Project Report No. 467

Maximising bioethanol processing yield of UK wheat: effects of non starch polysaccharides in grain

by

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CONTENTS

ABBREVIATIONS USED

AA	Amino acid
AX	Arabinoxylan
AMG	Amyloglucosidase
AY	Alcohol yield
CO_2	Carbon dioxide
DM	Dry matter
DP	Diastatic power
DU	Dextrinising units
FAN	Free Amino Nitrogen
ha	Hectare
HFN	Hagberg Falling Number
L	Litre
N	Nitrogen
NSP	Non Starch Polysaccharides
NG-NSP	Non-glucosic Non Starch Polysaccharides
RL	Recommended List
RV	Residue viscosity
SWRI	Scotch Whisky Research Institute
t	Metric tonne
SpWt	Specific weight

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1. ABSTRACT

The objectives of the project were to understand differences in alcohol yield (AY) between UK wheats typical of those which would be delivered to a biofuel distillery including hard endosperm and 1B1R wheats (i.e. those containing the 1BL/1RS translocation), to quantify the importance of non starch polysaccharides (NSP) in wheat grain with respect to variation in AY, and to use this information to guide plant breeders, growers, distillers and operators of UK bioethanol plants. The standard lab method for determination of AY (the 'wheat cook' method using barley malt as a source of enzymes and free amino N) was transferred to the ADAS lab, and later adapted to an enzyme-only method, representative of a biofuel distillery. The modified enzyme method was used to screen a set of samples from a variety x N response experiment harvested at ADAS High Mowthorpe, comparing two varieties Ambrosia (+1B1R) and Istabraq (-1B1R) with 6 levels of fertilizer N from 0 to 340 kg/ha and the NSP contents measured. Fermented and distilled samples were also assessed for their residue viscosity (RV) which gives an indication of processing quality. A set of 30 commercial samples at a fixed protein content of 11.5% was also selected from a larger dataset, to provide representation of the major nabim wheat classes, and their AY, RV and NSP contents determined. There was a significant negative relationship between AY and grain protein, but no significant effects of fertiliser N on NSP concentration, and no significant difference in average AY or NSP between Ambrosia and Istabraq. However, it was noted that Ambrosia is not entirely typical of the 1B1R wheats which have historically given processing problems, hence other 1B1R wheats may have higher NSP levels. Interestingly, RV increased significantly with increasing grain protein content (in response to fertiliser N) indicating that soluble proteins, rather than arabinoxylans, may be responsible for differences in poor processing quality in high protein wheats. For wheat samples studied at a fixed protein content, there was a negative relationship between average AY and NSP content when wheats were arranged by nabim classification, with Groups 3 and 4 soft wheats having the highest AY and lowest NSP levels. Group 1 wheats had the highest NSP contents. On average reduction of 2 g/100g in NSP content gave an increase in AY of 14 L/t at a fixed protein content. The analysis suggests that in order to fully understand variation in AY in wheat grain, more information is needed on the other non-starch, non-protein fractions such as oil and ash, and also data quantifying the rate and extent of starch digestibility.

2. SUMMARY

2.1. Objectives of the study

The objectives of the project were to establish a biofuel lab testing methodology, use this to investigate differences in alcohol yield (AY) between UK wheat varieties including hard wheats, to quantify the importance of non starch polysaccharides (NSP) in wheat grain with respect to variation in AY, and to use this information to guide plant breeders, growers, distillers and operators of UK bioethanol plants, thereby improving the competitiveness of UK-produced bioethanol from wheat.

2.2. Background

With a UK government target of 3.5% of transport fuels from renewable sources by 2010 and an expected 5% by 2013, a significant market for biofuels is emerging and wheat will be the principal feedstock for bioethanol production. Potentially the UK biofuels industry possesses advantages over its European competitors due to high yields and good quality grain, but it will be vitally important to maximise the yield of alcohol per tonne of grain, in order to maintain UK competitiveness in a world market for bioethanol. Currently varieties screened at Recommended List (RL) stage for the potable alcohol industry focus on a restricted range of soft wheats. However, for the emerging biofuel industry, it would be desirable to have information on a wider range of varieties.

Varieties in HGCA-funded RL trials and in the Green Grain LINK project (Project 2979) have been tested for AY by the Scotch Whisky Research Institute (SWRI), and there are some indicators of the best varietal types suitable for distilling (see HGCA Project Progress 14). We have shown that for each 1% decrease in protein content, there is an increase of 7 L/t in AY, which indicates direct replacement of protein with starch. However, for a given protein content, different wheat varieties do not give the same AY. For example at 11.5% protein, the range in alcohol yields is of the order of 50 L/t. This means that other components of the grain must influence potential alcohol yield.

Research Review No. 61 identified the key components of feedstock quality, which are likely to influence AY from wheat (starch, sugars and protein), and predicted the theoretical yield of alcohol for wheat at 11.5% protein of 478 L/t based on typical

starch, sugar and non-starch polysaccharide (NSP) values determined from various literature sources, and assuming a fixed loss of 8% of the sugars due to yeast growth. NSP (principally soluble and insoluble arabinose and xylose (arabinoxylans, AX), and to a lesser extent β-glucans) contribute up to 10% of the total grain dry matter (Smith *et al*., 2006). NSP may have negative impacts on alcohol processing yield and also the efficiency and throughput of the overall potable alcohol process: NSP can physically limit release of starch from the endosperm matrix and therefore reduce the yield of fermentable sugars. However to date, for UK wheats, we have little data on the actual NSP contents in grain in the range 8-12% protein or for different varieties, and therefore do not know with any certainty what the theoretical yield should be at these lower protein contents and for different varietal types. To date there has not been a comprehensive study of UK wheats, where both protein, NSP and AY have been measured on the same samples.

There was clearly a need to study in a systematic way the requirements of wheat for bioethanol production, by (i) screening varieties using methodology not constrained by traditional potable alcohol processes e.g. using enzymes and a lower temperature of mashing, and (ii) understanding the changes in grain composition and AY over a range of grain N contents (iii) studying a wider range of varieties than is currently studied by the potable alcohol industry at a fixed protein content. Through judicious choice of wheat varieties and samples, the work brought these three elements together.

2.3. Materials and methods

The standard lab method for determination of AY (the 'wheat cook' method using barley malt as a source of enzymes and free amino N (FAN)) was transferred to the ADAS lab, and later adapted to an enzyme-only method, typical of a biofuel distillery. The modified enzyme method was used to screen a set of samples from a variety x N response experiment harvested at ADAS High Mowthorpe, comparing two varieties Ambrosia (+1B1R) and Istabraq (-1B1R) with 6 levels of fertilizer N from 0 to 340 kg/ha and the NSP contents measured. Fermented and distilled samples were also assessed for their residue viscosity (RV) which gives an indication of processing quality. A set of 30 commercial samples at a fixed protein content of 11.5% was also selected from a larger dataset, to provide representation of the major nabim wheat

classes, which might express the full range of NSP contents seen in UK elite varieties of wheat, and their AY, RV and NSP contents determined.

2.4. Results and Discussion

2.4.1. Establishment of alcohol yield lab methodology

The initial step was to replicate the SWRI alcohol yield method and establish it in the ADAS lab. This involved an extensive period of testing and modifications to the methods. The method was validated using a number of commercial wheat samples which had been initially screened on an Infratec NIR by FOSS representing the full range of proteins typically found in wheat (7.7 to 14.6 g/100g). These represented the typical feed and other wheats available at the time of the project and in particular, in the region surrounding the planned Vivergo bioethanol plant. The results from the two labs only differed by *ca*. 15 L/t (approximately 3.5% of the average AY of 435 L/t). The method also gave sensible estimates of AY when plotted against grain protein content similar to that reported previously with a typically high adjusted R^2 value of 0.78. For others wishing to adopt this methodology, various steps were identified where particular attention is required; specifically attention must be given to milling, ensuring presence of sufficient thermostable enzyme and employing a rapid heating procedure during the distillation step. All determinations of AY and RV were carried out in duplicate.

Next, using commercial enzymes, a protocol was developed to assess AY of wheat by an 'enzyme-only' method, avoiding the use of barley malt. It was found that the AYs were very low using the simple enzyme method. For four samples of Robigus taken from an ADAS N response trial, the average AY (349 L/t) was only 80% of the value obtained for the same samples using the barley malt method (438 L/t). It was assumed that insufficient starch conversion was achieved. Hence the process conditions were lengthened, the temperatures increased by including an autoclave step, and an additional protease enzyme employed to aid dissolution of the endosperm matrix and release the starch granules. Finally, the concentrations of enzymes used were increased by a factor of 10 above commercial rates, to ensure they were present in excess. This gave an average AY of 418 L/t. The enzyme-only procedure was then used to screen wheat samples in the following two sections. Further modifications could undoubtedly be made to refine this method, for instance

using more thermostable amylases, but this was beyond the scope of the current project. Until a commercial bioethanol supplier is active, and more is understood of the typical process and AY it is premature to adapt a definitive lab AY method. Further dialogue on this subject will be required with bioethanol producers.

2.4.2. Impact of 1B1R translocation and N fertiliser on bioethanol processing yield and NSP content

Samples of Ambrosia and Istabraq from an N response trial at High Mowthorpe were assessed for AY and RV using the enzyme only method. Both varieties had shown significant responses of both grain yield and grain protein to fertiliser N. When AY was plotted against grain protein the slope was close to the theoretical slope of 6.6 (based on a 1:1 replacement of starch and sugars with protein). When the variety effect was assessed separately, it was seen that Istabraq had a slightly higher average AY than Ambrosia (+2 L/t), greater than that expected based on the differences in grain protein between the two varieties. However, analysis of variance showed that this variety effect was not statistically significant.

Residue viscosity was also measured following distillation. There was no significant effect of variety, but a significant effect of N fertilizer. A linear regression analysis was carried out to investigate the relationship between RV and grain protein. RV was shown to be positively related to grain protein content and the regression was highly significant.

The samples of Ambrosia x Istabraq from the N response trial described above were analysed for their total NSP concentrations. Analysis of the sugars was determined following the digestion and removal of starch and hydrolysis of NSP. There were very small effects of variety or N level on total NSP or the concentrations of individual constituent sugars, and no significant interactions between variety and N. The sum of arabinose and xylose (the two major constituents of wheat NSP) were significantly higher in Ambrosia (by 0.3 g/100g) which was due to a higher xylose level in this variety. It was noted that Ambrosia is not entirely typical of the 1B1R wheats which have historically given processing problems, hence other 1B1R wheats may have higher NSP levels. However, since most breeders now avoid the use of 1B1R wheats in their breeding programmes (because of negative associations with the rye translocation and feed quality in particular) 1B1R is less relevant to elite varieties on

the RL, and few such varieties are now available for study, particularly in N response trials. Regression analysis indicated that there was no significant relationship between grain protein and NSP content. The observation of a lack of relationship between NSP content and applied fertiliser N supports recent work on Belgian varieties, published since the start of this study.

It is concluded that if NSP varies little in response to fertiliser N, then any other variation in AY not explained by changes in starch concentration, must be due to variation in other non-starch, non-protein components, specifically the oil and ash fractions (associated with germ and bran layers respectively). Alternatively, other variation in AY at a given level of protein may be due to differences in the proportion of starch converted (extent of starch digestibility).

An interesting observation was that RV appeared to increase significantly with increasing grain protein content (and hence applied fertiliser N). Hence rather than soluble AX being the main factor influencing processing quality (the viscosity of which can be controlled by commercial xylanase enzymes in a biofuel distillery), it may be that soluble proteins are more significant in impacting on RV and hence processing quality of wheats for distilling. Since there is appreciable variation in protein sub unit composition within wheat germplasm, which is well understood by breeders, further work on the effects of protein content and composition on the processability of wheats may be valuable in the future, once a definitive lab biofuel methodology is established.

2.4.3. Alcohol yield, residue viscosity and NSP of wheat varieties at fixed protein content

Commercial samples of wheat were collected from a national survey at 2008 harvest, which allowed selection of 30 wheats of the same protein content (11.5% typical of a feed wheat fertilized at the economic optimum), which were contrasting in terms of their classification by nabim group i.e. Groups 1 & 2 (milling wheats), 3 (soft/biscuit wheats), 4 hard and 4 soft (feed wheats). Samples were analysed for AY and RV using the enzyme method described above, as well as for neutral sugar composition and NSP content.

