PROJECT REPORT No. 271

DEVELOPMENT OF A BLUE STARCH/AGAROSE GEL METHOD FOR RAPID INTAKE AND FIELD TESTING FOR CEREAL *ALPHA*-AMYLASE ACTIVITY

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by

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1 Abstract

The aim of this one-year project was to produce a rapid test for assessing levels of *alpha*-amylase in wheat and barley grains. The principal requirement of such a test is that it would be suitable for use at mill intake, in grain stores and in the field. Ideally, the new diagnostic test would be simple, effective and affordable, and thus provide an alternative to the WheatRite™ kit that has, so far, not gained widespread acceptance in the UK.

The Strip Test has been developed to address these needs of the home-grown cereals industry. This new test is based on a recent CCFRA innovation, the "grain-blotting" assay for visualising *alpha*-amylase activity in cut sections of germinated wheat grain, using Phadebas (blue starch) powder suspended in an agarose gel. Several possible formats were examined and a range of commercially available blue-starch or blue-amylose/amylopectin substrates tested. The most promising format features the release of blue dye from the Phadebas test substrate by enzyme action, and its subsequent separation and visualization on a white strip of special filter paper.

The test displays a blue coloration on the white strip, and the intensity is directly related to the rate of hydrolysis of the Phadebas substrate by *alpha*-amylase extracted from wheat flour. The performance of the device was evaluated under laboratory conditions with a total of 125 stored samples of ground wheat from the 1999 and 2000 HGCA Cereal Quality Surveys, and whose HFN (Hagberg Falling Number) values were established. The *alpha*-amylase activity within these samples was measured by the Ceralpha (Megazyme) test method. The blue coloration produced on the white strips was judged visually by two independent observers, and the intensity noted according to the scoring system:

0 - no colour; **1**- very faint blue; **2** – slightly blue; **3** – moderate blue; **4** – dark blue.

The two sets of samples were evaluated on two separate occasions using the Strip Test. Overall, there was good agreement between the two observers and the duplicate evaluations. The results showed that all the samples with HFN value of less than 210 gave a detectable blue colour, i.e. were given a score of 1 or more. The performance of the new test was found to be marginally better when compared with Ceralpha results, than with HFN data. While the test in its present form lacks precision in assessing samples with HFN values between 190 and 250, it is effectively able to indicate whether *alpha*-amylase activities in wheat samples are either high or low. This would be most useful for screening purposes. The Strip Test is not suitable for assessing barley samples owing to a loss of sensitivity found when dealing with barley extracts. This is probably due to presence of high levels of β-glucans, which interfere with the assay.

2 Summary

2.1 Introduction

Pre-harvest sprouting in wheat and barley can lead to serious economic losses to growers, and to the country as a whole due to increased need for imported grain. The poor quality of sprouted or rain-damaged grain is mainly due to excessive levels of *alpha*-amylase, a principal starch-degrading enzyme. The deleterious effects of high *alpha*-amylase activity on breadmaking include darkened crust, plus a weak and sticky crumb causing slicing problems. Biscuits made with sprout-damaged flour can exhibit unsatisfactory surface structure. Sprout damage in barley can reduce the useful storage life of malting barley and leads to rejection for malting as this process depends on controlled germination of grain. There are a number of tests for measuring *alpha*-amylase levels in grain. However, many of them are fairly sophisticated laboratory-based tests, requiring expensive instrumentation, and often require a substantial amount of operator input and skill both in performing the test and interpreting results. These types of tests are often time-consuming and can require significant quantities of material. The WheatRite™ test, which has recently become available in the UK, has been devised for assessing *alpha*-amylase levels under field conditions. This test has been developed in Australia and evaluated for that market. However, it has yet to gain widespread acceptance in the UK.

The aim of this project was to develop a new simple, reliable and affordable diagnostic test for *alpha*-amylase for use in intake laboratories and in the field.

2.2 Methods

The starting point for a new assay for *alpha*-amylase was based on the performance of a novel assay, featuring a blue starch/agarose gel (previously created by CCFRA), that visualises *alpha*-activity activity directly in cut surfaces of germinated grain. Several formats of a device that could display *alpha*-activity semi-quantitatively were designed and evaluated for their suitability. Different commercially available types of blue-starch or blue-amylose/amylopectin substrates were compared for their characteristics (particle size, colour intensity, enzyme susceptibility, etc.) using a fungal *alpha*-amylase preparation. Several procedures for rapid extraction of enzyme from wheat flour were examined and the prototype kit was calibrated with selected samples of known enzyme activity. Sets of 125 ground wheat wholemeal samples and 100 ground barley wholemeal samples, possessing a range of enzyme activities, from the HGCA Cereal Quality Survey from the 1999 and 2000 harvests, were used to evaluate the performance of the test kit. The *alpha*-amylase enzyme activity within the wheat wholemeal samples was measured by the Ceralpha (Megazyme) method, and Hagberg Falling Number (HFN) values were also determined. The enzyme activity within the barley samples was determined by a modified Randox assay. Colour intensities within the strips used for a selected set of wheat wholemeal samples were quantified using the Phoretix 1-D Advanced, densitometric analysis software designed for electrophoresis gels.

2.3 **Results**

The Phadebas™ blue starch was identified as being the most suitable of all the substrates. The test format that looked the most promising featured the chromatographic separation of solubilized blue dye from the insoluble starch granules using a strip of special filter paper. The Strip Test filter papers were cut to give 2.2 x 0.5 x 0.1cm strips, and a small block of siliconised rubber containing a 2mm hole was attached near the bottom (Figure 1) and is the sample well. The enzyme from wheat flour was extracted by mixing 1g of wholemeal with 10ml of 85mM sodium chloride inside a capped glass tube and shaken by hand periodically over five minutes, and then allowed to settle for a further five minutes. The extract was mixed with an equal volume of the Phadebas™ blue starch (1.32%) mixed with low melting point agarose (0.66%) in a buffer (189mM maleic acid, 2mM calcium chloride pH 6.0) and incubated at 37°C for 10 minutes inside a small chamber. The reaction was stopped by the addition of 500mM sodium hydroxide and 50mM di-sodium EDTA. A 20µl aliquot was pipetted into the sample well. After one minute, 200µl of the extraction solution (85mM sodium chloride) was applied to the bottom of the filter paper strip to mobilise any liberated blue dye upwards to produce a blue coloration of the white filter paper.

Figure 1. Schematic diagrams of sample application and colour visualisation on a Strip Test

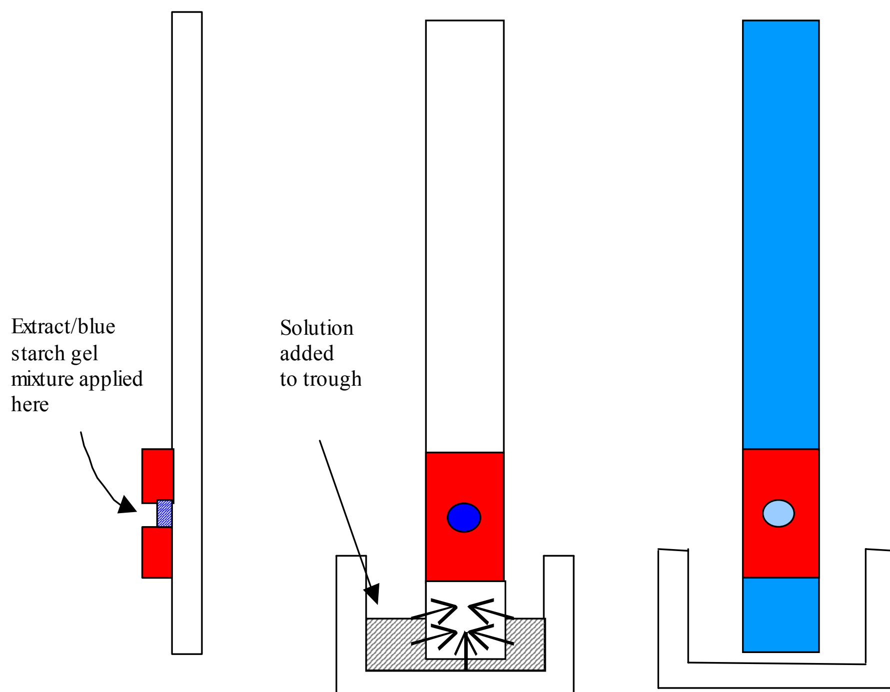


Figure 1(a) shows the side view, in which the reaction (incubated extract and substrate) mixture has been applied to the sample well.

Figure 1(b) shows the front view, in which the strip is placed into a trough and extraction solution is added.

Figure 1(c) shows the migration of free dye, following absorption of the liquid by the filter paper strip.

The blue coloration produced on the test strips was judged visually by two observers, and the intensity noted using the scoring system below:

Test Strip Score	Colour Intensity Description
0	No colour
1	Very faint blue
2	Slightly blue
3	Moderate blue
4	Dark blue

The two sets of ground wheat samples from the HGCA Cereal Quality Surveys of 1999 and 2000 were evaluated in duplicate, on separate occasions using the Strip Test. The resultant Strip Test scores were analysed with respect to the corresponding enzyme activity values measured by the Ceralpha method (performed at CCFRA), and Hagberg Falling Number values (performed at CCFRA and at Andover Analytical Laboratories). The visual sensitivity of the Strip Test was examined using 9 samples of wheat whose HFN values ranged from 71 to 386 (Figure 2). Samples 0 and 10 were reagent blank and fungal *alpha*-amylase, respectively. The intensity of the blue colour was measured visually and by densitometry. Table 1 shows the intensity scores together with HFN and Ceralpha data. Overall, the Strip Test produced blue coloration that was reasonably consistent with the *alpha*-amylase levels within these samples.

Figure 2. Samples used for densitometric analysis

Sample no.	0	1	2	3	4
HFN	-	386	373	249	224

Sample no.	5	6	7	8	9	10
HFN	189	170	153	120	71	-

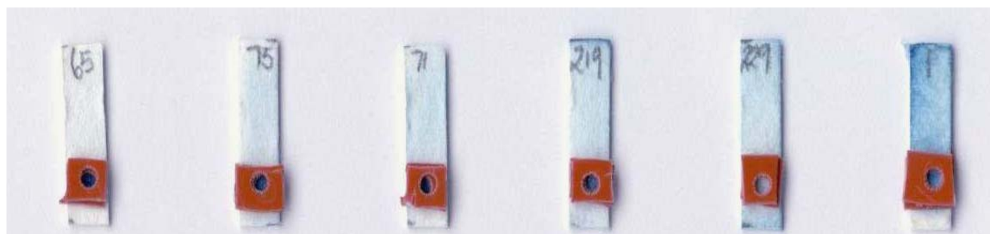


Table 1. Scanned (densitometric) intensity values and other measured values of 9 selected samples (Figure 2)

Strip Test No.	1	2	3	4	5	6	7	8	9
Sample No. CM/52804/	23	16	73	36	29	33	31	67	70
Ceralpha Activity (CU/g)	0.145	0.150	0.330	0.455	0.625	1.350	1.515	2.685	4.990
HFN (seconds)	386	373	249	224	189	170	153	120	71
Strip Test Scores	0	0	1	1	1	3	4	4	4
Densitometric Intensity (optical density units)	2.53	3.30	5.24	5.21	4.94	7.42	7.98	8.64	9.00

The performance of the test strips relative to Ceralpha values of the 1999 harvest samples is shown in Figure 3. Generally, there is good agreement between evaluation 1 and 2 scores. Four of the evaluation 1 scores were found to be invalid due to false positives observed for blanks included as controls and these were subsequently omitted (sample numbers 13, 49, 59 and 71).

Figure 4 shows the performance of the test strips relative to Hagberg Falling Number determinations of the 1999 harvest samples, performed by CCFRA. A number of HFN values supplied by Andover Analytical Laboratories were found to be inconsistent with the Strip Test results. A comparison between the HFN values determined in the two laboratories, plotted against Ceralpha enzyme activity, is shown in Figure 5. With the exception of the five very obvious outliers highlighted with circles, HFN values obtained from Andover Analytical (tested at harvest) tended to be lower than those measured by CCFRA. Inspection of the CCFRA HFN/Ceralpha values in Figure 5 shows that the data scatter is the greatest between HFN range of about 150 – 230 seconds.

There were only 6 samples with low HFN (<250 seconds) from the 2000 harvest set, in contrast with the 1999 harvest set where 32 samples produced HFN values below 250 seconds. This was reflected by the Strip Test results in that all bar two samples produced colour intensity scores of 0 or 1 (data not shown). Figures 6 and 7 show Strip Test scores plotted against HFN (CCFRA) and Ceralpha values, respectively, using the combined (second evaluation) data for 1999 and 2000 harvest wheat samples. Using a cut-off HFN value of 250 seconds to provide an 'accept/reject' assessment of the performance of the Strip Test, it was found that of the 86 samples with HFN values >250 seconds, 82 samples scored 0. Only 8 out of the 40 samples with HFN values <250 seconds scored 0.

