VALUE ADDED WHEAT CRC PROJECT REPORT

Identification of Variety and Quality Type by Protein Analysis: Recommendation of Methods

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SUMMARY

A range of methods of protein fractionation has been investigated to select the most promising methods for identifying variety and quality-type.

For variety identification ...

- Micro-gel electrophoresis of gliadins at pH3 suits the need for variety verification at a regional laboratory, providing rapid run time (~17 minutes), and taking advantage of the modest capital cost of the equipment and the modest requirements of operator expertise.
- Capillary electrophoresis was selected as being best, at a central laboratory, for the rapid (automatic) identification of individual samples, and for identification of large numbers of samples.
- For use out of the laboratory, the lab-on-a-chip versions of capillary electrophoresis are the most promising, but there is considerable development needed to achieve the promising ideal that is envisaged.

For quality attributes ...

- Genetic potential for dough properties are best determined as the composition of high-and low-molecular-weight subunits of glutenin (HMW-GS and LMW-GS) by capillary electrophoresis. The role of SDS-gel electrophoresis for this purpose is still valid, provided the appropriate skills are available for its performance and interpretation.
- For actual dough strength, appropriate analyses determine the proportions of very large glutenin polymers, and the ratios of glutenin-to-gliadin and/or of high-to-low-molecular-weight glutenin subunits. Methods for determining these quantities are provided, but there is a need to pursue promising leads to provide simpler methods based on these research procedures.

Need for interpretative software

- The value of the methods proposed will be enhanced if computer methods can be up-dated and improved, so as to facilitate the use of the methods in practice.

INTRODUCTION

The declaration of variety for wheat deliveries is used in many countries for defining quality type. Australia has a tradition of effective segregation of its wheat crop on the basis of grain quality, especially involving the grouping of varieties of appropriate quality type. As early as 1911, wheat growers cooperated to segregate strong Farrer wheats. The formation of the Premium Wheatgrowers' Association began a lasting effort to segregate wheat for its quality, again based particularly on varietal identity.

Identification of variety

The identification of wheat variety will gain further importance in the future with the introduction of point-of-delivery royalty payments under Plant Breeders' Rights (PBR) (Wrigley and Bekes, 2002). The growth of novel wheat varieties has further increased the requirement for varietal identification.

Varietal identification originally involved visual examination. However, this approach is subjective, relying on the experience and expertise of the inspector. Electrophoretic methods have been available since the 1960s in various formats. Chromatographic methods, and more recently capillary electrophoresis, have been used as alternatives to meet the increasing requirements for variety identification.

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Identification of quality type

In many situations, quality type is the primary requirement of identification, and knowledge about the variety is secondary to this goal. An important aim of this project has thus been to develop methods that indicate quality attributes, mainly via testing for specific proteins that can act as quality markers. These include

- Specific subunits of reduced glutenin as markers of dough properties:
    - high-molecular-weight (HMW) subunits, using the *Glu-1* scoring system of Payne (1987) and Vawser *et al.* (2002) to predict dough strength;
    - low-molecular-weight (LMW) subunits (*Glu-3* alleles) as indicators of dough properties, based on the results of Vawser *et al.* (2002).
- Specific isoforms of granule-bound starch synthase (GBSS) indicate starch properties, there being three homoalleles on chromosomes 7AS, 4AL and 7DS.
    - Of special relevance to general quality type is the 'Null-4A' gene, indicating suitability for the manufacture of white-salted (udon) noodles. Wheat varieties that are 'null' for this gene (the *Wx-B1b* allele) have advantages in their starch properties for noodle production. The presence of this gene is the *Wx-B1a* allele.
    - Waxy and semi-waxy wheats are indicated by the absence, respectively, of one or two of the remaining GBSS genes on chromosomes 7AS (*Wx-A1*) and 7DS (*Wx-D1*). Although waxy and semi-waxy wheats are not yet in commercial cultivation, when they are, it will be very important to have the capability to identify them.
- Puroindoline isoforms (puroindolines a and b), relevant to grain hardness and varietal identity.
- Rye-translocation lines, in which the short arm of the rye number 1 chromosome has been substituted for the short arm of wheat's chromosome 1B or 1A (lines 1B/1R or 1A/1R). More recently, iD/iR lines have also become available.
- Other potential targets for analysis include isoforms of polyphenol oxidase, late-maturity alpha-amylase and serpins.

Earlier recommendations to be implemented

This report describes the initial out-working of the requirements and recommendations coming from workshops involving industry representatives (Batey and Wrigley, 2001) and a Feasibility Study, required by the Management of the VAW CRC, to determine the best opportunities for protein analysis to provide effective diagnostic tests for variety and quality type. These recommendations, described by Batey and Wrigley (2002), were:-

1. The analysis of grain composition provides valuable opportunities for the identification of variety and/or quality type, because grain protein is readily extractable, and because suitable methods of extraction and fractionation can detect marker proteins that are indicative of variety and/or quality attributes.
2. Better methods of protein analysis are potentially available to suit industry needs. The methods considered most worthwhile developing are:-
    a. Capillary electrophoresis (CE) with research-level equipment to screen for quality in a breeding program;
    b. Capillary electrophoresis with research-level equipment to screen for variety in a centralised situation, and to provide a basis for simpler CE methodology;
    c. Capillary electrophoresis with simple equipment for deployment at regional centres, the laboratories of grain processors, and possibly in field situations;
    d. Micro-gel electrophoresis for varietal identification in a regional centre;
    e. Mass spectroscopy for varietal identification in a central laboratory.
3. Develop simpler methods for the routine analysis of marker proteins (existing ones, and those being identified in ongoing research) for a range of quality attributes. Capillary electrophoresis appears to be capable of providing this flexibility.

4. Manage the information about varieties and results, re-developing and utilising the programs *WhatWheat* and *PatMatch*, and assembling sets of authentic grain samples for use throughout relevant CRC projects.
5. Trials and implementation of methods developed, in collaboration with the most relevant parts of the wheat industry, especially member organisations of the VAW CRC.

Implementation of these recommendations requires the employment of two Post-Doctoral Fellows. On the basis of encouragement from the Grains Research and Development Corporation (GRDC), grant proposals were submitted in January, 2002, for three-year funding to commence in July, 2002. It now appears unlikely that these grants will be provided, so funding is now requested from the VAW CRC. If this funding provides only one Post-Doctoral Fellow, the scope of these recommendations must be re-evaluated.

The recommended methods in Paragraph 2 (above) have been pursued during the past year. This report describes those that are proving to be effective, together with Appendices detailing the experimental procedures to carry them out. The first appendix lists progress against the proposed practical steps listed in the Batey-Wrigley CRC Report of April, 2002.

'Industry-pull' research

This research is prompted primarily by 'industry pull', rather than 'science push', although the ideal situation is to have these two factors meet in a mutual balance. Market requirements for 'tighter quality specifications' have been predicted by Nicoll (2003), quoting the following statement from Gerard McMullen (AWB Ltd):

'Buyers will require a highly differentiated product based on a range of quality parameters. It means that we will need to be able to segregate grain at receival and determine quality right then and there.'

The need to segregate received grain calls for rapid and effective test systems, covering the customers' specifications. The title of the Nicoll (2003) article ('2010 – What will grain markets demand?') suggests that there are up to seven years to perform the research and development to get these quality-testing methods into practical use routinely.

DEFINING INDUSTRY NEEDS

A. Industry needs for variety identification

These needs are described in priority order below.

1. Following harvest

- There is the need for on-the-spot identification, when grain must be assessed on delivery for variety within a few minutes, with minimal facilities.
- Next comes the role of a regional laboratory, with modest equipment and modest expertise expected of the staff, where questionable samples may be sent for a prompt result to be provided, possibly over-night.
- Ultimately, identification involves a major centralised laboratory, with sophisticated equipment and trained staff, where the emphasis is on the efficient analysis of large numbers of samples. This third scenario suits the approach of having farmers' samples taken at the time of grain delivery, for subsequent analysis to verify the declaration of variety made at delivery.

These three scenarios are summarised in Table 1 with regard to the specific requirements and the suitability of the various methods to these needs. Visual examination has long been used for on-the-spot identification, but it requires great experience, and its discrimination is poor.

The use of antibody specificity holds great promise, and this is being exploited in Project 1.1.2 of the CRC. Developments with the other methods are described below.

Table 1. The relative suitability of methods for the three major situations where variety/quality identification is required. Adapted from Wrigley and Bekes (2002).

	1. On-the-spot	**2. Regional lab**	**3. Central lab**
Examples of these situations:-	Grain receival at mill or silo	Back-up to mill or silo; breeder's or seed lab; export terminal	Contract lab for large numbers of post-harvest samples
Requirements:-	Speed	Over-night results	Efficiency for large numbers
Visual examination	✓✓		
Immuno-assay kit	✓✓	✓	
ELISA immuno-assay			✓✓
Micro-gel PAGE		✓✓	✓
RP-HPLC		✓✓	✓
Capillary electrophoresis		✓✓	✓✓
Lab-on-a-chip	✓✓	✓	
Head-space analysis	✓✓	✓	
Mass spectrometry			✓✓

Traditionally, these requirements have been for the determination of quality to establish the grade for which a load qualifies. Ideally, identity is needed within minutes, before the load is tipped into the appropriate grade bin. However, the retention of growers' samples permits the identification process to proceed at a more leisurely pace, either with an overnight check or with subsequent identification at a central laboratory.

More recently, there is the additional need for identity to be established in relation to PBR royalty payments. This information can be determined subsequent to delivery, based on retained samples.

2. Breeding and pure-seed production

- The breeder needs to verify that the same genotypes are being sown and tested.
- At the time of registration, the breeder needs to know how readily a new variety can be distinguished from existing varieties.
- It is essential for the pure-seed producer to establish the identity of seed for sowing.
- Identification is needed by the grower to establish that the appropriate variety is chosen for sowing.

In these c ases, speed of identification is not essential; rather, efficiency is the requirement. Laboratory facilities needed at a breeding station might approximate those of a regional laboratory. Seed producers and growers need a laboratory to which they can send samples for identification (regional or central).

3. In trade, transport and processing

- Verification of identity may be required at various stages downstream of harvest, to ensure quality type is as expected.
- Processors' requirements for identification often relate to cases of trouble-shooting, to see if production problems are due to errors in the procurement of raw materials.

Identification at these stages is generally complicated because grain shipments are mixtures of varieties, requiring the identification of many grains individually.

B. Industry needs for quality-type identification

These needs are described in priority order below.

1. Breeding

- Determination of quality type is the main need of the breeder, preferably at an early stage of the selection process, so that progeny of poor quality can be discarded, avoiding the cost of further propagation.
- The range of quality attributes depends on the specific objectives of the breeding program, but (apart from feed-wheat breeding) dough properties would be essential. Other important attributes include grain hardness, Null-4A starch quality, rye-translocation proteins and late-maturity amylase. Soon, the other two GBSS-null proteins will require identification.

The very large numbers of lines involved in breeding make efficiency of testing to be an essential requirement. Speed of testing is not critical, but low labour costs are. Precision of determination may not be greatly important, as the aim at early generation is the elimination of much of the unsuitable material. Automatic interpretation of results is an important added feature required.

2. After harvest

- Rapid determination of quality attributes at receival would revolutionise the process of grade determination, facilitating the allocation of grain loads to appropriate bins and price categories, avoiding the need for varietal identification.

Ideally, speed of testing is needed. The range of possibilities for quality type is much less than for variety determination, but disputes are more likely because the determination of quality characteristics involves quantitative scales, and varying degrees of accuracy. Historically, disputes involving the determination of sprout damage illustrate the potential for such difficulties.

The identification of quality type is likely to become more important with the introduction of genotypes with very distinct characteristics, including waxy endosperm, extreme dough properties and genetic modification (GM). In fact, the statement of a local grain expert is notable, namely, that the mandatory introduction of test methods at harvest will be led by the need segregate for GM grain.

3. In trade, transport and processing

- Knowledge about quality type would provide great advantage to potential buyers, permitting local millers to select grain appropriate to their needs, and assisting exporters

with the logistics of transport. In addition, such knowledge would provide growers with marketing advantages.

- Processors' requirements for identification, relating to trouble-shooting, primarily involves quality type, not variety.

One such scenario is the assessment of dough strength for many grain lots by a bulk handler or trader, permitting guarantees of quality to be provided to buyers. Speed of analysis may not be required, because this scenario involves the analysis of samples from stored grain, but obviously efficiency is needed.

REVIEW OF POTENTIAL METHODS

The range of methods suited to the identification of variety and quality has been described in the preceding report by Batey and Wrigley (2002). In the light of our further development of most of them (reported here), Table 1 summarises their relative abilities to suit the constraints of the three major situations in which the identification must be conducted, and Table 2 lists the relative merits of the methods. These three scenarios in Table 1 are:-

1. on-the-spot identification, generally at delivery within a few minutes, with minimal facilities;

2. overnight testing at a regional laboratory, providing modest equipment and modest staff expertise;

3. multi-sample testing at a central laboratory, providing trained staff and good equipment.

CHOICE OF SAMPLES AND STANDARDS

For variety identification, it is essential that authentic samples are available to cover the relevant range of varieties. Ideally, these are foundation seed or original breeders' stock obtained from the Australian Winter Cereals Collection, RMB 944, Tamworth, NSW 2340. A set of 31 varieties was selected to evaluate the discriminating power of the variety-identification methods trialled in this study. They are listed in Table 3. Single-plant selections were used to avoid possible complications from discriminating between biotypes within a variety.

