis* (endo 1-3(4) ß-D glucanase); Bioprotease N from *Bacillus subtilis*. The extracts were pre-incubated at room temperature with each enzyme separately and then sequentially in different combinations. The foaming potential and foam stability of the extracts was then determined.

2.2.22 Determination of the protein content of samples prepared for SDS-PAGE

The determination of the protein content of samples containing SDS, tris and mercaptoethanol is notoriously difficult. The European Brewing Convention and the American Society of Brewing Chemists have both accepted a spectrophotometric method for the determination of soluble protein in unhopped beer (ASBC, 1990; Lie, 1994). This method was adapted to measure the protein composition of samples prepared for SDS-PAGE.

The protein used for the standards was taken from a plant source as similar as possible to the sample to be loaded on the gel, for example wheat gluten was used as the standard curve for proteins extracted from barley endosperms.

A Philips PU8700 UV/Vis scanning spectrophotometer was zeroed at both 215 nm and 225 nm and a baseline stored with a NaCl (0.5 %) blank in matched semi-micro quartz cuvettes with a 1 cm path length. The absorbances of serial dilutions of sample buffer containing protein were measured at both wavelengths using the Dual 1 program and the difference between the absorbances calculated.

A linear calibration was then performed by calculating the regression line equation ($y=a+bx$) for the data. The difference in absorbance (A215 - A225) was used as the x variable and the protein content ($\mu g\ mL^{-1}$) was used as the y variable. This equation was then used to calculate the protein content in the samples prepared for SDS-PAGE.

2.2.23 SDS-PAGE

Protein samples (10 μg) and molecular weight standards (Mr = 200, 116, 97.4, 66 and 45) were loaded into the wells of duplicate 7.5 % SDS- polyacrylamide gels in a BioRad Mini Protean II electrophoresis cell. Each gel consisted of 3.5 mL of main gel {7.5 % acrylamide : bis-acrylamide (36.5 : 1), 0.1 % (w/v) SDS, 0.375M tris/HCl (pH 8.8), 0.5 mgmL-1 ammonium persulphate and 0.5 μLmL-1 TEMED}, and 1 mL of stacking gel {3.75 % acrylamide : bis-acrylamide (36.5 : 1), 0.1 % (w/v) SDS, 0.125M tris/HCl (pH 6.8), 0.25 mgmL-1 ammonium persulphate and 0.5 μLmL-1 TEMED}. The electrophoresis cell was filled with running buffer: {0.05 M tris / 0.38M glycine (pH 8.3), 0.1 % SDS} and "run" at 25 V to concentrate the proteins in the stacking gel and then at 100 v for 90 min. One of the duplicate gels was reserved for Western blotting and the other was stained for 1 h with Coomassie staining solution {25 % (v/v) propan - 2 - ol, 10 % (v/v) acetic acid, 0.1% (w/v) Coomassie brilliant blue}. After staining the gel was destained in {25 % (v/v) propan - 2 - ol, 10 % (v/v) acetic acid} for 2 h changing the "destain" every 15 min. The gel was then fixed in 7 % acetic acid and photographed.

The used "destain" was recycled by passing it through a foam rubber column to remove the Coomassie brilliant blue.

2.2.24 The assessment of the effectiveness of protease inhibitors as protection against proteolytic degradation during protein extraction for SDS-PAGE

A cocktail of protease enzyme inhibitors was assessed for its effectiveness as a broad spectrum protection against proteolytic degradation during protein extraction for SDS-PAGE.

2 g of sample (barley, *H. vulgare cv.* Chariot; malt; friable flour and non-friable flour) were extracted in 10 mL of 0.5 M NaCl for 1 h at room temperature with and without a cocktail of inhibitors: PMSF (200 μM), Leupeptin (1 μM), EDTA (100 μM) and Pepstatin (1 μM). NB. PMSF was prepared as a 100 mM stock solution in 100 % propan-1-ol, Pepstatin was prepared as a 1 mg mL^{-1} stock solution in methanol.

Soluble casein solution (0.5 mL), buffer (0.25 mL; pH 5) and extract (0.25 mL) were incubated at 37 °C for 2 h. After cooling on ice for 20 min, ice-cold 5% TCA (1.5 mL) was added and the mixture centrifuged. Absorbance of the supernatant was measured at 280 nm against a reagent blank. A tyrosine standard curve was prepared and the results expressed as mg tyrosine h^{-1} g^{-1} tissue.

2.2.25 Scanning Electron Microscopy (SEM)

Wheat grains were fractured transversely in corresponding areas using a stiff razor blade. Grain sections were mounted on stubs and coated with a gold film before they were examined in a Cambridge 2A Scanning Electron Microscope. The micrographs are representative of structural differences seen in greater detail during examination of the sections.

3.0 RESULTS AND DISCUSSION

The quality of wheat samples was assessed in terms of specific weight, thousand corn weight, starch and nitrogen content (**Table 2**). As expected, fungicide treatment resulted in samples with different total nitrogen contents. The fungicide-treated samples had a lower total nitrogen content than the untreated samples, with the exception of the cultivar Beaver in which the samples were the same regardless of treatment. The soft and hard wheat cultivars Riband and Brigadier had similar nitrogen contents which was useful for comparison purposes.

3.1 Wheat intake grain size after fungicide treatment

At grain distilleries, intake of wheat is according to hardness, nitrogen content and specific weight. The wheat must be soft with low nitrogen (<2%) and have a specific weight of 72 Kg/hL or higher. Allowances are negotiated for loads identified as being outside the specification. Specific weight tended to be higher for the fungicide treated samples, particularly cultivar Riband. The untreated sample of Riband had a specific weight of 65 Kg/hL which would have been rejected by a distiller outright, whereas all the other samples would have been accepted with reductions. This reflects the high yield response of Riband to fungicide treatment, which averages at 2.5 tonnes per hectare (the maximum yield response of Riband is 5 tonnes per hectare) compared to the average yield response of Beaver which is about 1.6 tonnes per hectare (Wale, 1995). Fungicide treatment also had a marked effect on thousand corn weight. The treated sample of Riband was over 10 g per thousand corns heavier than the untreated sample (**Table 2**). This is most likely related to the reduction in photosynthetic area of the leaves due to disease during grain filling. This would in turn

reduce the assimilate available for starch synthesis, leading to less starch deposition and hence smaller corn size. Starch and protein content were inversely related (corr. coeff. -0.755). Fungicide treated Riband also had the highest starch content (70.6%) and the lowest protein content (7.81%).

3.2 Endosperm structure of hard and soft wheats mealy and steely grains

Wheat endosperm was examined by the light microscopy to visually identify the so called mealy and steely areas prior to more detailed scrutiny by scanning electron microscopy. **Plates 1** and **2** are electron micrographs of the wheat cultivars Riband and Mercia respectively. **Plate 1** shows the mealy outer sub-aleurone endosperm tissue of Riband. Small starch granules are about 5 μm in diameter and the large starch granules range in size from about 10 - 30 μm. Comparatively, this outer endosperm tissue of Riband is less compact than the corresponding tissue of the steely area of Mercia shown in **Plate 2**. The starch granules appear to be loosely packed with little or no protein matrix between them. In contrast the starch granules in **Plate 2** are embedded in a dense protein matrix. These micrographs and details seen in greater detail in other micrographs indicate that the starch and protein content of a sample of grain appear to be more important factors in determining whether the grains will be mealy or steely than the hardness of the cultivar **(Tables 1,4 and 5)**.

Tables 3 and **4** show the proportions of mealy and steely grains for the samples grown in the years 1993/4 and 1994/5 respectively. Cultivars Admiral and Beaver had the largest proportion of grains in the piebald (mixed mealy/steely) category. Riband, which in the absence of fungicide was predominately steely, consistently had more than 50 % mealy grains when grown with the use of fungicide, this in turn resulted in a low nitrogen content of the

whole sample. The higher nitrogen, untreated samples had most grains in the steely category, particularly in the cultivar Brigadier. The classification of grain into these categories was largely dependent on the nitrogen content of wheat in contrast to the lack of correlation in barley.

Although the soft wheats Riband and Admiral contained the lowest percentages of steely grains and the highest percentages of mealy grains, it is difficult to link these characteristics of the grain to wheat variety. However, if we compare wheats of similar nitrogen values, it would seem that the soft wheat Admiral was more mealy than the hard wheat Brigadier. In contrast, Beaver, which is regarded as a soft wheat, when compared with the hard wheats Brigadier and Mercia, had the lowest percentage of mealiness. In this regard, Beaver may be different from the other soft wheats such as Riband and Admiral, which, at low nitrogen, may develop mealiness to a greater degree than hard wheats.

The light transmittance method for determining steeliness developed during this project is low cost and simple to carry out. With further development the method could be a very important tool for assessing wheat quality at intake.

3.3 Tests for hardness and softness in wheat

The first steps of this development have been carried out using an image analysis method for determining the steeliness of a wheat sample. The advantage of this system is that it enables not only the percentage of completely mealy or steely grains to be determined but it enables an accurate determination of the amount of steeliness in all the grains including the piebald grains. For example: 21 grains were measured and they had a total area of 1.019×10^6 μm^2,

there were 66 steely regions (including 3 completely steely grains) with a total area of 74 191 μm^2. Therefore the sample was 7.28 % steely. With the aid of automation the determination of steeliness should be possible in under 5 minutes allowing the method to be used at intake. Modern personal computers with image analysis software linked to video cameras can be utilised to limit the installation cost of the system.

Table 5 suggests that NIR hardness does not correlate with Stenvert hardness. However Stenvert hardness does correlate with known hardness grades of soft and hard wheats. In general, it would seem that hardness, unlike mealiness and steeliness, is under genetic (varietal) control. In consequence of this, hardness is not influenced to any significant degree by mealiness or steeliness or by the nitrogen content of the grain. Growing wheat with the use of fungicides had a dramatic effect in reducing NIR hardness, but not Stenvert hardness, in the soft wheats. The reasons for this are unclear.

3.4 Microscopic analysis of hardness and softness in wheat

Plates 1,3 and **6** show scanning electron micrographs of transverse sections of the soft wheat Riband. **Plate 3** is at the lowest power of magnification. **Plates 1** and **6** show the characteristically loose association of small starch granules to the protein matrix materials of the endosperm of this soft wheat. This loose structure facilitates fracturing **across** endosperm cells.