Figure 3. Strip Test scores versus Ceralpha, 1999 harvest

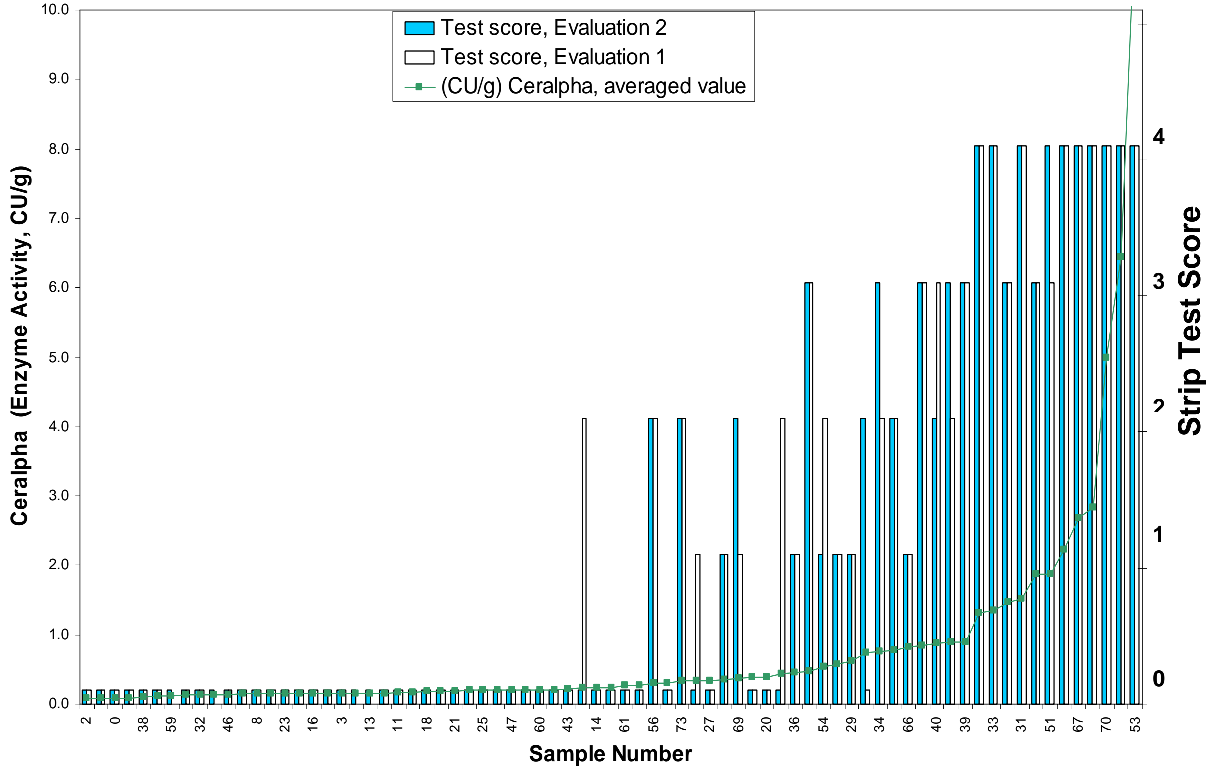


Figure 4. Strip Test scores versus HFN, 1999 harvest

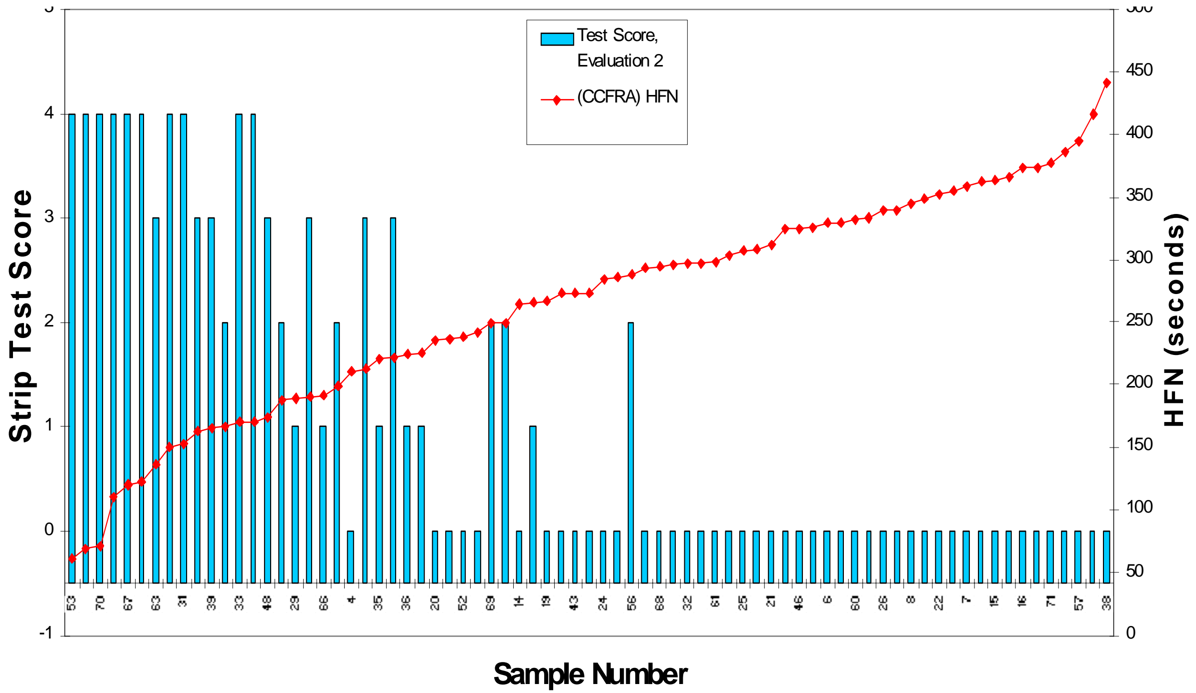


Figure 5. Andover/CCFRA HFN versus Ceralpha data, 1999 harvest samples

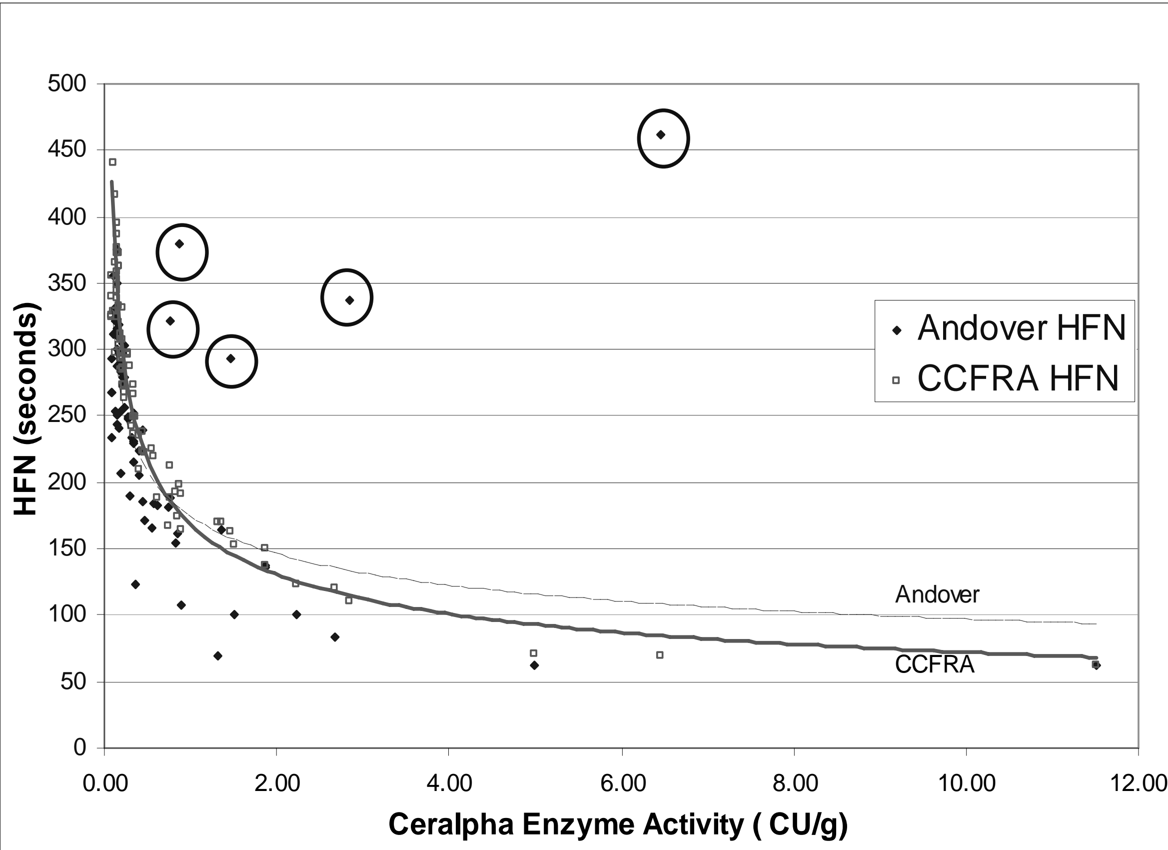


Figure 6. Strip Test scores versus HFN (CCFRA) data from 1999 and 2000 harvests

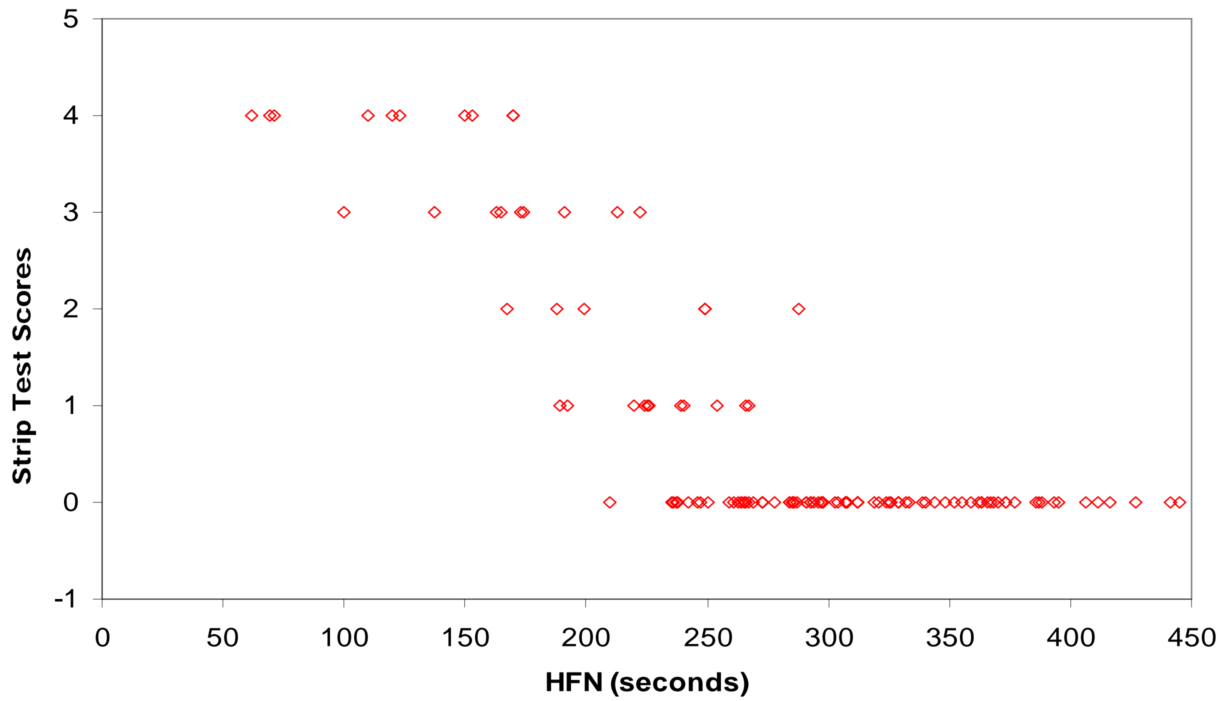
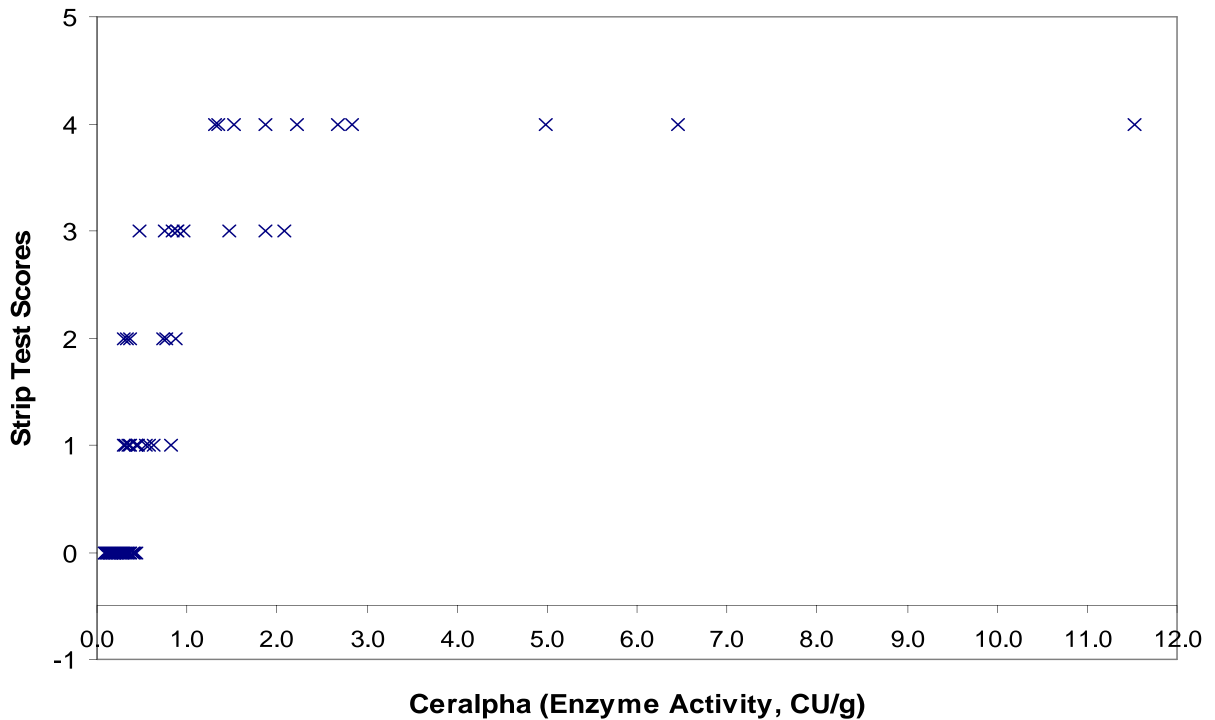


Figure 7. Strip Test scores versus Ceralpha data from 1999 and 2000 harvests



Attempts to use the Strip Test with barley samples proved to be unsuccessful. The extracts were highly viscous and difficult to pipette accurately. The blue coloration was minimal and inconsistent for all the barley samples examined, despite the Randox test data indicating the presence of significant levels of *alpha*-amylase in many of the samples. Minor modifications made to the test format did not improve the sensitivity, and it was assumed that high levels of β-glucans present in the sample extracts were responsible for the increased viscosity, which interfered with the assay.

