

Standardisation Of Diastatic Power Method For Barley Breeding Programs

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Introduction

Since the early 1990s Australian barley breeding programs have concentrated on selecting and releasing varieties with high diastatic power (DP). These efforts were focused on regaining the lost ground of the export barley and malt markets in Asia, particularly Japan. In a forum on diastatic power at the 1993 6th Australian Barley Technical Symposium, a number of issues were highlighted including diastatic power and starch degrading enzymes, industry methods, industry processing to produce malt with desirable levels of DP and environmental effects on DP (Evans & McLeod, 1993; Fox, Inkerman *et al.*, 1993; Henry, 19993; Allan *et al.* 19993; Cole, 19993; Arends *et al.*, 1993; Lance *et al.*, 1993). At the same conference, Stuart (1993) reviewed a number of methods used within barley quality laboratories and suggested that methods for measuring starch degrading enzymes be standardised and correlated to industry methods. One of the recommendations of this forum was the need for a rapid, small-scale method for breeding programs that was correlated to the industry method (Oliver, 1994).

Following a national review of Barley Quality Evaluation Laboratories in 1995, it was recommended that the methods used by laboratories for key quality parameters should be standardised (Enright *et al.*, 1995). Further, DP would be the first of these methods to be addressed. Personnel from all Australian Barley Quality Laboratories met to identify key areas of difference in the DP assays used by these laboratories. Concurrently, a trial was designed to test a wide range of variables (Stage 1 trial). Based on the results of this trial, a set of standard parameters were identified. Thereafter, a second trial (Stage 2 trial) was conducted to assess the reproducibility of this standard method between laboratories.

Industry methods for the determination of DP use large amounts of malt and are time consuming for the application in breeding programs. Consequently, small-scale procedures have been developed for the rapid extraction, assay and determination of reducing sugars (Henry, 1984; Lance *et al.*, 1989; Inkerman *et al.*, 1993) with the Henry (1984) method being the most widely adopted to date by Industry (ASBC, 1990; 1991).

This paper presents the results of this study and discusses the implications for barley quality evaluation in Australia.

Material And Methods

Stage 1

The aim of this part of the study was to test a number of variables to establish which were most suitable for incorporation into a standard method. The variables tested included:

- malt particle size
- extractant type
- extraction time
- starch substrate

Malt Samples

Three commercially produced malt samples of Australian barley varieties were obtained, representing low (L), medium (M), and high (H) levels of DP as determined by the industry methods.

Reagents

All reagents used were analytical grade.

Sodium acetate buffer pH 4.6: 20 mM glacial acetic acid and 0.5 M sodium acetate trihydrate.

Buffered starch solution pH 4.6: A 2% soluble starch solution to which 10mL sodium acetate buffer solution was added. The pH was adjusted to 4.6. The starch solution was prepared fresh each day.

Extracting solutions: (i) 0.5% sodium chloride, (ii) 6mM ammonium hydroxide

Stopping solution: 0.5 M sodium hydroxide.

Alkaline diluent: 0.5 M trisodium citrate, 0.1 M calcium chloride and 0.5 M sodium hydroxide.

PAHBAH solution: *p*-Hydroxybenzoic acid hydrazide (PAHBAH) was dissolved at a rate of 5 g/L in alkaline diluent. PAHBAH solution was prepared each day.

Maltose Standard: 0.1%, stored at 4°C between use.

Extraction

Enzyme extraction was carried out on finely milled malt (setting 0.2 mm) as described in EBC method 4.12. Malt (1.000 g) was mix with 10 mL of extractant, dispersed by mixing, and incubated in a water bath for 30 min at 25°C. The samples were mixed at 5 min intervals during extraction and then centrifuged for 5 min at 2000g at the end of extraction.

Assay

An aliquot (5 mL) of buffered starch solution was dispensed into test tubes and pre-incubated for 5 min at 25°C. An aliquot of the enzyme extract supernatant was added to the starch solution, vortexed to mix thoroughly and incubated at 25°C for exactly 10 min. The reaction was terminated with the addition of 500 µL of stopping reagent. A substrate blank containing 100 µL distilled water and 5 mL buffered starch was included with each batch.

Measurement of reducing sugars.

The reducing sugars were measured using *p*-hydroxybenzoic acid hydrazide (PAHBAH) as a number of previous researchers have identified the reliability and precision of this method (Lever, 1972; Blakeney & Mutton, 1980; Henry, 1984; Delcour & Verschaeve, 1987; Bajomo & Young, 1990 and Buttimer & Briggs, 1998).