Plates 2,4 and **7** show scanning electron micrographs of transverse sections of the hard wheat Mercia. **Plate 4** is at the lowest power of magnification. Compare with **Plate 3** and note that cellular patterns are more prominent in the hard wheat Mercia. This pattern is indicative

of fracturing **along** endosperm cell walls in Mercia, rather than across cells as was noted in the soft wheat Riband. **Plates 2** and **7** show endosperm areas which correspond to those shown for the soft wheat Riband in **Plates 1** and **6**. Note the cell walls and that the small starch granule - protein matrix compaction, or rigidity, is effected by an inter-connecting lattice work of protein matrix materials.

Plates 5 shows a scanning electron micrograph of a transverse section of the hard wheat Brigadier. Although sectioning revealed a cellular structure similar to that of the hard wheat Mercia, cellular definition is not as sharp and to a limited degree is closer to the loose structure of the endosperm of the soft wheat Riband, especially in areas of high nitrogen. Despite this, the typical compact hard wheat structure can be seen. In soft wheats, small starch granules are often dislodged with associated matrix protein. In the hard wheat Mercia, the small starch granules are held firmly in place by adhering protein matrix material. In wheats, hardness may be related to the rigidity of the matrix protein which tends to remain in place, even when the starch granules are dislodged. This important observation is being investigated further.

3.5 Mashing (wort) properties of hard and soft wheats

The properties of wort extracted from mashes of the hard wheats Mercia and Brigadier and the soft wheat Riband were compared **(Table 6).** The hard wheats gave higher degrees of mash thickness than the soft wheat Riband. **Fig 1** shows that the worts of the hard wheat Mercia produced foams which were more stable than the corresponding foam of Riband, confirming observations reported above in **Table 6**.

The results of mashing studies presented in **Table 7** indicate that although Riband contained lower levels of protein than Mercia in the grain, the worts of Riband contained more soluble protein. These results suggest that the greater foaming potential of Mercia was not related to greater levels of soluble proteins in the wort. It is worth noting that Brigadier, which has many of the structural characteristics of a hard wheat produced levels of soluble nitrogen which were greater than that of the hard wheat Mercia, suggesting that although the endosperm structure of Brigadier is similar to that of Mercia, it processed like the soft wheat Riband as regards, soluble nitrogen and spirit yield. Brigadier also had a separate processing advantage over Riband and Mercia in terms of its lower pentosan content **(Table 8)**. SDS-PAGE of the proteins extracted from Riband, Mercia and Brigadier **(Fig 2)** shows that the proteins extracted from the three wheats were similar except when extracted using 60 % propanol, with 1 % mercaptoethanol and 1 % acetic acid, *i.e.* the Prolamin II fraction. The Mercia showed an additional band of about 90 000 kDa molecular weight. The significance of this band, which was absent from Brigadier and Riband is not clear. However it cannot be ruled out that such a protein could be involved in the differing foaming properties of the three wheats and / or in endosperm hardness.

3.6 Mashing properties of wheat bran wort separation and foam development

Wheat bran is an important component of wheat grains. The bran is composed of the aleurone layer (which may have some adhering starchy endosperm material), the pericarp and the overlying testa. The aleurone layer contains as much pentosan (65-70%) as the cell walls of the starchy endosperm. The protein content of the bran was higher than that of the endosperm cell walls.

A detailed series of mashing trials (only a summary of the results are presented) were performed using commercial wheat bran, hard wheat and soft wheat flour and the flours of milled Mercia and Riband grains. **Table 8** and **Fig. 1,** confirmed that, for the whole grain flours, Mercia gave better foaming potential than Riband. In combination with hard wheat flours, the 10 % bran used, marginally depressed the foam potential of the hard wheat flour. The inclusion of 10 % bran into the soft wheat mashes increased the foaming by the end of the essential period of 15 minutes (i.e. the initial fermenter filling period during which foaming needs to be at its minimum).

Tables 8 and **9** show that hard wheat flour gave very thick mashes and that the inclusion of bran increased the thickness of the soft wheat mashes, which were normal before bran addition. These differences in mash-thickness development of the hard and soft wheat flours were also observed during mashes of the milled grains of Mercia and Riband. Neither the viscosities, extracts, or pentosan levels of the worts can explain the foaming differences reported from these worts in **Fig. 1**. Never the less it is possible that the slower wort separation when bran is mashed with the hard wheat flour may indicate that the slower rate of wort separation when bran is included may allow foam-forming agents to remain in the mash bed or be destroyed rather than be extracted into the wort.

The major component of the bran (the aleurone layer) is not digested during mashing and its percentage of the solid component of the spent grains increases as the starch and proteins are solubilised. Although the bran may not contribute to the development and stability of foam because of reduced solubility, it may, because of its hydroscopic properties retard evaporation of pot ale and may limit the rate at which the spent grains can be dried during animal feed

production.

Our work suggests that since hard wheats increased foaming and through their greater mash thickening and hydroscopic properties, may retard dewatering processes, it would be bad practice to use hard wheat, or soft wheat which had been contaminated with hard wheat. Also since the cell walls and the bran contribute to foaming and hydroscopic problems, use of wheats of small grain size would increase the percentage of cell walls and bran during processing.

In order to address this problem of hard wheat contamination of soft wheat the Stenvert hardness test was used to test for increasing contamination of soft wheat by hard wheat. **Fig. 3.** shows the effect of decreasing contamination of the soft wheat Riband by the hard wheat Mercia on the hardness score. There was a positive correlation between Stenvert hardness and the progressive adulteration of soft wheat by hard wheat. Therefore, with further development (calibration) this method could become a useful tool for detecting contamination.

3.7 Identification of wheat types using sedimentation tests

Further attempts were made to distinguish between hard and soft wheats and to detect contamination of soft wheat by hard wheat. The Palmer and Harvey (1977) sedimentation test for barley quality was adapted to distinguish between hard and soft wheats. However, the sedimentation test could only detect levels of contamination greater than 50 %. The sedimentation test could reliably distinguish hard from soft wheats. If the supernatant of the sedimentation test was cloudy then the wheat was soft and if it was clear then the wheat was hard **(Plate 8)**. The principle behind this test is simple when milled the rigid structure of

hard wheat yields flour with larger particles and with less free starch than soft hard wheat. Therefore the hard wheat flour drops out of suspension faster than the soft wheat flour.

During the course of preparation of the flours for the sedimentation test it was observed that different wheats yielded different amounts of each flour fraction. The hard wheats, in contrast to soft wheat, yielded lower amounts of fine flour, *i.e.*, $<250\ \mu m$, for the sedimentation test (**Table 10**). The flour fraction >1 mm and <2.2 mm gave a correlation with increasing contamination of soft wheat by hard wheat. Therefore the detection of adulteration of soft wheat by hard wheat can be detected at significantly less capital cost than by purchasing a Stenvert mill.

3.8 Spirit yield potential of hard and soft wheats

Since one of the aims of the project was to relate the structure of the endosperm to its ability to be converted in the distillery, the spirit yield from each sample was determined. The potential yield was determined using an excess of fungal enzymes (Ambazyme, Nervanase and Wheatzyme). The yield using kilned malt as the enzyme source was also measured to give a closer indication of performance in the distillery. There was a high negative correlation (-0.946) between potential spirit yield and nitrogen content of the grain (**Table 11**). This was probably due to the inverse relationship between nitrogen content and starch content.

The cultivar Riband, which was the highest starch containing sample, gave the highest yields with both enzymes and malt (**Table 11**). The hard wheat cultivar Brigadier also gave a high potential yield (450 L of alcohol/tonne) whereas when malt was used as the enzyme source,

it was the lowest yielding of the fungicide treated samples. The mash, following cooking, was very viscous for this sample and it may be that the enzymes from the ground malt were less able to gain access to the wheat starch substrate than they were in the less viscous soft wheat mashes. The levels of proteins and pentosans and their possible effects on wort separation and spirit yield were examined in greater detail.

The protein composition of the samples was assessed by SDS-PAGE following sequential extraction with 0.5M NaCl, 60% propanol (prolamin I fraction) and 60% propanol and 2% mercaptoethanol; (prolamin II fraction). The banding patterns of the four wheats Riband, Beaver, Admiral and Brigadier were similar, as can be seen from the gels in **Figs 9, 10, 11** and **12**. There was an extra band in the ω-gliadins of the prolamin I fraction of the cultivars Riband and Brigadier (c 66 kDa) when compared with Beaver and Admiral. Other differences were also observed in the lower molecular weight proteins (<45 kDa). These differences in the protein bands may be related to the 1B1R translocation from rye. The 1B1R translocation from rye involves the replacement of the short arm of the first chromosome of the B genome of wheat with the short arm of the first chromosome of the R genome of rye. The original purpose of this translocation was to transfer the disease resistance of rye to wheat. However, the transformed wheat also produces rye proteins (secalins) which are stickier than the original wheat proteins (gliadins). There may also be effects on other components of the grain which have yet to identified. The possible effect on the amount and nature of the pentosans should be investigated.

The relationship between spirit yields and pentosan levels of spent wash residues of hard and soft wheats was examined. **Table 12** suggests that except for Riband, the two hard wheats

Brigadier and Mercia gave better spirit yields than the soft wheats Admiral and Beaver, despite higher nitrogen levels. Except for Brigadier, the pentosan contents of the spent wash residues of both hard and soft wheats appeared to be similar. The reason why the hard wheat Brigadier gave a high spirit yield and low pentosan level in the spent wash residue may be related to underlying softness which is not detected in hardness tests. This statement supports the scanning electron microscopic evidence already presented.

3.9 Method for determining pentosans

A method was developed for the measurement of pentosan levels in mealy, steely and piebald wheat grains. Optimisation of the acid hydrolysis stage of the phloroglucinal method for total pentosan determination, showed that soft wheat pentosans required TWO hours for complete hydrolysis, while hard wheat pentosans required THREE hours (**Fig. 5**). This suggested that extracted hard wheat pentosans can resist acid hydrolysis. This was found to be an important consideration in the determination of pentosan levels in this work.