Grain samples must be crushed or milled before extraction. For gliadin extraction, but it is not critical that the grain should be reduced to fine particle size. For initial analyses of identity, wholemeal or flour samples are recommended, as the grinding step has the effect of 'averaging out' the composite varieties if the sample is a mixture. On the other hand, the analysis of single grains individually must be interpreted in terms of the statistical significance of how their identities relate to the composition of the whole consignment (Wrigley and Batey, 1995).

For breeding studies, it is valuable to be able to cut off the brush-end of the grain and crush it, leaving the germ end to be grown on after analysis of protein composition. In this case, crush the grain (or brush end of half grain) using pliers or with a hammer and metal plate.

Table 2. The comparative effectiveness of rapid methods of variety identification based on protein analysis. Adapted from Uthayakumaran *et al.* (2003).

FACTORS	PRE-CAST MICRO GELS	RP-HPLC	CE	Lab-on-a-chip
Time to set gel or regenerate column	12 min	7.5 min	5 min	2 min
Sample extraction	2 min	2 min	2 min	2 min
Sample run time	17 min	25 min	18 min	4 min
Protein visualisation	70 min	Instant	Instant	Instant
Data interpretation	Side by side visual comparison	Automated	Automated	Automated
Through-put in 24 hours	10/gel*	30	72	400
Health risk to operators	Low	Low-medium**	Low	Low
Costs – equipment	Low	High	High	Medium
Costs – consumables	Medium	Medium	Medium	Medium
Costs – labour	Moderate	Low	Low	Low
Distinguishing ability***	85%	All	All	Not yet determined

* 100samples with a 2-gel unit in an 8-hour day
** Limited health and environmental risks from solvents used.
*** The proportion of combinations of 30 current Australian varieties that could be distinguished.

Table 3. Variety samples used in this study. SP-1 denotes grain samples obtained from single-plant selections (SARDI).

No	Variety	No	Variety	No	Variety
1	Anlace SP-1	11	DM5367*B8	21	Snipe SP-1
2	Arrino SP-1	12	Dollarbird SP-1	22	Sunbri SP-1
3	Blade SP-1	13	Frame SP-1	23	Sunsoft SP-2
4	Bowie SP-1	14	H45 SP-1	24	Sunvale SP-1
5	Brookton SP-1	15	Harrier SP-1	25	Thornbill SP-1
6	Cadoux SP-1	16	Kirchauff SP-1	26	Thriller SP-1
7	Cook SP-1	17	Kukri SP-1	27	Wylah SP-1
8	Corrigin SP-1	18	Lorikeet SP-1	28	Wyuna SP-1
9	Datatine SP-1	19	Perenjori SP-1	29	Qual Bis
10	Diamondbird SP-1	20	Rosella SP-1	30	Qual Club
				31	Qual 2000

A. Methods for variety identification
IMPROVING ON TRADITIONAL APPROACHES
1. Gel electrophoresis
For many years, identification of wheat varieties has involved acidic polyacrylamide gel electrophoresis (A-PAGE) to determine the composition of the readily extractable gliadin fraction. Under the acidic conditions of pH-3 electrophoresis, the gliadins move cathodically (to the negative electrode). In Australia, this method has become a standard/routine procedure approved in 1988 as Method No 08-01, one of the Official Methods of the Cereal Chemistry Division of the Royal Australian Chemical Institute (RACI) (Black, 2003). It involves the use of pre-cast polyacrylamide gels.

An alternative is SDS-PAGE of fully reduced flour extracts, providing patterns of glutenin subunits as well as gliadins and water-soluble proteins (Method 08-03 in Black, 2003). This method is more complex to perform than A-PAGE. Furthermore, the resulting patterns are very complex, so that specific identification of varieties is difficult, apart from the simpler pattern of HMW-glutenin subunits at the top of the pattern. The value of the glutenin subunit patterns relates mainly to the evaluation of quality type, as described below, but the HMW-subunit patterns are of limited discriminating value for variety identification.

Advantages of traditional A-PAGE
- Standard methods are established for both A-PAGE and SDS-PAGE.
- Both provide good distinction between varieties.
- Patterns are specific for genotype, with negligible interference from growth conditions.
- The equipment is relatively inexpensive.
- Many samples may be analysed in parallel, providing a good throughput of analyses.
- The use of pre-cast gels overcomes the safety problem of handling the neurotoxic acrylamide monomer.

Disadvantages of traditional A-PAGE
- Established PAGE methods are relatively slow, usually requiring a few hours for the electrophoresis step, plus overnight for the visualisation of protein zones.
- Even using pre-cast gels to avoid the time and labour of gel casting, the method is labour intensive.
- Interpretation of results generally involves subjective visual comparison of patterns.
- Automatic interpretation is only possible after the difficulty of scanning or image analysis of the gels with expensive equipment. As a result, many laboratories have adopted visual examination as the standard procedure.

Consequently ...
The challenge has been to capitalise on these advantages, and to overcome the disadvantages of the method being slow and lacking automatic interpretation. This has involved the use of very short gels (about 25 mm length) and scanning with a desktop scanner.

The pre-cast micro gel permits rapid electrophoretic analysis of gliadin composition. The capabilities of these gels were reported by Wrigley *et al.*, (1991), but it is only recently that they have become commercially available from Gradipore (Sydney, at www.gradipore.com). Gliadin bands are not resolved so well in the micro-gels as in larger gels, but the patterns are sufficient to see similarities or differences between sample patterns, and thus to verify identity, checking side-by-side with patterns for authentic samples (Figure 1). According to the

procedure in Appendix 2, the electrophoresis step takes only 17 minutes. With sample extraction and gel staining, the overall analysis time is less than 120 minutes.

A computer program has been written to permit the rapid scanning of these or other gels on a desktop scanner for automatic interpretation. Nevertheless, at this stage of these developments, we prefer to indicate that this method should be restricted to verification of identity, involving the side-by-side comparison of patterns visually.

The micro-gel method was applied to the set of 31 Australian varieties listed in Table 3. Most (85%) of the many combinations of these varieties could be distinguished (Table 2). Complete details of which distinctions can be made are provided by Tonkin *et al.* (2002).

The value of micro-gel A-PAGE for routine analysis

The micro-gel A-PAGE system is suited to providing a rapid answer for verification of varietal identity at a regional centre, taking advantage of the modest capital cost of the equipment and the modest requirements of operator expertise. In addition, it may have a role at a central laboratory where the short run time takes good use of the equipment.

Figure 1. Precast micro-gel patterns of gliadin composition for ten Australian varieties.

2. Reversed-phase high-performance liquid chromatography (RP-HPLC)

RP-HPLC has also been available for some time as a routine method to determine varietal identity, based on gliadin composition. RP-HPLC fractionates proteins largely on the basis of differences in hydrophobicity; this separation principle is distinct from that of gel electrophoresis (charge and size), so RP-HPLC is thus likely to provide distinctions between varieties that give similar electrophoretic patterns.

RP-HPLC has not achieved the popularity of gel electrophoresis for routine analysis, but there is a standard method, approved in 1995 as Official Method No 08-02 of the RACI Cereal Chemistry Division (Black, 2003).

Advantages of traditional RP-HPLC

- Automatic loading of samples overnight provides the analysis of many samples.
- Modest speed of analysis for gliadin proteins – about 25 minutes per sample for satisfactory distinction.
- Modest speed of regeneration between samples. 7.5 min.
- Instant visualisation of results as a profile of peaks.
- Automatic interpretation of results.
- Standard method available.

Disadvantages of traditional RP-HPLC

- Moderately high capital cost, and significant operating costs.
- Rather slow analysis, limiting sample throughput.
- Some concern about Occupational Health and Safety, due to the hazard and environmental problem of the solvents required.

- Only one sample can be analysed at a time; this restricts the throughput of the method.

Consequently ...
The advantages above were considered valuable, provided that some of the disadvantages could be improved. This is not possible for the use of solvents, nor for one-at-a-time analysis, but it did prove possible to achieve modest reductions in the speed of analysis (to about 25 minutes per sample) and regeneration (to 7.5 minutes), without compromising distinguishing ability. The modified procedure is detailed in Appendix 3, matched to the Beckman Coulter Gold System. Examples of the resulting profiles are provided in Figure 2.

The procedure (Appendix 3) provided good distinction between Australian varieties, permitting discrimination among all of the 31 Australian varieties listed in Table 3. Using the advantage of automatic loading, analysis of about 30 samples should be possible in a 24-hour day (Table 2).

The value of RP-HPLC for routine analysis
The RP-HPLC procedure appears to be suited to variety analysis at a central laboratory, taking advantage of automatic loading and interpretation of results.

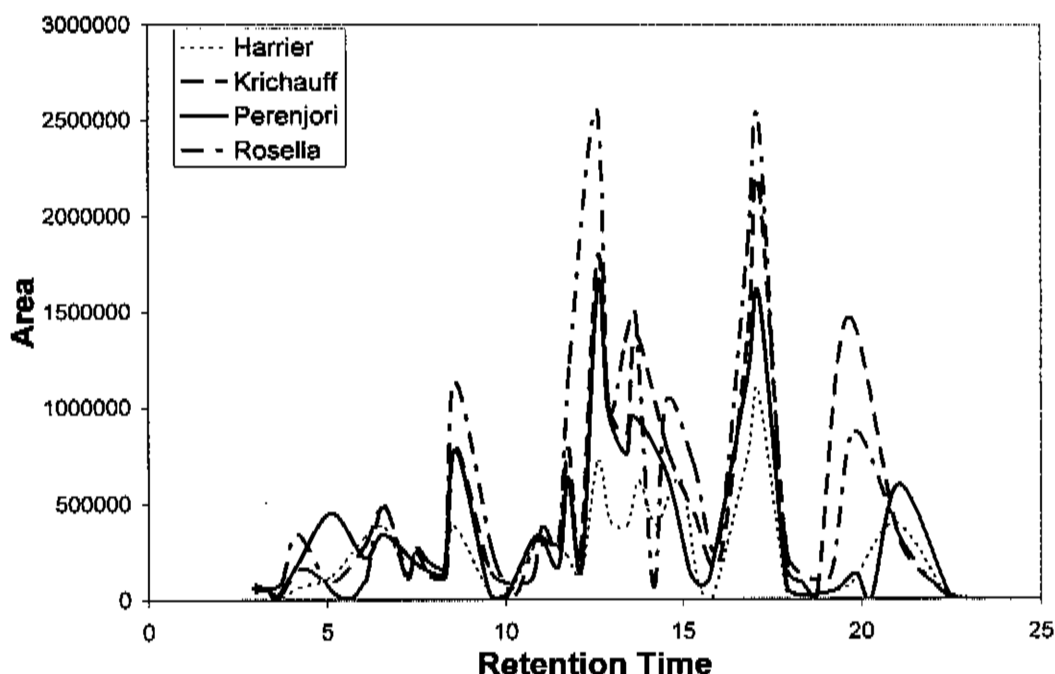


Figure 2. RP-HPLC profiles for Australian wheat varieties.

NEWER APPROACHES

3. Capillary electrophoresis (CE) in the Beckman MDQ equipment

The first research publications on CE analysis of wheat proteins appeared in the mid-1990s (e.g., Lookhart and Bean, 1995; Bean and Lookhart, 2000), so CE is not really a new method, but it does not have the established status of PAGE or RP-HPLC. The first Australian use of CE involved the BioRad BioFocus 3000 equipment, which did not prove to be reliable. Nevertheless, it provided a good indication of the potential of CE to distinguish Australian varieties (Siriamornpun *et al.*, 2001). These studies provided a CE method for gliadin composition that could distinguish between most of a modest set of 13 Australian wheat

varieties, and that the resulting profile shapes are largely independent of variations in the protein content of the grain samples (Batey *et al.*, 2002).

The installation of new CE equipment (Beckman Coulter P/ACE™ MDQ Series) at Food Science Australia in October 2002 provided the first opportunity of testing the Beckman-CE method for wheat proteins in Australia.

Advantages of capillary electrophoresis with the Beckman MDQ equipment

- Published methods available for wheat proteins.
- Reasonably fast analysis times for gliadin proteins.
- Automatic loading, permitting 24-hour operation.
- Instant visualisation of results as a profile of peaks.
- Automatic data capture, providing the potential for automatic interpretation.
- No significant safety or environmental problems.
- Low operating costs.
- Environmentally 'friendly' reagents.
- One capillary can be used many times (about 75), and the cost of the capillaries for the method developed is small (about $375.00 for a 5 metre length of capillary).
- The promise of very small, portable equipment offers potential to extend the use of this methodology.

Disadvantages of capillary electrophoresis with the Beckman MDQ equipment

- High capital cost. The MDQ unit at Food Science Australia, North Ryde, cost about Aust$110,000.
- Only one sample is analysed at a time; this restricts the throughput of the method.

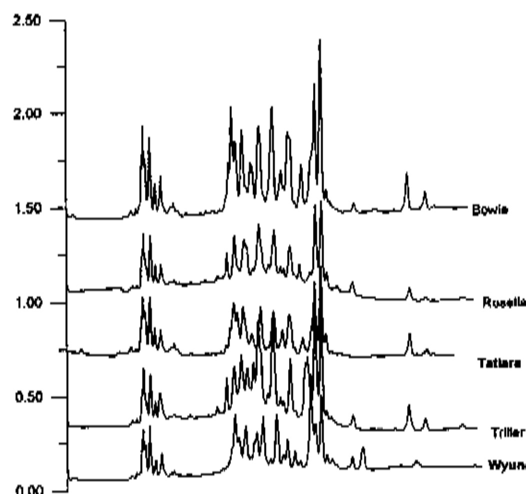


Figure 3. Capillary electrophoresis profiles for gliadin proteins from Australian wheat varieties.