2.4 **Conclusions**

A prototype simple test for assessing *alpha*-amylase levels in wheat has been developed. The Strip Test features the Phadebas™ blue starch substrate and allows visual assessment of enzyme activity without the need for a spectrophotometer. The test requires 10 minutes for sample extraction from ground wheat, 10 minutes reaction time and a further minute to visualize the blue colour. While the test can operate at room temperature, the sensitivity and repeatability are enhanced when it is performed at 37ºC.

The performance of the new test was evaluated using 125 samples of wheat. In comparison with the Hagberg Falling Number test, the Strip Test gives:

1. a strong blue coloration with samples with HFN values of between 62 – 169 seconds
2. a range of blue coloration from moderate to none with samples with HFN values of between 170 – 289 seconds
3. no blue coloration with any of the samples with HFN values of between 290 – 445 seconds.

These results suggest that the prototype Strip Test is effectively able to distinguish between high and low *alpha*-amylase activities in wheat samples, which would be most useful for screening purposes. Ways of improving the performance of the test, in terms of speed and sensitivity, have been identified, and discussions with potential manufacturers will attempt to address these issues. The test is not suitable for use with barley flour samples, in its present format.

2.5 **Implications**

The performance of the prototype Strip Test indicates that it could form the basis of a commercial diagnostic test for detecting excessive *alpha*-amylase activity in wheat grain samples. The sensitivity of this assay could be enhanced through a number of modifications, e.g. by the inclusion of an overlay of starch on top of the Phadebas™/agarose substrate to act as partial barrier to the enzyme and which would provide a simple "positive or negative" result for a threshold HFN value of 250 seconds. The availability of a simple, rapid and portable test, for use in the field, grain stores and at mill intake, would be of great benefit to the cereals industry.

The average annual losses to UK farmers due to excessive levels of *alpha*-amylase in rain-damaged wheat are estimated at about £17M. Clearly, the availability of a robust and reliable field test kit that would permit the grower to make harvesting decisions should reduce a useful proportion of these losses. By giving early warning to the farmer, an effective test would allow growers to make judgements about final crop quality and meeting marketing requirements. The new test could also allow the farmer to segregate poor and sound crop within a field at harvest.

Breeders would be able to screen large numbers of grain samples of limited quantities in a shorter time compared to conventional laboratory-based testing services. At mill intake, or elsewhere in the grain trade, a simple, relatively inexpensive, semi-quantitative test for *alpha*-amylase would save time and resources through screening grain lots, reserving the more precise laboratory-based methods for examining those examples identified as being borderline cases.

Discussions are currently taking place between CCFRA and interested manufacturers regarding commercial development of the Strip test. The time-scale for uptake of the final commercial product is expected to be about two years. While the raw materials used in the prototype Strip Test are relatively inexpensive and readily available compared to those of the antibody-based WheatRite™ kit, it is not possible to quantify the eventual mass production cost at the present time. It is anticipated that the selling price of a commercial version of the Strip Test would be significantly cheaper than that currently quoted for the WheatRite™ kit – about £7 per wheat sample (excluding labour costs).

2.6 **Acknowledgements**

The author wishes to gratefully acknowledge the Home-Grown Cereals Authority for providing guidance and financial support for this study. I am indebted to the significant contributions made by Nick Saunders and Jonathan Gibbons through their dedicated efforts and technical skills. I would like to thank Dr Philip Greenwell for the many stimulating ideas and advice. My thanks go to Mrs Sue Salmon, Dr Martin Whitworth and Dr Simon Hook for guidance and their helpful advice. I would also like to thank Miss Jennie Hands, Miss Paula Stabler, Neil Buck and Miss Charmaine Dudfield for their excellent technical input, and Mrs. Pip Chappell for performing the statistical analysis.

3 Technical Detail: Development of a blue starch strip test for rapid *alpha*-amylase determination

3.1 Introduction

Rain at or just before harvest can lead to pre-harvest sprouting in grains of wheat and barley. The presence of a small quantity of sprouted grain can significantly lower the market value of the entire batch. The poor quality of sprouted grain is largely due to excessive levels of *alpha*-amylase, a hydrolytic enzyme that has a major role in degrading starch. *Alpha*-amylase acts on starch through the hydrolysis of α-(1-4)-glycosidic linkages between adjacent residues within the starch polymers to produce soluble dextrins and maltose. It is believed that some other enzymes, including arabinoxylanases, glucanases and glycosidases (Marsh *et al*., 1988), as well as some proteolytic enzymes which break down functional endosperm proteins, also contribute to the deterioration in grain quality (Lukow & Bushuk, 1984). The levels of *alpha*-amylase can increase by 20-1000 fold, in pre-harvest sprouted grain, depending on variety, location and assay method used.

The deleterious effects of excessive levels of *alpha*-amylase on breadmaking quality include loaf volume collapse, darkening of the crust, lack of crumb resilience and poor crumb texture that can be uneven and sticky, causing slicing problems. After a harvest featuring high levels of *alpha*-amylase, the UK milling industry demand for sound home-grown wheat can exceed the supply, and so the balance has to be made up with imported grain. Similarly with barley, excessive levels of pre-germination *alpha*-amylase renders it unfit for malting purposes.

There are many tests for assessing *alpha*-amylase activity either directly or indirectly. They are classified as colorimetric, nephelometric (e.g. Modified AACC Method 22-07) and viscometric methods. The colorimetric and nephelometric methods are based on the use of standardised defined substrates (e.g. soluble starch, amylopectin, β-limit dextrin and dextrin-anthranilate); these include the Phadebas and Ceralpha (Megazyme) tests. The substrate for Ceralpha is a non-reducing end blocked p-nitrophenyl maltoheptaoside and the Phadebas is an insoluble cross-linked potato starch with a blue dye bound to it. The direct colorimetric methods for measuring *alpha*-amylase (e.g. Ceralpha assay) are usually time consuming or expensive to perform, and are not suitable for testing wheat in the field or at mill intake laboratories which require rapid and simple methods.

The most widely used test, the Hagberg Falling Number (HFN), is a viscometric method that measures the enzyme activity indirectly. This method is used in breeding and in the marketing and processing of cereals. Although it is relatively simple to use, it does have a number of disadvantages. The results of the HFN test are partly dependent on the water binding capacity of the flour, influenced by the pasting properties of the gelatinised starch and by various proteins and pentosans present in the samples. The relationship between *alpha*-amylase activity and HFN values is curvilinear, and the test is less precise at both extremes of the

activity scale. Also, the Falling Number method has relatively low sample throughput, and there is an initial outlay of about £8,000 to £11,000 for the purchase of the instrumentation. Other viscometric methods include the Rapid Visco-Analyser (RVA) which can be used to mimic the Falling Number test by measuring Stirring Number or to produce a full pasting curve over a defined temperature range.

A number of studies have reported the use of antibodies for recognising the high and low pI groups of isoenzymes of *alpha*-amylase (Daussant & Renaud, 1976; Sargeant, 1980; Lazarus *et al*., 1985). Antibody technology has been commercially exploited by the Wheat Quality Co-operative Research Centre (Australia) in the form of the WheatRite card test kit. This test has been designed to allow the farmer to assess wheat and barley grains for rain damage in different fields or areas before harvesting, and thus to segregate the crop accordingly. WheatRite is devised to permit the quantification of *alpha*-amylase protein (rather than activity) under field conditions (Ellis *et al*., 1999; Rathmell & Wrigley, 1999). While this test has been developed in Australia and evaluated for that market (Skerritt & Heywood, 2000; Skerritt *et al*., 2001) its performance and suitability for use in the UK has yet to be proven, and has not gained widespread acceptance in this country.

A new method for the direct detection of *alpha*-amylase activity in halved wheat grains was recently developed by CCFRA (Bhandari *et al*., 2001). This simple grain-blotting assay, which has the Phadebas substrate (an insoluble cross-linked partially hydrolysed potato starch powder, labelled with the Cibacron Blue dye) mixed into an agarose gel layer, is able to display germination activity in cut grain halves, in about 15 minutes. The enzyme located in the cut face of the grain releases the blue dye from the starch substrate in the agarose gel. The blue dye diffuses from the region of enzyme action, leaving a lighter coloured patch or "imprint" in the otherwise dark blue background. The size of the lighter patch is related to the *alpha*-amylase activity in the grain.

It became apparent that this same principle could also be used for the direct visual measurement of the enzyme activity from extracts of grain samples. Such a method has potential for modification into a practical amylase assay for use in the field and intake laboratories, in which incubation of the extract with a pre-fabricated blue gel device could lead to a visual assessment of amylase activity.

3.2 **Materials and Methods**

Ground wheat and barley samples from the HGCA Cereal Quality Surveys of 1999 and 2000 were obtained from Andover Analytical Laboratories. The 1999 harvest set consisted of 75 wheat samples which were selected to cover a wide range of Falling Number values, and 50 barley samples. The 2000 harvest set consisted of 50 wheat samples and 50 barley samples. The *alpha*-amylase activity within the wheat flour samples was determined directly by the Ceralpha (Megazyme) method, and indirectly by the Hagberg Falling Number method. CCFRA Guideline No.3, updated in 1999, gives the details of the Ceralpha assay (Method

No. 0018) and the Hagberg Falling Number method (Method No. 0006). The modified Randox test (Randox Laboratories Ltd, N. Ireland), using the micro-titre plate format (Bhandari *et al*., 2001), was used for determining the relative *alpha*-amylase activities within the barley samples. Fungal *alpha*-amylase was obtained from Novo Nordisk.

Unbuffered Phadebas blue starch powder was obtained from Pharmacia Upjohn Ltd (Sweden). The Phadebas tablet assay (Barnes and Blakeney, 1974) kit and the filter paper strips were obtained from Amersham Pharmacia Biotech Ltd. Amylose Azure, Amylopectin Azure and Starch Azure substrates (all containing Remazol Brilliant Blue R dye) and Type IX ultra-low melting point agarose were purchased from Sigma Chemical Company Ltd. The Megazyme AZCL-Amylose substrate was obtained from Megazyme International Ireland Ltd. All the other reagents were of standard laboratory grade. The self-adhesive siliconised rubber sheeting was obtained from Molecular Probes (Oregon USA). Strip test wells were purchased from J & P Agar Aids Ltd. A Denley Well Warm-1 incubator was used for performing the assays involving the strip test.

Quantification of blue colour development within the strip test was carried out using the Phoretix 1-D Advanced software from Phoretix International. Linear discrimination analysis was performed using Minitab version 13.0. Reproducibility and repeatability were examined by Run Chart analysis (Minitab version 13.0) which allows any patterns evident in the process data to be seen and performs two tests for non-random behaviour. The Run Chart plots all of the individual observations versus the subgroup number, and draws a horizontal reference line at the median. The programme allows one to see whether one operator consistently measures higher than the other does, or if the measurements on certain samples vary more when compared to other samples.

3.3 **Results**

The effectiveness of several commercially available types of blue starch or blue-amylose/amylopectin substrates available from Pharmacia (Phadebas), Megazyme and Sigma were compared for their characteristics (particle size, colour intensity, enzyme susceptibility, etc.), in order to optimise the performance of the various test formats using fungal *alpha*-amylase preparations. The Phadebas blue starch was identified as being the most suitable of all the various substrates examined (data not shown). Several formats of a device were evaluated for their ability to display *alpha*-amylase activity semi-quantitatively following the incubation of aqueous extracts from ground grain or flour. The earlier designs were cassettes made of glass microscope slides and featured clarification of the blue substrate/agarose layer, which depended upon the speed of starch hydrolysis and the amount of applied amylase diffusing in from the grain extracts. However, it was concluded that the cassette format lacked the level of sensitivity necessary for further development as a practical diagnostic test (data not shown). Also, the cassette devices were not

suitable for producing on a large-scale for testing all of the Cereal Quality Survey samples of wheat and barley. The format was re-designed to visualise the blue dye liberated from the cross-linked starch substrate following enzyme hydrolytic action.