Although Group 3 and Group 4 soft (4s) wheats had the highest AY, there was no significant difference between groups in terms of average AY, specific weight and Hagberg falling number. Total NSP differed significantly between groups, with Group 1 and Group 4 hard (4h) wheats having higher NSP contents than those of the Groups 3 and 4s wheats The individual sugars contributing to these differences were principally arabinose and xylose, and hence the total AX which were significantly greater in Group 1 and 4h wheats. Within the total NSP (which normally includes glucose from cellulose and β-glucan) the non-glucosic NSP (NG-NSP) showed highly significant differences between varieties with Groups 1, 2 and 4h wheats having significantly higher NG-NSP concentrations than Groups 3 and Group 4s. Further work would be required to see whether this was a consistent marker for these types of wheat. There was a negative relationship between average AY and concentration of NG-NSP when assessed by variety group.

Results from this study are consistent with a previous study (HGCA Project report 448) which indicated that Group 1 wheats might have higher NSP levels, and this may have been due to inadvertent selection for high water absorption in milling wheats. In contrast Group 3 wheats which are used for biscuit and batter production require low water absorption, and may indirectly have been selected with lower NSP concentrations.

If NSP concentration in wheat could be decreased by 2 g/100g for a fixed grain protein level, then this should increase AY by *ca*. 14 L/t given no other compensatory changes in grain composition. Other solutions to assessing wheat varieties at the same protein content would be to grow samples in a variety trial with say two or three N levels and after harvest, and blend samples from the different N treatments of each variety, to give a standard protein content sample for testing, or alternatively to study isogenic lines. However, since most 1B1R wheats have now been removed from the testing system (both for feed and distilling purposes) it seems more pertinent to focus on the AY of the hard wheats (Groups 1 and 4h) rather than 1B1R in the future.

2.5. Key conclusions

1. Methods are in place to screen wheats for AY and RV using traditional methods appropriate for the distilling industry using barley malt, and also using enzyme-only methods appropriate for the biofuels industry.
2. An enzyme-only method gave results with the same relationship between AY and grain protein seen in previous work, but slightly lower average AY in absolute terms than using the potable alcohol distilling method.
3. Further work needs to be done in liaison with commercial biofuels distilleries to ensure that lab screening methods correctly mimic their processes and give similar AY as the commercial processes; in particular, further work to mimic a biofuel type process is required, by considering the inclusion of thermostable amylases.
4. Using samples of varieties Ambrosia and Istabraq harvested from an N response trial, there appeared to be no difference in AY or RV between these wheats contrasting in presence of the 1B1R translocation, and no difference in NSP content.
5. NSP concentration did not appear to be affected by N fertilizer rate (or grain protein concentration) but interestingly RV appeared to increase with increasing grain protein content, suggesting that soluble proteins rather than AX may be implicated in causing high RV (and hence poor processing quality) in wheat.
6. Analysis of wheats at a fixed protein content indicated that wheats of nabim Groups 1, 2 and 4h had lower AY, and that Groups 1 and 4h had the higher AX and NSP concentrations, compared to the soft wheats in Group 3 and Group 4.
7. Analysis of the data for wheats averaged by nabim class indicated that high AY wheats tended to have lower NSP, and were associated with Groups 3 and 4s, (containing those varieties which currently are given distilling ratings on the RL).
8. Further work is required to investigate whether hard wheats do give consistently poorer AY than conventional distilling wheats, as Group 4h wheats are likely to be a major source of wheat to UK bioethanol plants in the near future, alongside high yielding 4s wheats.
9. Further work to study the variation in AY at a given protein content should focus on those other factors which might vary in wheat such as concentration of oil and ash, and the rate and extent of digestion of starch.

3. TECHNICAL DETAIL

3.1. Introduction

3.1.1. Objectives of the study

The aim of the project was to understand differences between UK wheat varieties, and to quantify the importance of non starch polysaccharides (NSP) in wheat grain with respect to variation in bioethanol yield, and to use this information to guide plant breeders, growers and operators of UK bioethanol plants, thereby improving the competitiveness of UK-produced bioethanol from wheat.

3.1.2. Background

With a UK government target of 3.5% of transport fuels from renewable sources by 2010 and an expected 5% by 2013, a significant market for biofuels is emerging and wheat will be the principal feedstock for bioethanol production. Potentially the UK biofuels industry possesses advantages over its European competitors due to high yields and good quality grain, but it will be vitally important to maximise the yield of alcohol per tonne of grain, in order to maintain UK competitiveness in a world market for bioethanol. Currently varieties screened at Recommended List (RL) stage for the potable alcohol industry focus on a restricted range of soft wheats. However, for the emerging biofuel industry, it would be desirable to have information on a wider range of varieties.

Previous HGCA studies

Varieties in HGCA-funded RL trials and in the Green Grain LINK project (Project 2979) have been tested for AY by the Scotch Whisky Research Institute (SWRI), and there are some indicators of the best varietal types suitable for distilling (see Project Progress 14 and Kindred *et al*., 2007). We have shown that for each 1% decrease in protein content, there is an increase of 7 L/t in AY, which indicates direct replacement of protein with starch (Smith *et al*., 2006; Kindred *et al*., 2008). However, for a given protein content, different wheat varieties do not give the same AY. For example at 11.5% protein, the range in alcohol yields is of the order of 50 L/t. This means that other components of the grain must influence potential alcohol yield.

Smith *et al*. (2006) identified the key components of feedstock quality, which are likely to influence AY from wheat, and predicted the theoretical yield of alcohol for wheat at 11.5% protein based on typical starch, sugar and non-starch polysaccharide (NSP) values determined from various literature sources. The NSP fraction is analogous to the dietary fibre fraction in foods, and comprises both insoluble fibres (originating mainly in the bran layers) and soluble fibres (from bran and more significantly, the endosperm). However to date, for UK wheats, we have little data on the actual NSP contents in grain in the range 8-12% protein, and therefore do not know with any certainty what the theoretical yield should be at these lower protein contents. While Weightman *et al*. (2008a, 2009) have studied the arabinoxylan (AX) contents of milling fractions of wheats, these data were not available at the start of this project and to date there has not been a comprehensive study of UK wheats, where both protein and NSP contents together with AY have been measured on the same samples.

3.1.3. Importance of non-starch, non protein components

Of the non-starch, non-protein grain components, NSP are perhaps the most important: NSP (principally soluble and insoluble arabinoxylans, and to a lesser extent β-glucans) contribute up to 10% of the total grain dry matter (Smith *et al*., 2006). Commonly, NSP have negative impacts on alcohol processing yield and also the efficiency and throughput of the overall potable alcohol process. NSP can physically limit release of starch from the endosperm matrix and therefore reduce the yield of fermentable sugars. However, this has not been studied to date in UK wheats, partly because NSP is rarely quantified and is difficult to measure.

Relatively low concentrations of AX in the co-products can lead to high viscosity and processing problems in the co-product stream, due to their high water binding capacity. This leads to a high energy requirement in the plant, as a result of removal of water on drying the residues (DDGS). NSP also fundamentally affect the rate of ethanol production, because the processing problems which they cause limits the number of batches of grain which can be processed within a given time period.

Viscosity in wheat is known to be closely associated with presence of the 1B1R translocation, and the presence of soluble NSP (Weightman *et al*., 2001). Once the liquid biofuel market is established in the UK, growers may find over time that certain

feed wheats (which give viscosity problems) are rejected by bioethanol producers. The industry needs advanced warning if this is to be the case.

Looking to the future it is also important to understand the possible variation in NSP levels in UK wheats, as there is interest in reducing their content in the distillers dried grains and soluble (DDGS) co-product, and eventually the NSP may themselves be substrates for fermentation to ethanol in bran (Palmarola-Adrados *et al*., 2005) and also straw. Conversely, NSP can be extracted from bran to produce novel food ingredients (Du *et al*., 2009; Misailidis *et al*., 2009).

3.1.4. Relationship between grain protein and NSP

As noted above, there is appreciable variation in AY even at a fixed protein content, but screening diverse samples e.g. from CEL trials (which vary both in terms of variety and the environmental conditions under which they were grown) means it is difficult to quantify the importance of grain traits (other than protein content which is known to vary widely due to residual N in soil, and N uptake by the crop). Therefore, there is a need to approach the problem in two different ways; firstly to study variation in AY and NSP at a fixed protein content (e.g. 11.5 g/100g), where variation in other traits is minimised, and secondly to assess samples varying widely in protein content, but where other environmental factors have been eliminated e.g. by taking such samples from a N response trial at a single location.

At the outset of the project there was little data on the relationship between N fertilisation and NSP level, or between grain proteins and NSP. More recently Dornez *et al*. (2007a,b) working in Belgium reported that N fertilization had no effect on AX level in wheat, although N fertilizer was shown to increase the levels of endogenous xylanase inhibitors in the grain (for further information on this topic see Verhoeven *et al*., 2005). However, in Dornez's studies, N was only assessed at three levels of N (0, 150, 300 kg/ha) rather than in a full response trial. Further information is therefore still required for UK varieties over a full range of N fertilization levels. While harvest year, and pre-harvest conditions (e.g. sprouting) were both shown to affect the proportions of water soluble AX, these factors appeared to have little effect on the concentrations of total AX.

3.1.5. Review of enzyme technology for bioethanol production

The current method for measuring alcohol yield (AY) is based on the traditional process for potable alcohol in whisky, using barley malt as the source of enzymes and free amino nitrogen (FAN) to provide nutrients for fermentation. A typical bioethanol distillery does not use barley malt but obtains the enzymes (necessary for liquefaction and hydrolysis of the wheat grain) from commercial enzyme suppliers (such as Genencor and Novozymes). These enzymes are of microbial origin – generally fungal enzymes. It should be noted that commercial enzymes do not possess single enzymes activities, but rather, a mix of enzymes to provide overall robustness in application. This robustness is required because each batch of cereal processed will vary slightly in starch and protein characteristics and the process will vary day by day from one distillery to another in heating rate, residence times, pH etc. Importantly, the barley malt in the Scotch Whisky method also provides an appreciable source of free amino nitrogen (FAN), which the yeast needs for growth (in addition to sugars). In the bioethanol (biofuel) distillery, the nitrogen source for the yeast is provided by urea and proteases. A schematic diagram of the bioethanol process is shown in Figure 1.

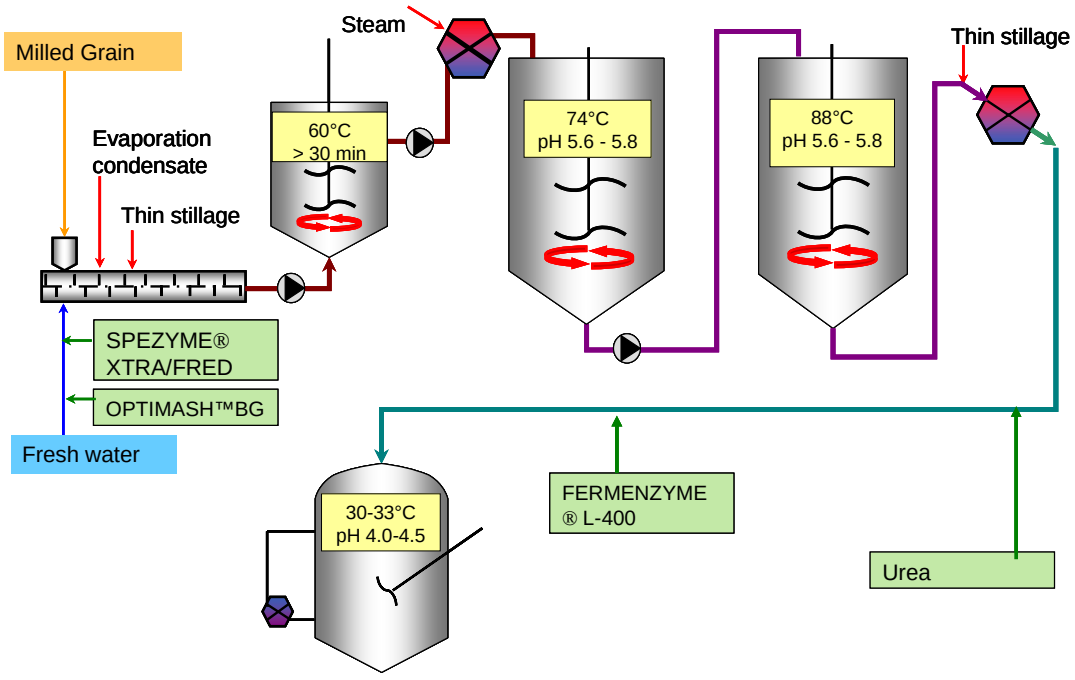

Figure 1 Schematic diagram of typical bioethanol process with points indicated for addition of enzymes and urea (green boxes). Image courtesy of Genencor.

