VALUE ADDED WHEAT CRC PROJECT REPORT

Protein-Composition Analysis to Determine Variety and Quality Type: Principles and Practice

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Protein-Composition Analysis to Determine Variety and Quality Type: Principles and Practice

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INTRODUCTION

This report describes the needs of the Australian wheat industry with respect to the identification of variety and quality type, with further reference to the scientific possibilities, based on world trends in relevant technologies. The report was originally produced in December, 2001, as a Feasibility Study, requested as a preliminary to the full implementation of VAW CRC Diagnostics Project 1.1.1, entitled 'Protein-composition analysis'. However, the contents of the report have relevance to a much wider range of projects in the VAW CRC, particularly, all of the Diagnostics Projects (Program 1), plus the proteomics and genomics projects in Program 3. The implementation of the research results is likely to find application in most other projects. Minor additions have been made to the original version, based on further visits to facilities of the wheat industry, and newer developments in relevant methods.

AIMS OF THE FEASIBILITY STUDY

- Identify the best opportunities to develop quality-test methods to improve the quality of the Australian wheat crop, based on the analysis of grain-protein composition.
- These 'quality-test methods' would involve the analysis of aspects of protein composition that would provide identification of variety, or of specific quality attributes, or of both at the same time.
- Identify ways for Project 1.1.1 to ...
    - support the research of Project 1.1.2 on immuno-diagnostics;
    - provide quality-selection methods for CRC breeding projects (Program 4);
    - interact profitably with Project 2.1.9 on gluten modification;
    - provide practical application of research findings in other CRC projects, particularly proteome and molecular-marker studies in Program 3.
- Identify possible opportunities to enhance the status of the VAW CRC, and to generate royalty income.

SUMMARY OF RECOMMENDATIONS OF FEASIBILITY STUDY

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.
6. 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.

Section A. The Analysis of Protein-Composition: Principles

The declaration of variety for wheat deliveries is used in many wheat-producing countries as a basis for defining quality type. This long-established practice has worked well in Australia, but there has always been the recognition that, at best, it is only a surrogate for quality. The main deficiency of the practice is that it ignores the considerable likelihood that growth conditions will modify the genotypic aspects of grain quality that have been 'built in' by the breeder.

The protein composition of the grain provides a readily-accessible source of information about the genetic origins and identity of the sample, as well as providing the potential to indicate the combined effects of genotype and growth environment. **(Recommendation #1).** Each of the possible methods for determining protein composition provides a significant number of components (peaks or bands) whose presence and/or quantity contributes to the ability of that method to provide distinction between varieties, and also in some cases to indicate quality attributes.

It is generally agreed that, in commercial situations, tests for quality attributes would be preferable compared to the analysis of variety *per se*, but until recently this approach has been regarded as being hardly practicable. Difficulties include deciding which of the many aspects of quality should be evaluated, and also determining how this testing could be performed under the rushed and primitive conditions of grain receival, if this situation is a major focus. Nevertheless, the objective of quality evaluation is now within reach, given

current advances in the identification of marker proteins and in the development of new methods of protein analysis.

Varietal identification *per se* is now assuming greater importance, with the current introduction of point-of-delivery royalty payments under Plant Breeders' Rights (PBR). Under this system, royalties for a particular PBR variety are paid according to the volume of grain harvested and delivered, with the payment being assessed according to the declaration of the variety at the time of delivery. There is thus the temptation for the declaration to be a variety that does not attract a royalty payment. Verification of the declaration is thus seen to be critical to the implementation of PBR by this system. However, this form of verification testing can be implemented at any time subsequent to delivery; the verification of the declaration is not necessarily related to the immediate decision about where the load is to be dumped.

End-point royalties are currently being levied for five wheat varieties in Western Australia (also for varieties of other grain crops). These wheats are Ajana, Camm, Karlgarin, Wyalkatchem and Harrismith. The level of royalty payable is up to $1 per tonne, currently estimated to total about $500,000 in a season for WA Agriculture (a significant income, even initially, that warrants the input of technological effort to ensure its collection). Given the concern of the WA Department of Agriculture about this royalty income, a staff member (Nadine Morgan, at Three Springs) has the task of determining what methods can be used to identify these varieties. We have had extensive exchanges of information with Nadine, including interaction on a GRDC application by WA Agriculture for partial funding of the development and application of better methods of variety identification. This application was not successful, but ongoing collaboration is planned nevertheless.