Development of the Strip Test

The Strip Test is based on the principle of chromatographic separation of solubilized blue dye from the insoluble starch using a strip of thick filter paper as the solid phase medium (Figure 8). The dimensions of the filter paper strips are 2.2 x 0.5 x 0.1cm. The extract from a ground wheat sample is mixed with Phadebas blue starch and agarose and an aliquot is pipetted into a 2mm diameter sample well (made from self-adhesive siliconised rubber sheeting) at the base of the filter paper. Once the mixture has soaked into the paper, either extraction solution is applied to the bottom of the strip, causing the liberated blue dye to migrate upwards and stain the paper blue (Figure 9). The procedure for performing the strip test was developed initially by using highly dilute preparations of fungal *alpha*-amylase (26600 Farrand Units/mg). Thereafter, selected samples of wheat of known HFN values were used routinely for subsequent modifications and improvements made to the kit.

Figure 8. Schematic diagram of Strip Test

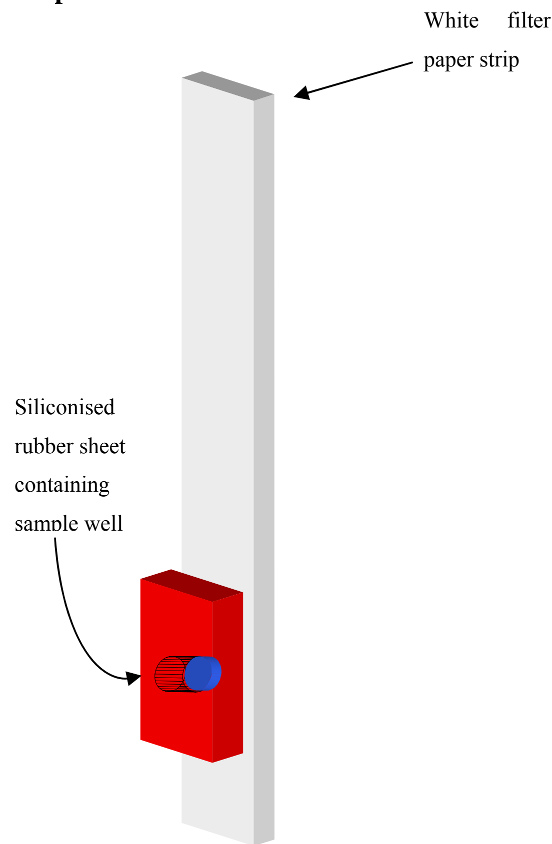


Figure 9. Schematic diagrams of sample application and colour visualisation on a Strip Test

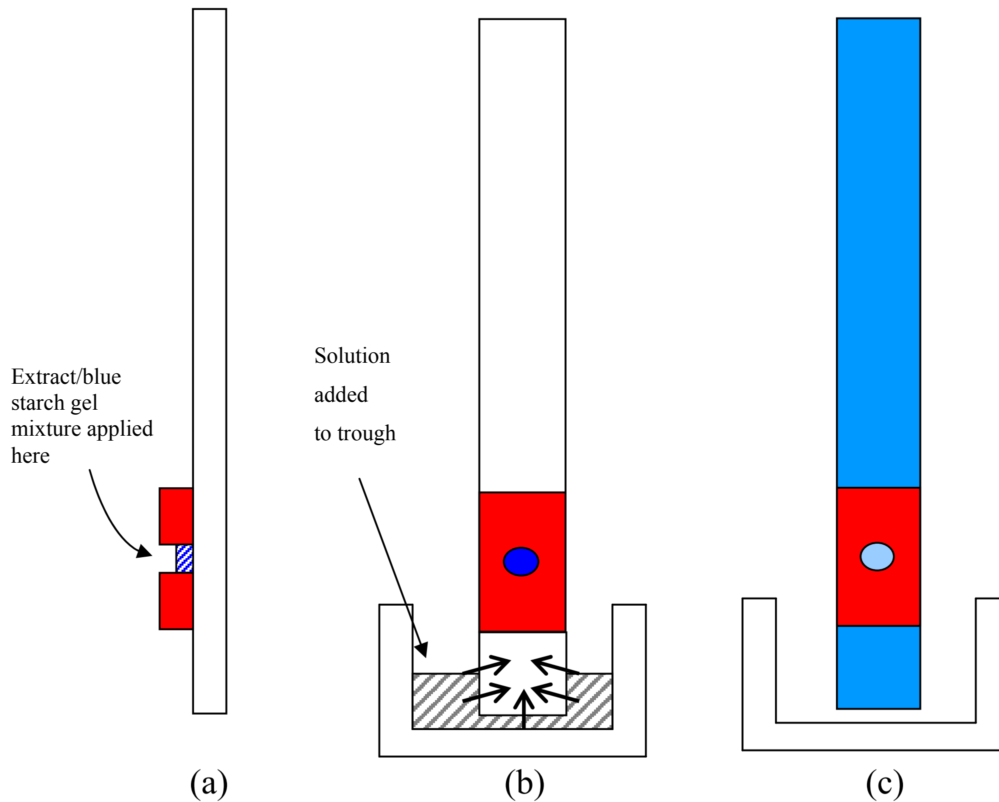


Figure 9(a) shows the side view, in which the reaction (incubated extract and substrate) mixture has been applied to the sample well.

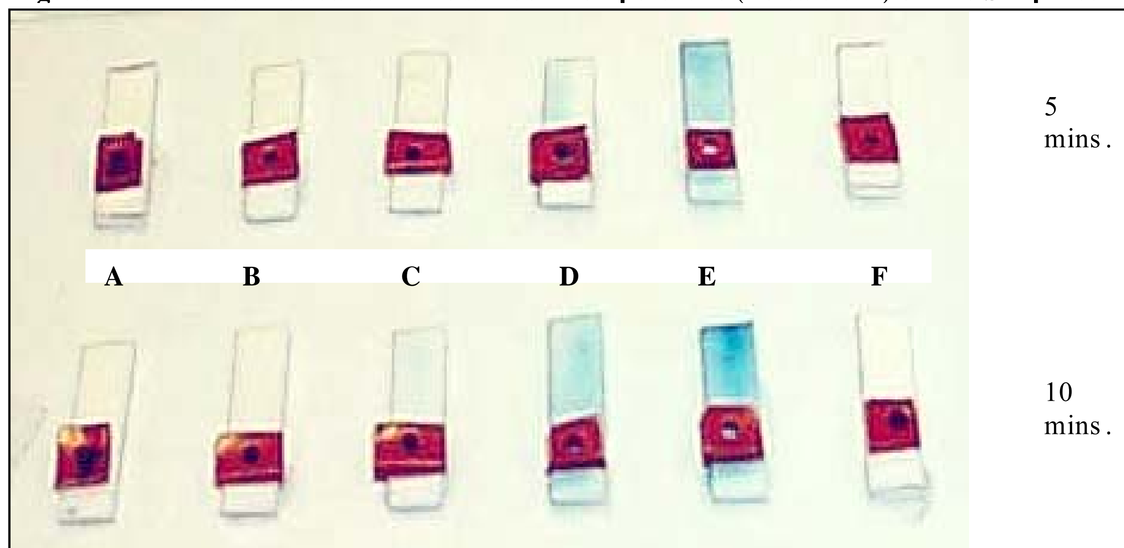
Figure 9(b) shows the front view, in which the strip is placed into a trough and extraction solution is added.

Figure 9(c) shows the migration of free dye, following absorption of the liquid by the filter paper strip.

Optimisation of reaction conditions and sample extraction

It was found that presence of 2mM calcium chloride and incubation of the reaction mixture at 37°C improved the performance of the assay quite significantly (Figures 10 and 11). Extending the time of incubation from 5 minutes to 10 minutes also helped to increase the sensitivity of the test.

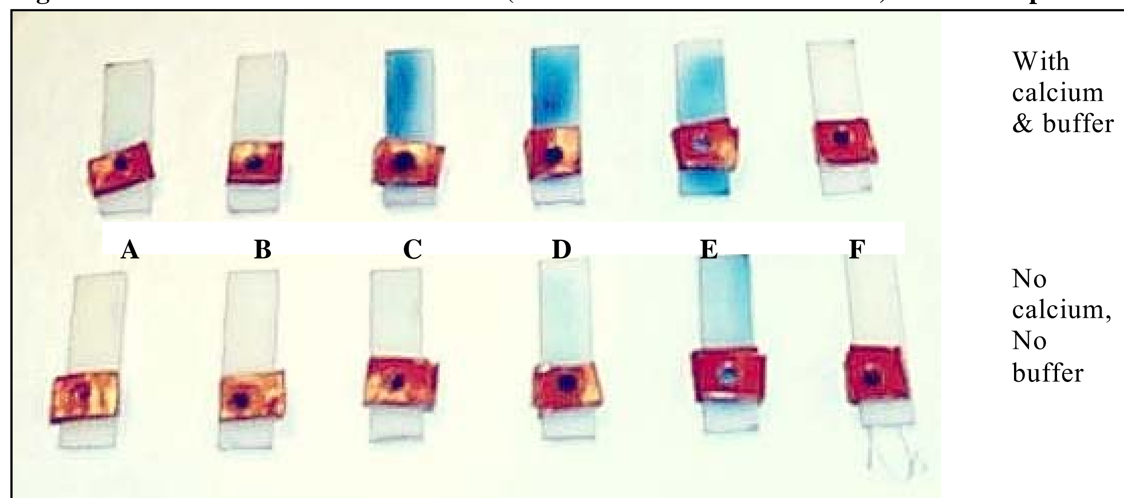
Figure 10. Effect of incubation time at room temperature (no calcium) on the Strip Test



5 mins.

10 mins.

Figure 11. Effect of calcium and buffer (5 minutes incubation at 37°C) on the Strip Test



With calcium & buffer

No calcium, No buffer

Sample	HFN	Enzyme activity (Absorbance - Ceralpha method)	Content
A	224	0.40	Wheat flour
B	250	0.28	Wheat flour
C	110	2.84	Wheat flour
D	62	11.52	Wheat flour
E	-		Fungal *alpha*-amylase
F	-		Reagent blank

In order to establish a rapid and efficient method of extraction of *alpha*-amylase for use with the test, three different solutions were studied using a wheat flour sample with a Falling Number value of 62. The extraction solutions used in the commercially available diagnostic tests for measuring *alpha*-amylase activity- Kodak Ektachem (Osborne *et al*., 1990); Phadebas (Barnes and Blakeney, 1974); WheatRite (Skerritt and Heywood, 2000), were examined for their suitability. The efficacy of the three extraction methods was determined by assaying for the level of *alpha*-amylase activity recovered using the Phadebas tablet assay (Barnes and Blakeney, 1974), and their relative absorbance values were compared. The composition of these extraction solutions were as follows:

Ektachem test: 5g sodium chloride and 0.2g calcium chloride per litre of water
Phadebas test: 50mM maleic acid, 50mM sodium chloride and 2mM calcium chloride pH 5.2
WheatRite test: 85mM sodium chloride

The WheatRite test extraction solution was shown to yield consistently higher *alpha*-amylase levels with both diluted and undiluted samples (see Table 2). This solution contains no calcium chloride, and as it is widely accepted that *alpha*-amylase activity is dependent on calcium levels; the effect of calcium addition to this solution was investigated.

Table 2. Comparison of extraction buffers on enzyme activity (expressed as absorbance values at 405nm) in the Strip Test

Flour type and dilution	Ektachem	Phadebas	WheatRite	Temperature
HFN: 62 (undiluted)	2.143	2.054	2.133	20°C
	2.097	2.131	2.163	
Average	**2.12**	**2.09**	**2.15**	
HFN: 62 (x 2 dilution)	1.486	1.833	1.897	20°C
	1.522	1.833	1.934	
Average	**1.50**	**1.83**	**1.92**	

However, it was found that the presence of calcium in the extraction solution lowered the measured enzyme activity (Table 3), possibly by reducing the release of *alpha*-amylase from flour matrix. The extraction solution consisting solely of 85mM sodium chloride was, therefore, adopted as standard in all the experiments involving wheat samples.

Table 3. Effect of calcium in the WheatRite extraction buffer (expressed as absorbance values at 405nm) in the Strip Test

Flour type and dilution	2mM calcium	No calcium
HFN: 110 (undiluted)	0.456	0.596
	0.457	0.614
	0.495	0.589
	0.493	0.606
Average	**0.48**	**0.60**
HFN: 110 (x 2 dilution)	0.295	0.318
	0.302	0.333
	0.273	0.318
	0.275	0.331
Average	**0.29**	**0.33**

Protocol for the wheat *alpha*-amylase assay:

(i) The Phadebas blue starch/agarose mixture was prepared by mixing 0.066g agarose in 5ml calcium/maleate buffer (189mM maleic acid, 2mM calcium chloride pH 6.0) and adding 0.132g of Phadebas powder dispersed in 5ml calcium/maleate buffer, maintained at 40°C on a stirring hot plate.

(ii) *Alpha*-amylase was extracted by adding 10ml of extraction solution (85mM sodium chloride) to 1 g of ground wheat in a 20ml capped glass tube for 5 minutes with occasional agitation by hand, and allowed to settle for 5 minutes.