Consequently ...

The published methods were examined and modest improvements were made to suit the likely needs of the Australian wheat industry, resulting in the procedure detailed in Appendix 4. Rapid analysis was achieved, involving a run time of less than 18 minutes; with the added time for sample extraction, overall analysis time is less than 30 minutes.

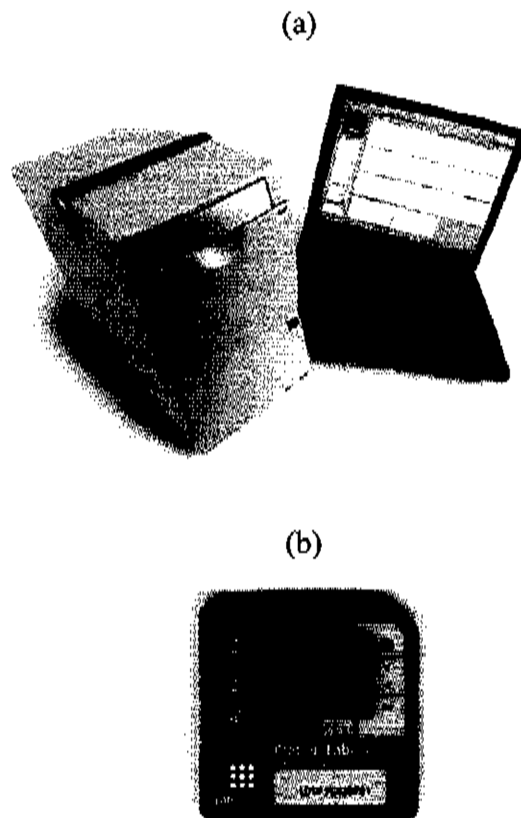
The resulting profiles for the gliadin composition of several varieties are shown in Figure 3. The procedure has provided good distinction between Australian varieties, permitting discrimination of all 31 test varieties (Table 3). Using the advantage of automatic loading, analysis of about 72 samples should be possible in a 24-hour day (Table 2). The integral data capture of CE lends itself to automatic interpretation of results by matching the CE profile of an unknown sample against a library of profiles for authentic variety samples, such as with the *PatMatch* program (Gore *et al.*, 1990).

This assessment of CE is based on the analysis of gliadin composition. Other classes of grain proteins can be used effectively for variety identification. For example, Siriamporpun *et al.* (2002) showed that CE analysis of proteins of bran or germ may be more effective in providing distinction between varieties than extracts of endosperm proteins. In addition, a procedure has been devised for the analysis of serpin composition, as another class of endosperm proteins that are known to provide polymorphism in Australian wheats (Skylas, personal communication).

The value of capillary electrophoresis for routine analysis

The Beckman CE equipment and the procedure of Appendix 4 are suited to variety analysis at a central laboratory, taking advantage of automatic loading and interpretation of results. Its speed of analysis also suits it to rapid identification of single samples when the need is urgent.

(a)



(b)

Figure 4. (a) The Agilent lab-on-a-chip instrument, with the lap-top computer required for its operation. (b) Lab chip used for separation of proteins (size 5 cm X 5 cm).

4. Lab-on-a-chip

Recent developments with capillary electrophoresis promise to overcome the two disadvantages listed above for the Beckman CE system, namely, its high capital cost and the

restriction that only one sample can be analysed at a time. This new development has been termed the 'lab-on-a-chip' concept. We are collaborating with one research program on this concept at the Chemistry Department of Kansas State University. A version of the 'lab-on-a-chip' concept (already commercially available) is the Agilent 2100 Bioanalyzer; this 'chip' and its analysis equipment were investigated for its suitability to the needs of the Australian wheat industry.

The instrument is based on the principles of capillary electrophoresis, and it comprises a power supply and detector in a small box (Figure 4), into which a 5-cm-square plastic 'chip' is placed. This chip contains a series of capillaries through which up to 16 samples may be run. It is usual to include standards with unknown samples, so the practicable number of test samples is less than 16. It was hoped that this instrument would provide distinctions between varieties, in a much faster time and for lower cost than the larger laboratory-based capillary electrophoresis units.

A demonstration of the instrument was arranged, and a number of different varieties were tested. The instrument, in the configuration demonstrated, separated proteins on the basis of molecular weight. Thus, a set of proteins of different molecular weight was also prepared and run on the instrument. The profile of this sample is shown in Figure 5.

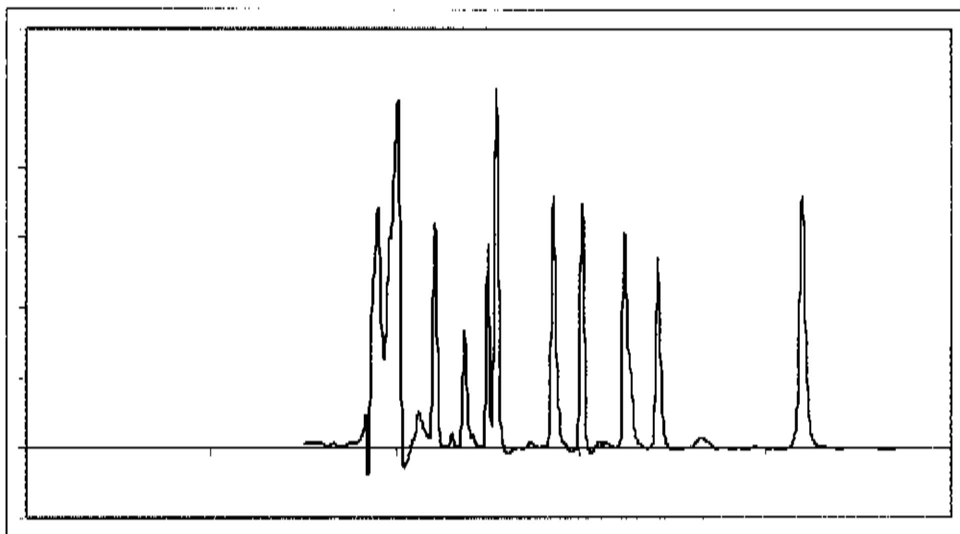


Figure 5. Fractionation of non-wheat standard proteins of differing molecular weight on the 'lab-on-a-chip' of the Agilent 2100 Bioanalyzer.

The instrument showed some distinctions between samples of several varieties. Figure 6 shows a comparison of gliadins extracted from flour of the varieties Grebe and Wyuna separated on the Agilent instrument. Distinctions are not as obvious as on charged-based separations normally used for gliadin electrophoresis on gels and CE. However, the separation on the basis of molecular weight shown in Figure 5 indicates its potential for distinguishing high-molecular-weight glutenin subunits in this configuration. An offer by the instrument manufacturer for an extended trial in our laboratory has been made, and this will give the opportunity for further investigating its potential for variety identification and quality characterisation.

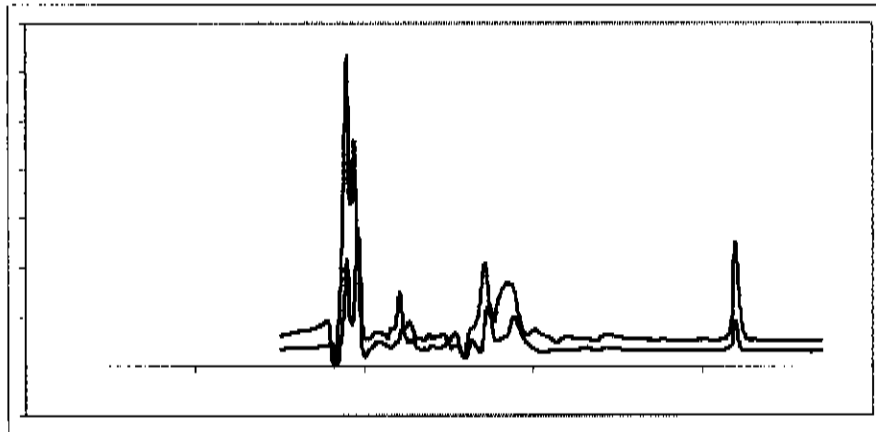


Figure 6. Fractionation of gliadin from the varieties Wyuna (lower trace) and Grebe (upper trace).

Advantages of capillary electrophoresis with the Agilent system

- Rapid analysis.
- Multiple samples analysed simultaneously.
- Portable equipment.
- No need for laboratory backup.
- Modest cost of equipment.

Disadvantages of capillary electrophoresis with the Agilent system

- Lower expectations of distinguishing ability.
- Modest cost operating costs per sample if the full set of samples are used per chip.
- Not yet fully evaluated.

The value of lab-in-a-chip CE systems for routine analysis

There appears to be excellent potential of the lab-in-a-chip system for variety analysis beyond and within the laboratory, but further study is needed to establish this potential. We are pursuing a few international collaborations to further study this potential.

5. Portable Gas Chromatograph

A promising approach to variety identification was pursued as part of a GRDC project involving Food Science Australia (Werribee) and collaborators. The project involved seeking applications in grain quality for equipment for aroma sensing. Initially, promising results were obtained as discussed at a Workshop entitled, 'Aroma Sensing Technology for Early Detection of Grain Quality' (see Wrigley, 2002). The preferred equipment was a portable gas chromatograph (GC) (the 'Z-nose'), used to analyse the composition of the head space above grain samples. This technique showed significant differences between two non-aromatic rice varieties (Figure 7). It had also been applied to a pair of near isogenic lines of wheat differing in hardness (hard and soft Falcon). These wheats also showed differences with the Z-nose equipment. This work was carried out at Food Science Australia (FSA) in Werribee.

The instrument appeared especially suitable for deployment in a grain-receival centre, as it is portable, giving a chromatogram in seconds (Table 1). Its cost is less than the NIR instruments currently being used for measurement of protein content at most Australian silos, and does not require laboratory facilities for its operation. Accordingly, its potential use was investigated further in collaboration with Brian Young, FSA, Werribee.

A larger set of wheat samples of individual varieties was provided for Z-nose testing. This set included eight different varieties plus a second sample of two of these varieties from a different growth environment. These results did not live up to the promise of the initial results. While there were obvious differences between some samples, the differences between samples of the same variety were as great or even greater than some of the differences between varieties.

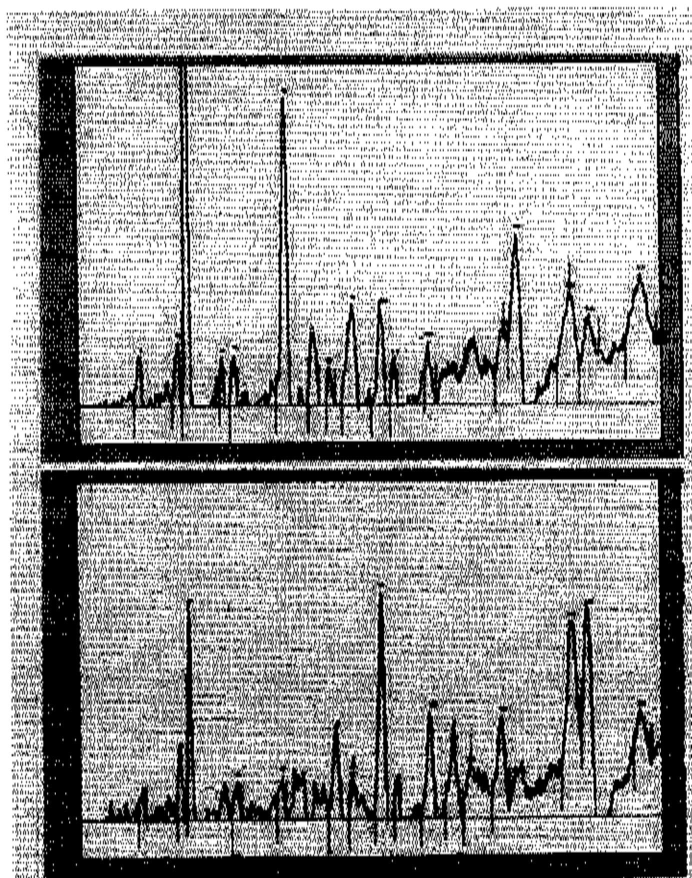


Figure 7. Comparison of GC trace for two rice varieties

Advantages of the portable gas chromatograph

- Very rapid analysis.
- Portable equipment.
- No need for laboratory backup.
- Modest cost of equipment.
- Low operating costs.

Disadvantages of the portable gas chromatograph

- Interference from non-variety factors.
- Poor distinguishing ability.

The value of the portable gas chromatograph system for routine analysis

For variety identification, this instrument could not provide the distinction necessary for reliable identification, based on these studies. However, its use in receival centres could still be appropriate for other purposes. For example, identifying grain samples with various

diseases, such as black point, or the detection of unacceptable pesticide application may be possible. While these applications are important, they are outside the present scope of Project 1.1.1 and they have not been pursued further.

6. Mass Spectrometry

Mass spectrometry (MS) is another method that has been reported to provide efficient varietal identification, based on grain-protein composition. Although MS equipment is expensive, it can provide very accurate analyses of the molecular mass of molecules, even extending in size to include proteins of modest size. Recently, MS of gliadin composition has been used to distinguish among all of 16 Canadian wheat varieties, representing five classes of quality type, and including multiple samples of each variety, grown at different sites (Ens *et al.*, 2000). These studies, conducted in the Department of Physics at the University of Manitoba, used MALDI TOF laser equipment, with three channels, in the range 30 to 38 kiloDaltons. Growth environment had little impact on the spectra. Analysis took only two minutes per sample manually, or 15 seconds per sample if automated. The method requires only 10^{-16} mole of sample. This approach to variety identification may suit a central laboratory where large numbers of samples would justify the high costs of the equipment. MS has been applied successfully to the identification of cereal varieties at Murdoch University, Western Australia.