Table 13 outlines the analysis of the pentosan content of different kinds of grains selected from different wheat varieties. It can be seen that there is no general trend between pentosan levels and grain type or wheat varieties. However, the steely grains of Admiral, Beaver and Brigadier, all of which carry the 1B1R translocation from rye, had higher levels of pentosans than the corresponding grains of Riband and Mercia, which do not carry the 1B1R translocation. Although further work is in progress, to clarify these observations, **Table 13** distinctly shows that the hard wheat Mercia had an overall higher level of pentosan than all the other varieties.

3.10 Structural (and viscosity) properties of pentosans of hard and soft wheats

Solutions of isolated and purified pentosans were prepared from the soft wheat Riband, and the hard wheat Mercia. The "purity" of the pentosan preparations was determined by paper chromatography of acid hydrolysates of the pentosans. As only arabinose and xylose were detected in the acid hydrolysates the pentosans were not contaminated with -glucans or galacto-mannans. The pentosans were also found to be nitrogen free. Solutions of hard wheat pentosan had slightly higher viscosities than those of the soft wheat (**Fig. 6**). Viscosity is important in distilling as it reduces flow rate from vessels. The addition of pure Arabinofuranosidase (Megazyme) reduced the viscosity of the hard wheat pentosan to that of the soft wheat pentosans. Arabinofuranosidase has debranching activity, suggesting that the pentosan of the hard wheat may be more complex than that of the soft wheat, or at least that the hard wheat pentosans had a different structural configuration.

The ratios of the total carbohydrate to the number of reducing sugars in the isolated and purified pentosans of the hard and soft wheat were 1:60 and 1:200 respectively. The ratio of total carbohydrate to the number of reducing sugars is an indication of the degree of polymerisation of the pentosans. It is possible that the hard wheat pentosans had a higher degree of polymerisation and therefore had more complex molecular structure than the soft wheat pentosans. Size exclusion column chromatography (**Fig. 7**) of the pentosans showed that the molecular structures of the hard and soft wheat pentosans were significantly different. The most significant conclusion from the chromatogram (**Fig. 7**) was that the hard wheat (Mercia) pentosans passed more rapidly through the column and may have had larger or more complex molecules than the soft wheat (Riband) pentosans.

As expected, xylanase reduced the viscosity of both pentosans to virtually that of water. In a neutral spirit distillery the addition of Bioxylanase accelerates flow rates. Protease had no effect on the viscosity of solutions of the purified pentosans.

In contrast to these viscosity experiments, foaming experiments revealed that purified pentosans of hard wheat foamed significantly more and the foam was more stable than similar pentosans of soft wheat (**Fig. 8**). Only the addition of xylanase had any significant effect on the foaming or foam stability of either hard or soft wheat pentosans which had been extracted and purified.

3.11 Role of ß-glucan in foam development of hard and soft wheats

Distillers have always assumed that commercial pentosanases can reduce viscosity and limit foaming. On the basis of these experiments it would appear that they may be correct.

However, to test this assumption a more commercially relevant approach was adopted. Extracts were made from isolated cell walls and they were not subjected to further steps of purification. The cell walls of soft and hard wheats were cooked under similar conditions to that in a distillery. The composition of the resulting extract was surprising.

The extract of the soft wheat cell walls was now more viscous than that of the hard wheat (**Table 14**). The nitrogen release was similar. But the soft wheat cell wall extract contained more pentosan and more ß-glucan than the corresponding extract of the cell walls of hard wheat.

The surprising results here are that soft wheat cell walls released more pentosan than the hard wheat cell walls. Also, the ß-glucan levels of the extracts were higher than those of the pentosan levels for both wheats. This was unexpected because the "established or text book" pentosan content of wheat cell walls is supposed to be three to four times that of the ß-glucan.

One of the important technological consequences which emanates from this simple observation is that pentosanases may have been assumed to be very important in foam destruction because the commercial pentosanases used may have unwittingly contained ß-glucanases.

The effects of commercial enzymes on the crude extracts of the cell walls of hard and soft wheats were examined (**Fig. 9**). Bioglucanase was more effective than the BioXylanase in reducing foaming and foam stability. This suggests that commercial enzyme complexes which are intended to reduce viscosity and foam should contain required levels of ß-glucanase for optimal effectiveness, especially as regards foam destruction.

Although the protein nitrogen content of the cell wall extracts were very low. It is known that proteins form part of the structural make up of the endosperm cell walls of cereals. It can also be seen (**Fig.9**) that commercial proteases also reduced foaming significantly, and like the ß-glucanase, should be included in pentosanase enzyme complexes which are intended for use in neutral spirit production where wheat as an important carbohydrate source.

It is important to note that the extracts of soft wheat cell walls foamed more than those of hard wheat cell walls. This confirms similar patterns of foaming observed in worts extracted from hard and soft wheat flours (**Fig. 1**).

4.0 CONCLUSIONS

Wheat grown with the aid of fungicide had lower nitrogen content and higher thousand corn weight, specific weight and starch content and gave higher spirit yield than wheat grown without the use of fungicides. Fungicide grown wheat also had a higher proportion of mealy grains. Therefore to ensure maximum spirit yields and limit processing problems in the distillery farmers must apply sufficient fungicide at the correct growth stages (*i.e.*, GS32, GS39 and GS59). Insufficient or incorrect timing of fungicide applications leads to disease which in turn limits the photosynthetic capacity of the leaves thus reducing the available assimilate for incorporation into starch.

The steeliness of a wheat sample is a reflection of its thousand corn weight, nitrogen and starch content. Since these characteristics are mainly due to the environmental conditions under which the wheat was grown it can be concluded that steeliness is also a function of the conditions of growth. The variety Riband may have a genetic predisposition to be mealy but, without favourable growing conditions, Riband will be predominantly steely. A light transmittance method has been developed for the rapid determination of the steeliness of a wheat sample. Advancements made to this method of image analysis may have commercial applications.

Hardness is more reliably determined using the Stenvert hardness mill. NIR was found to be an unreliable method as it is susceptible to variations in the nitrogen content. A sedimentation test was developed during the course of this project which enables hard wheat to be distinguished from soft wheat. The advantages of the sedimentation test are

its low capital costs, simplicity and reliability.

The hardness and softness of wheat are varietal characteristics which are not, to any large degree, altered by the mealy - steely structure of the starch endosperm. The distribution of mealiness and steeliness in a population of grain appears to be influenced by the nitrogen content of the grains. Low nitrogen samples of soft wheats appeared to have more mealy grains.

Scanning electron microscopic studies of the soft wheat Riband, and the hard wheats Mercia and Brigadier suggest that the association between the starch granules and protein matrix material of the starch endosperm determines the manner in which the grain fractures during sectioning. Soft wheats fracture mainly across their cells, while hard wheats fracture mainly along their cell walls. An important observation which emerged from comparative microscopic studies of their starchy endosperm of soft and hard wheats was that dislodgement of small starch granules from the endosperm of soft wheats was associated with removal of protein matrix material. In contrast, dislodgement of small starch granules from the endosperm of Brigadier left the matrix protein in situ. In Mercia, there was a very firm association between the small starch granules and their protein matrix materials. This study is being extended. However, at present our concept of softness and hardness of wheat varieties is that hardness reflects structural (plastic) rigidity of the matrix protein. These proteins also tend to have high levels of adhesion, especially in hard wheats such as Mercia. Protein studies of different wheat varieties will be developed to complement the pentosan work described in this report.

Steely wheat grains tend to have higher levels of pentosans than mealy grains. The hard wheat Mercia had the highest levels of pentosans in all grain types. It is not clear why Mercia had higher levels of pentosans than Brigadier. However, both varieties released higher levels of pentosans into worts than the soft wheat Riband. Pentosans of hard wheat had higher apparent molecular weight, a complex molecular structure and were more resistant to acid hydrolysis than soft wheat pentosans. The isolated pentosans of Mercia were more viscous and produced a more stable foam than those produced by the soft wheats Admiral and Riband (**Fig. 8**). The extraction procedure may have enhanced the foaming properties of the pentosans of hard wheat.

The foam of the worts extracted from milled Mercia grains were more stable than the foam of corresponding grains of Riband (**Fig. 1**). An important deduction from this work is that injudicious mixing of wheat varieties could cause unexpected foaming problems. Notwithstanding, the foaming patterns of milled wheat grains were different from the foaming patterns of the corresponding wheat flours and cell walls (**Fig. 1 & Fig. 9**). Thus, the flours and cell walls of soft wheat gave more foam than those from hard wheats. Our explanation is that the bran and / or the germ of the hard wheat may have very positive foaming properties. Differences in the pentosan content of varieties which carry the 1B1R translocation from rye and those which do not carry it, suggest that the short arm of chromosome 1 may carry genes which are responsible for defining the physico-chemical properties of the pentosans in wheat.

The bran fractions, the flours and the endosperm cell walls of wheat play significant roles in determining mash thickness and the foaming and viscosity properties of wort. An

important role of bran resides in its hydroscopic properties which lead to problems in animal feed production.

ß-glucanase and protease may have important complementary roles to pentosanases in foam management in distilleries which produce spirit from wheat. Further work is in progress to identify the most effective ß-glucanases, proteases and pentosanases required to eliminate foam and viscosity problems in both the brewing and distilling industries.

This work suggests that definite requirements, for optimal processing efficiency in industry requires that the nature of the raw materials and their derived extracts should be understood. This kind of knowledge tends to lead to rapid technological development.

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Table 1. Wheat cultivars obtained from the variety trials of the Scottish Agricultural College. All samples were grown with and without the use of fungicide. Samples marked with * carry the 1B1R translocation from rye. For details on growing conditions and treatments see materials section.

Year Location	 East Lothian	1993/4 East Lothian	1994/5 East Lothian	1995/6 Aberdeenshire
Soft	Riband	Riband	Riband	Riband
	Admiral*	Admiral*	Encore*	Encore*
	Beaver*	Beaver*	Consort	Consort
		Hunter*	Hunter*	Hunter*
Hard	Brigadier*	Brigadier*	Brigadier*	Brigadier*
		Mercia	Mercia	Mercia
			Lynx	

Table 2. The effect of fungicide (+ or -) on the quality of wheat. The data are +/- the standard error of the mean. For experimental details see methods.