(iii) 20µl of the enzyme was added to 20µl of the Phadebas/agarose mixture in a 1.5ml Eppendorf tube, mixed and incubated for 10 minutes at 37°C in a Denley Well Warm-1 micro-titre plate incubator.

(iv) The reaction was stopped by the addition of 20µl of stopping reagent (500mM sodium hydroxide, 50mM di-sodium EDTA).

(v) 20µl of the incubated extract and substrate mixture was pipetted into the sample well at the base of the filter paper strip (Figure 2a).

(vi) After 1 minute the filter paper strip was placed in a trough, measuring 12 x 5 x 3mm, within a rubber block (J & P Agar Aids Ltd) and 200µl of extraction solution was added to mobilise any liberated blue dye upwards along the filter paper strip.

(vii) The colour reaction was observed and recorded after 1 minute.

Two observers judged the blue coloration produced on the Strip Tests visually, and the intensity was recorded using the scoring system below:

Strip Test Score	Colour Intensity Description
0	No colour
1	Very faint blue
2	Slightly blue
3	Moderate blue
4	Dark blue

Wheat samples from the 1999 and 2000 harvests were evaluated in duplicate, on separate occasions using the Strip Tests. The resultant Strip Test scores were analysed with respect to the corresponding enzyme activity values measured by the Ceralpha method (performed by CCFRA), and Hagberg Falling Number values (performed by CCFRA and by Andover Analytical Laboratories).

1999 Harvest Wheat Samples

The performance of the Strip Test relative to Ceralpha values is shown in Figure 12. Generally there is good agreement between evaluations 1 and 2 scores. Four of the evaluation 1 scores were found to be invalid due to false positives observed for blanks included as controls and these were subsequently omitted (sample numbers 13, 49, 59 and 71).

Figures 13 and 14 show the performance of the Strip Test relative to Hagberg Falling Number determinations, performed by CCFRA and by Andover Analytical Laboratories, respectively. While there is a broad agreement between the two sets of data, a number of exceptions are noted. A comparison between the HFN values determined in the two laboratories, plotted against Ceralpha enzyme activity, is shown in Figure 8. With the exception of the five very obvious outliers highlighted with circles, HFN values obtained from Andover Analytical (tested at harvest) tended to be lower than those measured by CCFRA. Inspection of the CCFRA HFN/Ceralpha values in Figure 15 shows that the data scatter is the greatest between HFN range of about 150 – 230 seconds.

Figure 12. Strip Test scores versus Ceralpha, comparison of two evaluations, 1999 harvest

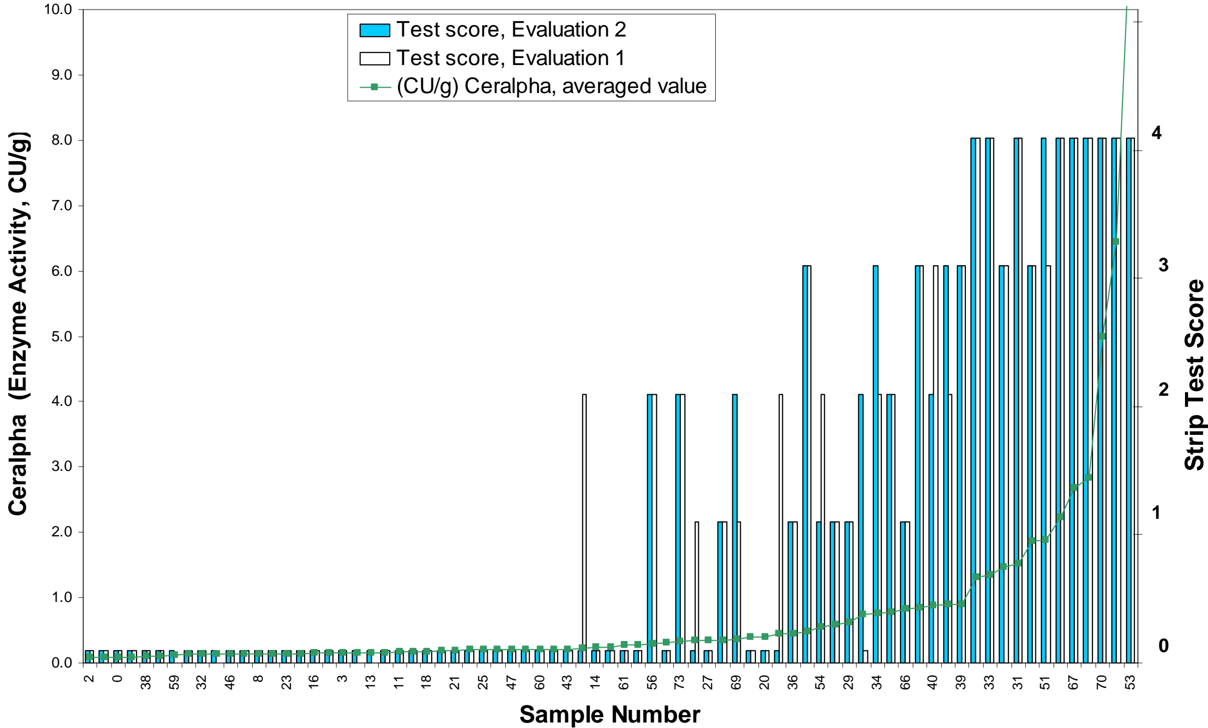


Figure 13. HFN (CCFRA) versus Strip Test (2nd Evaluation)

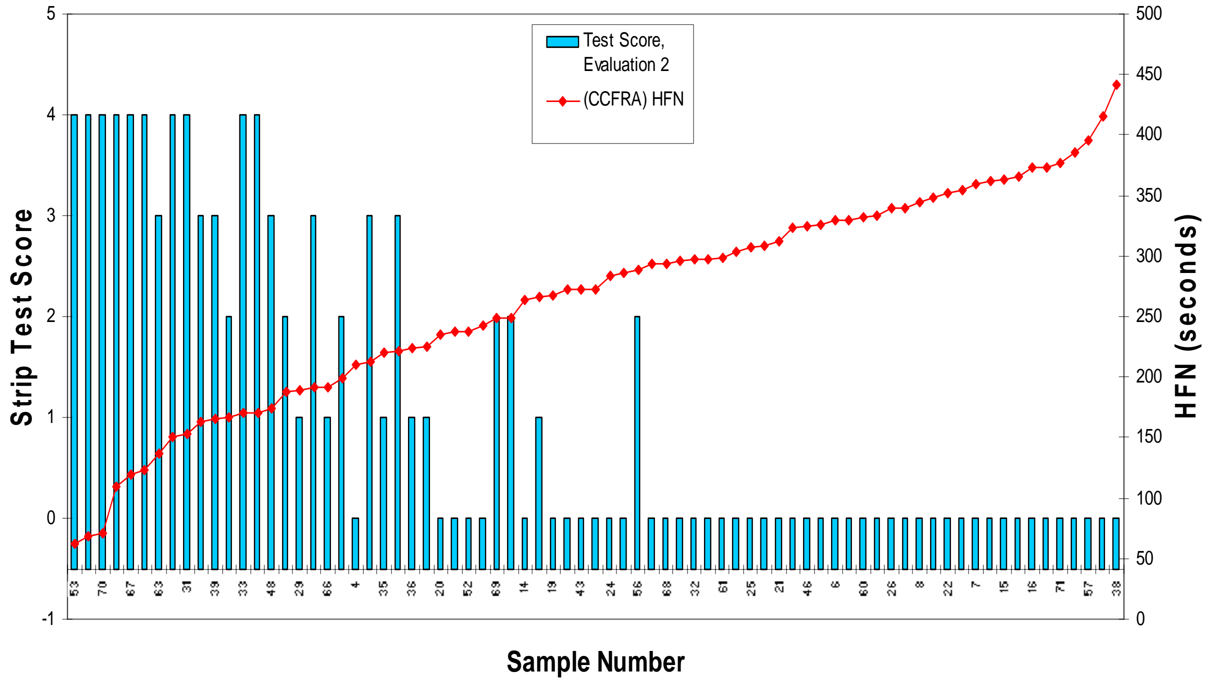


Figure 14. HFN (Andover) versus Strip Test (2nd Evaluation)

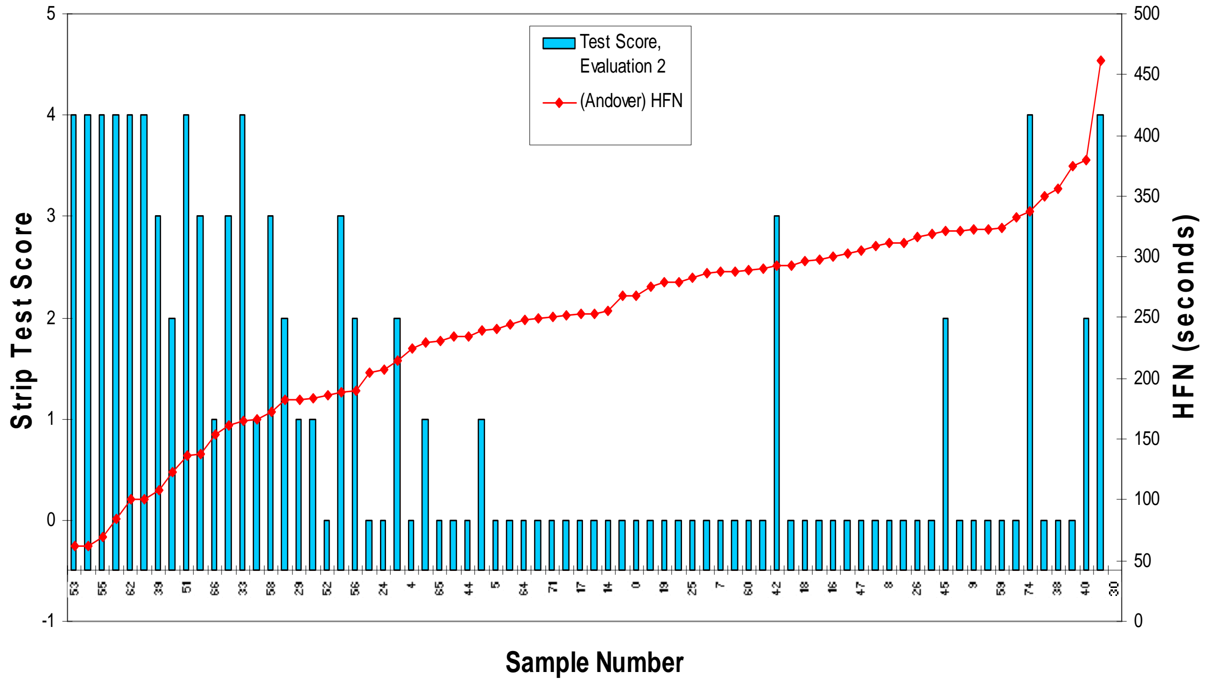
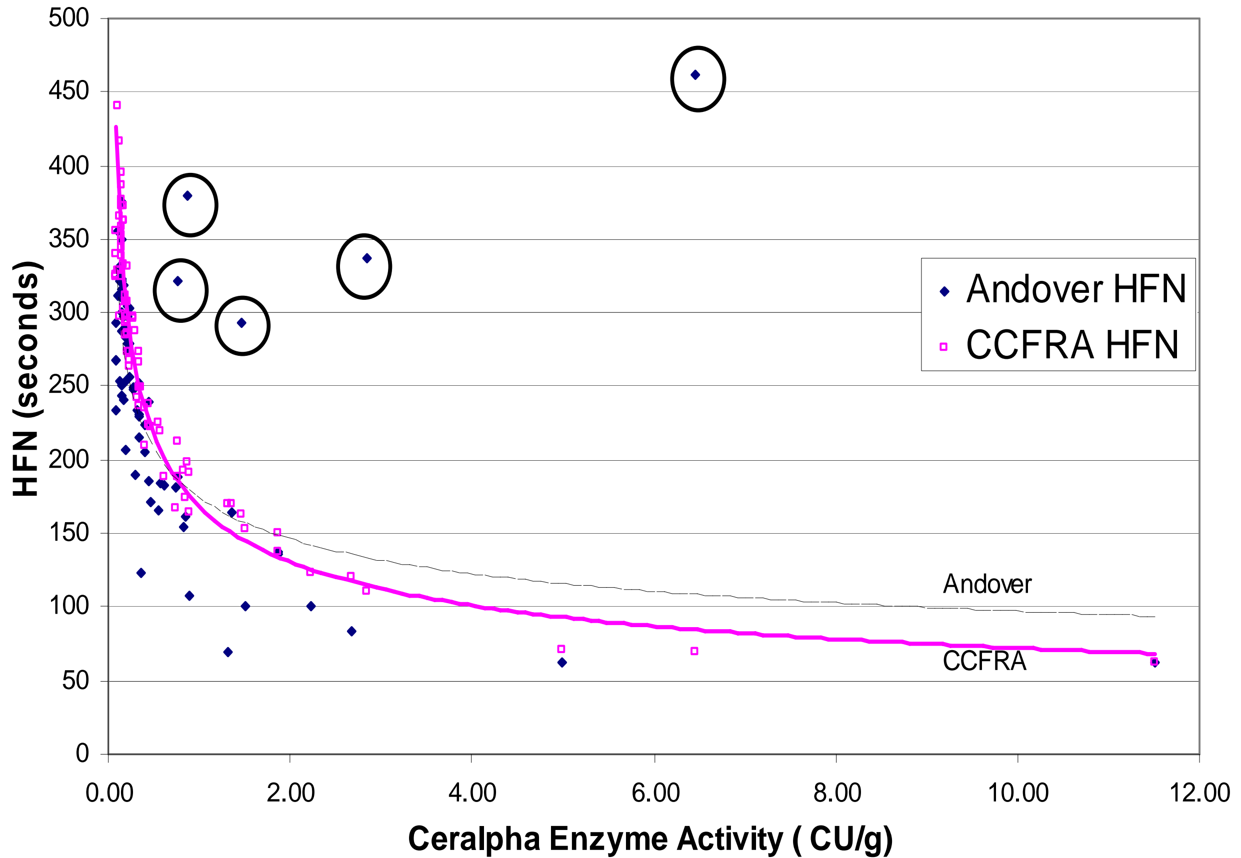


Figure 15. Andover/CCFRA HFN versus Ceralpha data, 1999 harvest samples



2000 Harvest Wheat Samples

There were a limited number of samples with high *alpha*-amylase activity within this set. Figure 16 shows the two evaluations of the Strip Test against HFN (CCFRA) values determined by CCFRA. Again there is broad agreement between the two evaluations, with the only two minor exceptions being samples 42 and 45 which had scores of 1 for evaluation 1, but scores of 0 for evaluation 2. Otherwise, all the samples with HFN >270 gave a score of 0. The plot of HFN values from the two labs versus Ceralpha values are given in Figure 17.