However, disappointing results were obtained when the method of Ens *et al.* (2000) was applied to gliadin preparations extracted from the flour of some Australian varieties using equipment available at APAF. It is planned to continue with this approach using an alternative matrix for the proteins.

Advantages of mass spectrometry

- Rapid analysis.
- Good distinction between Canadian varieties reported.

Disadvantages of portable gas chromatograph

- High cost of equipment.
- Distinguishing ability for Australian varieties could not be demonstrated in initial trials.
- Several steps required to prepare samples for analysis.

The value of mass spectrometry for routine analysis

These initial investigations may not have indicated the potential of MS for routine analysis, and the literature claims were not substantiated. At this stage, MS does not appear to be worth pursuing, but this approach is claimed to have proved worthwhile in Western Australia.

B. Quality-type identification: Methods available

1. Dough properties (genetic potential)

There is extensive evidence of the value of glutenin-subunit composition to predict genetic potential for dough properties. There are two distinct groups of glutenin subunits, those of high- and of low-molecular weight (HMW and LMW). They are coded by genes at the *Glu-1* and *Glu-3* loci, respectively. The HMW subunits are especially important in this respect, as indicated by the *Glu-1* scoring system of Payne (1987), backed up by many publications about Australian varieties (e.g., recently Cornish *et al.*, 2001 and Vawser *et al.*, 2002). LMW-subunit composition complements that of the HMW subunits (Vawser *et al.*, 2002).

a. SDS gel electrophoresis

For many years, SDS-PAGE (RACI Method 08-03 in Black, 2003) has been used almost exclusively to indicate glutenin-subunit composition. Whilst the HMW subunits appear at the top of the SDS-PAGE pattern, well separated from the other polypeptides, the analysis of the LMW subunits requires pre-extraction of non-glutenin proteins prior to gel electrophoresis.

Advantages of SDS-PAGE to identify glutenin subunits

- Long adoption as the method of choice.
- Published and standard methods are available.
- Modest cost of equipment and low operating costs.

Disadvantages of SDS-PAGE to identify glutenin subunits

- Patterns are difficult to interpret for the allocation of subunit numbers or allele designations.
- The traditional SDS-PAGE method is slow and labour intensive.
- OH&S concerns about use of the neurotoxic acrylamide monomer, unless pre-cast gets are used.

Consequently ...

It is apparent that the micro-gel system would be inadequate for determining subunit composition, so alternative methods were sought. Therefore, the standard SDS-PAGE method must remain as the primary reference method for identifying glutenin subunit composition. A procedure for doing so is provided in Appendix 5.

Knowledge about the glutenin alleles of parent and progeny lines has become essential information for breeders. However, the practical disadvantages of SDS-PAGE make it difficult to deploy for analysis of the large numbers of lines that must be characterised in a breeding program. Alternative methods must therefore be found that would provide automatic interpretation and lower levels of labour and cost. The most promising methods available are RP-HPLC and CE.

b. Reversed Phase-HPLC

An established RP-HPLC method is available for characterising glutenin subunits. The procedure in Appendix 6 is based on that of Marchylo *et al.* (1989). The respective advantages and disadvantages of RP-HPLC are set out above for gliadin fractionation. These considerations also apply to the RP-HPLC procedure for analysing glutenin subunits.

Further advantages of RP-HPLC for identifying glutenin subunits

- Established method available.
- Both HMW and LMW subunits are shown in the same profile from the same extract.
- Integral data capture will permit automatic interpretation of results.
- Modest speed of analysis (72 min.).

Further disadvantages of RP-HPLC for identifying glutenin subunits

- Complex extraction procedure, requiring significant labour input.
- Cannot distinguish between HMW-GS 5 and 2 (an important combination).

c. Capillary electrophoresis in the Beckman MDQ equipment

Appendix 7 details charge-based CE procedures for the characterisation of HMW (Figure 8) and LMW glutenin subunits using the Beckman MDQ equipment. These methods are based on reports of Bean and Lookhart (2003) and Yan *et al.* (2003). It has proved possible to allocate peaks with specific mobilities to individual HMW subunits, using varieties of known HMW-subunit composition and also with multiple-deletion lines. However, it is more difficult to allocate allele designations for the LMW subunits (Figure 9).

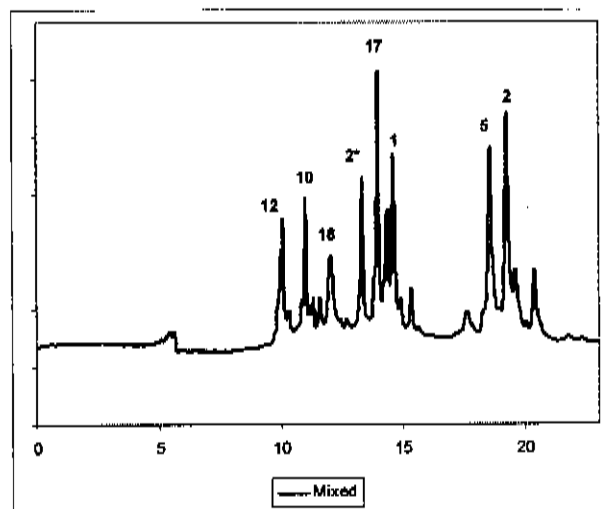


Figure 8. Charge-based capillary electrophoresis profiles of all HMW-GS from a mixture of Cadoux (2*, 17+18, 2=12) and Diamondbird (1, 17+18, 5+10) showing the separation of 5+10 and 2+12

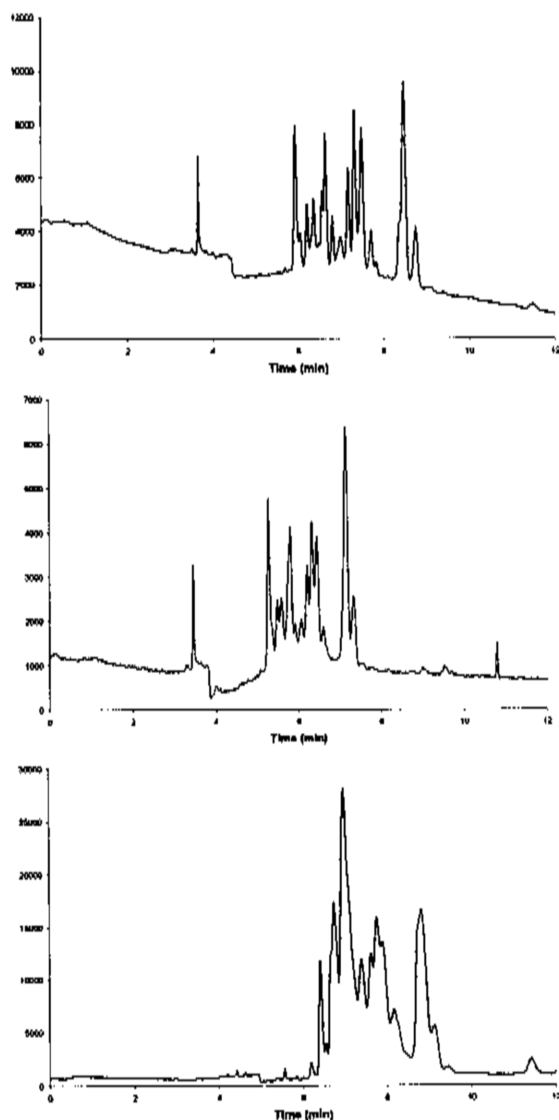


Figure 9. Charge-based CE profiles of HMW-GS (top), LMW-GS (middle) and total extract (bottom) from Frame.

d. Size-based capillary electrophoresis in the Beckman MDQ equipment

Appendix 8 details size-based CE procedures for the characterisation of total glutenin subunits (Figure 10) using the Beckman MDQ equipment. This method is based on the report of Bean and Lookhart (2003). Both HMW-GS and LMW-GS can be analysed with one injection, but in its present form, the analysis takes a long time (about 45 min) and not as many LMW-GS are distinguished as in the charge-based separation. This procedure might be improved to overcome these problems, but we are waiting for the specialised running buffer reagent to be supplied by Dr Bean.

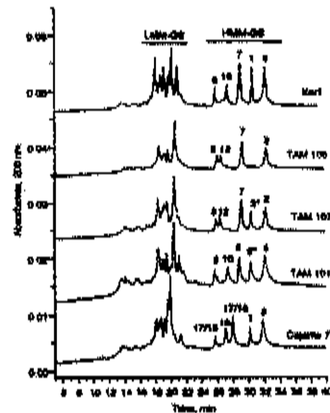


Figure 10. Size-based separation of total glutenin extract (Bean and Lookhart, 2003).

The respective advantages and disadvantages of the Beckman CE system are set out above for gliadin fractionation. These considerations also apply to the CE procedure for analysing glutenin subunits.

Further advantages of CE for identifying glutenin subunits

- Relatively rapid analysis.
- Distinction between HMW-GS 5 and 2 is readily done (unlike RP-HPLC).
- Integral data capture will permit automatic interpretation of results.

Further disadvantages of CE for identifying glutenin subunits

- High capital cost.
- Separate extraction and fractionation procedures for HMW- and LMW-subunits.

The value of the CE for routine analysis of glutenin-subunit composition

This approach shows definite promise, and it will be pursued, to ensure that procedures are reproducible, to provide peak identification and to arrange programming for automatic interpretation.

e. Capillary electrophoresis – lab-on-a-chip

The ability of the Agilent 2100 bioanalyzer to fractionate proteins on the basis of molecular size (as shown in Figure 5) offers a promising approach to the characterisation of glutenin subunits. This potential is clearly worth pursuing, via further research with the Agilent version of this principle and via the collaboration with Kansas State University.

2. Dough properties (actual) and gluten-protein composition

Characterisation of glutenin-subunit composition has already proven valuable in breeding to predict the genetic potential of lines for appropriate dough properties. However, there is the additional important need to know what are the actual dough properties of a wheat sample, given the particular environmental contributions of growth and storage conditions, as these have already modified the genetic potential of the harvested sample.

The breeder particularly needs this information at the stage of evaluating advanced lines that have been grown out at relevant sites. Prediction of actual processing quality is also needed by

the grower, the buyer, the exporter and the miller to facilitate the processes of trade, transport, blending and milling.

The basis of this information goes beyond subunit composition to the extent of polymerisation of the subunits, and the consequent content of very large glutenin polymers and their proportion in relation to the gliadin fraction. Aspects of composition that are relevant for this aspect of quality thus relate to the size distribution of the gluten fraction. Procedures for determining this information range from the classical Osborne solvent fractionation, through determining the proportions of gliadin and glutenin, to recent methods such as field-flow fractionation, which characterises extracted protein according to size distribution with no upper limit to the distribution span.

Of these many methods, only a few have the potential to provide worthwhile predictive information, whilst also being sufficiently simple as to consider for the routine analysis of large numbers of samples. The situations where this type of analysis is realistically required is in the categories of regional or central laboratories (as defined in Table 1), but it would also be extremely valuable if this aspect of quality could be determined at grain receival. The following three methods have been selected for further research as being those most worthy of consideration for determining actual dough strength. These are the ratio of to glutenin to gliadin, the determining the percentage of 'unextractable polymeric protein' (%UPP) by size-exclusion HPLC (SE-HPLC), SDS sedimentation, the ratio of high- to low-molecular-weight subunits of glutenin, and an extraction system combined with Dumas method for total nitrogen analysis (using Leco equipment).

a. SE-HPLC to determine the ratio of glutenin to gliadin

HPLC columns have long been available for size-exclusion chromatography, thereby to characterise protein mixtures on the basis of size distribution. These have been adapted to characterising extracted flour proteins. The most basic of the indications of size distribution for the gluten proteins is the ratio of glutenin (polymeric proteins) to gliadin (monomeric proteins). The extraction and SE-HPLC procedure is described in Appendix 10.

b. SE-HPLC for % UPP

In adapted SE-HPLC to the characterisation of extracted flour proteins, the extraction step has been critical to this characterisation. Ultrasonic treatment has been used for this purpose, on the basis that it is the least extractable very large glutenin material that is most indicative of dough strength.

Accordingly, the determination of percent 'unextractable polymeric protein' (%UPP) has involved two sequential extractions of flour proteins into SDS solution, the first involving modest mixing of the suspension, and the second extraction of the resulting residue involving sonication to force the largest glutenin polymers into suspension. Both supernatants are then characterised according to size, using SE-HPLC (Figure 11), and the proportion of the largest protein peak in the second extract is calculated in relation to the total amount of glutenin. This is explained further in. This procedure is detailed in Appendix 10, based on the method of Batey *et al.* (1991).