Other technologies are available for alcohol production, for instance low temperature processing, whereby saccharification and fermentation occur simultaneously, as in the 'Stargen' process by Genencor (Anon, 2010). Such processes reduce energy inputs as well, and therefore the potential costs of the distilling process. Another reported advantage of this process is that the yeast does not suffer a high osmotic shock (as occurs in a traditional process where, after the starch is hydrolysed, the yeast is added to a medium containing a high concentration of sugars). Fermentation under such conditions is deemed to be more efficient than conventional processing. However as far as we are aware, none of the planned distilleries in the UK are going to use a low temperature process because the engineers are employing standard process technology as used in most US corn ethanol plants, so it is not considered further in this report.

Description of enzymes

It will be seen from Table 1 that a wide range of enzyme activities are needed to fully disperse the wheat grain and endosperm, to break down the protein matrix and to release all the starch granules so they can be hydrolysed prior to fermentation.

Table 1. Source of activities in commercial enzymes provided by Genencor for use in conventional distillery systems.

Product name	**Activities supplied (recommended addition rate[1])**
NSP-ases	
Optimash BG	β-glucanase/xylanase for barley/wheat (0.025-0.05 kg/t)
Optimash XL	cellulase/xylanase for wheat/rye (0.05-0.1 kg/t)
Optimash VR	xylanase/cellulase for rye/triticale (0.05-0.10 kg/t[2])
Proteases	
Fermenzyme L-400	saccharifying enzyme (gluco-amylase + protease) (0.06-0.1 kg/t[3])
Fermgen	acid fungal protease for ethanol production (0.1-0.6 kg/t)
Amylases	
Spezyme Xtra	high performance alpha amylase (0.2-0.4 kg/t)
Spezyme FRED	high thermostability alpha amylase (0.2-0.4 kg/t)

[1] kg enzyme product per tonne of wheat grain unless otherwise stated

[2] kg enzyme per tonne triticale

[3] kg enzyme per tonne starch

Xylanases

Given the presence of NSP, enzymes such as Optimash BG are required to provide xylanase activity to reduce the viscosity of arabinoxylans, particularly the water-soluble AX in the wheat endosperm. Other more powerful products supplying xylanase activity are available e.g. Optimash XL for wheat and rye and Optimash VR for rye and triticale. This reflects the higher AX content of rye and the higher viscosity of rye and also triticale (Davis-Knight and Weightman, 2008). It is likely that these only affect the ease of processing rather than starch yield *per se*, although if viscosity was very high during the early stages of processing, it could change the rates of heat transfer and mixing and hence rate of gelatinization of starch granules and access of amylase enzymes to the starch. These are currently unknown until commercial bioethanol plants are in operation and their exact processes more widely understood.

Proteases

The main purpose of the *cooking* and *mashing* processes in the distillery are to saccharify the starch and provide the glucose necessary for fermentation of alcohol by the yeast. In wheat, the starch granules are embedded in a protein matrix. The toughness of the endosperm varies between hard and soft wheats (see Kindred *et al*., 2007) and on milling, this influences the particle size distribution and degree of starch damage. During *cooking*, the starch is gelatinized and is released from the protein matrix. The high temperature gelatinizes and solubilises the starch, and at the end of cooking, there is a release of pressure as the temperature is reduced, which helps to mechanically break up the structure of the grain and release any tightly bound starch. In commercial bioethanol production, protease enzymes are added to accelerate the breakdown of the proteins and hence release of starch. For this reason, a product such as Fermenzyme L-400 is added to the process (Figure 1). Following cooking, and once the temperature has dropped sufficiently, the barley malt is added in the traditional whisky process and during *mashing*, the malt enzymes begin to hydrolyse the gelatinized starch.

As discussed above, the yeast requires a source of nitrogen to grow. In part this is provided by urea, but a second protease is generally added at the end of the process to provide amino-nitrogen rather than simply supplying mineral N. This further breakdown of protein occurs at the same time as fermentation is taking place, but action of the yeast reduces the pH of the fermentation culture to a degree. Therefore a protease is required which works under acidic conditions, and typically an acid-

protease such as Fermgen is added at the latter part of the process (although not indicated in Figure 1).

Amylases

Amylases (e.g. Spezyme Xtra, Table 1) are the most important part of the process as they provide the sugars for fermentation. Alpha-amylase is an important enzyme which attacks linear chain of amylase, attacking alpha (1-4) glycosidic linkages at random releasing maltose (and also yielding maltotriose and limit dextrins).

Alpha-amylase cannot deal with all the structures found in starch, for instance the alpha 1-6 branch points found within amylopectin which are attacked by pullulanases and also amyloglucosidase. Unlike other forms of amylase, amyloglucosidase (as found in Fermenzyme L400) is most active at low pH and is therefore added at the end, prior to fermentation. Amyloglucosidase releases glucose as well as maltose, and also attack the final glycosidic bond at the non-reducing end of a glucan chain.

Clearly amylases need to be able to perform over a wide range of temperatures and pH conditions (Figure 1). Again by combining different sources of alpha amylase activity within a commercial enzymes product, some robustness to process change is built in. One problem in particular is presented by high temperature, and products such as Spezyme FRED (Table 1) or Termamyl from Novozymes provide thermostable alpha amylases. Termamyl is a widely used thermostable amylase and is particularly suited to use in the SWRI 'wheat cook' laboratory method for determination of alcohol yield.

In whisky distilling, a second form of amylase, so-called β-amylase (a plant enzyme supplied in the barley malt) is present, which sequentially releases maltose units from glucans by attacking from the non-reducing ends of a linear chain (in a similar fashion to amyloglucosidase). In brewing it is considered that β-amylase produces more fermentable sugars, whereas alpha-amylase produces more non-fermentable sugars (and hence a sweeter product). It was beyond the scope of this project to study the performance of amylases, but it should be noted that one major difference between bioethanol distilling and whisky distilling may be that both α- and β-amylase activity are supplied by barley malt in the latter, and that their relative proportions are important in ensuring maximum efficiency.

3.1.6. Summary

There is clearly a need to study in a systematic way the requirements of wheat for bioethanol production, by (i) screening varieties using methodology not constrained by traditional potable alcohol processes e.g. using enzymes and a lower temperature of mashing, (ii) understanding the changes in grain composition and AY over a range of grain N contents and (iii) studying a wider range of varieties than is currently studied by the potable alcohol industry at a fixed protein content. Through judicious choice of wheat varieties and samples, the work brought these three elements together.

3.2. Materials and methods

3.2.1. Samples

Commercial wheat samples were provided by Frontier Agriculture. All samples were collected after harvest and scanned by NIR for determination of moisture and protein using standard FOSS calibrations.

Experimental wheat samples from a N response trial at ADAS High Mowthorpe in 2006 (HGCA project 3084) with 6 levels of N treatment, and 2 varieties contrasting in either the presence (Ambrosia) or absence (Istabraq) of the 1BL/1RS translocation, were obtained. Two replicates of each variety x N rate treatment were studied (n=24). Further details of the field experiment have been reported by Sylvester-Bradley *et al*. (2008).

3.2.2. Alcohol yield determination

Standard procedure using barley malt
AY determination was based upon the SWRI 'wheat cook' method (Agu *et al*., 2006). In summary:

- Finely ground wheat (Miag setting 0.2mm) is gelatinised and pre-liquefied at 85 ^{o}C with Termamyl, a thermostable endoamylase, to rapidly break down starch to oligosaccharides and reduce viscosity,
- The wheat is cooked at 142 ^{o}C and then given a second treatment of Termamyl at 85 ^{o}C to minimise retrogradation,

- The cooked slurry is then mashed at 65°C with a high DP/DU malt which contains a relatively high α- and β-amylase content and also supplies modified starch to the process (which is taken into account when calculating the AY of the wheat),
- The slurry is fermented for 68 +/- 2 hours and then distilled.

The process conditions are summarised in Table 2.

Table 2 Summary of process conditions for standard laboratory procedure for determination of alcohol yield of wheat, using 'wheat cook' method.

Process step	**Temp**	**Time**	**Enzyme dosages**
1. Slurry make-up	45 °C	Heating to 85 °C over 30 minutes	Termamyl 12.5 µL
2. Primary "Liquefaction"	85 °C	30 minutes	
3. Secondary "Autoclave"	126 °C	11 minutes	
4. Tertiary "Liquefaction"	85 °C 65 °C	30 minutes then cool to 65 °C 60 minutes	Second dose of Termamyl 12.5 µL Barley malt (20% DW addition)
5. Fermentation	30 °C	68 hours	
Post fermentation steps			
6.Distillation	Distil alcohol	*ca*. 30 min	Make to volume and measure alcohol strength at 20°C
7. Extract viscosity	Centrifuge	Recover s/natent	Determine viscosity (U-tube capillary) at 20 °C

This standard method was followed in the present study with the exceptions that in the ADAS lab the following changes were made:

- the wheat grain was milled using a Glen Creston hammer mill fitted with a 2 mm screen.
- the moisture content of the flour was determined on a subsample by drying overnight at 100 °C.
- quantities of flour tested (15 g) in each determination of AY were half that of the SWRI method, but samples were analysed in duplicate (single analyses at SWRI).
- The autoclave at ADAS reached 126 °C, rather than 142 °C at SWRI.

The full method as applied in the ADAS lab was therefore as follows:

ADAS standard (barley malt) method

Wholemeal flour (15 g fresh weight basis) was placed in a stainless steel beaker with 40.5 mL of water and 12.5 µL of a thermostable alpha-amylase (added in excess) to rapidly break down starch to oligosaccharides (Termamyl 120L, Novozyme). The slurry was then heated in a waterbath to 85 °C with frequent stirring, before being autoclaved at 126 °C for 11 min. The sample was returned to the waterbath and further 250 µl of the amylase was added when the slurry returned to 85 °C, to minimise retrogradation. The cooked slurry was then reduced in temperature and mashed at 65 °C for an hour with inclusion of barley malt that contains a relatively high α and β amylase content and also supplies modified starch and free amino nitrogen to the yeast (20% malt to 80% wheat on a dry weight basis). The slurry was pitched with distillers yeast (0.4% w/w; ABMauri) and fermented at 30 °C for 68 hours before being distilled and the distillate measured for alcohol content using an Anton Paar density meter. The residue after distillation was adjusted to 125 mL with water before being centrifuged and the supernatant filtered twice through GF/A filter papers. Viscosity of the supernatant was determined at 20 ^{o}C using a U-tube viscometer (PSL-BS/U B, Poulten Selfe & Lee, Essex, UK).