Following starch hydrolysis, 100 μ L of the hydrolysate (in duplicate) was added to 5 mL PAHBAH solution in a 20 mL boiling tube and heated in a boiling water bath for exactly 4 min. The contents were rapidly cooled to room temperature. The sample was diluted with 10 mL distilled water, mixed thoroughly and the absorbance read at 415 nm. The maltose standard and a PAHBAH reagent blank were included in each batch.

Statistical Analysis

For Stage 1, the tests were carried out each day for three days. The raw data (absorbances) were used in the analysis. Data were analysed using analysis of variance.

Stage 2

The aim of this part of the study was to test the between-laboratory variability in the standard method identified from the Stage 1 trial.

Malt samples

Ten commercially produced malt samples of Australian barley varieties, including two double blinds samples, were used for Stage 2. The method was carried out each day for three consecutive days.

The reagents and procedure were as for Stage 1, with the extractant being sodium chloride and the substrate being ASBC starch.

Moisture content

Malt moisture was determined in duplicate by EBC method 3.1.

Statistical Analysis

Stage 2 statistical analysis used diastatic power results Units per gram (on a dry basis). Each laboratory used a different randomisation order each day. Samples were analysed daily for three days and individual DP values were used in the analysis. Data was analysed to calculate means, standard deviation and standard error.

Results and discussion

Stage 1 Establishing variables in "new" method

The results for the first stage showed that there were up to six factor interactions (Table Ia). The factor which contributed the most variance, after the individual laboratory (Table Ib), was the starch substrate. The ASBC starch providing the most consistent results, while the Merck starch provided consistently higher blank values. The longer extraction time (30 min) produced the lowest variance which supports previous reports (Inkerman *et al.* 1993; Fry & Inkerman, 1997). In the case of extractant, there was no significant difference ($p < 0.05$). However, for convenience of conducting additional enzyme assays such, eg. sodium chloride was the preferred extractant (Fry & Inkerman, 1997). For particle size, the Miag mill (0.2mm) gave least variance. (Results for the comparison of extraction times, extractant types, starch substrates and particle sizes are not presented due to limited printing area).

Stage 2 Establishing level of error in new method

The second stage established the level of error for then new method through a validation study. A summary of statistical results for the new method is presented in Table II. The standard deviation for this method was 37 U/g for the sample range 317 - 762 U/g. This is equivalent to 8% with the mean of the samples tested at 493 U/g. The means for each sample

across laboratories are shown in Table II. Laboratories one, two and three ranked the samples in the same order. Laboratories four and five had some minor differences in the ranking of samples in the mid range. However, all laboratories ranked the highest and lowest samples in the same order.

Table 1a. Lab*treatment interactions in order of significance

Lab*sample	**
Lab*starch	**
Lab*grinder	*
Lab*time	*
Lab*extractant	*
Lab*grind*sample	*
Lab*grind*time	*
Lab*sample*time	*

Table 1b. Within Lab variance

lab	<0.0001*
starch	<0.0001*
lab*starch	<0.001*
time*grinder	<0.01*
lab*time	<0.01*

* Level of significance

Table 2. Summary of DP results from each laboratory

Sample	Laboratory					Mean	SD
	1	2	3	4	5		
1	464	459	546	507	484	492	40
2	708	695	762	710	744	723	43
3	399	387	455	405	393	408	33
4	450	440	533	497	504	485	36
5	333	317	401	339	337	346	40
6	406	400	450	394	391	408	30
7	374	365	410	361	356	373	27
8	414	405	507	456	426	441	44
9	672	654	701	673	650	670	38
10	500	493	566	525	435	524	35

The relationship between the new method and the EBC method will be evaluated in the near future. However, an equation to express the DP results from the new method in grams maltose equivalents per 30 min per 100 g of malt is available. This will allow laboratories to convert to the industry DP unit. This follows suggestions from the 1993 DP forum (Stuart, 1993; Oliver, 1994) and the GRDC review of barley quality laboratories (Enright *et al.*, 1995).

Conclusion

The results of this study suggest that the application of the new method within Australian barley quality laboratories provides reproducible data for DP. This will ensure that cultivars are ranked similarly between the different laboratories. Additionally, with an equation to convert our data to industry units, both breeders and industry can assess data in terms of industry requirements.

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