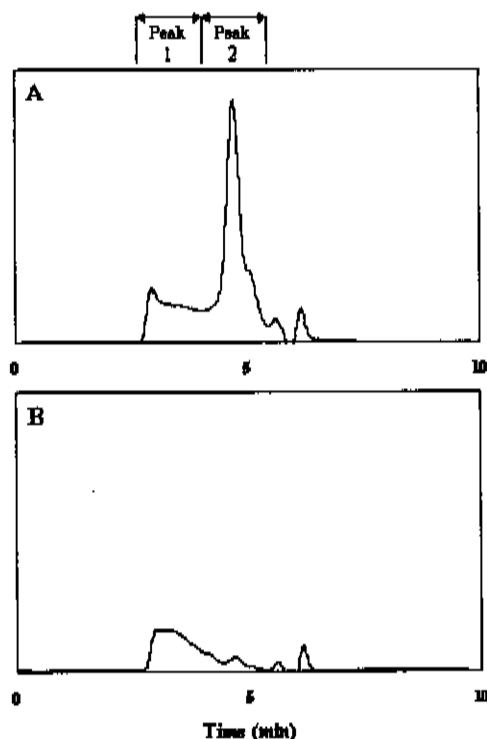


Figure 11. SE-HPLC profiles for both steps, (A) unsonicated and (B) sonicated, of extraction for %UPP determination.

Advantages of SE-HPLC to determine %UPP

- Established method.
- Established correlations to dough strength.

Disadvantages of SE-HPLC to determine %UPP

- Long and tedious method.
- Modestly expensive for equipment and operating costs.
- Operator experience needed to get reliable results.
- Unsuitable for routine analysis of large numbers of samples in its present form.

The value of the %UPP method to predict dough strength

The determination of %UPP is too complex for routine analysis of large numbers of samples, but it may serve as a reasonable 'bench-mark', with which to compare other methods. Furthermore, the principle could serve as a basis for developing a simpler measure of the proportion of the gluten protein of large molecular weight.

A related measure of actual dough properties is the ratio of glutenin to gliadin. This is actually also a measure of size distribution, especially if it is determined by SE-HPLC. It is also worthy of consideration as the basis of a simpler test procedure.

c. SDS sedimentation

One of the oldest methods to predict baking quality is based on the extent of swelling of flour in suspension. Various reagents have been used. Most recently an SDS solution has been used for this purpose. The method involves shaking a sample of finely ground wheatmeal in this solution and allowing the flour to swell and settle. The volume of the settled residue is read in

a volumetric cylinder as the 'sedimentation volume'. This value may be adjusted for the protein content of the sample, thereby to correct for this important factor.

The SDS sedimentation method has been used successfully by Goodman Fielder Mills for some years to select wheat consignments that are suited to their requirements for milling and baking. Several variations of the procedure for sedimentation tests are provided as AACC Standard Methods (AACC, 2000):

- Method 56-61A, using 3.2 g wheatmeal, mixed with 50 mL water, then with 25 mL lactic-propanol solution.
- Method 56-60, using 3.2 g flour, but otherwise the same as for Method 56-61A.
- Method 56-62, 'Modified', using 3.2 g wheatmeal, mixed with 100 mL solution (0.33M lactic acid – 13% iso-propanol).
- Method 56-63, 'Micro' method, using 0.32 g wheatmeal, mixed with 10 mL lactic-propanol solution.
- Method 56-70, suited to durum wheats, using 6.3 g wheatmeal, mixed initially with 50 mL water, and later 50 mL SDS-lactic solution is added (SDS is 3% and lactic acid is 0.024M).

The last-mentioned method (AACC #56-70) was adapted for use in Goodman Fielder Mills (collaboration with Dr N.Hehir), and subsequently in Allied Mills, Summer Hill. The procedure is described in Appendix 11 for wholemeal.

Advantages of the SDS sedimentation method

- Simple.
- Minimal equipment needed (grinder and volumetric cylinders).
- Modest speed, satisfactory for a laboratory method.
- Minimal operator skills required.
- Practical value established by Goodman-Fielder experience.

Disadvantages of the SDS sedimentation method

- Biochemical basis of the test is only partly understood.
- Problems with repeatability were experienced in Goodman-Fielder.
- Labour-intensive.

d. Ratio of HMW-GS to LMW-GS

Another potential indicator of actual dough properties is the proportion of the glutenin subunits in the respective classes of high- and low-molecular-weight groups. This proportion is generally determined by RP-HPLC after fully reducing disulfide bonds. This measure can be determined from the profiles provided by the method for identifying the individual HMW and LMW subunits (Appendix 6). The area under the respective curves for the HMW and LMW subunits are used to calculate the ratio.

e. Fractional extraction and Leco analysis

In an attempt to devise a simplified method to estimate the proportion of very large glutenin in a wheatmeal or flour sample, an extraction procedure has been developed and trialled, using an SDS reagent for extracting all protein except the large glutenin plus Leco analysis to determine the remaining protein content. The results gave mixed promise, depending on the particular set of samples to which the method was applied. It thus has promise, but is at present unsatisfactory for general use.

3. Starch properties

The ratio of amylose to amylopectin affects the processing quality of wheat starch, especially for noodle production. Of special relevance in this respect is one of the isoforms of granule-bound starch synthase (GBSS). The absence of this isoform corresponds to the 'Null-4A' gene, which is indicative of superior noodle wheats. The absence of one or both of the remaining GBSS isoforms indicates partial double waxy wheat or full waxy wheat, respectively.

The original identification of these isoforms of GBSS involved isolation of starch granules, followed by protein extraction from them and SDS-PAGE. This procedure is too tedious for routine analysis. Therefore we are attempting to devise a simple extraction method for wheatmeal or flour, followed by capillary electrophoresis of the extract to identify wheats according to GBSS status. This procedure would extend the value of the Beckman MDQ equip,ment for use in breeding. Hopefully, the method could be extended for use with the Lab-on-a-chip equipment that is also under development.

4. Rye translocations

Most of the methods mentioned above have been used to identify the presence of rye secalins that would be indicative of rye translocation lines, either 1B/1R or 1A/1R types. In particular, such a method has been devised for the Beckman CE equipment (Lookhart *et al.*, 1996). This important factor in the screening of breeding lines could thus be included in the application of CE in a breeding program. Nevertheless, this may not be needed because of the efficient immunoassay that is provided to breeding programs by the Wheat CRC.

RECOMMENDATIONS

Of the various methods investigated, the following are recommended for further research to suit the respective roles of identifying variety and quality-type.

For variety identification

- Micro-gel electrophoresis of gliadins at pH3 suits the need for variety verification at a regional laboratory, providing rapid run time at modest capital cost and modest operator skill, combined with good distinction ability for verification of identity.
- Capillary electrophoresis was selected as being best, at a central laboratory, for the rapid (automatic) identification of individual samples, and for identification of large numbers of samples, taking advantage of good distinguishing ability and potential for automatic interpretation of results.
- For use out of the laboratory, the lab-on-a-chip versions of capillary electrophoresis are the most promising, but there is considerable development needed to achieve the ideal that is envisaged.

For dough-quality attributes (genetic potential)

- Genetic potential for dough properties are best determined as the composition of high-and low-molecular-weight subunits of glutenin by capillary electrophoresis. Further work is needed to enable the allocation of alleles to peaks in the profile of LMW subunits.
- The ability of the Agilent Lab-on-a-chip system to provide size-based separation for standard proteins suggests that it may be suited to the fractionation of glutenin subunits.
- The role of SDS-gel electrophoresis for this purpose is still valid, provided the appropriate skills are available for its performance and interpretation.
- RP-HPLC also serves this purpose, except for its inability, at present, to distinguish between HMW subunits 2 and 5.

For actual dough-quality
For actual dough strength, appropriate analyses determine the proportions of very large glutenin polymers, and/or the ratios of glutenin-to-gliadin and/or of high-to-low-molecular-weight glutenin subunits. Methods for determining these quantities are provided, but there is a need to pursue promising leads to provide simpler methods based on these research procedures.

Amounts of glutenin subunits
Current methods of identification for glutenin subunits (e.g. SDS-PAGE) indicate whether specific subunits are either present or absent, but the interpretation assumes that the amount of each subunit is constant. A potential advantage of CE is that the amounts of subunits are readily determined. This information should be applied to the prediction of dough properties, because there are outstanding examples of over-expression of some glutenin genes, resulting in significantly enhanced dough quality, well beyond the values predicted by the *Glu-1* score. For example, the transgenic insertion of multiple copies of the *Glu-D1* encoded subunit Dx5 into lines with an Olympic/Gabo background, resulted in 'over-strong' dough (Rooke *et al.*, 1999).

Furthermore, some cultivars show a natural over-expression of the *Glu-B1*-encoded subunit Bx7, thereby conferring high dough strength to cultivars such as Red River and Glenlea (Lukow *et al.*, 2002) and the Australian cultivars Kukri and Chara (Butow *et al.*, 2002). In addition, a critical balance between the x- and y-type HMW subunits is needed for normal dough-mixing behaviour (Butow *et al.*, 2003). Therefore, consideration of the amounts of specific subunits should further improve the predictive value to analyse genetic potential, based on glutenin-subunit composition (Juhasz *et al.*, 2003). This consideration may also relate to the analysis of subunits for actual dough quality.

Automatic interpretation of results
The potential advantages of methodologies such as CE and HPLC are that results are captured for immediate interpretation, provided suitable programs are available to do so. Such programs were developed over a decade ago, especially *PatMatch, Allele* and *WhatWheat* (e.g., Gore *et al.*, 1990). These programs need to be up-dated to suit current and future computing systems.

A traditional disadvantage of gel electrophoresis has been the difficulty in taking up the results in digital form to suit computer programs. To overcome this problem, we have developed a procedure and program to permit the scanning of stained gels on a desk-top scanner. This program must be added to the interpretative program during their re-development.

Furthermore, these various programs need to be integrated to form an overall package to suit the needs of the various branches of the wheat industry that have been described in the Introduction to this report.

Suiting the breeder's needs
These interpretive programs must be suited to the systems being introduced into Australian breeding, particularly the system of using of glutenin-subunit composition in breeding for dough quality as described by Eagles *et al.* (2002a and 2002b). In this system, genotypic values for Rmax, dough extensibility and dough-development time can be predicted for allelic combination using all six glutenin loci, based on data from over 10,000 analyses of dough quality for nearly 5,000 genotypes (varieties and breeding lines) grown in 324 environments in field trials of the southern Australian breeding programs.

A total of 31 alleles are listed for the six glutenin loci. Theoretically, 1,440 genotypes could be formed from combinations of these alleles, and dough quality has been predicted for each of these. In practice, the breeding strategy is to select suitable parent material and then to segregate progeny for the most suitable combination of glutenin alleles from that cross, which would of course result in a much smaller combination of glutenin alleles. The challenge is next to develop efficient means of characterising the alleles present in each of the many lines resulting from a cross. A computer program has been developed to assist in the interpretation of results.

Immediate products for waiting collaborators

Immediate goals of the project are to provide the Beckman MDQ equipment with procedures and programs developed for transfer to the University of Sydney's Plant Breeding Institute at Narrabri. In addition, GrainCorp at Narrabri are planning to buy equipment for HPLC, providing another immediate industry focus for the development and trialling of the procedures found to be most worthy of pursuing.

APPENDIX 1. PROJECT 1.1.1 PLANS, PROPOSED BY BATEY AND WRIGLEY (2002)

The subtitles below summarise the plans proposed by Batey and Wrigley (2002) in the VAW CRC Report, required by CRC Management. Text below the subtitles indicates progress towards rhese objectives. In the original plan, two Post Doctoral Fellows were to be provided, with resources. However, only one PDF was appointed, so achievements may be expected to be correspondingly less. First-priority items were indicated by an asterisk (*) in the original version.

A. INFORMATION MANAGEMENT

1. Assemble data on Australian wheat varieties

This task has been achieved by the production of two CRC Reports (Wrigley *et al.*, 2001, and Cornish *et al.*, 2002). This information has also been assembled ready for the VAW CRC Web Site or a CD ROM. Similar information might later be warranted for overseas sets of varieties if methods are to be marketed internationally.

2. Choose subsets of varieties with special need

A set of soft varieties was assembled for early studies, based on the identification requirements of industry partners. CE analysis of these wheats was reported by Batey *et al.* (2002). More recently, a set of 30 wheats currently grown in Australia was selected for the Diagnostics Program generally, and authentic samples of these were grown as single plants.

3. Up-grade and up-date *WhatWheat* software to manage results

The *WhatWheat* program was originally devised by Dr Batey, and has already been set up for older Australian varieties (Gore *et al.*, 1990). It provides a system for recording the results from applying several methods of variety identification to many varieties, and the means of interrogating this database. The up-grade and up-date of the program will be possible with the appointment of Dr Bekes (part-time) and a computer-trained vacation student late in 2003.

4. Up-grade and up-date *PatMatch* software to sort results

The *PatMatch* program was originally devised by Dr Bekes, and has been used for many sets of protein-analysis data, mainly from HPLC (Gore *et al.*, 1990). This program provides an efficient means of comparing the protein profile of an unknown variety against a library of profiles. It also offers several other features. The up-grade and up-date of the program is also planned to be carried out, with that of the *WhatWheat* program, with the appointment of Dr Bekes (part-time) and a computer-trained vacation student late in 2003.

5. Assemble sets of authentic samples

A set of of 30 wheats, currently grown in Australia, has been selected for study by all researchers in the CRC's Diagnostics Program. Single-seed authentic samples of these have been grown as single plants at SARDI, Adelaide, and the progeny has been the basis of initial CRC studies of methods of variety identification, e.g., Tonkin *et al.* (2002).