ADAS enzyme-only method

Alcohol yield and viscosity were determined in duplicate on a subset of five flour samples using an ADAS method adapted from that of the Scotch Whisky Research Institute (SWRI; Agu et al., 2006). For the original grain (Fraction 1) the wheat grain was milled using a Glen Creston hammer mill fitted with a 2 mm screen. Flour (15 g, fresh weight basis) was placed in a stainless steel beaker with 40.5 mL of water to which 53 µL of a thermostable alpha-amylase (Spezyme Xtra, Genencor), 75 µL of a protease (Fermgen, Genencor) and 6.8 µL of a β-glucanase (Optimash BG, Genencor) were added (in excess) to rapidly break down starch to oligosaccharides. The slurry was then heated in a water bath set at 60 °C for 35 minutes with frequent stirring, before the temperature of the water bath was increased to 74 °C and the sample was stirred for a further 60 min. The sample was then autoclaved at 126 °C for 11 min before being returned to the water bath set at 88 °C and a second dose (53 µL) of the alpha amylase added to minimise retrogradation. This cooked slurry was then mashed for a further 60 min at 88 °C before being removed from the water bath and allowed

to cool to approximately 30 °C. The slurry was then pitched with distillers yeast (0.4% w/w) as well as further enzyme additions; 75 µL of the protease, 13 µL of a saccharifying enzyme (Fermenzyme L-400, Genencor) and 1 mL of a 25 mg/mL solution of urea, before being fermented at 30 °C for 68 hours, after which the slurry was distilled, and the distillate measured for alcohol content using an Anton Paar density meter. The residue after distillation was adjusted to 125 mL with water before being centrifuged and the supernatant filtered twice through GF/A filter papers. Viscosity of the supernatant ('residue viscosity'; RV) was determined at 20 °C using a U-tube viscometer (PSL-BS/U B, Poulten Selfe & Lee, Essex, UK).

3.2.3. NSP determination

The procedure for determination of neutral NSP was adapted from the method of Englyst & Cummings (1984) reported by Weightman *et al*. (2009) and consisted of three major steps:

1. Removal of starch by enzymic digestion

All analyses were carried out in duplicate. To 100 mg finely milled wheat, 1.0 ml dimethylsulphoxide was added to completely wet the sample. Tubes containing the sample were placed in an oil bath at 100^{o}C for 30 min to gelatinize the starch. A solution of Termamyl enzyme and acetate buffer (4 mL) was added to each tube and the tube replaced in the oil bath for a further 10 min. Tubes were removed and placed into water bath at 50 ^{o}C before the addition of a mixed amyloglucosidase and pullulanase solution. Tubes remained in the 50 ^{o}C bath for a further 30 min. Samples were then cooled and 100% ethanol added to achieve a final concentration of 85% alcohol then refrigerated to all polysaccharides to precipitate. After repeated washing and removal of supernatants a dry residue was achieved.

2. Hydrolysis of NSP

Hydrolysis to individual monosaccharides was carried out based on the procedure of Saeman *et al*. (1954). The dry residue from step one was taken and 0.125 mL of 72% H_2SO_4 added. This was left to stand for an hour in a 30 ^{o}C waterbath. 0.4 mL of an internal standard (IS) of inositol (solution stored in 50% saturated benzoic acid) was then added along with 2.3 mL H_2O, before mixing and heating for 2 hours at 100 ^{o}C. Samples were then cooled prior to derivatization of the released monosaccharides (preparation of alditol acetates).

3. Derivatisation of neutral sugars and quantification of alditol acetates

Reduction of the sugars was achieved by adding 0.2 mL of NH_3 solution with 3mL sodium borohydride solution to 1 mL of the hydrolysate before vortex mixing. The tubes were left for 1 hour at 30 ^{o}C before being cooled on ice and neutralized with ~0.1 mL glacial acetic acid. To 0.3 mL of the above acidified solution 0.2 mL of methylimidazole and 3 mL acetic acid were added before leaving at 30 ^{o}C for 30 min and cooling on ice again. Water (5 mL) and dichloromethane (3 mL) were added before repeated washing and then transfer of the organic layer to a fresh test tube prior to analysis by gas chromatography using an Agilent DB-225 column, and detected using a mass spectrometer operating in selective ion mode (Agilent 6890 GC with a 5975 Mass Selective Detector; Agilent Technologies, Cheshire, UK). Each sample of alditol acetates was injected in duplicate onto the GC. Response factors for individual sugars were determined using a standard solution of monosaccharides plus IS and hydrolysed using 2N sulphuric acid in the same conditions as above. Concentrations of monosaccharides were converted to their anhydro-polymer equivalents by multiplying by the following factors: 0.896 (rhamnose), 0.88 (arabinose, xylose) or 0.9 (mannose, galactose, glucose) before correcting concentrations to a 100% DM basis. It should be noted that total NSP in this report is defined as total neutral sugars (uronic acids were not measured).

3.2.4. Statistical analysis

Analysis of Variance and linear regression analyses were carried out using Genstat 8 (VSN International Ltd, © 2005 Lawes Agricultural Trust). For studies involving wheat samples from N response trials, two-way ANOVA was carried out with treatment effects; Variety (df=1) and N level (df=4) and their interactions (df=4) compared against the residual variation (df=12) within treatments between blocks. For the study of commercial wheat samples at fixed protein content (n=30), one-way ANOVA carried out to test for the significance of differences between the means of 5 different wheat classes according to their nabim classification.

3.3. Results

3.3.1. Establishment of standard alcohol yield method

The initial step was to replicate the SWRI alcohol yield method (the 'wheat cook' method which uses barley malt as a source of enzymes and FAN) and establish it in the ADAS lab. This involved an extensive period of testing and modifications to the methods (data not presented here). In order to validate the method, a number of commercial wheat samples were sourced. These samples had been initially screened on an Infratec NIR by FOSS, using a prototype AY prediction calibration, developed under the 'Green Grain' project (HGCA project 2979). The raw data are shown in Table 3, representing the full range of proteins typically found in wheat (7.7 to 14.6 g/100g). These represented the typical feed and other wheats available at the time of the project and in particular, in the region surrounding the planned Vivergo bioethanol plant.

Table 3 Variety and source of samples with protein, moisture and alcohol yields from commercial samples of wheat (2007 harvest).

Variety/mix	Location	Protein (%DM)	Moisture (%)	ADAS AY (L/t DM)	SWRI AY (L/t DM)
Humb/Oakley	DN17 (S.Yorks)	7.8	17.0	444	470
Glad/Oakley	DN21 (Lincs)	10.1	16.4	432	457
Cordiale	NG32 (Lincs)	14.1	14.1	399	mv
Cordiale	NG32 (Lincs)	13.9	14.1	392	mv
Hereward	NG32 (Lincs)	11.8	13.7	434	440
Einstein	LN11 (Lincs)	10.7	16.1	440	455
Alchemy	S26 (S.Yorks)	7.7	15.9	444	468
Xi19	YO42 (E. Riding)	11.8	14.0	408	427
Xi19	YO42 (E. Riding)	11.7	14.8	404	428
Solstice	LS22 (W.Yorks)	11.8	18.7	417	438
Solstice	LS22 (W.Yorks)	12.1	18.5	419	439
Paragon	DN7 (S.Yorks)	14.6	16.4	383	404
Solstice	NE15 (T&Wear)	12.0	19.9	414	434
Einstein	CV13 (Warwicks)	10.4	15.3	420	449

mv, missing value

It can be seen that there is reasonable agreement between the two laboratories (Figure 2) with the points located close to the 1:1 line but with the ADAS lab underestimating AY by ca. 15 L/t compared to the SWRI measurements.

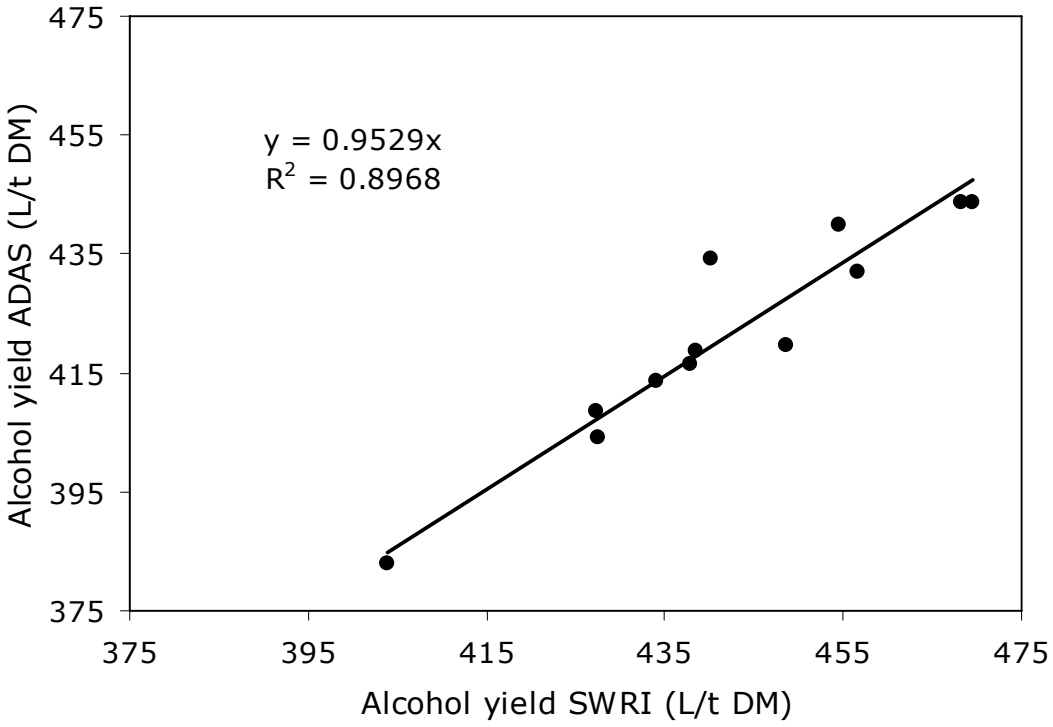

Figure 2 Comparison between laboratory determinations of AY of wheat samples at ADAS and SWRI, using the standard ('wheat cook') method using barley malt. Fitted line has intercept forced through zero (further details on variety and origins in Table 3).

While the adjusted R^2 is only 0.9 in Figure 2, it should be noted that there will inevitably be uncertainty in the measurements at both laboratories, and so it is very unlikely that the adjusted R^2 would approach 1.0 for such a complex and lengthy procedure.

Figure 3 shows the AY plotted against grain protein content. The data show a typical negative relationship, similar to that reported elsewhere (e.g. Smith *et al*., 2006) with a typically high adjusted R^2 value of 0.78. A similar regression analysis carried out to describe the relationship between AY measured at SWRI and protein gives the equation:

AY = -9.28 x grain protein + 545 (R^2 = 0.90).

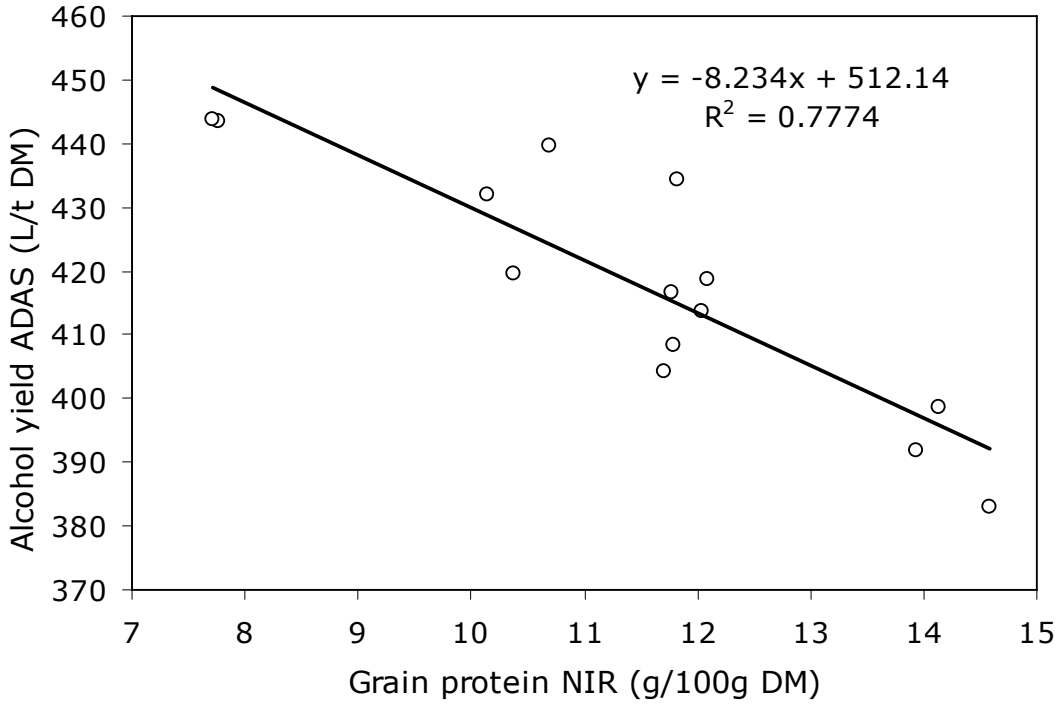

Figure 3 Relationship between alcohol yield measured in laboratory at ADAS and grain protein content determined by NIR for commercial wheat samples (further details on variety and origins in Table 1).