Further requirements for varietal identification are coming due to the increasing growth of wheats with unusual quality characteristics, termed "ingredient wheats". Such specialty wheats are not intended for admixture with wheat of conventional quality, but rather for specific processing needs. Accordingly, they must be identified at delivery so that they can be kept segregated ("identity preserved"). Segregation may be achieved by identification of the specific variety, or of the distinguishing quality attribute. These wheats arise from advances in breeding technologies that have led to the development of novel quality types, such as:

- Waxy wheats, with all the starch as amylopectin.
- Wheats with very strong dough properties, suited for blending with weaker wheats or for frozen-dough production, e.g. the variety Kukri, transgenic lines with over-expression of glutenin subunits resulting from Australian-overseas collaborations, and also wheats grown overseas such as the Canadian variety, Glenlea.
- Wheats with unusual endosperm colour, such Krichauff, which has a high level of yellow pigment in the endosperm. Although this is desirable for the production of some types of noodles, it is not favoured for the production of conventional bread.
- Wheats with very extensible dough properties, e.g. based on the UK variety Galahad 7.
- Wheats with extreme proportions of large (A-type) or of small-sized starch granules.
- Finally, there is the coming need for genetically modified (GM) wheats to be kept segregated from conventionally bred wheats. This requirement has already caused considerable difficulties in identification and segregation for other grains, such as maize and canola.

VARIETAL IDENTIFICATION *versus* VERIFICATION

It is important to establish, at the outset, what questions need to be answered. There are three main possibilities with respect to varietal identity (Wrigley and Batey, 1995).

Question 1. Is this sample true to the declaration? This question, requiring *verification of identity*, is the easiest to answer, and the resulting answer is sufficient for most industry needs. A satisfactory answer involves the application of a discriminating test (or tests) to compare the 'unknown' sample with an authentic sample of the variety declared, resulting in a 'yes-no' answer depending whether the two sets of results are similar or not, respectively.

Question 2. Is this sample variety A, B, or C? This question is similar to the first, except that more than one reference sample may be needed for analysis, in parallel with the 'unknown' sample. The degree of discrimination of the test(s) applied needs to be known, to ensure that this specific combination of alternative varieties can be distinguished from one another.

Question 3. What is the identity of this sample? This 'open' question is very difficult to answer, because it presumes knowledge of the characteristics of all possible varieties. An obvious place to start is to obtain knowledge of the range of possibilities, thereby reducing the range to the level of Question 2, above. Nevertheless, the provision of an answer will require knowledge of the results for many varieties. This can most efficiently be provided by the use of the software programs *WhatWheat* (a catalogue of the results for many varieties with many methods) and *PatMatch* (a process for automatically matching the results of an 'unknown' sample against those of reference samples). Wrigley and Batey (1995), in reviewing the use of these programs, point out the need to make judicious combinations of simple and more complex test methods to achieve efficiency. This approach will be adapted to the present research study. **(Recommendation #4.)**

NON-HOMOGENEOUS SAMPLES

The task of identification is greatly complicated if there is the likelihood that the sample for analysis contains a mixture of varieties, or even a mix of biotypes, each an authentic part of the variety but providing different results with the test chosen. In this case, different results may be given for each of several single seeds.

For Questions 1 and 2, a likely solution is to analyse ground wheatmeal samples of both the 'unknown' and reference samples. In this case, the range of varieties is averaged in the 'unknown' sample, resulting in a protein profile that is not exactly the same as that for the pure authentic sample, warranting a negative answer to the question. This approach may not detect the presence of admixture for smaller levels (e.g., 5 to 10%) of a contaminating variety, depending on the method of analysis and the characteristics of the varieties involved.

For Question 3, analysis of a wheatmeal sample may not be readily interpretable, if the sample is a mixture of varieties. As a result, it is probably necessary to analyse many grains individually, with the added need to apply statistical analysis to the results (Wrigley and Batey, 1995).

ANALYSIS OF QUALITY TYPE

In many cases, the analysis of varietal identity relates solely to segregation of loads of similar quality type, and for the corresponding premium payment. It would thus be preferable to have test methods that determined quality attributes, thereby also taking into account the effects of growth environment, which are essentially neglected by the concept of varietal identification to indicate quality grade. Already, there are several marker proteins with the potential to indicate specific quality attributes, as listed in Table 1. Additions to the collection of marker proteins are being made by on-going study of the Australian National Wheat Molecular Marker Program – both at the DNA and protein levels (Appels *et al.*, 2001). Extension of these studies is planned for the current project (1.1.1), in collaboration with other projects of the VAW CRC, especially in Program 3.

Table 1. Specific marker proteins for use in the determination of quality type. Adapted from Wrigley and Bekes (2002).

Quality trait	**Marker proteins**	**Methods of identification**
Dough strength	HMW and LMW subunits of glutenin	SDS-PAGE, RP-HPLC or CE
Dough extensibility	Gliadins of *Gli-2* loci	pH 3 PAGE or CE
Grain hardness	Purindolines *a* and *b*	Complex extract'n + SDS-PAGE
Null 4A (noodle)	GBSS 4A or not	Complex extract'n + SDS-PAGE
Waxy (ingredient)	No GBSS	Complex extract'n + SDS-PAGE

Table 2. The relevant suitability of deploying methods of variety identification in three industry situations. 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 silo or mill; breeder or seed lab; export terminal	Contract lab for many post-harvest samples
Requirements:-	Speed, simple method	Over-night results	Efficiency for large numbers
On-the-spot methods			
Visual examination	✓✓		
Image analysis		✓	✓
NIR for quality or variety	(✓)	(✓)	(✓)
Multi-grain (slow) method			
Phenol test		✓	
Immuno-assay			
Test card (ICT)	✓	✓	
Microtitre plate (ELISA)		✓	✓✓
Protein composition methods			
PAGE		✓✓	✓
RP-HPLC		✓✓	✓
Capillary electrophoresis	(✓)	✓✓	✓
Mass spectroscopy			✓✓
DNA method			
DNA analysis	(✓)	(✓)	✓✓

(✓) = research still needed.