B. RESEARCH-SCALE METHODOLOGY

Use CE (CRC-SydUni Beckman equipment; UNSW BioRad unit; FSA,Werribee Beckman; USDA, Manhattan, KS Beckman) **and RP-HPLC** (CRC's at FSA, N Ryde) **to determine distinction capability, based on:**

1. Gliadin composition

Improved faster versions have been developed, based on the CE method of Siriamornpun *et al.* (2001), on the micro-gel electrophoresis method of Wrigley *et al.* (1991), and on the RP-HPLC method (Official Method No 08-02 of the RACI Cereal Chemistry Division, in Black, 2003). Details of the methods are provided in the following appendices (numbers 4, 2 and 3, respectively). These have been applied to the sets of variety samples described above.

2. Glutenin subunit composition

Improved CE methods have been devised for analysis of HMW- and LMW-subunit composition, based largely on the procedure of Bean and Lookhart (2003). This method (detailed in Appendix 7) has been applied to the set of Australian wheats. This CE method complements the established RP-HPLC method (Marchylo *et al.*, 1989).

3. Glu:Gli ratio; %UPP

The established SE-HPLC methods are considered to be the main possibilities for determining these ratios (Batey et *et al.*, 1991). See Appendix 9.

4. Protein markers

The analysis of marker proteins is described in the body of this report. Developments for new markers are being watched in contemporary research. The recent publication of Cornish *et al.* (2001) relates.

C. ROUTINE ANALYSIS METHODS

1. Develop simplified extraction methods

In developing the methods described in this report, we have tried to provide the simplest possible extraction methods.

2. Adapt research-scale CE methods to small-scale CE unit

Original plans for a small-scale CE unit involved collaboration with CE Resources, Singapore. However, their plans have not yet extended to a model capable of handling proteins. Instead, we have pursued the Agilent equipment. In addition, we have established collaboration with the Chemistry Department at Kansas State University, where Lab-on-a-chip methodology is being developed.

3. Field-test simplified CE systems

Plans for this aspect are longer-range, obviously dependent on success in the preceding item.

4. Develop simple CE methods for protein markers (From other CRC projects)

There has been initial success in developing methods for the analysis of some marker proteins, as described in the body of this report. Developments for new markers are being watched in contemporary research.

5. Trial Gradipore MicroGels

A routine procedure was developed (Tonkin *et al.*, 2002; Uthayakumaran *et al.*, 2003; Appendix 2). It was demonstrated at the Breeders' Workshop prior to the 53rd Australian Cereal Chemistry Conference in Adelaide in September, 2003.

6. Trial mass spectroscopy for identification

The method of Ens *et al.* (2000) was trialled at the Australian Proteome Analysis Facility, but satisfactory results were not obtained. Further trials are in progress.

D. MARKER IDENTIFICATION

1. Characterize distinguishing peaks from CE and RP-HPLC

Not yet pursued.

2. Provide protein-chemistry back-up for immuno-assay Project 1.1.2

Provided to a limited extent, as requested.

3. Interact with Project 3.1.3 on proteomics to identify protein markers

Interactions have led to joint publications.

4. Determine protein composition of suitable sets of genotypes

This task has been pursued for the varieties described in this report, but not yet to identify quality markers in characterised sets, such as the National Molecular Marker Wheats.

APPENDIX 2. MICRO-GEL ELECTROPHORESIS OF GLIADINS (pH3)

Equipment needed

The MicroGels are compatible with many gel tanks designed for minigels, because their cross-section has not been altered, only the length of the gel. According to Gradipore instructions, compatible gel tanks include:-

- Gradipore Mini 4-cell or MicroGel unit
- Bio-Rad Mini-Protean
- Novex XCell I and II

The MicroGels are available from Gradipore (info@gradipore.com). The gradient range used in this method is 3 – 20%T (Gradipore Catalogue Number CB 21-320). The gels measure 80 X 25 X 1 mm, contained in a cassette that is 100 mm wide, 50 mm deep (measured top to bottom in the direction of migration), and 4.5 mm thick. They are provided with ten sample wells, each holding up to 50 µL of sample. Routinely, store at 4°C; do not freeze! Shelf life is about five months from the date of manufacture. The gels are manufactured with Tris-borate buffer (pH 8.6) in the gel, so a pre-electrophoresis step is necessary to introduce the sodium lactate buffer (pH 3) into the gel. Note the instructions provided with the gel, especially guidance concerning trouble-shooting. A power supply is needed, capable of delivering at least 300 volts (direct current).

PROCEDURE

1. Sample preparation

Weigh the crushed sample (15–100 mg) into an Eppendorf tube.

Add extracting solution at about ten times the weight of the dry sample, using ethylene glycol (Reagent 1) or 6% urea solution (Reagent 2). For example, if there is 20 mg of crushed grain, add 200 µL of ethylene glycol. **N.B.** Ethylene glycol is viscous; use a pipette with a large tip. A small amount of crystal violet (Reagent 3) or other suitable acid marker dye (such as methyl green) may be added to indicate the progress of electrophoresis, and to ensure that the electrodes are connected with the correct polarity (migration towards the cathode – negative electrode).

Vortex the sample.

Centrifuge for 2 minutes at about 14,000 rpm. The supernatant is now available for loading into the gel slots.

2. Electrophoresis

Fit the MicroGel cassette into the gel-holder tank, so that the loading wells can be seen above the gasket and within reach of the sample-loading syringe. Remove air bubbles from wells and from spaces above the gel using sodium lactate buffer. Follow the instructions provided with the MicroGels.

Fill the apparatus with sodium lactate buffer (Reagent 4), with the gel and upper electrode compartment in place. Use the cooling unit if available, set to an initial temperature of 25–30°C, mainly to ensure reproducibility of run conditions. Connect the tank to the power supply so that the lower electrode is negative (reversed polarity compared to pH-8 anodic electrophoresis, such as SDS electrophoresis) and set to 300 volts. The current should be about 70 mA per gel. Run for about ten minutes or longer to start the process of equilibrating the gel with the sodium lactate buffer.

Load 5 µl of sample into each well using a micro-syringe, after the gel is equilibrated and the power is switched off. Rinse the syringe with buffer between each sample. Each well may

be loaded with 1 µl of crystal violet dye, if a marker dye has not been included in the extracting solution.
Switch on the power and continue electrophoresis for about five minutes after the marker dye has run off the bottom of the gel (about 17 minutes). The dye moves ahead of the proteins. The best run time must be determined experimentally for the apparatus in use.
Remove gel cassette after power is switched off and electrodes are disconnected. Gently prise the cassette open. Mark the gel corner corresponding to lane #1.

Staining, interpretation and drying
Immerse the gel in dye solution. Use Gradipure stain (Reagent 5) or Reagent 6 at room temperature. To accelerate staining, heat the dye to 50°C and immerse for only about 15 minutes.
Destain the gel by rinsing in Reagent 7 (6 % v/v acetic acid) if Gradipure stain was used, or in Reagent 8 (methanol-acetic acid) after the standard Coomassie stain. This is recommended to enhance contrast. Destaining after Gradipure stain takes about twice the stain time.

3. Interpretation

Examine gel patterns, to see if there is a match between the authentic sample and the test samples declared to be that variety. If these patterns are seen as being similar by visual inspection, the sample is likely to be correct as declared, with the rider that there may be other varieties that give the same or similar pattern. Table 4 indicates varieties with patterns that are indistinguishable by this method. If a test sample does not show the pattern as declared, it may be another variety or it may be a mixture of varieties with different patterns. In such cases, identification is much more difficult than verification of identity. Initial steps towards identification involve a repeat of this method including authentic samples of varieties likely to be involved, plus the analysis of individual grains if a mixture of varieties is suspected.

Dry gel by soaking in GradiDry II solution (Reagent 9, ~20 mL per gel, plus cellophane sheets) for up to 20 minutes (no longer), if the gel is to be stored dry. The gels can also be dried after soaking in 3% glycerol for 20 minutes. The cellophane sheets can alternatively be soaked in Reagent 10. Place a cellophane sheet on frame and apply about 1 mL GradiDry II or in soaking solution. Place the gel on the sheet and remove any bubbles. Cover with the second cellophane sheet and remove any more bubbles. Clamp the drying frame holder down and stand the gels upright to dry overnight, or place in drier for at least 2 hours at about 45°C.

REAGENTS

Reagents for sample extraction

Reagent 1. Ethylene glycol
This reagent (ethanediol, glycol, ethylene alcohol) is recommended for the extraction of gliadin proteins, using it undiluted (< 4 % water content).
CAUTION: This reagent is hazardous; see the MSDS for it.

Reagent 2. 6% Urea solution

Urea (reagent grade)	0.6 g
Deionised water	9.5 mL

This reagent is an alternative to ethylene glycol for gliadin extraction, as specified in RACI Standard Method 08.01 (Black, 2003).

Reagent 3. Crystal Violet 1%
Dissolve 1 g crystal violet in about 90 mL of 50 % sucrose solution and make up to 100 mL with sucrose solution.

Reagents for electrophoresis

Reagent 4. Sodium lactate electrophoresis buffer, pH 3.1

NaOH (sodium hydroxide)	170 mg
Concentrated lactic acid	~ 1.7 mL
Deionised water	to 1 L

Dissolve 170 mg NaOH in ~ 900 ml water. Add concentrated lactic acid (~1.7 mL) until the pH is 3.1, and dilute to 1 L. The buffer may be made up at ten times concentration and diluted as needed. Store in refrigerator (4°C).

CAUTION: These reagents are hazardous. Wear gloves, cover clothing, eye protection, and use fume hood. See MSDS information.

Reagents for staining and drying

Reagent 5. Gradipure stain
Obtainable from Gradipore, Sydney.

Reagent 6. Coomassie stain

Methanol	100 mL
Acetic acid (glacial)	100 mL
Coomassie Blue R-250	1 g
Deionised water	to 1 L

Dissolve Coomassie Blue R-250 in 100 mL water. Dissolve methanol and acetic acid in about 500 mL water, stirring well. Add Coomassie solution, make up solution to 1 L, store in a dark bottle.

Reagent 7. Destaining solution (for use with Reagent 3.1, Gradipure stain)

Acetic acid (glacial)	60 mL
Deionised water	to 1 L

Add acetic acid to ~ 900 ml water and make up to 1 L, stirring well.

Reagent 8. Destaining solution (for use with Reagent 3.2, Coomassie stain)

Acetic acid (glacial)	100 mL
Methanol	100 mL
Deionised water	to 1 L

Add acetic acid and methanol to about 750 mL water, make up to 1 L, stirring well.

CAUTION: These reagents are hazardous. Wear gloves, cover clothing, eye protection, and use fume hood. See MSDS information.

Reagent 9. GradiDry II Gel Drying Solution
Obtainable from Gradipore, Sydney.

Reagent 10. Gel-Drying Solution

Ethylene glycol	40 mL
Ethanol	350 mL
Deionised water	610 mL

As described in the Gradipore instruction sheet:- Add the water, then ethanol, then ethylene glycol to a container and mix for 10 minutes. Store in a tightly sealed container.

Reagent 11. Acid Wash solution

Hydrochloric acid (33%)	100 mL
Deionised water	900 mL

This reagent is for cleaning equipment. Add the acid to the water. Store the reagent in a glass bottle. This gives a 3% HCl solution; if the concentrated HCl is of a different grade, adjust the formula accordingly.

CAUTION: HAZARDOUS CHEMICALS! Use heavy gloves, acid-resistant apron, face shield and fume cupboard. Beware of hot solutions. Take all safety precautions!

Table 4. Discrimination ability of the MicroGel method for 30 wheat varieties currently grown in Australia. A tick indicates that the two varieties give patterns that are visually distinguishable. Similarity of pattern is indicated by a cross. Reproduced from Tonkin *et al.* (2002).

	Anlace	Arrino	Bowie	Brook ton	Cadoux	Camm	Corrigin	Data Tine	Diamon dbird	Frame	Grebe	Kala nnie	Krich auff	Kukri	Lorikee
Anlace	-	✓	✗	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Arrino	✓	-	✓	✓	✗	✓	✗	✗	✓	✓	✓	✓	✓	✓	✓
Bowie	✗	✓	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Brookton	✓	✓	✓	-	✓	✓	✓	✓	✓	✓	✗	✓	✓	✓	✓
Cadoux	✓	✗	✓	✓	-	✓	✗	✗	✓	✓	✓	✓	✓	✓	✓
Camm	✓	✓	✓	✓	✓	-	✓	✓	✓	✗	✓	✓	✓	✓	✓
Corrigin	✓	✗	✓	✓	✗	✓	-	✗	✓	✓	✓	✓	✓	✓	✓
Datatine	✓	✗	✓	✓	✗	✓	✗	-	✓	✓	✓	✓	✓	✓	✓
Diamond Bird	✓	✓	✓	✓	✓	✓	✓	✓	-	✓	✓	✓	✓	✗	✓
Frame	✓	✓	✓	✓	✓	✗	✓	✓	✓	-	✓	✓	✓	✓	✓
Grebe	✓	✓	✓	✗	✓	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
Kalannie	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	-	✓	✓	✓
Krichauff	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	-	✓	✓
Kukri	✓	✓	✓	✓	✓	✓	✓	✓	✗	✓	✓	✓	✓	-	✓
Lorikeet	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	-
Perenjori	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Rosella	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✗
Snipe	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✗
Sunsoft	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✗
Tammin	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Thornbill	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Triller	✓	✓	✓	✓	✓	✗	✓	✓	✓	✓	✓	✓	✓	✓	✓
Westonia	✓	✓	✓	✗	✓	✓	✓	✓	✗	✓	✓	✓	✓	✓	✓
Wyuna	✓	✓	✓	✓	✓	✓	✓	✓	✗	✓	✓	✓	✓	✗	✓
Yanac	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Yitpi	✓	✓	✓	✓	✓	✗	✓	✓	✓	✗	✓	✓	✓	✓	✓
Gundaroi	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Kamilaroi	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Wollaroi	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Yallaroi	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