Variety		Nitrogen (%)	1000 Corn wt (g)	Specific wt (Kg/hL)	Starch (%)
Riband	+	1.37	42.0	70	70.6 +/-1.28
	-	1.61	31.1	65	62.6 +/-0.79
Admiral	+	1.46	40.3	68	65.9 +/-1.07
	-	1.64	34.1	68	62.1 +/-0.99
Beaver	+	1.54	37.2	69	65.9 +/-0.81
	-	1.54	33.2	68	65.9 +/-0.61
Brigadier	+	1.40	40.4	70	64.2 +/-1.37
	-	1.60	31.1	67	64.0 +/-0.47

Table 3. The proportions of mealy, steely and piebald grains in wheat samples grown with and without the use of fungicide during the 1993/4 season. For experimental details see methods. Additional samples were obtained from Nickerson Seeds Ltd. (NSL).

Variety		TN (%)	Population (%) Mealy	Steely	Piebald
Riband	+	1.57	51	13	33
	-	1.61	16	57	21
	NSL		64	7	29
Admiral*	+	1.46	26	15	56
	-	1.64	4	37	53
	NSL		26	17	57
Beaver*	+	1.54	12	23	63
	-	1.54	9	37	51
Brigadier*	+	1.40	16	22	59
	-	1.60	2	64	25
	NSL		4	25	71

Table 4. The Steeliness of the wheat varieties grown with the use of fungicide during the 1994/5 season. For experimental details see methods. (* IBIR Translocation)

Variety	TN (%)	Population (%) Mealy	Steely	Piebald
Riband	1.57	51	9	37
Admiral*	1.65	37	17	46
Beaver*	1.83	8	42	50
Brigadier*	1.73	18	41	41
Mercia	1.82	34	24	42

Table 5. Comparison of the hardness of wheat varieties grown with (+) and without (-) the use of fungicide. For experimental details see methods.

Variety		Moisture (%)	Nitrogen (%)	Hardness NIR	Hardness Stenvert	Hardness Grade
Riband	+	12.6	1.41	6.2	26	1
	-	12.1	1.73	2.1	20	1
Admiral*	+	12.0	1.65	2.8	25	1
	-	11.4	1.71	0.5	25	1
Beaver*	+	11.1	1.83	1.7	25	1
	-	11.4	1.78	0.4	26	1
Brigadier*	+	11.6	1.73	11.6	34	3
	-	11.6	1.93	7.8	38	3
Mercia	+	11.6	1.82	14.2	32	2
	-	11.2	1.87	15.4	35	3

* IBIR Translocation

Table 6. The wort properties from mashing different wheats with 10 % malt. The final weight of grist was 50 g, *i.e.* 45g of wheat was mashed with 5 g of malt.

Sample	Relative mash Thickness	Filtrate volume (mL)	Wort viscosity	Extract
Mercia	++++	210	1.7	354
Brigadier	+++	215	2.0	372
Riband	+	200	1.8	379

++++ is more viscous than +++ >++ > +

Table 7. The effect of the relative solubility of proteins from hard and soft wheat on the maximum potential spirit yield. The data are the means of four replicates +/- the standard error of the mean.

Variety	Nitrogen (%)	Soluble Nitrogen (%)	Spirit yield La/tonne
Riband	1.40	18.13 +/-0.64	442
Mercia	1.79	14.97 +/-0.20	433
Brigadier	1.70	17.03 +/-0.18	443

Table 8. The properties of worts extracted from mashing hard and soft wheat, hard (HWF) and soft wheat flour (SWF) and hard and soft wheat flour with 10 % added bran. The mash was carried out with a cocktail of commercial enzymes Ambazyme, Nervanase and Wheatzyme (supplied by ABM). For experimental details see methods.

Grist	Nitrogen (%)	Mash Thickness	Wort Viscosity	Extract	Pentosan (mg/mL)
Mercia	1.82	++++	1.3	360	1.31
Riband	1.41	+	1.3	366	1.24
HWF	2.12	+++	1.3	375	1.11
SWF	1.45	+	1.3	3.86	1.12
HWF + 10% Bran	-	+++	1.4	386	1.10
SWF + 10% Bran	-	+++	1.4	359	1.10

Table 9. The properties of worts extracted from mashing hard (HWF) and soft (SWF) wheat flour with increasing amounts of bran. The mash was carried out with a high DP malt (DP = c200) supplied by Crisp Maltings Ltd. For experimental details see methods.

Flour	% Bran	Mash Thickness	Wort Viscosity	Extract	Pentosan (mg/mL)
HWF	5	+++	2.1	383	1.3
HWF	10	+++	2.2	385	1.3
HWF	15	++++	1.9	363	1.5
SWF	5	+	2.1	400	1.2
SWF	10	+	2.1	385	1.1
SWF	15	+++	2.2	380	1.5

Table 10. The quality characteristics and the weight of flour < 250 μm produced from 16g of grain during preparation of samples for the sedimentation test. The samples were grown with (+) and without (-) the use of fungicide in the north east of Scotland.

Variety	Screenings < 2.2 mm	1000 corn weight	Weight of flour < 250μm (g)
Riband +	1.72	54.62	1.42
Riband -	4.90	46.72	1.37
Consort +	1.74	47.63	1.33
Consort -	3.27	44.79	1.69
Encore +	2.21	51.88	0.97
Encore -	3.03	50.83	1.23
Hunter +	1.52	50.30	1.12
Hunter -	5.80	49.00	1.32
Mercia +	4.27	45.29	0.60
Mercia -	5.74	44.55	0.71
Lynx +	1.43	52.14	0.70
Lynx -	1.53	54.61	0.86
Brigadier +	2.64	48.65	0.62
Brigadier -	6.48	50.29	0.65

Table 11. The effect of growing wheat with (+) and without (-) the use of fungicide on the spirit yield. The maximum potential spirit yield was determined using commercial enzymes (Ambazyme, Nervanase and Wheatzyme) and the yield obtainable in a distillery was determined using high DP malt (DP = c.200, 11% original barley). The data are expressed as Litres of alcohol per tonne (La/ tonne). For experimental details see methods.

Variety		Spirit Yield (La/tonne)	
		Enzymes	Malt
Riband	+	458	446
	-	437	427
Admiral	+	446	438
	-	437	430
Beaver	+	445	439
	-	445	439
Brigadier	+	450	433
	-	436	432
$Corr^n$. with N		-0.946	-0.697

Table 12. Potential alcohol yields from the 1994 crop wheat grown with fungicide and the composition of the pot ale and dark grains fractions of the spent wash.

Variety	Alcohol Yield (L/tonne)	Suspended Solids in spent wash (%)	Moisture content dark grains	Pentosan content	
				Pot ale (mg/mL)	Dark grains (%)
Riband	442	3.7	23.1	6.3+/-0.1	25.7
Admiral*	428	4.1	23.5	6.1+/-0.1	25.8
Beaver*	424	4.3	24.8	5.0+/-0.3	27.4
Brigadier*	443	3.6	23.7	4.3+/-0.1	25.2
Mercia	433	4.8	24.3	5.6+/-0.1	26.3

* IBIR Translocation

Table 13. The pentosan content (%) of mealy, steely, piebald and whole grain samples. The data are +/- standard errors of the mean. For experimental details see methods.

Variety	Pentosan content (%)		
	Mealy	Steely	Piebald
Riband	4.6+/-0.0	4.7+/-0.4	5.97+/-0.5
Admiral*	4.0+/-0.3	7.0+/-0.1	5.39+/-0.6
Beaver*	5.9+/-0.4	7.5+/-0.2	5.40+/-0.1
Brigadier*	4.4+/-0.0	6.3+/-0.0	4.56+/-0.4
Mercia	8.3+/-0.2	5.6+/-0.6	7.3+/-0.0

* IBIR Translocation

Table 14. The composition of extracts of isolated hard and soft wheat endosperm cell walls. For experimental details see methods.

Component	Soft Wheat	Hard Wheat
Viscosity (cp)	2.6	2.0
Nitrogen (mg mL-1)	0.16	0.15
Pentosan (µg mL-1)	0.5	0.3
β - glucan (µg mL-1)	17.7	6.2

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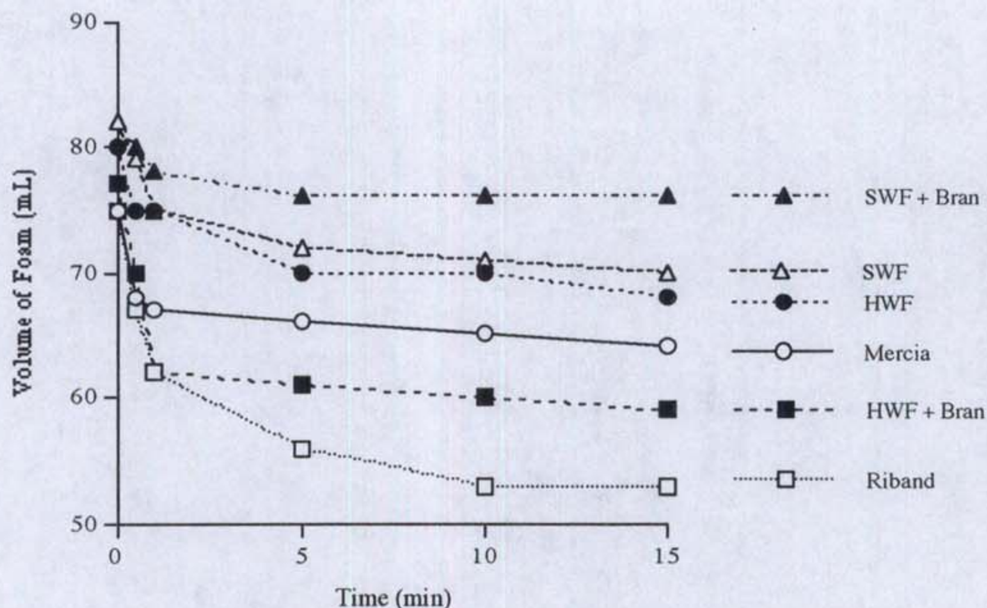


Fig. 2. Coomassie brilliant blue stained SDS - polyacrylamide gel of proteins extracted from Riband, Mercia and Brigadier. Molecular weight markers were loaded into lanes 1 and 10. Each lane was loaded with 10 μg of protein. Lanes 2,3 &4: propanol extract of Riband, Mercia and Brigadier ; lanes 5,6 & 7: propanol + 1% mercaptoethanol +1% acetic acid; lanes 8 & 9: Riband and Mercia. For further experimental details see methods.