Analysis of Combined Data from 1999 and 2000 Harvest Wheat Samples

Figures 18 and 20 show Strip Test scores plotted against HFN (CCFRA) and Ceralpha values, respectively, using the combined (second evaluation) data for 1999 and 2000 harvest wheat samples. Using a cut-off HFN value of 250 seconds to provide an 'accept/reject' assessment of the performance of the Strip Test, it was found that of the 86 samples with HFN values >250 seconds, 82 samples scored 0. Only 8 out of the 40 samples with HFN values <250 seconds, scored 0.

Figure 16. Strip Test Scores versus HFN (CCFRA), 2000 harvest samples

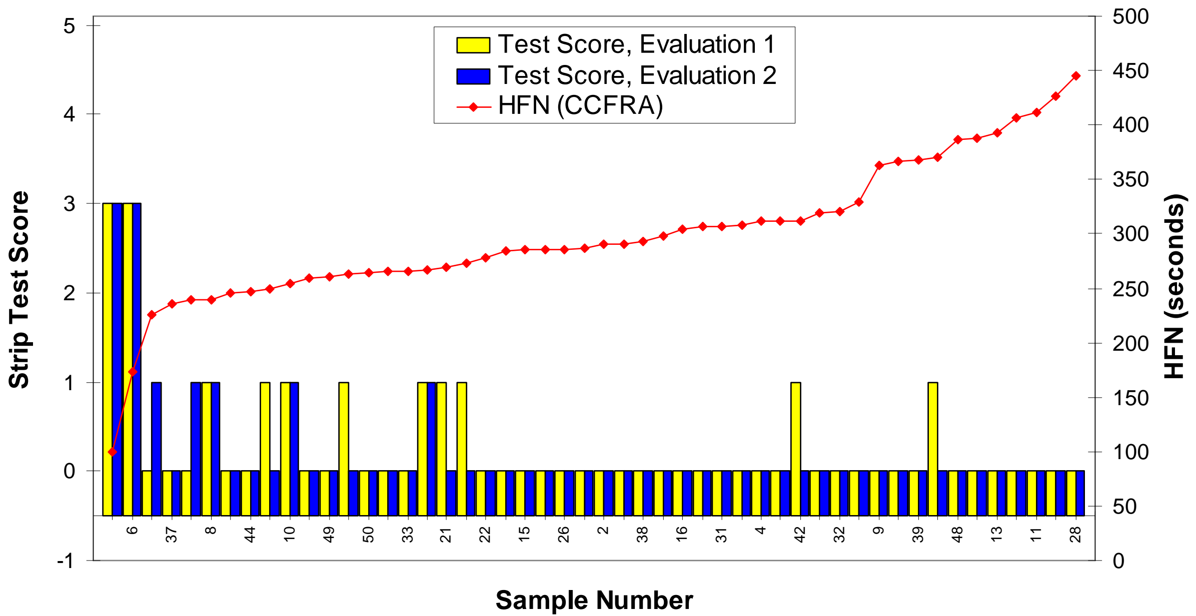


Figure 17. Andover/CCFRA HFN versus Ceralpha data, 2000 harvest samples

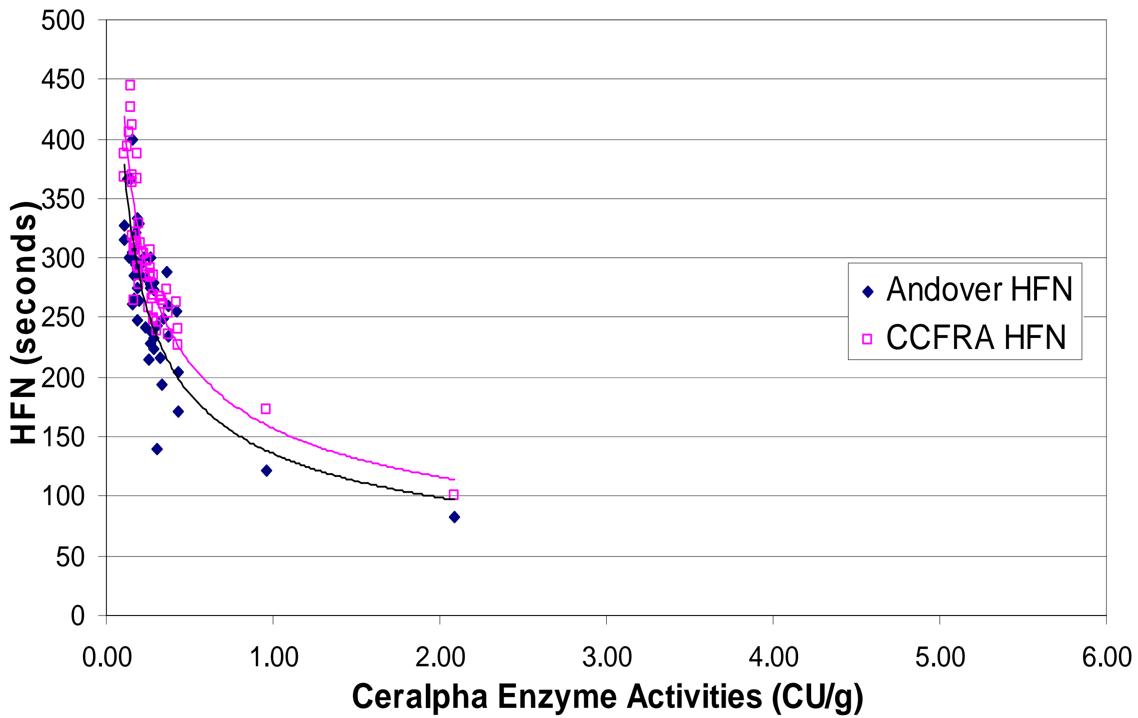


Figure 18. Strip Test scores versus HFN (CCFRA) data from 1999 and 2000 harvests

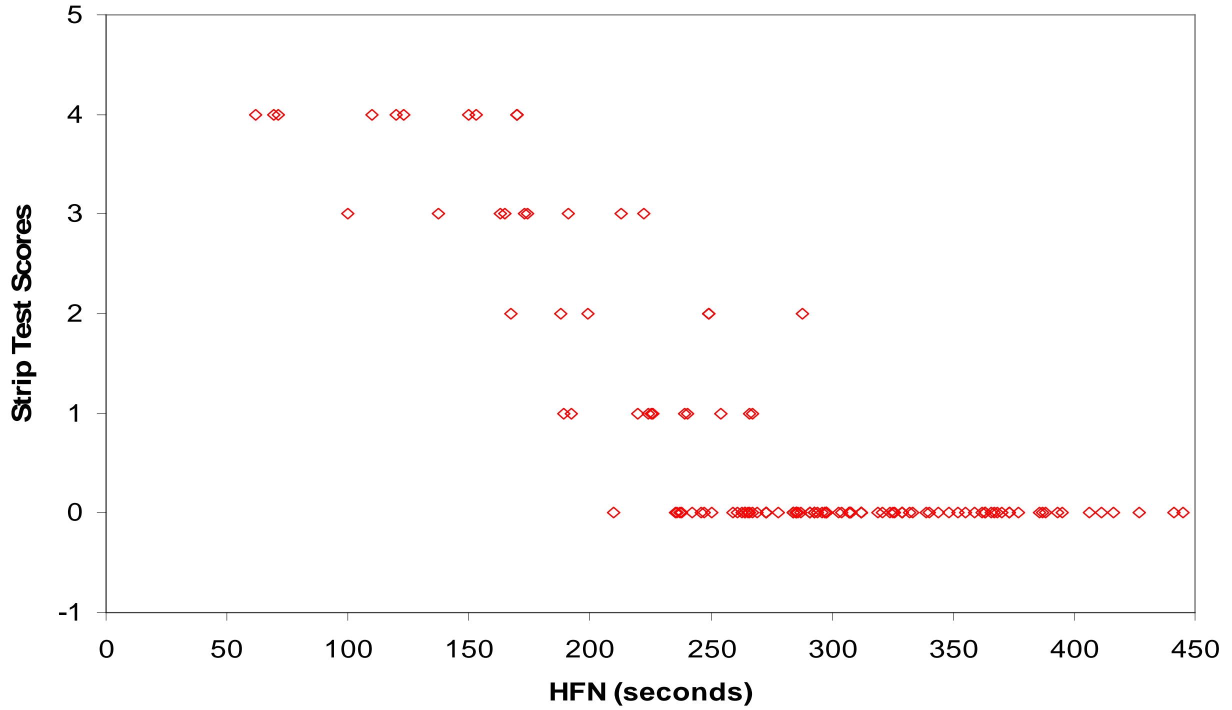
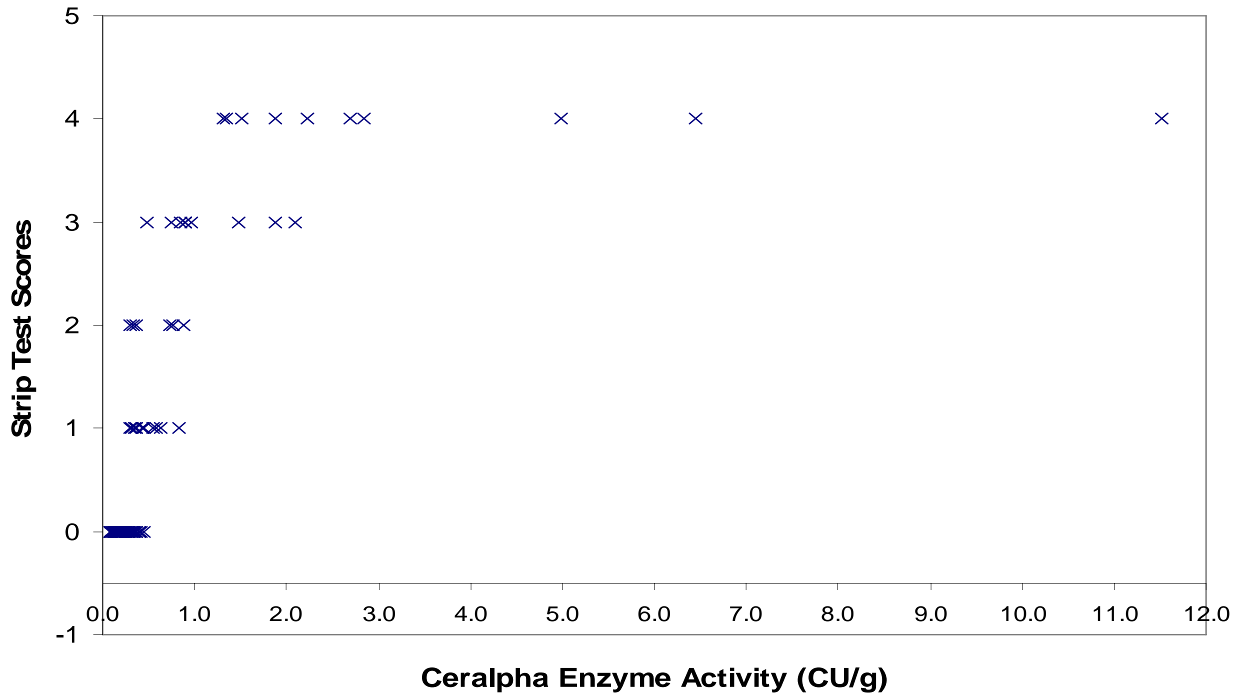


Figure 19. Strip Test scores versus Ceralpha data from 1999 and 2000 harvests



Linear discriminant analyses of the Strip Test scores versus HFN and Ceralpha values were carried out. Table 4 details the prediction of Strip Test scores from Ceralpha data, and how the predicted ("correct") class values relate to the actual classification. Table 5 has the corresponding details for Strip Test versus HFN data.