Table 4. (continued)

	Peren jori	Rosella	Snipe	Sunsoft	Tamm in	Thorn bill	Triller	West onia	Wyuna	Yanac	Yitpi	Gund aroi	Kamil aroi	Woll aroi	Yall aroi
Anlace	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Arrino	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Bowie	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Brookton	✓	✓	✓	✓	✓	✓	✓	×	✓	✓	✓	✓	✓	✓	✓
Cadoux	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Camm	✓	✓	✓	✓	✓	✓	×	✓	✓	✓	×	✓	✓	✓	✓
Corrigin	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Dataline	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Diamond Bird	✓	✓	✓	✓	✓	✓	✓	×	×	✓	✓	✓	✓	✓	✓
Frame	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	✓	✓	✓	✓
Grebe	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Kalannie	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Krichauff	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Kukri	✓	✓	✓	✓	✓	✓	✓	✓	×	✓	✓	✓	✓	✓	✓
Lorikeet	✓	×	×	×	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Perenjori	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Rosella	✓	-	×	×	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Snipe	✓	×	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Sunsoft	✓	×	✓	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Tammin	✓	✓	✓	✓	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Thornbill	✓	✓	✓	✓	✓	-	✓	✓	✓	✓	✓	✓	✓	✓	✓
Triller	✓	✓	✓	✓	✓	✓	-	✓	✓	✓	✓	✓	✓	✓	✓
Westonia	✓	✓	✓	✓	✓	✓	✓	-	✓	✓	✓	✓	✓	✓	✓
Wyuna	✓	✓	✓	✓	✓	✓	✓	✓	-	✓	✓	✓	✓	✓	✓
Yanac	✓	✓	✓	✓	✓	✓	✓	✓	✓	-	✓	✓	✓	✓	✓
Yitpi	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
Gundaroi	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	-	✓	✓	✓
Kamilaroi	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	-	×	×
Wollaroi	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	-	×
Yallaroi	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	×	-

APPENDIX 3. REVERSED-PHASE HPLC OF GLIADINS

Different companies offer HPLC instruments with various configurations. The important factors to be considered are the cost of the equipment, the presence and capacity of the auto sampler, the available injection and detection modes, buffer vial capacity, the ease of using the instrument and the ability to maintain the temperature of the capillary.

The following procedure for analysis of gliadins was optimised for a Beckman-Coulter Gold System.

PROCEDURE

1. Sample preparation

Samples must be crushed or milled before extracting the gliadin proteins.
Weigh the crushed sample (50 mg) into an Eppendorf tube.
Add extracting solution (30% ethanol) of 1 mL to the weighed sample.
Vortex the sample for 10 sec.
Centrifuge for 10 min at about 14,000 rpm.
Filter sample into the sample vial (2 mL vial) using a 0.45 μm filter.

2. Sample application

Inject an aliquot of 20 μL into a Vydac C 18 column, 300 Å, 250 x 4.6 mm (The Separations Group, Hesperia, CA, USA). Analysis should be carried out at 70°C using two solvents: Solvent A (0.05% trifluoroacetic acid in deionised water) and Solvent B (0.05% trifluoroacetic acid in acetonitrile). A gradient of 25% to 50% of Solvent B is used over a period of 26 min and the proteins are detected at 214 nm.

Gradient program for separation of gliadins by reversed-phase high performance liquid chromatography. The analysis was carried out over a period of 32 min (flow rate 2 mL/ min)

Time of gradient segments (min)	Gradient composition (% acetonitrile)	
	Start	End
0.00-23.00	25	50
23.00-25.50	50	50
25.50-26.50	50	25
26.50-32.00	25	25

3. Interpretation

Compare the profile for the test sample with that of an authentic sample of the nominated variety, either visually, of using the equipment's software. These profiles should preferably be obtained at about the same time, to avoid problems of lack of reproducibility.

The program *PatMatch* (Gore *et al.*, 1990) permits the automatic interpretation of results by comparing the profile of the test sample with a library of profiles for many varieties, plus normalising the test profile to match those in the library. This program needs to be up-dated to current computing procedures.

APPENDIX 4. CAPILLARY ELECTROPHORESIS OF GLIADINS IN THE MDQ SYSTEM

Various types of equipment are offered commercially for high-performance capillary electrophoretic with various configurations. The important factors to be considered are the cost of the equipment, the presence and capacity of the auto sampler, the available injection and detection modes, buffer vial capacity, the ease of using the instrument and the ability to maintain the temperature of the capillary.

The analysis of gliadins described below is optimised for the Beckman Coulter P/ACE™ MDQ Capillary Electrophoresis System.

PROCEDURE

1. Sample extraction

Samples must be crushed or milled before extracting the gliadin proteins.
Weigh the crushed sample (100 mg) into an Eppendorf tube.
Add extracting solution (30% propan-1-ol) of 1 mL to the weighed sample.
Vortex the sample for 10 sec.
Centrifuge for 10 min at about 14,000 rpm.
Filter sample into the sample vial (500 μL vial) using a 0.45 μm filter.

2. Capillary preparation and sample application

All separations are carried out in uncoated fused silica capillaries of a length of 27 cm (20 cm to the detector) with an internal diameter of 50 μm. New capillaries are washed with 0.1 M NaOH (filtered through 0.45 μm filter) for 20 min followed by 10 min rinse with the running buffer (Reagent 2.1). Between the sample runs, the capillaries must be rinsed for 2 min with 0.1 M NaOH followed by 3 min rinse with running buffer (Reagent 2.1).

Separation conditions

Optimum separation conditions established for 0.1 M phosphate running buffer (Reagent 1) is 22 kV at 40° C for 18 min. Proteins detected at 200 nm. The amount of sample injected is at 0.5 psi for 5 sec.

3. Interpretation of data

Examine electrophoretic pattern to see if there is a match between authentic sample and the test sample declared to be the variety. The patterns could be overlaid using the software and if the patterns match the sample is likely to be correct as declared. If test samples do not match as declared, it may be another variety or it may be mixture of varieties. In this case variety identification is much more difficult and DNA analysis is needed for variety identification.

REAGENT

Reagent 1. Running buffer

100mM sodium phosphate buffer, pH 2.5 containing 0.05% hydroxypropylmethyl-cellulose (HPMC).

Phosphoric acid 85%	0.919 mL
Monobasic sodium phosphate (highest possible purity)	4.2 g
HPMC	250 mg
Acetonitrile	100 mL

Mix ingredients and bring to 500 mL final volume with highest purity water available. There may be the need to stir overnight to dissolve the HPMC. Store the reagent at 4° C.

APPENDIX 5. SDS GEL ELECTROPHORESIS OF GLUTENIN SUBUNITS

The formation of a 10% polyacrylamide gel in the laboratory is recommended, although suitable pre-cast gels are available. Specific details depend on the electrophoresis apparatus available. Simple extraction of reduced proteins is sufficient for analysis of HMW-subunits of glutenin using SDS-PAGE. For analysis of LMW subunits, it is necessary to perform preliminary extraction to remove other proteins that mask the presence of the LMW subunits.

A discontinuous gel system is used to give very thin starting zones. It requires the formation of two gel layers: the resolving gel, in which band-separation takes place, and the short stacking (upper) gel, on which samples are applied and in which the protein zones are concentrated. The resolving gel occupies the main space in the gel slab. It is formed by mixing 56.4 mL Tris buffer (121.1 g Tris, plus HCl to pH 8.8 in one litre) with 86.3 mL gel solution (19.6 g acrylamide + 0.26 g bisacrylamide, made to 90 mL). The solution is degassed and 3.8 mL 1% ammonium persulfate solution, 1.5 mL 10% SDS solution, and 0.075 mL TEMED is mixed in.

The stacking gel is a short layer (1 or 2 cm) into which are set the sample-loading wells. This gel consists of 10 mL 1 M Tris buffer (30.0 g Tris in water adjusted with HCl to pH 6.8 and made to 250 mL), 4.0 g acrylamide and 0.07 g bisacrylamide made to 67 mL with water. The solution is also degassed and 3.0 mL 1% ammonium persulfate solution, 0.8 mL 10% SDS solution and 0.080 mL TEMED are mixed in. The electrode solution is a different composition, containing 3.0 g Tris, 14.1 g glycine, and 1.0 g SDS dissolved in water to a volume of one litre.

CAUTION. Great care must be exercised in the handling of acrylamide monomer to avoid contact with skin or inhalation of the powder, because the monomer (not the polymer) is neurotoxic.

PROCEDURE

1a. Sample preparation for analysis of HMW subunits

Weigh the crushed sample (15mg) into an Eppendorf tube.
Add extracting solution 160 μL of 4% extraction buffer (Reagent 1).
Sonicate suspension for 15 sec.
Reduce SS bonds by adding 2-mercaptoethanol (to 2% final concentration) heating for 30 min at 65°C.
Centrifuge for 10 min at 17,000 x g.

1b. Sample preparation for analysis of HMW and LMW subunits

Weigh the crushed sample (15mg) into an Eppendorf tube.
Pre-extract with 1 mL 50% propan-1-ol, agitate for 30 min at room temperature, centrifuged at 17,000 x g and discard the supernatant. Repeat the step.
Add extracting solution 160 μL of 4% extraction buffer (Reagent 1).
Sonicate suspension for 15 sec.
Reduce SS bonds by adding 2-mercaptoethanol (to 2% final concentration) heating for 30 min at 65°C.
Centrifuge for 10 min at 17,000 x g.

2. Electrophoresis

Load 12 μL sample into individual wells (for 1 mm thick 20-well comb) on the gel for discontinuous SDS-PAGE.

Electrophoresis should be carried out using running buffer (Reagent 2) in a 1 mm thick 10% acrylamide separating gel at a constant voltage range of 200 - 250 V for approximately 4 hours at 20 °C.

Stain gels overnight in Coomassie Brilliant Blue G-250 stain (Reagent 3).

Destain gels by rinsing in destaining (Reagent 1.4) detergent solution and then store in 20% (w/v) ammonium sulfate solution at 4 °C.

3. Interpretation

Compare the electrophoretic patterns with those of genotypes of known subunit composition to determine the subunit composition. Determination of subunit composition by visual comparison is difficult, requiring experience in doing so.

REAGENTS

Reagent 1. Sample extraction buffer

0.125 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 30% (w/v) glycerol, 0.002% (w/v) bromophenol blue)

Tris	3.027 g
SDS	16 g
Glycerol	60 g
Bromophenol blue	0.004 g
Deionised water	to 200 mL

Dissolve in 100 mL de-ionised water adjust pH to 6.8 with HCl and make up to 200 mL with more water.

Reagent 2. Buffer for electrophoresis

Electrode buffer (SDS Tris-glycine buffer, pH 8.3)

Tris	3.027 g
Glycine	14.4 g
SDS	1 g
Deionised water	to 1 L

Reagent 3. Coomassie stain

Methanol	110 mL
Acetic Acid (glacial)	63 mL
Coomassie Blue R-250	0.25 g
Trichloroacetic acid	57.8 g
Ethanol	70 mL
Deionised water	to 1 L

Dissolve Coomassie Blue R-250 in 30 mL water. Dissolve methanol, ethanol, trichloro and glacial acetic acid in about 500 mL water, stirring well. Add Coomassie solution, make up solution to 1 L, store in a dark bottle.

Reagent 4. Destaining solution

Acetic acid (glacial)	48 mL
Methanol	286 mL
Deionised water	to 1 L

Add acetic acid and methanol to about 500 mL water, mix well and make up to 1 L, stirring well.

CAUTION: These reagents are hazardous. Wear gloves, cover clothing, eye protection, and use fume hood. See MSDS information.

APPENDIX 6. REVERSED-PHASE HPLC OF HMW AND LMW GLUTENIN SUBUNITS

1. Sample preparation for analysis of HMW and LMW subunits

Sample extraction

Samples must be crushed or milled before extracting the gliadin proteins.

Weigh the crushed sample (25 mg) into an Eppendorf tube.

Extract with 1 mL of 70% ethanol for 30 min at room temperature, centrifuge at 17,000 x g and discard the supernatant. Resuspend the pellet in 1 mL 50% propan-1-ol, agitate for 30 min at room temperature, centrifuged at 17,000 x g and discard the supernatant. Resuspend the pellet in 1 mL of 50% propan-1-ol containing 1% DTT (w/v), maintain for one hour in a water bath at 60°C and centrifuge at 17,000 x g. The supernatant, containing the reduced subunits of polymeric proteins, is next alkylated with 10 µL of 4-vinylpyridine for 15 min in a water bath at 60°C.

Centrifuge for 10 min at about 14,000 rpm.

Filter sample into the sample vial (2 mL vial) using a 0.45 µm filter.

2. Fractionation of subunits

Inject aliquots of 20 µL into a Vydac C 18 column, 300 Å, 250 x 4.6 mm (The Separations Group, Hesperia, CA, USA). Carry out analysis at 70°C using two solvents: solvent A (0.07% trifluoroacetic acid in deionised water) and solvent B (0.05% trifluoroacetic acid in acetonitrile). A gradient of 24% to 48% of solvent B was used over a period of 55 min and the proteins were detected at 214 nm.