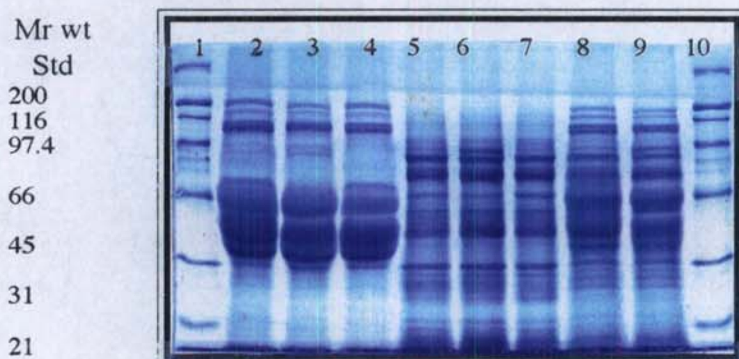


Fig. 3. The effect of decreasing contamination of the soft wheat Riband by the hard wheat Mercia on the Stenvert hardness score. For experimental details see methods.

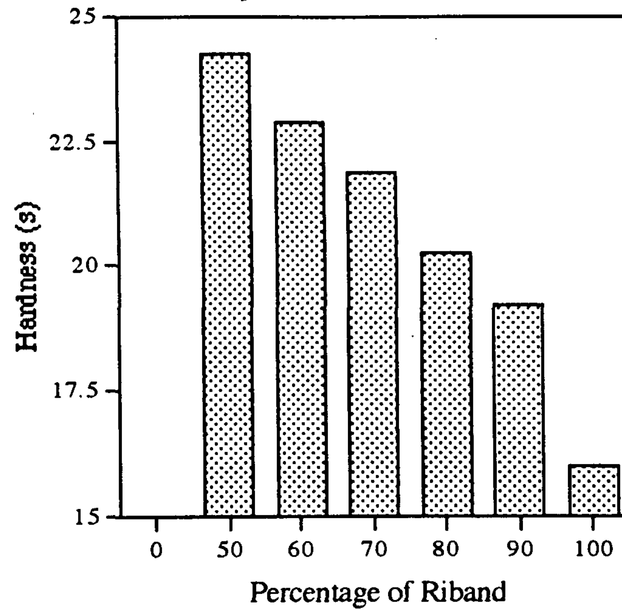
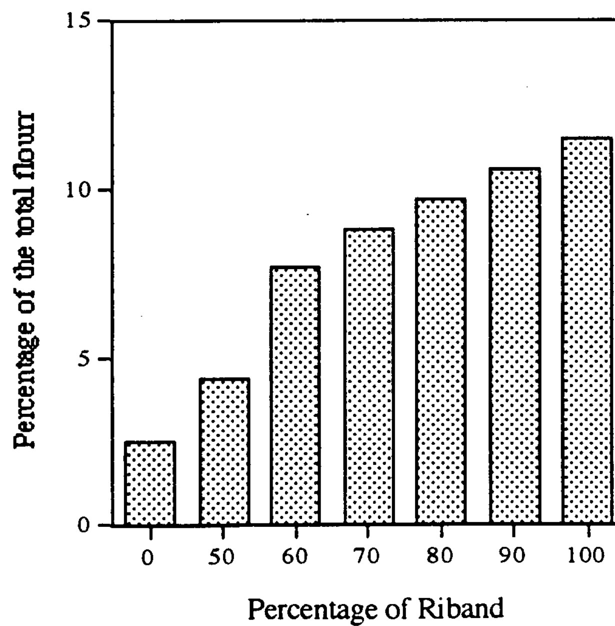


Fig. 4. The effect of decreasing contamination of the soft wheat Riband by the hard wheat Mercia on the percentage of the flour greater than 1 mm and less than 2.2 mm. The flour was prepared by milling the wheat samples in a coffee mill for 5 seconds. For experimental details see methods.



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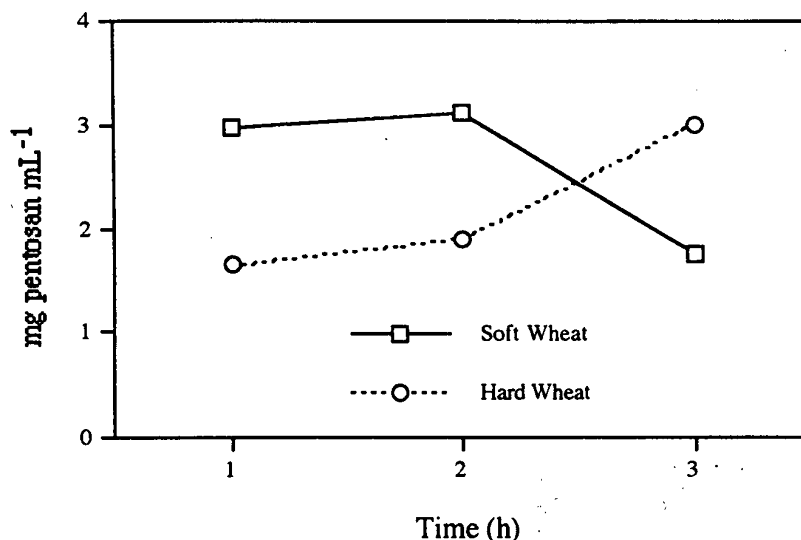

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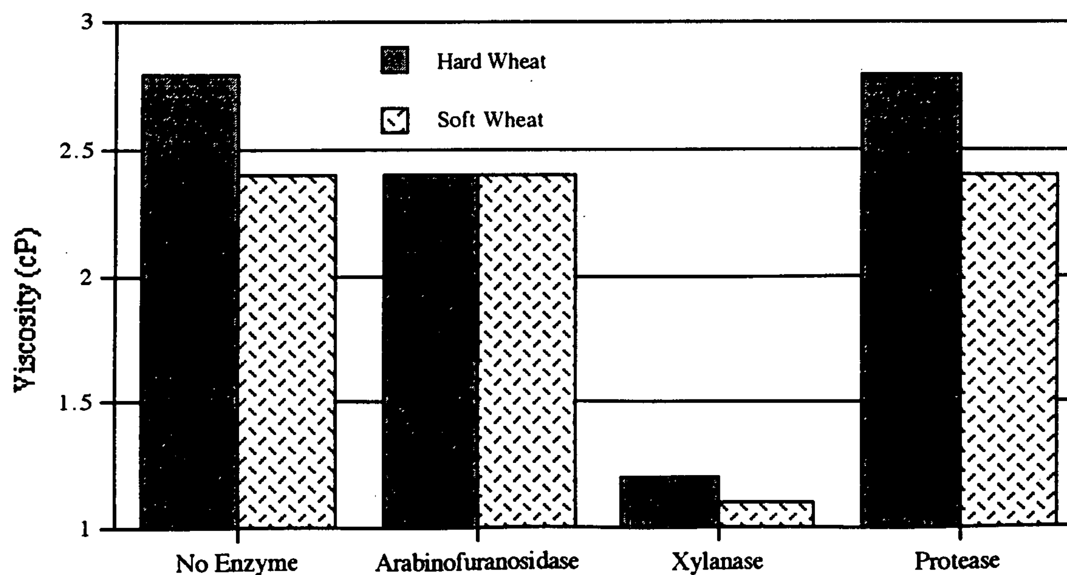

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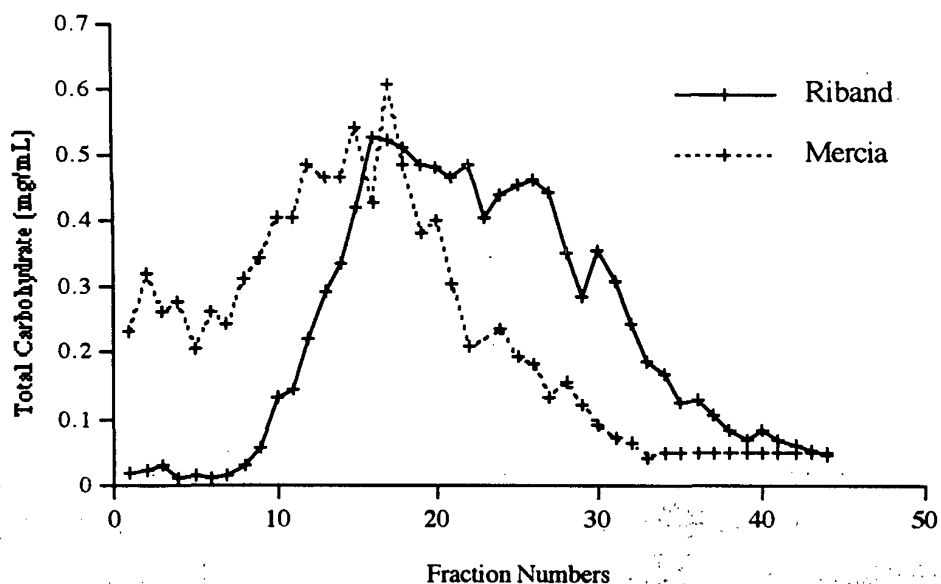