Table 4. Linear Discriminant Analysis: Strip Test Score versus Ceralpha

```
Linear Method for Response:   Strip Test Scores
Predictors:                   Ceralpha data

Group        0        1        2        3        4
Count       89       11        6        9       10

Summary of Classification

Put into         ............ True Group ............

Group              0        1        2        3        4
0                 82        2        2        0        0
1                  7        5        1        1        0
2                  0        4        2        2        0
3                  0        0        1        6        5
4                  0        0        0        0        5
Total N           89       11        6        9       10
N Correct         82        5        2        6        5
Proportion     0.921    0.455    0.333    0.667    0.500

N =  125     N Correct =  100     Proportion Correct = 0.800
```

Table 5. Discriminant Analysis: Strip Test Score versus HFN

```
Linear Method for Response:   Strip Test Scores
Predictors:                   HFN (CCFRA)

Group        0        1        2        3        4
Count       89       11        6        9       10

Summary of Classification

Put into         ............ True Group ............

Group              0        1        2        3        4
0                 67        0        1        0        0
1                 21        5        2        0        0
2                  1        4        1        2        0
3                  0        2        2        5        4
4                  0        0        0        2        6
Total N           89       11        6        9       10
N Correct         67        5        1        5        6
Proportion     0.753    0.455    0.167    0.556    0.600
N =  125     N Correct =   84     Proportion Correct = 0.672
```

The results of the linear discriminant analysis show that the Ceralpha data has a higher proportion of correctly classified Strip Test scores than the HFN data. In particular, the Strip Test class 0 places 92% correct for Ceralpha results, compared to 75% for the HFN results. The Ceralpha results also have a tighter range of the predicted classes than HFN values for this data.

Figures 20 and 21 show the respective boxplots of the classification of the Ceralpha and HFN data according to the Strip Test scores, 0 to 4. This type of plot summarises the information about the shape, dispersion and centre of the data. Each box represents the middle 50% of the observations, and the line drawn through the box represents the median. The whiskers extending from each box indicate the lowest and highest values in the data set, excluding the outliers (marked with an *).

Figure 20. Boxplot of StripTest score versus Ceralpha data

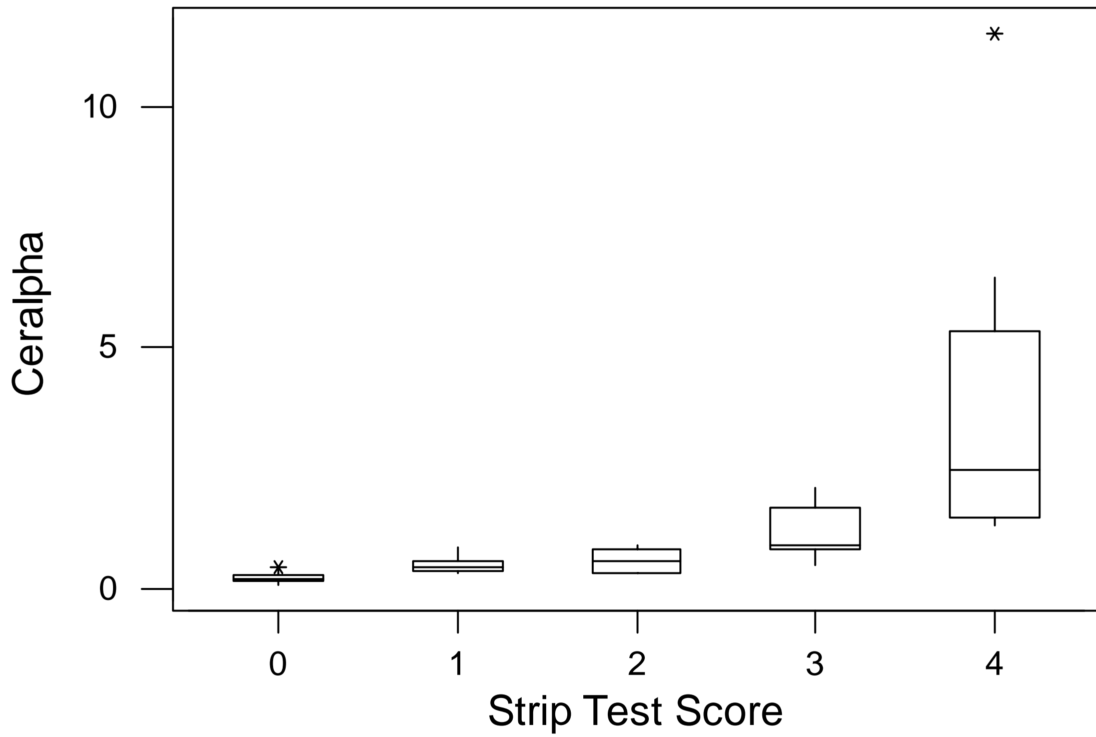
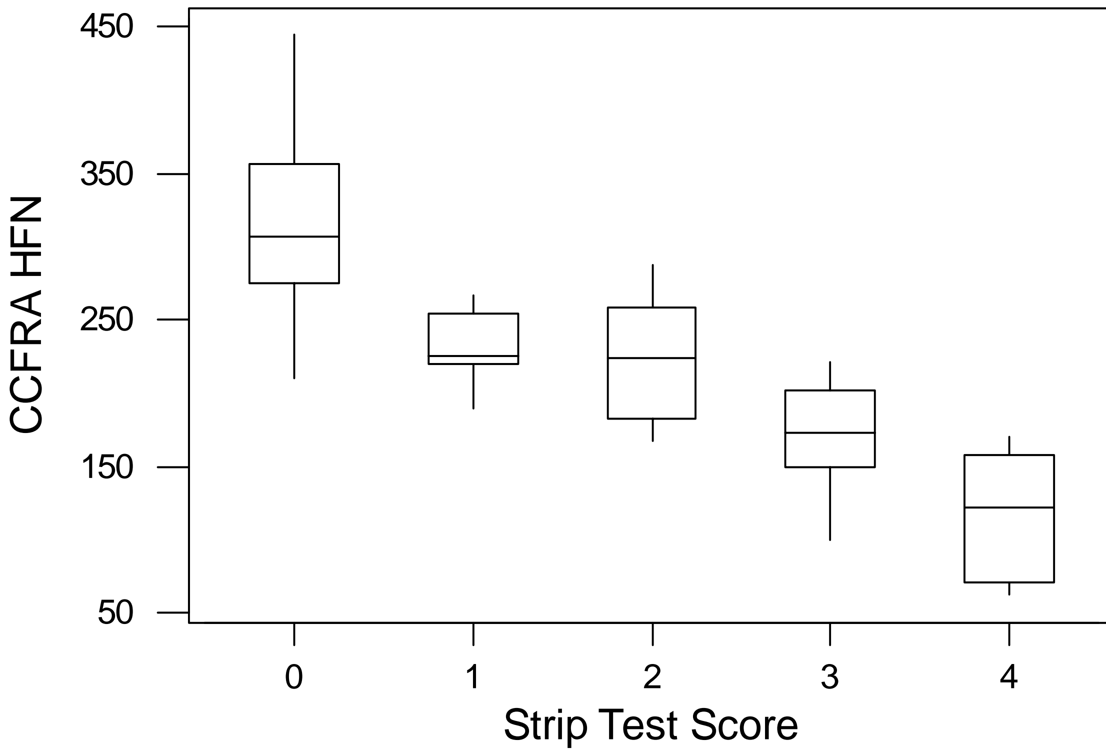


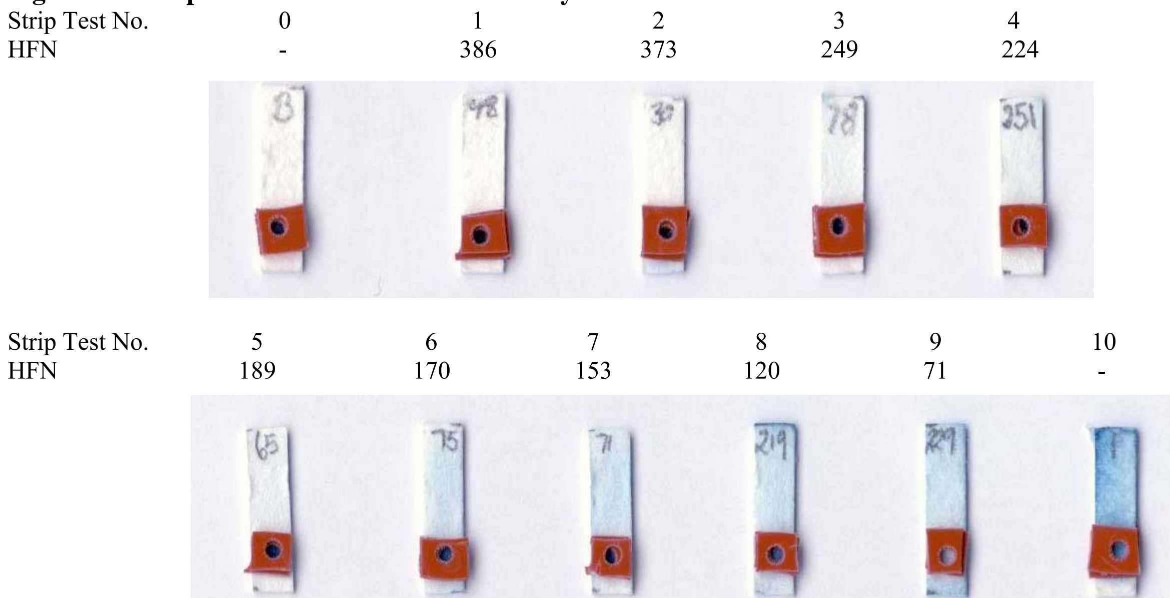
Figure 21. Boxplot of Strip Test score versus HFN data



Densitometric Evaluation of Strip Test

The performance of the Strip Test was assessed quantitatively using image analysis and densitometry (Phoretix 1-D Advanced software). Single determinations of 9 selected wheat flours were carried out, and the blue coloration was measured by the densitometric technique. Results of the Strip Test are shown in Figure 22 in which the reagent blank and a fungal alpha-amylase preparation are Strip Tests 0 and 10 respectively.

Figure 22. Samples used for densitometric analysis



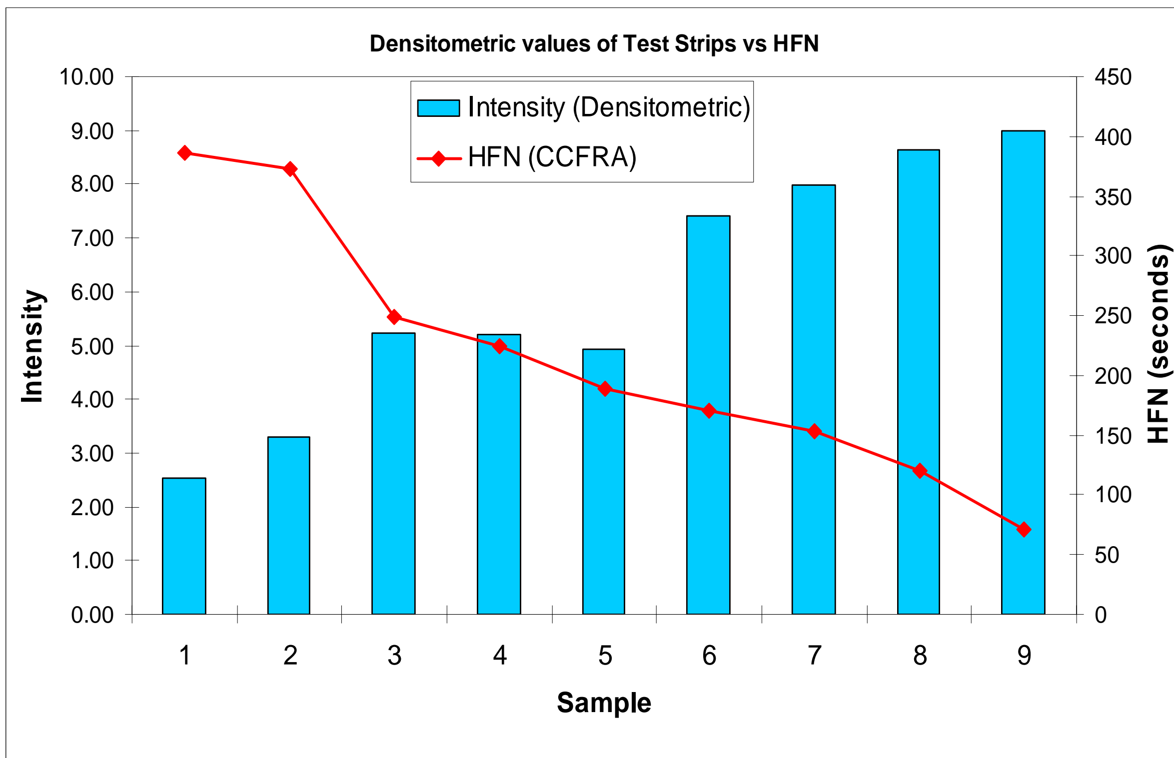
The results of the scanned intensities and the other measures of *alpha*-amylase are given in Table 6. The values given for the scanned intensities have been corrected by subtracting the intensity of the reagent blank. The relationship between the Strip Test scanned intensities and visual scores is consistent throughout the range of HFN values.