Viscosity determination

After distillation, the slurry was centrifuged, and the supernatant (liquid fraction) collected and its residue assessed. This 'residue viscosity' (RV) gives a means to identify any likely processing problems with particular varieties – a high RV being undesirable as it is indicative of poor processing characteristics. It can be seen that the SWRI RV values were lower than the comparative ADAS values (Table 4) which may be due to the higher cooking temperature at SWRI, or the source of barley malt.

Based on the performance of the laboratory method, in terms of its agreement with SWRI AY results and the ability to demonstrate a sensible relationship between AY and grain protein, it was deemed that the method could be confidently used to screen wheat varieties.

Table 4 Residue viscosity following distillation of wheat samples and assessed for their alcohol yield (wheat samples provided by Frontier agriculture, 2007 harvest).

Variety/mix	ADAS residue viscosity (mPa.s)	SWRI residue viscosity (mPa.s)
Humb/Oakley	1.67	1.28
Glad/Oakley	1.68	1.27
Cordiale	1.91	mv
Cordiale	1.86	mv
Hereward	1.81	1.20
Einstein	1.65	1.31
Alchemy	1.68	1.29
Xi19	1.71	1.33
Xi19	1.74	1.32
Solstice	1.57	1.28
Solstice	1.62	1.28
Paragon	1.81	1.33
Solstice	1.52	1.27
Einstein	1.60	1.29

mv, missing value

3.3.2. Development of enzyme-only method

Next, using enzymes supplied by Genencor as detailed in Table 1, a protocol was developed to assess AY of wheat by an 'enzyme-only' method, avoiding the use of barley malt. Initial studies employed the simple procedure shown in Table 5, which was devised to eliminate the need for an autoclave step and used enzymes at the recommended dosage rates as per manufacturer's instructions.

Table 5 Summary of process conditions for initial laboratory procedure for determination of alcohol yield of wheat, using commercial enzymes.

Process step	Temperature	Time	Enzyme dosages (mg enzyme/g wheat FW)
Slurry make-up	60 °C	35-40 min	SPEZYME®XTRA: 0.2 mg/g OPTIMASH BG: 0.05 mg/g
Primary "Liquefaction"	74 °C	60 min	No addition
Secondary "Liquefaction"	88 °C	60 min	Additional SPEZYME®XTRA; 0.2 mg/g
Fermentation	32 °C	72 hours	FERMENZYME®L-400: 0.06 mg/g + Urea

It was found that the AYs were very low using the simple enzyme method. For four samples of Robigus taken from an ADAS N response trial, the average AY (349 L/t) was only 80% of the value obtained for the same samples using the barley malt method (438 L/t). It was assumed that insufficient starch conversion was achieved. Hence the process conditions were lengthened, the temperatures increased by including an autoclave step, and an additional enzyme (Fermgen) employed. Finally, the concentrations of enzymes used were increased by a factor of 10 to ensure they were present in excess.

First it was necessary to check that increasing the enzyme concentration would not give high blank readings industrial enzyme preparations are often stabilised with sugars, which would make a contribution to the AY of fermentations. This was tested with a simple trial of varying inputs (Table 6) using enzymes added at up to 100x industrial recommendations.

Table 6 Inputs and measurements to assess effects of enzymes at 100x commercial addition rates, and urea on background contribution to alcohol yield measurements.

Treatment	Enzymes	Urea (25mg flask)	Glucose (5g/flask)	Yeast	Alcohol (%)
1	-	+	-	+	-0.3
2	+	-	-	+	0.0
3	+	+	-	+	0.1
4	+	+	+	+	2.6

The results in Table 6 show that a minimal amount of alcohol, 0.1% as measured, could be attributed to the enzymes (when urea was also included). Significant alcohol was only produced when glucose was available (incidentally confirming that the yeast was active). It was concluded that the enzymes may contribute a concentration of ~ 0.01% as measured, when included at 10x commercial concentration. As a proportion of total AY (0.18%) and with a constant contribution across all fermentations, this amount is considered negligible (*ca*. +1 L/t).

The final protocol chosen for use as an enzyme-only AY assessment method is shown in Table 7. Using this modified method, for the same four Robigus samples above, an average AY of 418 L/t was obtained; 95% of the AY value using the standard 'wheat cook' (barley malt) method. Given the extensive method development carried out, it was deemed that this method was suitable for use in screening samples. While it is accepted that there was a small bias between the two methods, this was similar to the bias seen between laboratories using the same method.

Table 7 Summary of process conditions of final laboratory procedure for determination of alcohol yield of wheat, using commercial enzymes.

Process step	Temperature	Time	Enzyme dosages (mg enzyme/g wheat FW)
Slurry make-up	60 °C	35 min	SPEZYME®XTRA: 0.2 mg/g OPTIMASH BG: 0.05 mg/g FERMGEN: 0.6 mg/g (**x10)**
Primary "Liquefaction"	74 °C	60 min	No addition
Secondary "Liquefaction"	Autoclave at 126 °C then 88 °C water bath	11 min + 60 min	Addition of more SPEZYME®XTRA 0.6 mg/g (**x10)**
Fermentation	32 °C	72 hours	FERMENZYME®L-400: 0.6 mg/g + Urea FERMGEN: 0.6mg/g (**x 10)**

3.3.3. Impact of the 1B1R translocation and N fertiliser on bioethanol processing yield and NSP content

Samples of Ambrosia and Istabraq from an N response trial at High Mowthorpe were assessed for AY and RV using the enzyme only method (Table 7). It should be noted that both varieties showed significant responses of both grain yield and grain protein to fertiliser N (data not presented here – see Sylvester-Bradley *et al*. 2008, p84).

Alcohol yield

When AY was plotted against grain protein (Figure 4) the slope was close to the theoretical of 6.6 (based on a 1:1 replacement of starch and sugars with protein). However, the intercept of the regression equation was lower than reported elsewhere for the standard 'wheat cook' method (e.g. Kindred *et al*., 2008). This means that the AYs were somewhat lower than expected with a sample of protein content 11.5 g/100g giving an alcohol yield of 406 L/t rather than *ca*. 435 L/t.

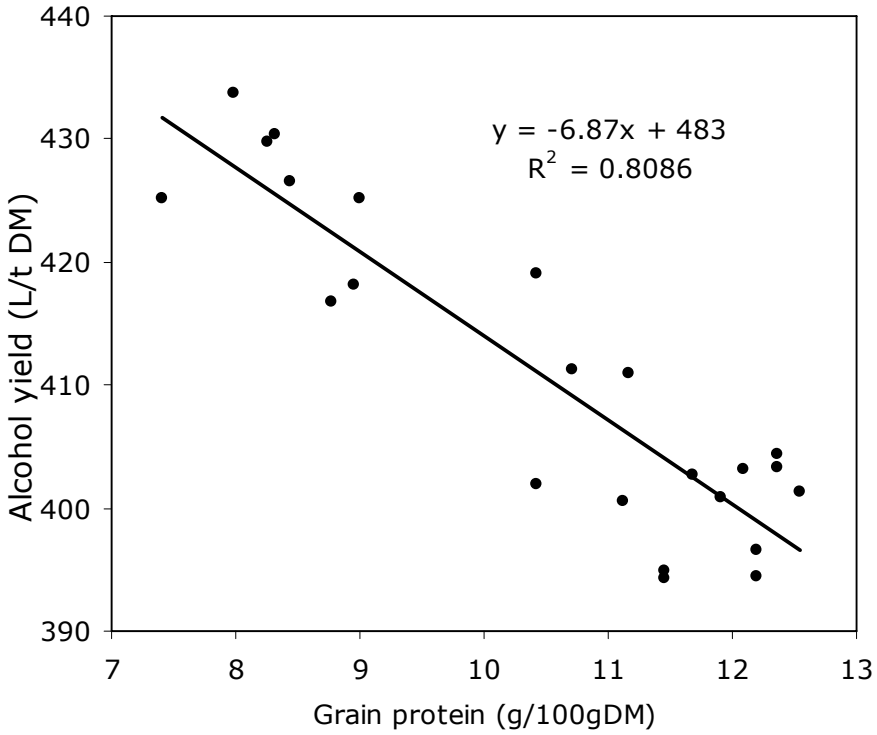

Figure 4 Alcohol yield plotted against protein for a series of Ambrosia and Istabraq for individual samples harvested from an N response experiment at High Mowthorpe 2006.

There was an overall significant effect of the regression ($p<0.001$). When the variety effect was assessed separately, it was seen that Istabraq had a slightly higher average alcohol yield than Ambrosia (+2 L/t), greater than that expected based on the differences in grain protein between the two varieties (0.11 g protein/100g would amount to a difference in AY of *ca*. 0.75 L/t). However, analysis of variance showed that this variety effect was not statistically significant. Figure 5 shows treatment means with a common slope fitted to the two varieties (regression analysis in Genstat showed there was no statistical justification for fitting separate lines to the two varieties).

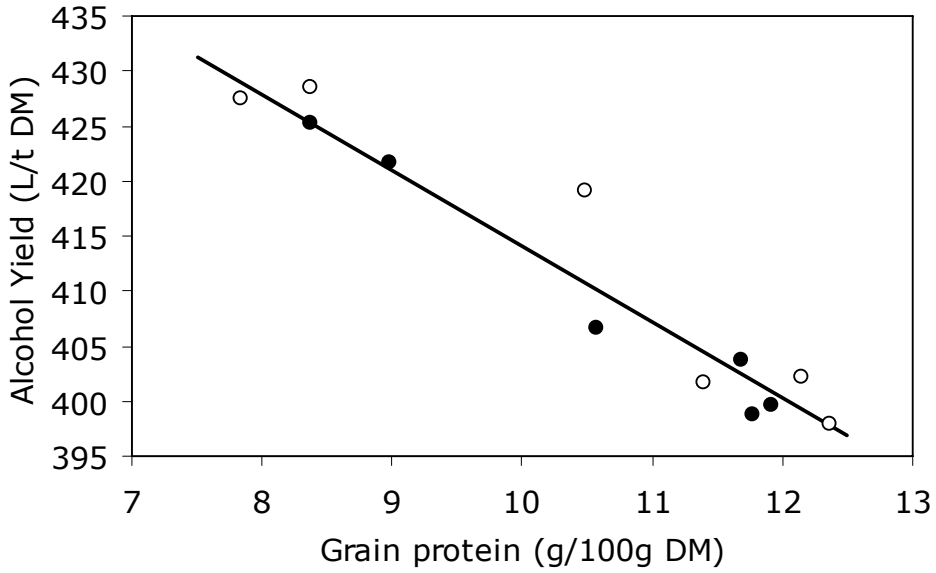

Figure 5 Alcohol yield plotted against protein as treatment means, for Ambrosia (●) and Istabraq (○) samples harvested from an N response experiment at High Mowthorpe 2006 (for slope equation see Figure 4).

Residue viscosity

Residue viscosity was also measured following distillation. There was no significant effect of variety, but a significant ($p<0.05$) effect of N fertilizer. A linear regression analysis was carried out to investigate the relationship between RV and grain protein. RV was shown to be positively related to grain protein content (Figure 6) and the regression was highly significant ($p<0.001$). As with the relationship between AY and protein, addition of variety as a grouping in the regression equation explained no more of the variation in viscosity. Therefore fitting separate lines to each variety could not be justified. The points for the individual variety means are shown in Figure 7 (fitted with the common line). In absolute terms, the RV values were low in this study (see values in Table 4 for comparison).