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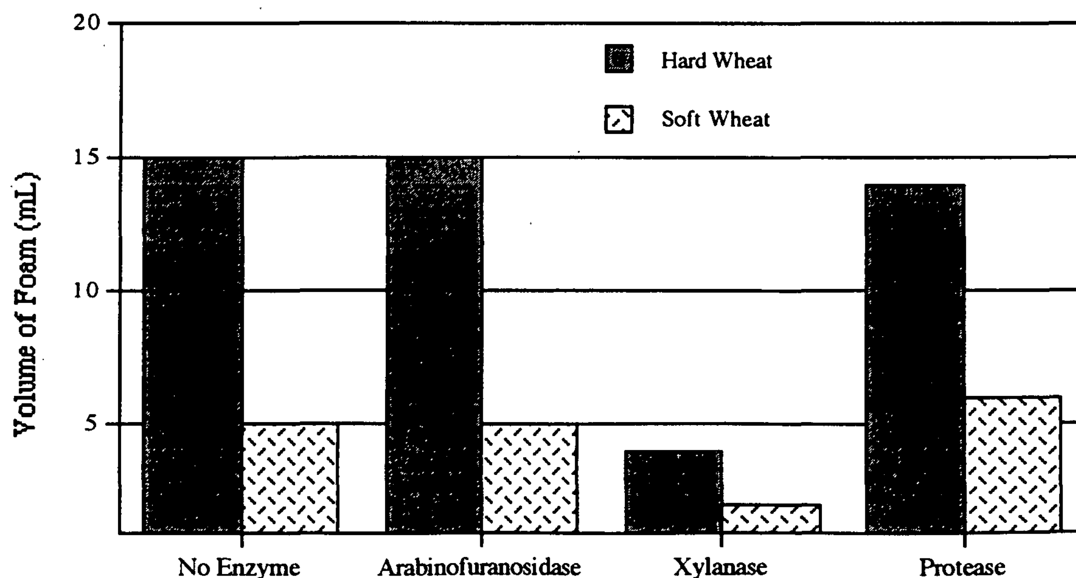

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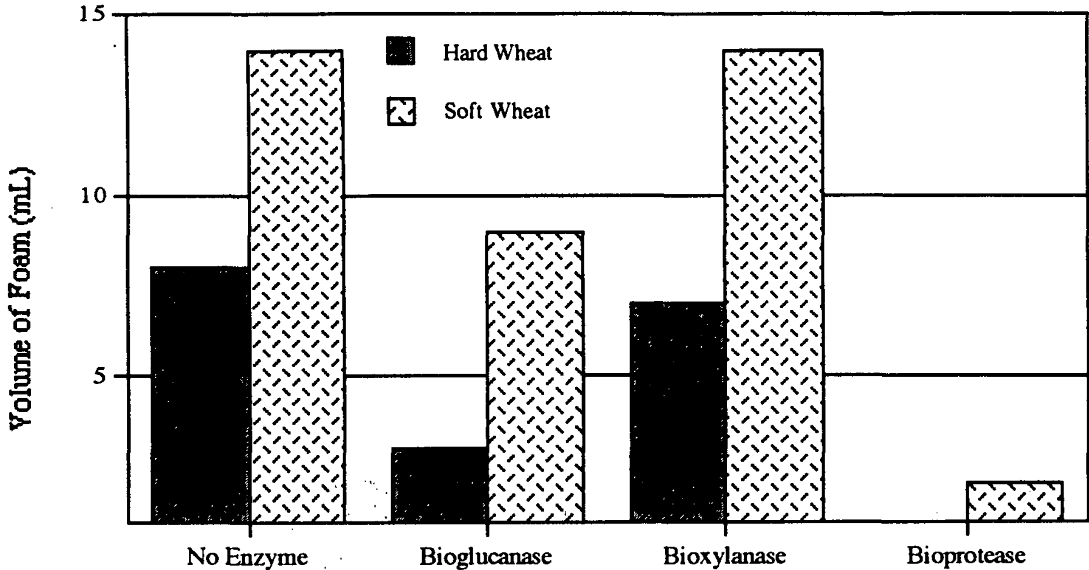

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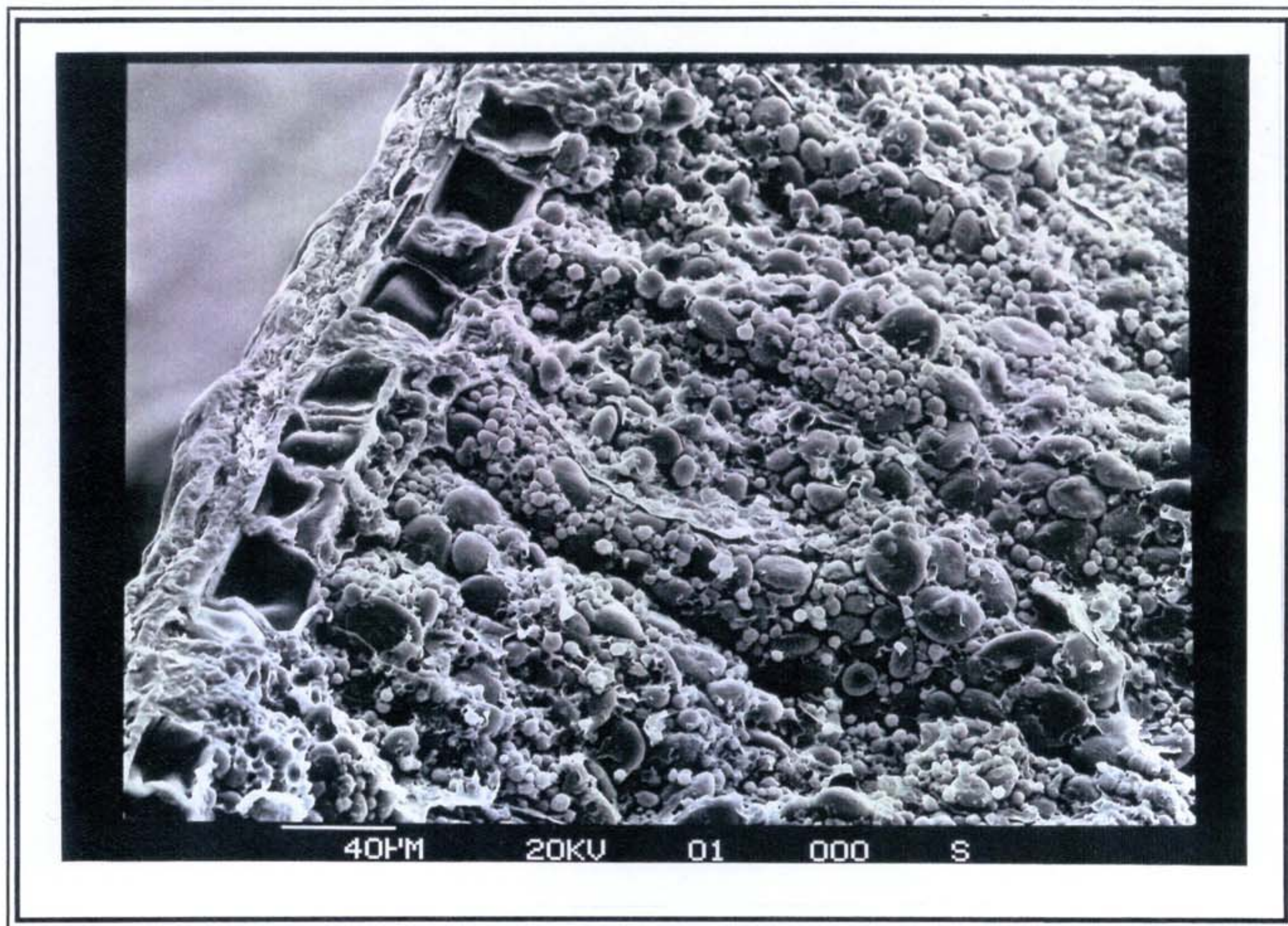

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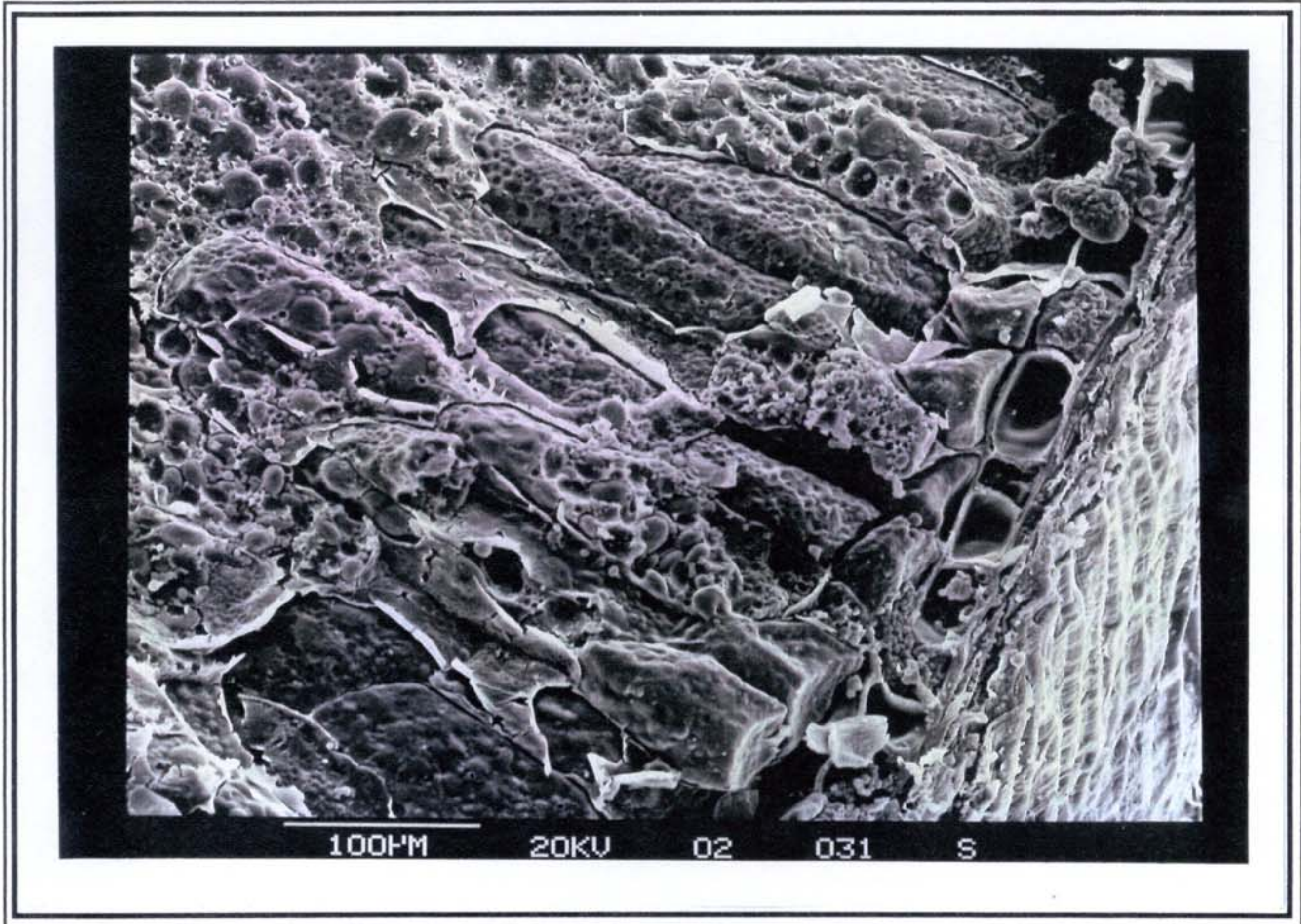