Table 6. Scanned intensity values and other measured values of 9 selected samples (Figure 22)

Strip Test No.	1	2	3	4	5	6	7	8	9
Sample No. CM/52804/	23	16	73	36	29	33	31	67	70
Ceralpha Activity (CU/g)	0.145	0.150	0.33	0.455	0.625	1.350	1.515	2.685	4.990
HFN (seconds)	386	373	249	224	189	170	153	120	71
Strip Test Scores	0	0	1	1	1	3	4	4	4
Densitometric Intensity (optical density units)	2.53	3.30	5.24	5.21	4.94	7.42	7.98	8.64	9.00

Both the visual and scanning methods for assessing intensities appear to be unable to discriminate between the samples with HFN values of 189, 224 and 249 seconds. Figure 23 shows the scanned intensity values plotted against HFN (CCFRA) values.

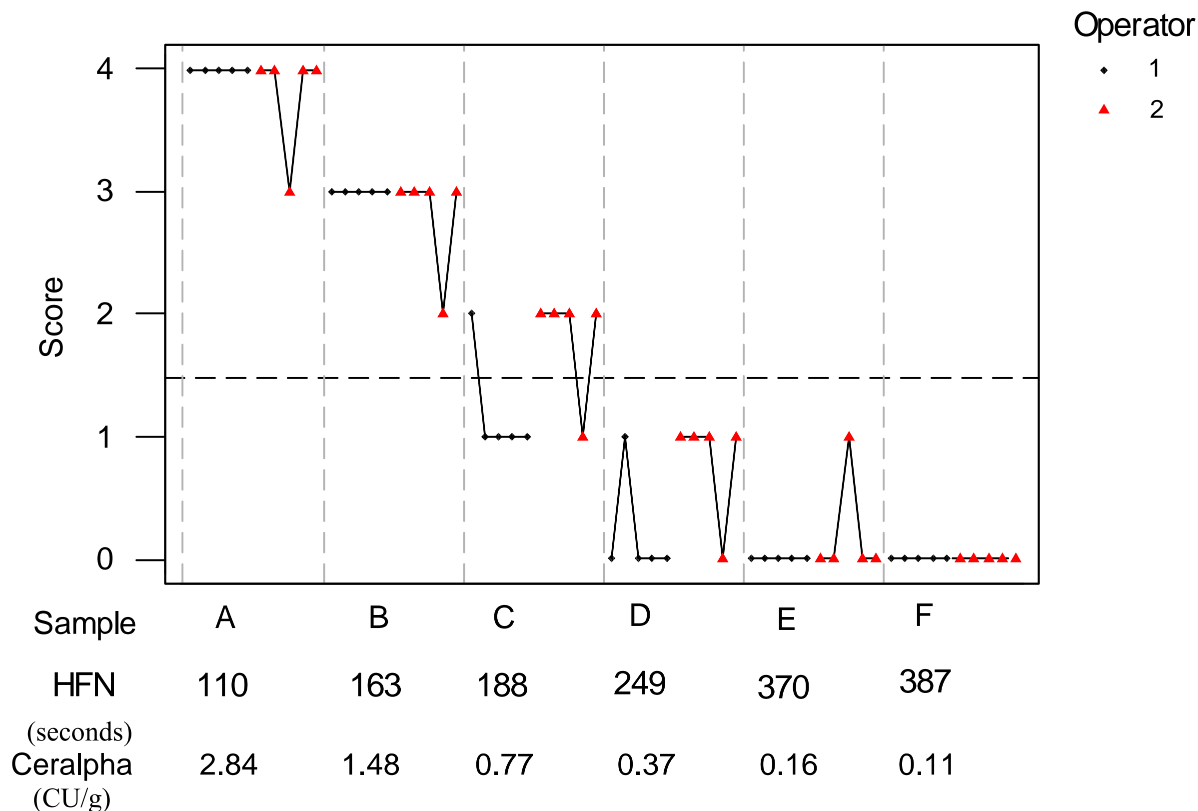
Figure 23. Relationship between scanned intensity values of Strip Tests and HFN



Repeatability and Reproducibility

A limited assessment of the repeatability and reproducibility of the Strip Test was performed with six wheat samples with HFN values ranging from 110 to 387 seconds. Two operators carried out five replicate determinations on each sample, on two separate days. Figure 22 shows a Minitab 13.0 Run Chart depicting the visual scores, together with HFN (CCFRA) and Ceralpha absorbance values. The results show that both operators produced highly repeatable scores for each set of the five replicates, operator 1 exhibiting a relatively higher degree of consistency that operator 2 when considering all samples. The main difference between the scores produced by the two operators can be seen with samples C (HFN of 188 seconds) and D (HFN of 249 seconds), where operator 2 scores higher than operator 1 does.

Figure 22. Run Chart of Strip Test Score by Sample, Operator



Assay of Barley Samples

Assaying barley extracts proved to be problematic. Results of preliminary experiments are given in Figure 25. The blue coloration of the Strip Test was minimal and inconsistent for all samples received from the 1999 harvest, despite Randox test data clearly showing that some of these samples (SB 174 and WB 137) possessed absorbances at 405nm, which are related to significant *alpha*-amylase activities (Table 7). For comparison, a winter wheat sample with a HFN of 167 seconds has a Randox absorbance value of 0.85. Design of the test kit was modified, from that used for wheat, in an attempt to increase sensitivity by:

(i) Reducing the ratio of extraction buffer to barley flour (3ml of buffer to 1g of flour was used) in order to increase the amount of *alpha*-amylase in solution.

(ii) Increasing extraction and incubation times.

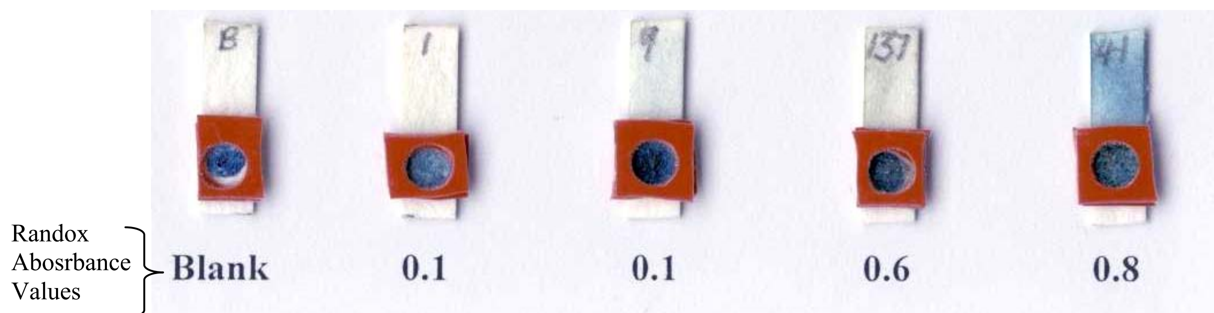
(iii) Increasing the volume of the sample application well of the Strip Test to accommodate 40µl of extract reacted with the Phadebas/agarose mixture (cf 20µl for wheat).

Table 7. Strip Test and Randox test results for Barley samples

Spring/Winter Barley	Sample No.	Randox Absorbance Value	Strip Test Colour	StripTest Score
Spring 1999	SB29	0.24	none	0
Spring 1999	SB49	0.22	none	0
Spring 1999	SB22	0.18	none	0
Spring 1999	SB21	0.17	none	0
Spring 1999	SB5	0.15	none	0
Spring 2000	SB174	0.36	none	0
Spring 2000	SB140	0.22	none	0
Spring 2000	SB13	0.18	none	0
Spring 2000	SB1	0.17	none	0
Spring 2000	SB27	0.15	none	0
Winter 1999	WB41	0.77	blue	3
Winter 1999	WB137	0.62	none	0
Winter 1999	WB7	0.15	none	0
Winter 1999	WB137	0.14	none	0
Winter 1999	WB5	0.13	none	0
Winter 2000	WB89	0.27	none	0
Winter 2000	WB18	0.21	none	0
Winter 2000	WB29	0.19	none	0
Winter 2000	WB30	0.16	none	0
Winter 2000	WB39	0.16	none	0

Unfortunately, problems arose in pipetting extracts due to the increased viscosity of the extracts following the reduction of buffer to flour ratio. It was found that very few of the samples from the 2000 harvest possessed adequate *alpha*-amylase activity according to the Randox assay, and none of these produced a detectable blue colour in the Strip Test. Further improvements were beyond the scope of this study, and therefore the remaining barley samples were not tested.

Figure 25. Preliminary results of assaying barley samples from the 1999 harvest



3.4 **Discussion**

A new simple test for measuring levels of *alpha*-amylase semi-quantitatively in wheat has been developed. The Strip Test features the Phadebas blue starch substrate. Unlike the commercially available test with Phadebas tablets which requires a water bath and a spectrophotometer to measure absorbance at 620nm, the new format has been designed for visual assessment of enzyme activity. The test requires 10 minutes for extraction from ground wheat flour, 10 minutes reaction time and a further minute to visualize blue colour development in the white filter paper strip. While blue colour was observed when the reaction was performed at room temperature, this was limited to those samples with very low HFN (about 62 seconds). Raising the temperature to 37°C increased the speed and sensitivity of the reaction, and hence the range of the Strip Test. A visual scoring system, on a scale from 0 to 4, was adopted to record the intensity of the blue colour.

The Strip Test was evaluated with a total of 125 samples of wheat from the 1999 and 2000 Cereal Quality Surveys. All samples with HFN<210 gave a detectable blue colour, i.e. were given a score of 1 or more. Overall, the performance of the new test method was found to be marginally better when compared with Ceralpha results, than with the HFN data when judged by linear discriminant analysis. This is consistent with the fact that both the Phadebas and Ceralpha assays are direct, colorimetric methods of measuring the amylase activity, whereas the HFN method is an indirect one. The test is less precise when assessing samples with HFN values between about 170 and 250 seconds. It should be noted that below 250 seconds, there is increased scatter in the data when HFN is plotted against Ceralpha measurements (Figures 15 and 17), and this region signifies the acceptability threshold for quality classification. It is generally accepted with the Hagberg Falling Number test that:

1. HFN values of less than 150 seconds indicate high *alpha*-amylase activity
2. HFN values of 200 to 250 seconds indicate moderate *alpha*-amylase activity
3. HFN values of more than 300 seconds indicate low *alpha*-amylase activity

In comparison with the Hagberg Falling Number test, the Strip Test gives:

4. a strong blue coloration with samples with HFN values of between 62 – 169 seconds
5. a range of coloration from none to medium blue with samples with HFN values of between 170 – 289 seconds
6. no blue coloration with any of the samples with HFN values of between 290 – 445 seconds

The above shows that in its present format, the Strip Test is clearly able to distinguish between high and low *alpha*-amylase activities in wheat samples, which would be most useful for screening purposes. In spite of some of the shortcomings discussed earlier, the new test shows considerable promise and has the potential to become more accurate through additional modifications, including faster sample extraction.

The Strip Test is simple to perform, and a putative commercial format could feature:

- a hand- or battery-operated grinder, such as a coffee grinder, for sample preparation
- disposable spoons to measure 1g of flour
- graduated capped tubes and an extraction solution
- dry Phadebas substrate (or equivalent), to be prepared as a suspension just prior to use
- disposable plastic pipettes or droppers
- an inexpensive battery-operated incubation chamber to maintain temperature at 37ºC (or possibly higher)
- filter paper strips with sample wells, possibly mounted on a plastic board containing troughs for application of the extraction solution to spread liberated blue dye along the strips
- photographic reproductions of intensities representing selected HFN values for reference
- a physical barrier to concentrate blue colour and improve sensitivity.

The Strip Test contains agarose mixed with the Phadebas substrate, to produce a consistent suspension of the blue starch material that can be transferred by pipette accurately. In a commercial product another reagent that is sufficiently viscous at room temperature could be used to replace the low melting point agarose. Alternatively, the un-reacted Phadebas substrate/agarose mixture could be pre-applied in a stable dry state inside the sample well of the Strip Test, and a drop of wheat extract placed upon it. The enzyme would digest the blue starch substrate inside the well, a drop of stop solution would be added, and the blue colour development visualized thereafter.

It is envisaged that sensitivity could be enhanced by the use of filter paper strips that could be thinner and narrower, and could be “whiter” than those currently used, to produce a better contrast with the faint blue coloration found with low enzyme activity. Another variation could involve exploring the use of other types of material for the strips, such as porous nitro-cellulose paper. It is believed that the Cibacron Blue dye liberated by enzyme action on the Phadebas starch substrate is negatively charged, and binds to charged groups present on the cellulose filter paper. This phenomenon could be exploited to trap and concentrate the free diffuse dye within a very small area of the cellulose filter paper and hence increase the intensity of the assay. The increased sensitivity could allow detection of low enzyme activity at lower temperatures than at present.

The Strip Test was found to be unsuitable for detecting moderate levels of *alpha*-amylase in barley samples. The most likely cause for this failure is β-glucans, which are extracted along with the amylase. The presence of β-glucans increases the viscosity of the solution, making pipetting difficult, as well as possibly interfering with enzyme action on the substrate. Some attempts were made to overcome these problems, but these proved to be unsuccessful, and were not explored any further.

In summary, a novel diagnostic prototype test has been developed for assessing *alpha*-amylase levels in wheat. The Strip Test features the release of blue dye from the Phadebas substrate and its subsequent separation and visualisation on a white strip of filter paper. This work demonstrates the principle of the Phadebas substrate in test strip form. Further refinement is required to increase both the speed and sensitivity to meet the needs of the grain industry and develop a commercial product. Discussions with potential manufacturers will attempt to address these issues.

3.5 **References**

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