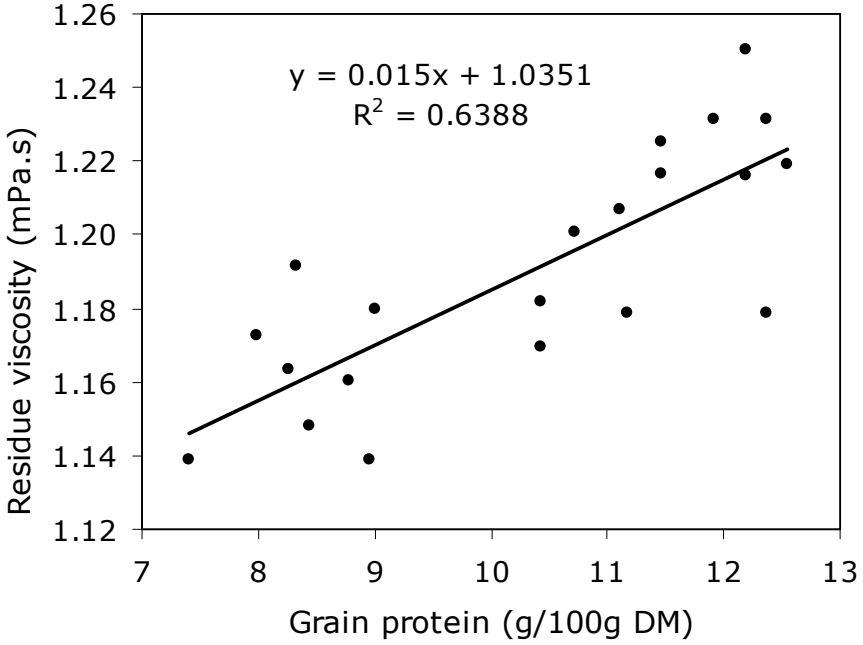

Figure 6 Residue viscosity plotted against protein for a series of Ambrosia and Istabraq individual samples harvested from an N response experiment at High Mowthorpe 2006.

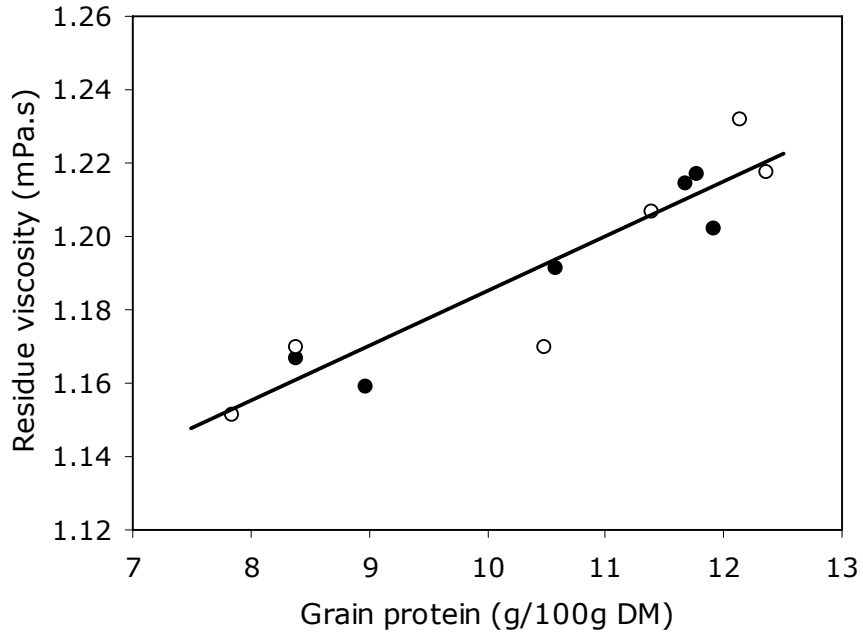

Figure 7 Residue viscosity plotted against protein as treatment means for Ambrosia (●) and Istabraq (○) samples harvested from an N response experiment at High Mowthorpe 2006 (for slope equation see Figure 6).

Non starch polysaccharides

The samples of Ambrosia x Istabraq from the N response trial described above were analysed for their total NSP concentrations (that is total neutral sugars, excluding uronic acids). Analysis of the sugars was determined following the digestion and removal of starch. It can be seen in Table 8 that were very small effects of variety or N level on total NSP or the concentrations of individual constituent sugars, and no significant interactions between variety and N. The sum of arabinose and xylose (the two major constituents of wheat NSP) were significantly higher in Ambrosia (by 0.3 g/100g) which was due to a higher xylose level in this variety. The arabinose:xylose ratio (a factor believed to influence solubility and possibly viscous properties of the AX) was significantly lower in the Ambrosia samples. Regression analysis indicated that there was no significant relationship between grain protein and NSP content.

3.3.4. Alcohol yield of feed wheats at fixed protein content

Commercial samples of wheat were collected from a national survey at 2008 harvest, which allowed selection of 30 wheats of the same protein content (11.5% typical of a feed wheat fertilized at the economic optimum), which were contrasting in terms of their classification by nabim group. Samples were analysed for AY and RV using the enzyme method described above, as well as for neutral sugar composition and NSP content. The raw data were analysed by one-way analysis of variance with nabim group as a factor, to enable comparison between group means (Tables 9 & 10). The full data set and sample identifiers are presented in Tables 11 & 12.

Although Group 3 and Group 4 soft wheats had the highest AY, there was no significant difference between groups in terms of average AY, specific weight and Hagberg falling number (Table 9).

Table 8 Concentration of total non-starch polysaccharides (NSP) and its constituent sugars, and ratio of arabinose to xylose, for two varieties of wheat harvested from an N response experiment at High Mowthorpe 2006.

		Concentration of anhydro sugars (g/100g DM)							
Treatment	**Rha[2]**	**Ara**	**Xyl**	**Man**	**Gal**	**Glc**	**Total NSP**	**Ara +Xyl**	**Ara:Xyl ratio**
Variety									
Ambrosia	0.154	2.42	3.95	1.13	0.543	2.40	10.60	6.37	0.611
Istabraq	0.151	2.42	3.65	1.04	0.529	2.46	10.24	6.07	0.662
SED (df=10)	0.0098	0.075	0.085	0.053	0.0193	0.135	0.286	0.150	0.0131
Sig[1]	ns	ns	**	P<0.1	ns	ns	ns	*	**
N treatment (kg/ha)									
0	0.159	2.46	3.81	1.14	0.555	2.32	10.44	6.27	0.648
70	0.162	2.45	3.79	1.10	0.563	2.40	10.46	6.24	0.647
140	0.142	2.18	3.53	0.92	0.486	1.99	9.26	5.72	0.620
210	0.161	2.54	3.98	1.11	0.557	2.44	10.78	6.53	0.639
280	0.149	2.44	3.93	1.05	0.509	2.73	10.81	6.37	0.621
350	0.140	2.37	3.74	1.15	0.535	2.59	10.53	6.11	0.635
SED (df=10)	0.0169	0.123	0.147	0.091	0.0334	0.234	0.496	0.259	0.0228
Sig	ns	ns	P<0.1	ns	ns	ns	ns	ns	ns

[1], Significance levels: * p<0.05; ** p<0.01; *** p<0.001; ns, not significant.

[2], Abbreviations: Rha, rhamnose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; NSP non starch polysaccharides.

Table 9 Alcohol yield, residue viscosity, specific weight and Hagberg falling number (HFN) of 30 commercial wheat samples at 11.5% protein content (for details of sample origins see Table 11).

Group	AY (L/t DM)	RV (mPa.s)	SpWt (kg/hl)	HFN (s)
1	388	1.14	74.1	178
2	399	1.15	75.5	195
3	405	1.17	72.6	192
4 hard	399	1.20	74.6	206
4 soft	408	1.16	73.5	107
Sig	Ns	**	ns	ns
df	25	25	25	24
SED*a*[1]	8.0	0.014	2.17	56.0
SED*b*	6.8	0.013	1.85	47.8

[1], Standard error of difference:

SED*a* for comparisons between means of all Groups, except;

SED*b* for comparisons between means of Group 2 & Group 4 hard with different numbers of observations

NSP content was determined as the sum of the individual constituent neutral sugars. Total NSP differed significantly between groups, with Group 1 and Group 4 hard wheats having higher NSP contents than those of the Group 3 and Group 4 soft wheats (Table 10). The individual sugars contributing to these differences were principally the arabinose and xylose, and hence the total AX which were significantly greater in Group 1 and Group 4 hard wheats ($p<0.001$). It was observed that the glucose concentrations were higher than in the previous study (section 5.3) and this may have been due to incomplete digestion of the starch. For this reason, the NSPs were re-calcuated as the sum of the remaining sugars, to give a value for 'non glucosic-NSP' (NG-NSP, Table 10), which showed highly significant differences between varieties ($p<0.001$) with Groups 1, 2 and Group 4 hard wheats having significantly higher NG-NSP concentrations than the Group 3 and Group 4 soft wheats.

Table 10 Non-starch polysaccharide (NSP) composition of 29 commercial wheat samples at 11.5% protein content (for details of sample origins see Table 11).

		Concentration of anhydro sugars (g/100g DM)								
Group	**Rha**	**Ara**	**Xyl**	**Man**	**Gal**	**Glc**	**Total NG-NSP[2]**	**Total NSP**	**Total Ara+ Xyl**	**A:X ratio**
1	0.171	2.64	4.21	1.13	0.61	3.65	8.75	12.41	6.85	0.63
2	0.168	2.47	4.04	0.10	0.59	2.96	8.26	11.22	6.51	0.61
3	0.145	2.26	3.49	0.99	0.55	3.20	7.43	10.63	5.74	0.65
4 hard	0.158	2.58	4.36	1.02	0.58	3.31	8.70	12.00	6.94	0.59
4 soft	0.163	2.22	3.64	0.99	0.56	3.34	7.58	10.92	5.86	0.61
Sig	ns	***	***	*	ns	ns	***	*	***	p=0.05
df=24										
SED*a*[1]	0.0110	0.089	0.193	0.046	0.026	0.534	0.304	0.653	0.264	0.020
SED*b*	0.0093	0.076	0.165	0.039	0.022	0.455	0.259	0.557	0.225	0.017

[1], Standard error of difference:

SED*a* for comparisons between means of all Groups, except;

SED*b* for comparisons between means of Gp 2 & Gp 4 hard

[2], NG-NSP (non glucosic-NSP) = sum of Rha + Ara + Xyl + Man + Gal (i.e. excludes Glc)

The relationship between AY and NG-NSP is shown in Figure 8 indicating that there is a negative correlation between these two traits. The slope is close to the theoretical of 6.6 L alcohol/10kg starch assuming that a change of 1 g/100g in NSP is reflected in a similar change in starch concentration (when no parallel changes in protein concentration take place).

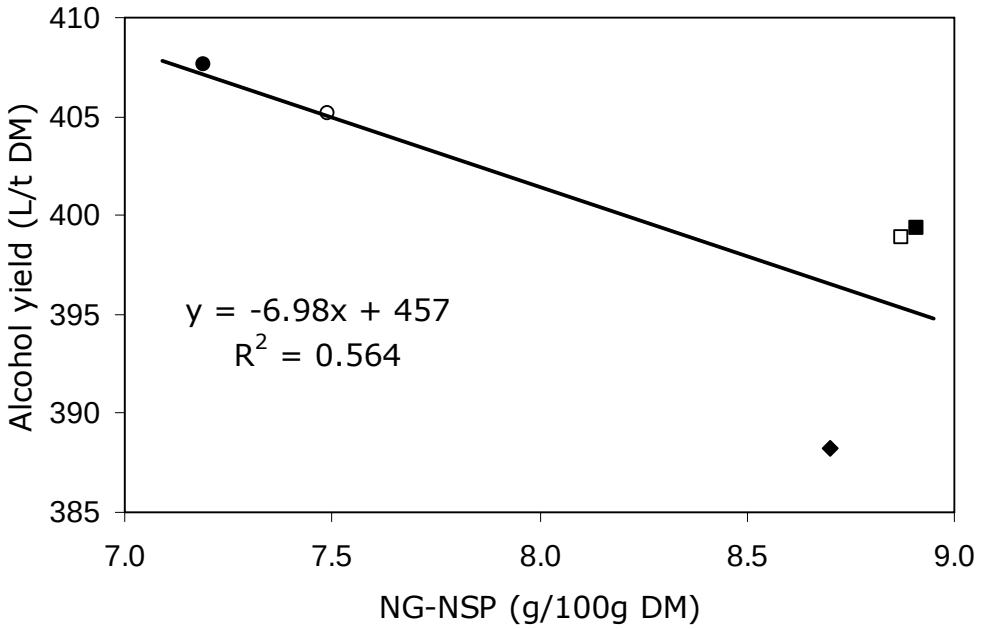

Figure 8 Average alcohol yield plotted against non-glucosic (NG-)NSP, for nabim Groups: 1 (◆), 2 (■), 3 (o), 4 hard (□) and 4 soft (●) for commercial wheat samples harvested in 2008 at 11.5% protein.