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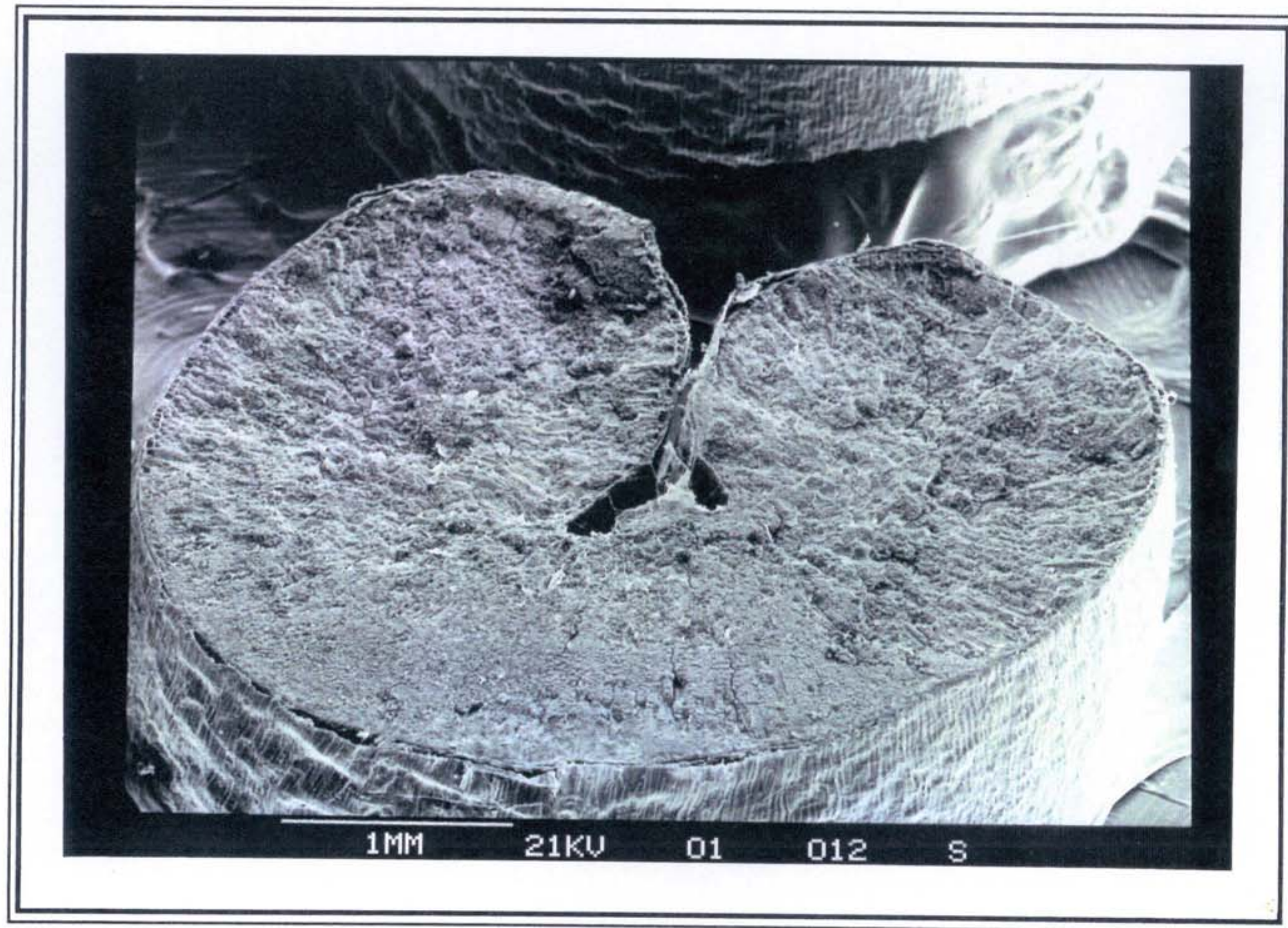

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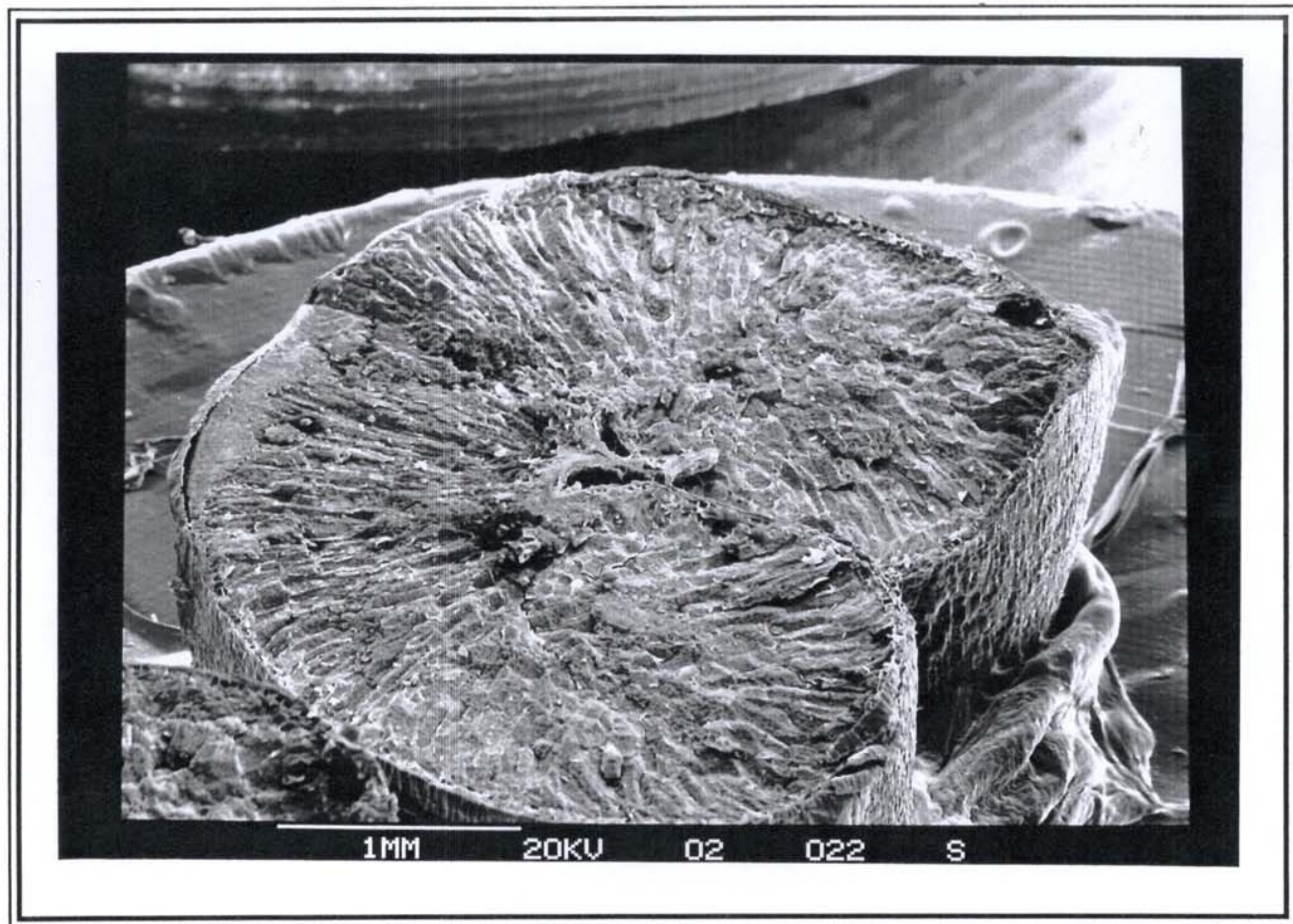