Gradient program for separation of alkylated HMW and LMW glutenin subunits by reversed-phase high performance liquid chromatography. The analysis was carried out over a period of 65 min (flow rate 1 mL/ min)

Time of gradient segments (min)	Gradient composition (% acetonitrile)	
	Start	End
0.00-1.00	24	24
1.00-26.00	24	31
26.00-51.00	31	48
51.00-54.00	48	60
54.00-55.00	60	24
55.00-65.00	24	24

3. Interpretation

Compare the HPLC profiles for test samples with those of genotypes of known subunit composition to determine the subunit composition. Determination of subunit composition by visual comparison of profiles is difficult. The provision of interpretative software should assist in this task.

APPENDIX 7. CHARGE-BASED CAPILLARY ELECTROPHORESIS OF HMW AND LMW GLUTENIN SUBUNITS IN THE BECKMAN MDQ

The analysis of glutenin subunits was optimised using a Beckman Coulter P/ACE™ MDQ Capillary Electrophoresis System.

PROCEDURE

1. Sample preparation for analysis of HMW and LMW subunits

Samples must be crushed or milled before extracting the proteins.
Weigh the crushed sample (100 mg) into an Eppendorf tube.
Extract 100 mg sample with 1 mL 50% propan-1-ol to remove gliadins, albumins and globulin.
Vortex the sample for 10 sec.
Centrifuge for 10 min at about 14,000 rpm and discard supernatant.
Repeat this preliminary extraction two times (a total of three extractions).
Add 1 mL of 50% propan-1-ol containing 1% dithiothreitol (DTT) (or 5% beta-mercaptoethanol) to the pellet and mechanically mix with spatula and maintain sample at 60°C for 1 hour, whilst frequently vortexing the sample. Centrifuge for 10 min at about 14,000 rpm.

Glutenin extract: The supernatant contains both HMW-GS and LMW-GS. The extract is filtered into the sample (200 µL vial) using a 0.45 µm filter. This extract is used for the subsequent steps

HMW subunit extract: Precipitate HMW-GS from the supernatant above by adding acetone to a volume of 40%. Centrifuge for 10 min at about 14,000 rpm and retain the supernatant for analysing LMW subunits (see below).
Resuspend pellet in 25% acetonitrile, 0.05% TFA in water.
Filter the extract into the sample vial (200 µL vial) using a 0.45 µm filter.

LMW subunit extract: After precipitation of HMW-GS by adding acetone (see above), the decanted supernatant is tipped into another tube and air dried supernatant.
Add pellet in 25% acetonitrile, 0.05% TFA in water.
Filter sample into the sample vial (200 µL vial) using a 0.45 µm filter.

2. Capillary preparation and sample application

The CE profiles of HMW and LMW subunits overlap in the following procedure, so they must be analysed separately. All separations must be carried out in uncoated fused silica capillaries of a length of 27 cm (20 cm to the detector) with an internal diameter of 20 µm. New capillaries are rinsed with 1 M phosphoric acid (filtered through a 0.45 µm filter) for 10 min followed by 20 min rinse with the running buffer (Reagent 1). Between the sample runs, the capillaries must be rinsed for 2 min with 1 M phosphoric acid followed by 3 min rinse with running buffer (Reagent 1).

Separation conditions

Optimum separation conditions established for 0.1 M phosphate-glycine running buffer (Reagent 1) are 17 kV at 40° C for 25 min. Protein fractions are detected at 200 nm. The amount of sample injected is at 0.5 psi for 30 sec.

3. Interpretation

Compare the CE profiles for test samples with those of genotypes of known subunit composition to determine the subunit composition. Determination of subunit composition by visual comparison of profiles is difficult. The provision of interpretative software should assist in this task.

Reagent preparation

Reagent 1. Running buffer

100mM sodium phosphate buffer, pH 2.5 containing 0.05% hydroxypropylmethyl-cellulose (HPMC).

Phosphoric acid 85%	1.6 mL
Glycine	2.0 g
HPMC	250 mg
Acetonitrile	100 mL
High-purity water	to 500 mL

Mix ingredients and bring to 500 mL final volume with highest purity water available.
The reagent may need to be stirred overnight to dissolve the HPMC. Store reagent at 4° C.

APPENDIX 8. SIZE-BASED CAPILLARY ELECTROPHORESIS OF HMW AND LMW GLUTENIN SUBUNITS IN THE BECKMAN MDQ

Both HMW-GS and LMW-GS can be analysed with one injection using this method. However, the overall analysis time is 45 min.

PROCEDURE

1. Sample preparation for analysis of HMW and LMW subunits

Samples must be crushed or milled before extracting the proteins.

Weigh the crushed sample (100 mg) into an Eppendorf tube.

Extract 100 mg sample with 1 mL 50% propan-1-ol to remove gliadins, albumins and globulin.

Vortex the sample for 10 sec.

Centrifuge for 10 min at about 14,000 rpm and discard supernatant.

Repeat this preliminary extraction two times (a total of three extractions).

Add 1 mL of 1% SDS containing 1% dithiothreitol (DTT) (or 5% beta-mercaptoethanol) to the pellet and mechanically mix with spatula and mix for 30 min (continuously shaking). Centrifuge for 10 min at about 14,000 rpm. Transfer the supernatant to a new Eppendorf tube.

Heat sample at 100 °C for 5 min. Centrifuge for 10 min at about 14,000 rpm.

Transfer the extract into the sample vial (200 µL vial).

2. Capillary preparation and sample application

All separations must be carried out in uncoated fused silica capillaries of a length of 27 cm (20 cm to the detector) with an internal diameter of 75 µm. New capillaries are rinsed with 1 M NaOH for 5 min followed by 1 M HCl for 5 min. The solutions are filtered through a 0.45 µm filter. The capillaries are then rinsed with BioRad SDS-CE/ ethylene glycol mixture (Reagent 1) for 5 min. Between the sample runs, the capillaries must be rinsed for 2 min with 1 M NaOH, followed by 1 min rinse with 1 M HCl and then with BioRad SDS-CE/ ethylene glycol mixture for 2 min.

Separation conditions

Optimum separation conditions established are 8 kV (reversed polarity) at 30° C for 45 min. The voltage is ramped up slowly to improve separation (Bean and Lookhart, 2001). Protein fractions are detected at 214 nm. The amount of sample injected is at 0.5 psi for 30 sec.

3. Interpretation

Compare the CE profiles for test samples with those of genotypes of known subunit composition to determine the subunit composition. Determination of subunit composition by visual comparison of profiles is difficult. The provision of interpretative software should assist in this task.

Reagent preparation

Reagent 1. Running buffer

BioRad SDS-CE reagent from Bio-Rad Laboratories Pty., Ltd. is mixed with ethylene glycol to give a final concentration of 15% (Bean and Lookhart, 1999). Mixing of reagents introduces bubbles. Degassing is done by centrifuging the mixture.

APPENDIX 9. SE-HPLC TO DETERMINE GLUTENIN-TO-GLIADIN RATIO

Size-exclusion HPLC (SE-HPLC) can be used to provide various measures of molecular-weight distribution. The most basic is the ratio of gliadin to glutenin (before rupture of disulfide bonds). The following procedure is a modified version of the method of Batey *et al.* (1991).

PROCEDURE

1. Sample preparation for determining glutenin/gliadin ratio

Weigh the sample (10 mg flour) into an Eppendorf tube. Wheatmeal samples may also be used, but the results cannot be compared directly to those obtained from flour.
Add extracting solution (1 mL of Reagent 1, 0.5% SDS phosphate buffer) to the sample.
Vortex the sample for 10 sec.
Sonicate the sample for 15 sec, ensuring that the sample is completely dispersed within the first five seconds.
Centrifuge for 10 min at about 14,000 rpm.
Filter supernatant into the sample vial (2 mL vial) using a 0.45 μm filter.

2. Fractionation of extracted gluten proteins

Use a Phenomenex BIOSEP-SEC 4000 column and a running time of 10 min (flow rate 2 mL/ min) instead of the standard 35 min run (0.5 mL/ min) as described by Batey *et al.* (1991). For eluent, use aqueous acetonitrile buffer (0.05% trifluoroactic acid in water and 0.05% in acetonitrile). Detect proteins at a wavelength of 214 nm. The areas of the glutenin peak (> 70,000 Daltons, Peak 1) and gliadin peak (< 70,000 Daltons, Peak 2, excluding albumins, Peak 3) were measured by Gold Nouveau software (Beckman Instruments, Inc., Fullerton, CA, USA). Use the ratio of the area of Peak 1 to the area of Peak 2 as the glutenin-to-gliadin ratio (polymeric protein-to-monomeric protein ratio).

3. Interpretation

A high ratio of glutenin-to-gliadin is associated with dough strength. Values obtained with wheatmeal samples are higher than those for flour samples, and this should be taken into consideration in interpreting results for both types of sample.

Reagent 1.
Sample extracting buffer – 0.5% SDS phosphate buffer

Solution A

Disodium hydrogen orthophosphate	1.775 g
SDS	1.25 g
Milli-Q water	to 250 mL

Solution B

Sodium dihydrogen orthophosphate	1.95 g
SDS	1.25 g
Milli-Q water	to 250 mL

Make up each solution to 250 mL with Milli-Q water. Add solution B to solution A until the pH falls to 6.9.

APPENDIX 10. SE-HPLC TO DETERMINE %UPP

The proportion of 'unextractable' polymeric protein (%UPP) is a good indication of the proportion of the glutenin protein that is very large, and thus that contributes in a greater way to dough strength than does the glutenin of more modest size. The term 'unextractable polymeric protein' sounds like a misnomer because the 'unextractable' protein must be extracted to be analysed by SE-HPLC. The point is that the term 'unextractable' is intended to indicate that this type of glutenin is the part that is more difficult to extract because of its very large size. This fraction of greatest glutenin polymers is known to make the greatest contribution to dough strength. The following procedure is a modified version of the method of Batey *et al.* (1991).

PROCEDURE

1. Sample preparation for determining %UPP

Suspend the flour sample (or wheatmeal) (10 mg) in 1 mL 0.5% SDS phosphate buffer (Reagent 1) by shaking the suspension for 10 min (vortexing initially). Centrifuge it for 10 min at 17,000 x g. Filter the supernatant through a 0.45 μm PVDF filter. This fraction is called 'SDS-soluble protein'.

Resuspend the insoluble residue in 1 mL 0.5% SDS phosphate buffer and sonicate for 30 sec, ensuring that the sample is completely dispersed within the first five seconds. Centrifuge for 10 min at 17,000 x g and filter the supernatant through a 0.45 μm PVDF filter. This fraction is called 'insoluble glutenin'.

2. SE-HPLC fractionation

A Phenomenex BIOSEP-SEC 4000 column is used for the analysis, with a running time of 10 min (flow rate 2 mL/ min) instead of the standard 35 min run (0.5 mL/ min) described by Batey *et al.* (1991). The eluent is aqueous acetonitrile buffer (0.05% trifluoroactic acid in water and 0.05% in acetonitrile). The proteins are detected at a wavelength of 214 nm.

The areas of the glutenin peak (> 70,000, Peak 1) and gliadin peak (< 70,000, Peak 2) are measured with the Gold Nouveau software of Beckman Instruments, Inc., Fullerton, CA, USA.

3. Interpretation

Determine the proportion of 'unextractable' polymeric protein (%UPP) as the ratio of the glutenin peak (Peak 1) of the 'insoluble glutenin' extract to the sum of the glutenin peaks of both 'insoluble' and 'soluble' extracts (Gupta *et al.*, 1993). When wheatmeal samples are used, the results cannot be compared directly to those obtained from flour, as in the case of glutenin-to-gliadin ratio.

Reagent 1.
Sample extracting buffer – 0.5% SDS phosphate buffer

Solution A

Disodium hydrogen orthophosphate	1.775 g
SDS	1.25 g
Milli-Q water	to 250 mL

Solution B

Sodium dihydrogen orthophosphate	1.95 g
SDS	1.25 g
Milli-Q water	to 250 mL

Make up each solution to 250 mL with Milli-Q water. Add solution B to solution A until the pH falls to 6.9.

APPENDIX 11. SDS SEDIMENTATION METHOD

The SDS sedimentation method is another procedure that is designed to provide an indication of the amount of glutenin. In this case, the glutenin is measured by its ability to swell in solution. The amount of the swollen fraction is measured by its volume after the suspension is allowed to settle in a measuring cylinder.

1. Sample preparation

Sample receipt: Grain samples must be labelled on receipt and sub-samples (about 100 g grain) must be cleaned free of husks and small grains.

Grain milling: Grind the cleaned grain samples in a Falling Number mill at low speed (to ensure a fine grind).

2. Sedimentation

Sedimentation: This step requires a set of 100-mL cylinders in a rack that permits simultaneous shaking and tipping.

1. Add 6.30 g wheatmeal to 50.0 mL distilled water in a stoppered 100-mL measuring cylinder, using an automatic pipette to dispense the water exactly (at 22°C) and using a wide-mouthed funnel to add the meal quickly to the water.

2. Shake the cylinder well and ensure that all meal is dispersed. Start timer and start mechanical stirrer inside cylinder (standing vertically). Stop stirrer after 15 seconds. Allow cylinder to stand for 4 minutes, 19 seconds.

3. Add 50.0 mL SDS solution (see below), using an automatic pipette.

4. Rock the cylinder gently from the horizontal position 15 times during 30 seconds. The timer should now show 4 minutes. Stand for a further 5 minutes.

5. Read the height of the sedimented layer and record the sedimentation volume.

3. Interpretation

Divide the sedimentation volume by the protein content of the wholemeal sample to obtain the Protein Quality Value (PQV).

Reagent

SDS solution

Dissolve 30 g sodium dodecyl sulfate in 970 mL distilled water; then add 20 mL lactic acid (concentrated).

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