Table 11 Origin, variety, nabim group, alcohol yield, residue viscosity, specific weight and Hagberg falling number of 30 commercial wheat samples at 11.5% protein content.

Sample (Frontier code)	Location	Variety	Group	AY (L/t DM)	RV (mPa.s)	SpWt (kg/hl)	HFN (s)
A08/11213	Wilts, BA12	Alchemy	4s	400	1.17	73.1	88
C08/13732	N.Yorks, YO19	Alchemy	4s	411	1.13	71.7	73
C08/13733	N. Yorks, YO19	Alchemy	4s	414	1.13	72.1	62
C08/14272	Staffs, DE13	Alchemy	4s	391	1.16	70.7	62
C08/15358	Warks, CV23	Alchemy	4s	404	1.21	76.5	207
C08/18046	Staffs, WS15	Alchemy	4s	427	1.16	76.7	149
A08/01651	Oxon, OX10	Gladiator	4h	409	1.21	75.8	280
A08/03137	Oxon, OX5	Gladiator	4h	407	1.14	79.3	172
A08/08828	Worcs, B61	Humber	4h	359	1.17	72.1	197
A08/10024	Hants, SO24	Timber	4h	405	1.23	71.6	128
A08/10035	Hants, SO24	Timber	4h	396	1.23	73.7	172
A08/11112	Hants, GU34	Humber	4h	404	1.20	74.3	228
C08/12475	Cambs, PE8	Brompton	4h	412	1.20	75.4	264
A08/03776	Wilts, SP4	Robigus	3	392	1.16	74.3	187
A08/03785	Dorset, D2	Robigus	3	403	1.17	72.9	101
A08/08823	Kent, DA4	Robigus	3	423	1.16	78.1	240
A08/10869	Kent, CT18	Zebedee	3	414	1.21	75.1	354
A08/11200	Wilts, SN14	Robigus	3	392	1.16	69.3	77
C08/14311	Staffs, DE13	Claire	3	407	1.18	65.8	mv
A08/03266	Gloucs, GL6	Cordiale	2	385	1.17	77.0	245
A08/06806	Berks, RG17	Einstein	2	404	1.14	74.2	88
A08/07245	Hants, SO24	Einstein	2	407	1.13	71.7	62
A08/09848	Hants, RG25	Battalion	2	388	1.18	74.9	97
A08/12530	Lincs, PE22	Cordiale	2	398	1.17	79.1	359
C08/13335	Notts, NG22	Cordiale	2	406	1.16	78.5	287
C08/14033	N. Yorks, DL10	Einstein	2	407	1.13	73.2	230
A08/03105	Gloucs, GL18	Solstice	1	391	1.13	75.4	152
A08/03288	Wilts, BA12	Solstice	1	387	1.15	76.9	196
C08/15313	Durham, DH6	Xi19	1	381	1.14	66.3	64
C08/18266	Warks, CV33	Solstice	1	393	1.15	77.8	300

Abbreviations: AY=alcohol yield; RV=residual viscosity; SpWt=specific weight and HFN= Hagberg falling number.

Table 12 NSP composition of 29 commercial wheat samples at 11.5% protein content (for location and variety details see Table 11).

		Concentration of anhydro sugars (g/100g DM)								
Sample[1]	**Rha**	**Ara**	**Xyl**	**Man**	**Gal**	**Glc**	**Total NSP**	**Total Ara+ Xyl**	**Total NG-NSP[2]**	**A:X Ratio**
A08/11213	0.152	2.11	3.50	0.904	0.528	2.62	9.81	5.60	7.19	0.603
C08/13732	0.160	2.15	3.60	0.947	0.533	2.82	10.21	5.75	7.39	0.596
C08/13733	0.174	2.44	4.03	1.025	0.586	2.87	11.12	6.47	8.26	0.604
C08/14272	0.168	2.21	3.54	1.011	0.587	4.86	12.38	5.75	7.52	0.624
C08/15358	0.162	2.22	3.57	1.026	0.570	3.53	11.07	5.79	7.55	0.622
C08/18046	0.159	2.21	3.60	1.029	0.566	3.35	10.91	5.81	7.56	0.615
A08/01651	0.139	2.60	4.50	1.047	0.588	3.32	12.19	7.10	8.87	0.578
A08/03137	0.145	2.71	4.53	0.945	0.545	5.01	13.88	7.24	8.87	0.599
A08/08828	0.167	2.64	4.62	1.035	0.586	3.02	12.08	7.27	9.05	0.572
A08/10024	0.161	2.45	4.02	1.023	0.584	2.81	11.05	6.47	8.24	0.611
A08/10035	0.163	2.62	4.13	1.036	0.598	2.82	11.37	6.76	8.55	0.634
A08/11112	0.158	2.52	4.56	1.023	0.578	3.29	12.13	7.07	8.83	0.553
C08/12475	0.168	2.49	4.19	1.061	0.560	2.86	11.33	6.68	8.47	0.593
A08/03776	0.160	2.29	3.50	0.990	0.555	2.73	10.22	5.79	7.49	0.654
A08/08823	0.122	2.19	3.39	0.895	0.497	2.18	9.27	5.58	7.09	0.645
A08/10869	0.176	2.23	3.68	1.139	0.593	2.65	10.48	5.91	7.82	0.605
A08/11200	0.095	2.13	3.02	0.848	0.499	4.47	11.06	5.15	6.59	0.704
C08/14311	0.171	2.45	3.84	1.060	0.628	3.98	12.13	6.29	8.15	0.638
A08/03266	0.158	2.57	4.70	0.920	0.564	3.84	12.75	7.27	8.91	0.547
A08/06806	0.177	2.43	3.81	1.053	0.624	3.49	11.58	6.24	8.09	0.638
A08/07245	0.177	2.85	4.47	1.059	0.660	2.16	11.38	7.32	9.22	0.638
A08/09848	0.143	2.27	3.56	0.896	0.535	2.02	9.43	5.83	7.40	0.640
A08/12530	0.181	2.38	4.12	1.044	0.580	4.52	12.82	6.50	8.31	0.578
C08/13335	0.177	2.20	3.68	1.085	0.561	2.62	10.33	5.88	7.71	0.597
C08/14033	0.161	2.55	3.96	0.934	0.581	2.06	10.25	6.51	8.19	0.645
A08/03105	0.173	2.71	4.06	1.170	0.591	3.46	12.16	6.77	8.70	0.668
A08/03288	0.167	2.70	4.11	1.194	0.585	4.10	12.86	6.81	8.75	0.655
C08/15313	0.182	2.65	4.67	1.132	0.695	3.99	13.32	7.32	9.33	0.567
C08/18266	0.161	2.50	3.99	1.033	0.550	3.07	11.30	6.49	8.23	0.627

[1], A08/03785 from Table 9: Constituent sugars of NSP not determined.

[2], NG-NSP = sum of Rha + Ara + Xyl + Man + Gal (i.e. excludes Glc)

3.4. Discussion

3.4.1. Establishment of AY laboratory method

Appreciable time was spent in establishing a laboratory at ADAS to measure alcohol yield using the SWRI 'wheat cook' method. Extensive details are not provided here, but the salient features required to achieve good repeatability may be of use to others, and are as follows;

Milling

Initially, poor repeatability of AY measurements was seen. The main source of this variability was determined to be in the milling step. This is because when wheat grain is milled, small particles such as those from the starchy endosperm pass through the screen first, whereas larger bran particles are retained behind the screen. Therefore fractionation of the wheat grain occurs in the mill. It is important, therefore, that all of the material which is ground is collected. This includes any material retained inside the mill which is then added to that which has passed through the screen. The whole sample is then thoroughly mixed prior to analysis.

Digestion

There is always a risk that the starch is not being fully hydrolysed, particularly because small laboratory autoclaves cannot provide enough energy to fully gelatinize it. Therefore it was important to have sufficient thermostable amylase (Termamyl), and this was increased to be twice that used in the SWRI method (on a per weight of flour basis).

Distillation

Initially it was thought that higher throughput (i.e. number of samples per day) could be achieved by using a multi-point heating mantle rather than a Bunsen burner to distil the alcohol. However, it was subsequently found that the more vigorous heating (more rapid boiling) provided by the bunsen was needed in order to distil over the alcohol efficiently, and that the power from the heating mantle was insufficient for this purpose.

Overall comments on standard AY methodology

It will be seen from Figure 2 that there was a slight bias in AY values, with ADAS underestimating the AY values measured at SWRI. For the samples in Table 5 (which had an average value of 11.5 g/100g grain protein) based on prediction equations from earlier work (e.g. Kindred *et al*., 2008) we would expect an average AY of 437 L/t. In the present study, the average AY at ADAS was lower (420 L/t) while the SWRI average AY was higher (442 L/t). Therefore it is likely that there are uncertainties in the methodology from both laboratories. With such a complex trait as AY, its determination is affected by so many variables, including variation in endosperm structure and release of starch granules, susceptibility of the starch to enzymes, performance of the yeast and the supply of N to the yeast etc.

Despite extensive efforts to standardise the procedure, in the present study, a number of features varied between laboratories. In addition to the obvious differences in equipment (sample size/digestion volumes, water baths/incubators etc), there were also differences in the sources of malt and yeast. It is possible that if agreement between laboratories needed to be improved in the future, then a common source of barley malt could be used and source of yeast standardised (this is supplied fresh weekly). Nevertheless, the fact that at the ADAS lab we saw a typical relationship between AY and protein in the results (Figure 3) and that the method was also used successfully to test triticale samples and AY of milling fractions (reported earlier: Davis-Knight and Weightman, 2008b; Weightman *et al*., 2009) it was concluded that the transfer of methodology was successful.

Comments on residue viscosity determination

The RVs determined on the validation data set (Table 4) were lower at SWRI (average 1.29 mPa.s) than at ADAS (1.67 mPa.s). This may be as a result of different cooking temperatures (higher at SWRI) and/or differences in source of barley malt. The ADAS samples were typical of the range seen previously, while the SWRI samples were all at the low end of the scale. For reference, a good distilling variety (e.g. Glasgow) might have a RV of *ca*. 1.5 - 1.6 mPa.s, and a bad sample (e.g. Kipling) >1.8 mPa.s. At the extremes of the range using the same methodology, triticale samples may give RVs in the range 2.11 - 3.53 mPa.s (Davis-Knight and Weightman, 2008b). Interestingly, the ADAS study shows that milling wheat varieties Cordiale, Hereward and Xi19 all gave relatively high viscosities (Table 4). This is an indication that RV itself may be influenced by soluble proteins rather than (or as well as) soluble NSP.

It is not clear why in this instance the SWRI lab gave lower RVs, although a different source of barley malt may be implicated, because barley malt will supply a range of enzymes including endogenous barley xylanases and glucanases which all serve to reduce viscosity.

For future purposes, it is concluded that if comparisons of varieties tested at different laboratories are required, then it is essential to include common reference samples, so that all results can be adjusted to a standard values for a common reference variety (e.g. a good quality distilling wheat).

3.4.2. Establishment of enzyme-only alcohol yield determination method

A method was devised which could successfully screen wheat grain across a range of protein contents, showing a typical response of AY (as reported by Smith *et al.*, 2006; Kindred *et al*., 2008). However the absolute values of AY were still somewhat lower than those assessed using the standard (barley malt) method. It was beyond the scope of the current project to come up with a biofuel-type method which would perfectly mimic a bioethanol distillery, if only because at the time of carrying out the project, the bioethanol distilleries were not yet active, and so we did not know what the typical AY was for UK wheat, or what the processing conditions would be (particularly temperatures, residence times and actual enzyme dosages).