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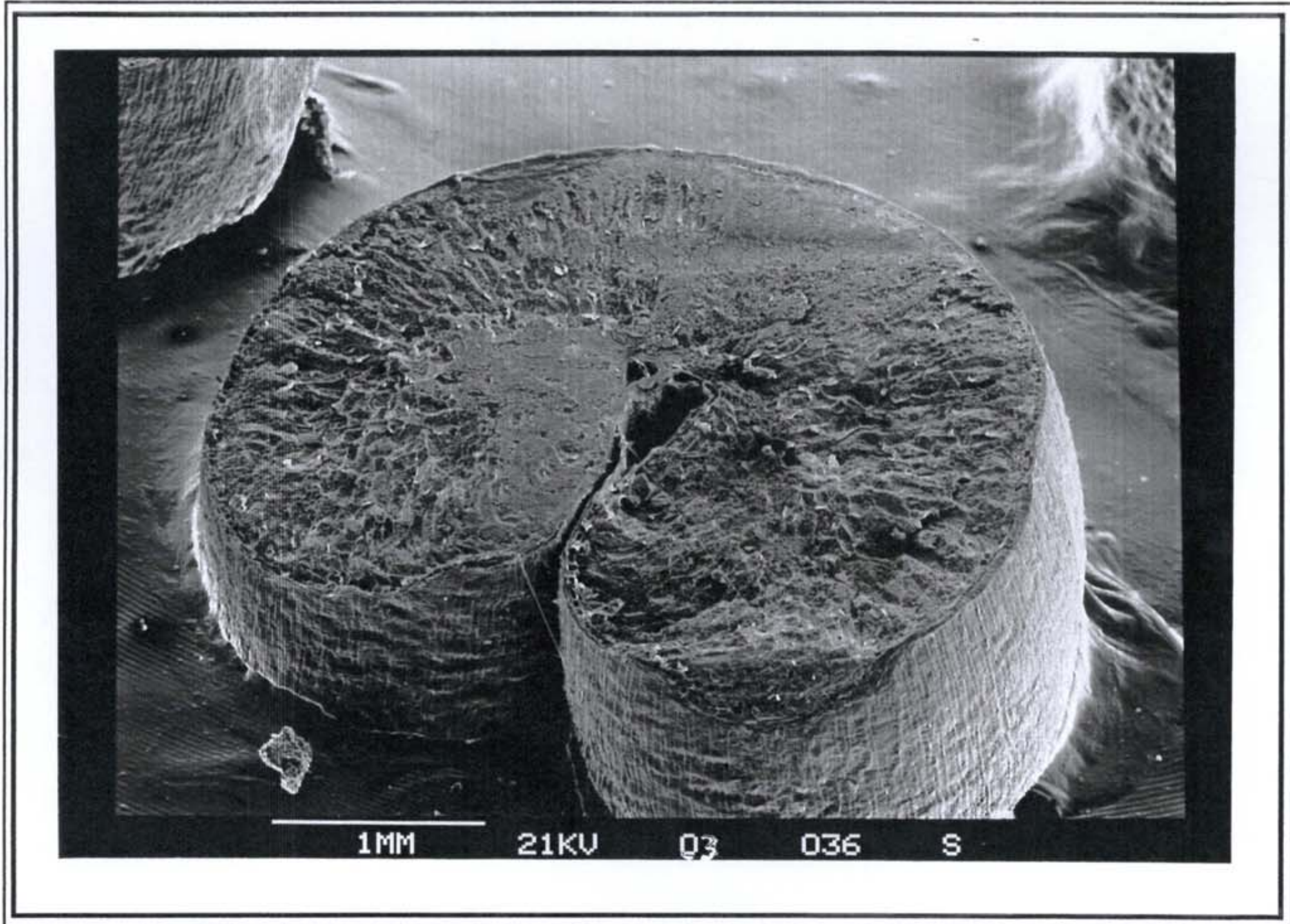

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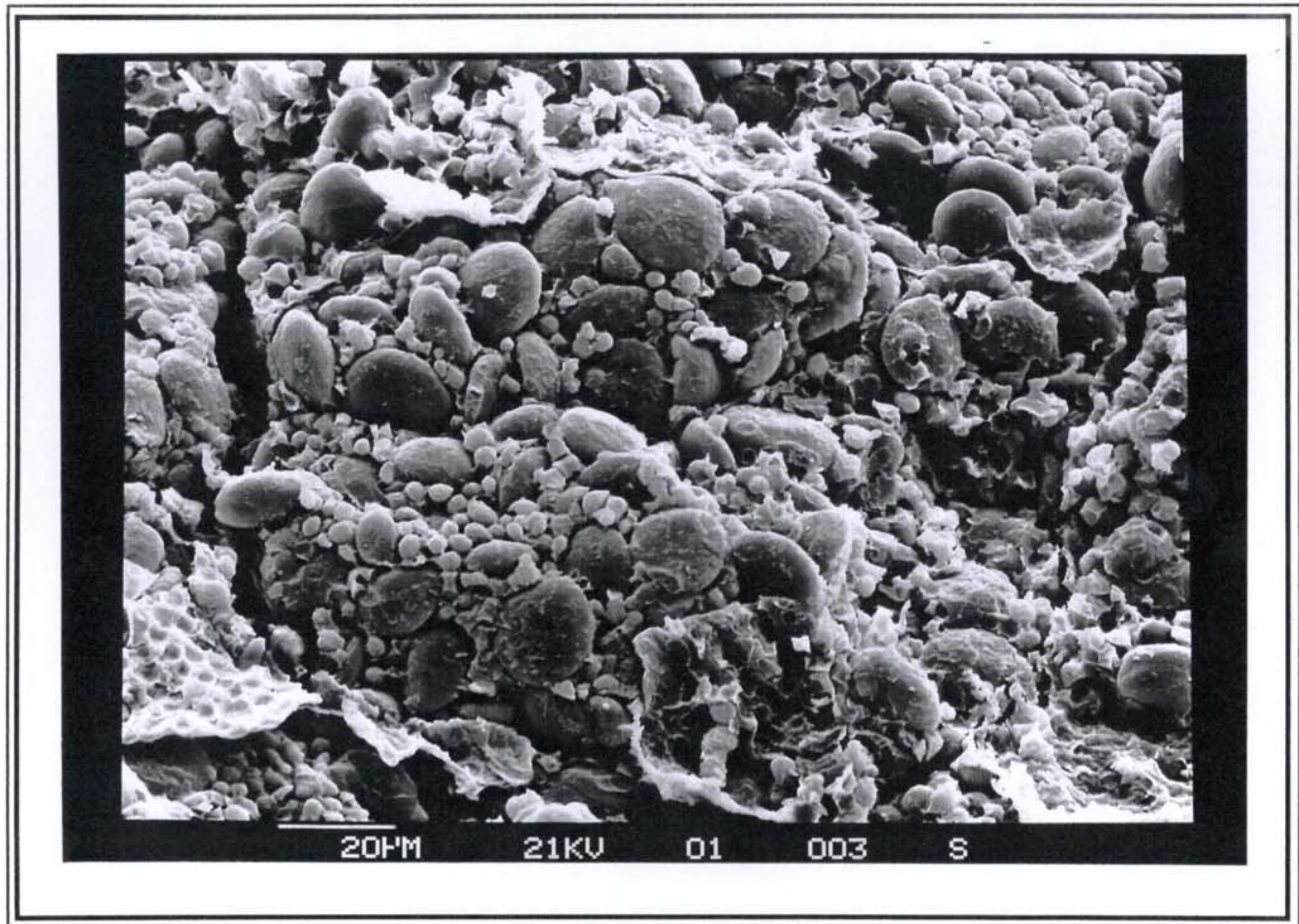

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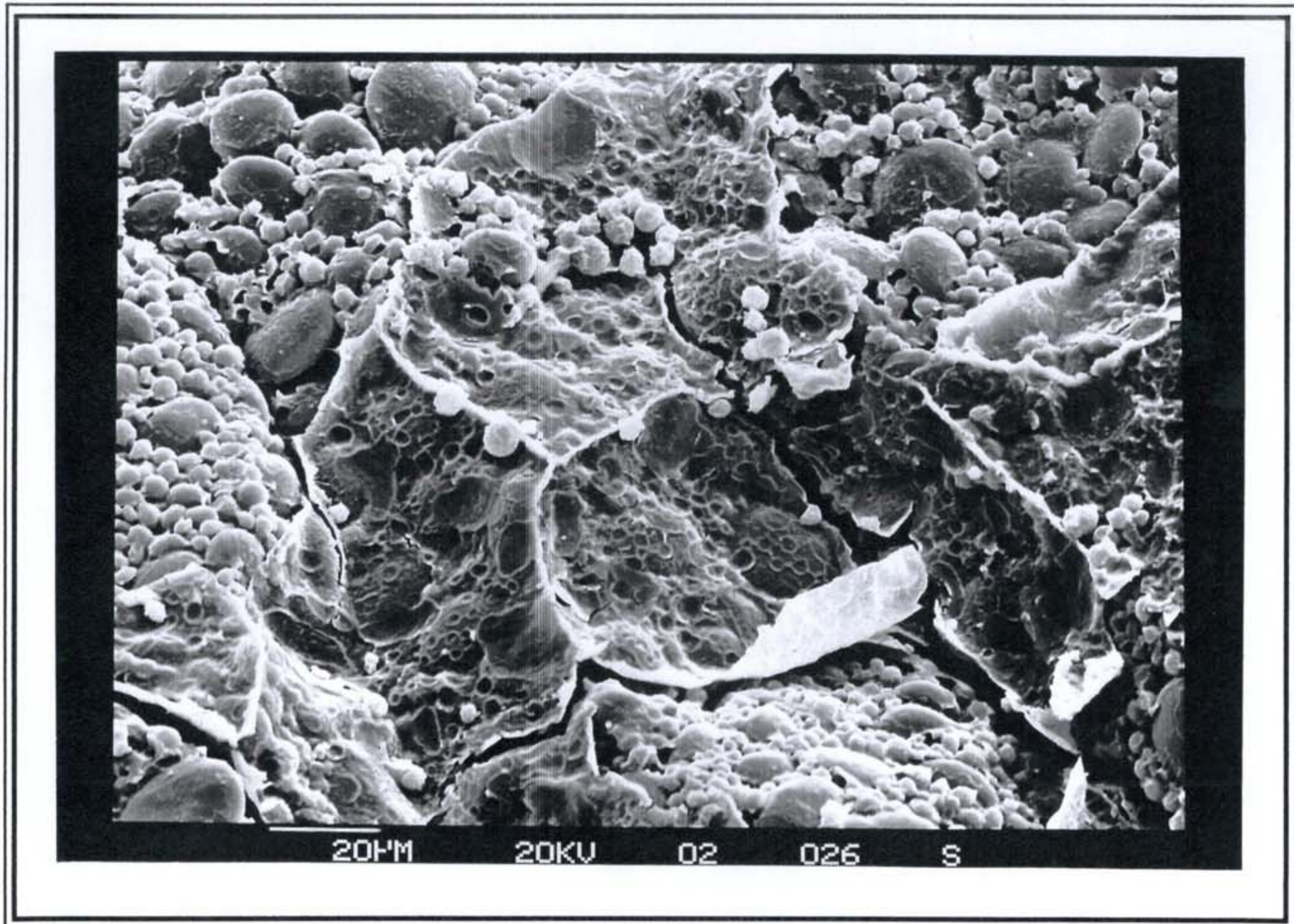

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Hard Wheat **Soft Wheat**

B L M R C E