Despite the inclusion of urea, it was clear that proteases were beneficial, both to supply FAN to the yeast and also to aid disruption of the endosperm protein matrix. Further work could be carried out to improve the lab method for AY by including more thermostable enzymes (e.g. Spezyme FRED; Table 1, or Termamyl). It is also not clear to what extent the β-amylase supplied by barley malt gives an advantage in the standard lab testing method.

There is one fundamental difference between an enzyme-only and a method which uses barley malt. That is that the contribution of sugars by the enzymes is trivial, whereas barley malt provides an additional source of sugars to the yeast. In the standard method, the contribution of AY from the barley malt is taken into account when calculating the actual AY of the wheat tested. The enzyme-only method gave

some advantages in use. It appeared to give less variable results (NB high R^2 in Figure 4) than the standard method and this is perhaps because of the additional errors associated with determining separately the AY and moisture content of each batch of barley malt.

Residue viscosity

Interestingly the enzyme-only method gave relatively low RVs. This is probably due to the presence of xylanase enzymes in the Optimash BG commercial enzyme which hydrolyse the AX during processing. This observation did reveal the possibility that RV is in part due to the presence of soluble proteins (discussed further below).

3.4.3. Relationships between alcohol yield, grain protein and NSP, in response to N fertilizer application

N Fertilizer effects

Large changes in starch content are seen with changes in wheat grain protein concentration (e.g. Kindred *et al*., 2007, 2008) and hence differences in grain protein are generally negatively related to AY (Swanston *et al*., 2005). At the outset of the project it was thought that the concentration of NSP might change with variation in grain protein content, thus explaining the observed difference between typical AY-protein relationship (7.3 L alcohol per 10 kg grain protein) and the theoretical relationship (6.6 L alcohol per 10 kg starch or sugars; Smith *et al*., 2006). However using samples from an N response trial in the present study, showed no significant change in NSP content with changes in protein (Table 8).

The results in the present study agree with the observations of Dornez *et al*. (2007b) published since the start of this project, that N fertiliser appears to have little effect on NSP content. This leads to the conclusion that any unexplained variation in the relationship between AY and grain protein may be due to changes in the concentrations of ash and oil, which together account for *ca*. 4.5% of the DM of a typical wheat grain (Smith *et al*., 2006). Such differences could be related to some feature of the bran layer or the size of the germ (in the case of ash and oil respectively) which may change as grain protein changes. Weightman *et al*. (2009) showed that there was variation in ash content (0.87-1.75 g/100g DM) between a group of ten UK wheats. However that was a small dataset, with each variety only represented once. There is little data on sources of variation ash content in bread

wheat, although Merah *et al*. (2002) have shown effects of variety or environmental conditions on ash content in durum wheat. Similarly there is no published information on variation in oil or germ content in wheat, although it is known in maize that there is genetic variation for these traits (Lambert, 2001) and that oil content is inversely related to starch content. Other differences in grain composition such as the amylose: amylopectin ratio of the starch may also influence AY, but the wheats studied were all assumed to have 'normal' or wild type starch, and it is not known whether fine variation in starch structure within UK wheats, would be significant in this context.

Residue viscosity (an indicator of processing performance) was shown to increase significantly with N fertilizer application (Figure 6), and there was a positive relationship between RV and grain protein (Figure 7). This is an important observation because while we did not distinguish in the present study between soluble and insoluble NSP, the fact that RV and protein increase together (but NSP does not), implies that RV is dominated by concentration of water soluble proteins. In fact in the present study because the enzyme-only method uses a commercial product containing xylanase and β-glucanase which would reduce or eliminate any viscosity due to AX or β-glucan (a minor component of the NSP in wheat grain, but one known to increase in oats in response to applied N, Weightman *et al*., 2004) the method may have exposed a more important effect of protein. This appears to support the evidence of the distilling industry that hard and/or Group 1 wheats are less desirable for alcohol production because of their greater tendency to present processing difficulties, particularly as Group 1 wheats tend to have higher grain protein. Recently Agu *et al*. (2009) have also reported a correlation between RV and grain N at certain sites for a set of UK soft wheats. Any starch which remains embedded in protein would not be available for hydrolysis and thus may also contribute to high RV. These observations are worthy of further study, in particular exploring wheats with a wider range of protein sub-unit composition, to see whether Group 1 varieties for example, respond more to applied N than other wheats, thus giving higher RV. If the hypothesis is correct, these wheats may need additional protease addition in order to improve processing characteristics when used in a biofuel distillery (or potable alcohol process where the use of enzymes is not constrained).

Variety effects

The choice of Ambrosia and Istabraq in the present study was made because Ambrosia possesses the 1B/1R translocation. It was thought that this would be a useful comparison between varieties, with 1B1R giving higher NSP content and lower AY. In fact Ambrosia did have a significantly higher AX concentration than Istabraq (Table 8) supporting the general hypothesis, but in absolute terms this difference was fairly small (0.3 g/100g DM) and as a consequence, the total NSP content and AY did not differ significantly between the two varieties. There is little published information on NSP levels in UK wheats. Englyst *et al*. (1992) reported an AX content of 7.8 for a sample of whole wheat flour (provenance unknown), slightly higher than the level of 6.6 g/100g for Swedish wheats reported by Aman (1988), 6.4 g/100g for French wheats by Saulnier *et al*. (1995) and the average of the Ambrosia/Istabraq in the present study (6.2 g/100g).

There has never been a distilling industry recommendation for 1B1R wheats in part due to their variability in performance and high probability of giving processing problems. Despite the relationship between presence of the 1B1R translocation and cold paste viscosity (Weightman *et al*., 2001) there was no effect here on RV, although it should be noted that Ambrosia is not the most 'extreme' +1B1R wheat in terms of this character, and not the best example for demonstrating this effect. However, since the project started, 1B1R wheats have become much less prevalent in the testing system due to their association with poor nutritional quality for animal feed (Wiseman *et al*., 2001). Hence few such varieties were available for analysis during the course of the project without specifically drilling field trials for the purpose. To extend this work, one approach would be to consider the use of near-isogenic lines for the 1B1R translocation, and to grow these under the controlled conditions of an N response trial. Alternatively, there may be samples from other groups (e.g. in France and Belgium) where there has been more extensive work on NSP and AX contents in wheat (Martinant *et al*., 1998; Dornez *et al*. 2007a; Saulnier *et al*., 2007). In particular, 1B1R wheats with their higher proportion of rye secalins (known to give problems of 'sticky doughs') would be useful models to test for the relationship between applied N and RV noted in the previous section.

3.4.4. Relationships between alcohol yield and NSP at fixed grain protein content

The previous section shows the importance of grain protein content on AY, supporting the earlier observations of Smith *et al*. (2006) and Kindred *et al*. (2008). Therefore when investigating sources of variation in AY in wheat, an alternative approach is to only screen wheats at a fixed protein content, thereby removing one of the major sources of variation in AY and allowing a greater chance of detecting varietal effects relating to NSP concentration. However it is not possible to produce wheat samples with protein contents to order; rather they have to be sourced from pools of wider survey samples, which although commercially relevant, inevitably come from a wide range of environments. Other environmental effects on grain quality and starch characteristics, such as wetting/re-drying of grain, affecting hardness and vitreosity (Weightman *et al*., 2008b) and pre-harvest sprouting, may also affect AY. Therefore when the 30 samples of wheat were screened, there was no apparent relationship between AY and NSP concentration. This is partly because the absolute differences in AX and NSP were relatively small. In the present study, the range for the average AX concentration for the nabim groups was 5.7 to 6.9 g/100g, sensible figures with respect to the earlier studies, but still a fairly small range. Total neutral NSP was in the range 10.6 to 12.4 g/100g (Table 10) indicating that Englyst's wheat sample was at the lower end of the range (10.6 g/100g). Rose *et al*. (2001) reported a range in NSP content of 9.6 – 12.5 g/100g for 16 samples of UK wheat (representing 4 varieties) similar to the range reported here. However a range of 2 g/100g NSP, if converted directly to starch, is not big enough to account for the observed differences in AY in commercial practice (*ca*. 50 L/t). Either other grain components such as ash and oil, as discussed earlier, are important or unexplained variation in AY is due to starch availability and/or rate of starch digestion.

When the samples were considered as their group means based on nabim classifications (Tables 9 & 10), significant differences were apparent between groups with the Group 4 hard wheats having higher RV than the other groups, confirming the anecdotal evidence based in distilling industry performance that Groups 1 & 4h have higher NSP, NG-NSP and AX levels.

It is perhaps not surprising the Group 4h wheats had higher NSP levels, given that they have principally been selected for their high grain yields and not been selected

for any other grain quality trait whereas the Group 4 soft wheats and the Group 3 wheats have been selected for ease of processing and low viscosity. In particular, Group 3 wheats used for processes like biscuit and batter production generally require low water absorption and low cold paste/batter viscosity. It is interesting that Group 1 wheats appear to have higher NSP levels, but in a different context this was noted in the study of Weightman *et al*. (2009) where the variety Hereward gave the highest AX levels. It may be that high AX level gives a higher water absorption, and that this has been inadvertently selected for in Group 1 wheats.

There was a positive relationship between the average AY and average NG-NSP level for the different groups (Figure 8) with the Group 3 and 4s wheats having the highest AY and the lowest NG-NSP levels. This tends to support the overall hypothesis that selection for low NSP content would be desirable in selecting for high AY at a given protein content (and has probably already taken place in soft distilling wheats). Although, the level of variation is fairly small (1.5 g/100g for NG-NSP, 1 g/100g for AX), if this gave an equivalent amount of starch, it would provide an additional 6.6 L alcohol/t of grain, which would be significant commercially. The problem for plant breeders is that NSP is not an easy character to select for, the assay methods being relatively expensive (and laborious) compared to a trait like grain protein content.

Other solutions to assessing wheat varieties at the same protein content would be to grow samples in a variety trial with say two or three N levels and after harvest, to blend samples from the different N treatments of each variety, to give a standard protein content sample for testing, or to study isogenic lines (cf Wiseman *et al*. 2001). However, since most 1B1R wheats have now been removed from the testing system, it seems more pertinent to focus on the AY of the hard wheats (Groups 1 and 4h) rather than 1B1R.

3.4.5. Conclusions

1. Methods are in place to screen wheats for AY and RV using traditional methods appropriate for the distilling industry using barley malt, and also using enzyme-only methods appropriate for the biofuels industry.
2. An enzyme-only method gave results with the same relationship between AY and grain protein seen in previous work, but slightly lower average AY in absolute terms than using the potable alcohol distilling method.
3. Further work needs to be done in liaison with commercial biofuels distilleries, to ensure that lab screening methods correctly mimic their processes and give similar AY as the commercial processes; in particular, further work to mimic a biofuel type process is required, by considering the inclusion of thermostable amylases.
4. Using samples of varieties Ambrosia and Istabraq harvested from an N response trial, there appeared to be no difference in AY or RV between these wheats contrasting in presence of the 1B1R translocation, and no difference in NSP content.
5. NSP concentration did not appear to be affected by N fertilizer rate (or grain protein concentration) but interestingly RV appeared to increase with increasing grain protein content, suggesting that soluble proteins rather than AX may be implicated in causing high RV (and hence poor processing quality) in wheat.
6. Analysis of wheats at a fixed protein content indicated that wheats of nabim Groups 1, 2 and 4h had lower AY, and that Groups 1 and 4h had the higher AX and NSP concentrations, compared to the soft wheats in Group 3 and Group 4.
7. Analysis of the data for wheats averaged by nabim class indicated that high AY wheats tended to have lower NSP, and were associated with Groups 3 and 4s, (containing those varieties which currently are given distilling ratings on the RL).
8. Further work is required to investigate whether hard wheats do give consistently poorer AY than conventional distilling wheats, as Group 4h wheats are likely to be a major source of wheat to UK bioethanol plants in the near future alongside high yielding 4s wheats.
9. Further work to study the variation in AY at a given protein content should focus on those other factors which might vary in wheat such as concentration of oil and ash, and the rate and extent of digestion of starch.

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