However, the analysis of many of these marker proteins still involves a diversity of complex processes (listed in Table 1), relying on the procedures by which they were discovered in the original research. There is thus a need to devise much simpler methods of extraction and fractionation to facilitate their routine analysis. Capillary electrophoresis appears to provide the flexibility to provide for the analysis of a range of marker proteins using only one fractionation procedure. **(Recommendation #3)** A desirable scenario would see the analysis of a diversity of marker proteins, some as indicators of specific quality attributes, and others as genotypic markers with no known relationship to grain quality.

INDUSTRY NEEDS AND SCIENTIFIC FEASIBILITIES

The feasibility of efficiently applying these principles was investigated by examining the needs of the Australian wheat industry at all levels, and by evaluating the range of methods available for use in this range of situations – both those now available, and those with suitable potential, given reasonable scope for research and development. These investigations involved the conduct of two workshops (Batey and Wrigley, 2001), review of recent literature, personal interactions and site visits, and interviews during an overseas trip (Appendixes 1 and 2).

LIMITATIONS OF INDUSTRY SITUATIONS

There is a considerable range of methods of identification (available or potential, as listed in Table 2). Faced with such a diversity of methods, it may initially be difficult to decide what is the most appropriate approach for a specific task of identification. This decision is partly based on the range of factors listed in Table 3, in relation to the constraints of the situation in which the identification must be conducted. Examples of such situations, listed in Table 2, include testing needs throughout the wheat business chain:-

- during breeding, involving the need to screen for quality type and to obtain certainty of genotype;
- at pure-seed production, to ensure that the correct variety is sold;
- for on-farm Quality Assurance, verifying that the correct variety of seed is sown;
- receival testing after harvest, when grain is delivered to the elevator or mill, thereby ensuring correct quality type, grade classification and possibly royalty payment under PBR;
- buying of grain, to verify quality type;
- during storage, transport, marketing and at the export terminal, again to ensure that the correct class is provided, but at this stage, probably as a variety mix;
- throughout processing, there is the need to verify variety mix, especially when there is the requirement of trouble shooting, to see if production problems are due to errors in the procurement of raw materials.

THREE SCENARIOS FOR IDENTIFICATION

This range of situations can be reduced to the three scenarios listed in Table 2, namely,

1. the need for on-the-spot identification, when grain must be assessed on delivery for quality grade with a few minutes, with minimal facilities;

2. 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;
3. 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.

THE RANGE OF TESTING METHODS

On-the-spot methods

Table 2 lists the main possibilities for methods of varietal identification (with the exception of 'the grape vine' – rumoured indications that certain loads may be incorrect declarations). The table shows that there are relatively few possibilities for effective identification of variety at the silo (Situation #1), given the short time-frame that is likely to be required. This difficulty is high-lighted in the report of the second workshop (Batey and Wrigley, 2001) and the report of visits to receival stands operating during the 2001 harvest (Appendix 1).

Visual examination of grain is well suited for this situation, but it requires experience, it is very subjective and it provides poor discrimination. During the 1970s and 1980s, Colin Wrigley led a project that provided reference publications of head and grain descriptions and colour illustrations. Varieties of wheat, triticale, barley and oats were described in a series of eight handbooks, the last covering recently registered varieties of all of these cereals (Fitzsimmons *et al.*, 1986). These publications were not continued after the Wheat Research Unit joined CSIRO Plant Industry, but the information is provided in the registration of new varieties, and it is listed for many wheats in the Wheat CRC Report by Wrigley *et al.* (2001). Information about crop varieties is now available on the CD ROM (Variety Selector, available from PO Box 2054, Hotham Hill, Vic. 3051) provided by the Australian Crop Accreditation System (ACAS), and on the ACAS web site (www.acas.on.net).

The need for objective methods on-the-spot is more likely to be provided by the immuno-assay research of Project 1.1.2 (assay of a limited number of protein markers), than by the more complete analysis of protein content. Nevertheless, the short time-frame may not be a complete barrier to the deployment of simple equipment at the silo, since there is the opportunity for analysis to be performed after a questionable delivery, thus to provide assurance of the correctness (or otherwise) of suspect declarations of variety or of quality type. The simple CE equipment is thus a candidate for this role. **(Recommendation #2c.)**

A significant comment from the second workshop on industry needs (Batey and Wrigley, 2001) was the statement that an emphasis on variety identification methods for use at receival would arise with the requirement to test for GMO varieties, because in this case, there would be the immediate need to segregate such loads. Commercial kits for GM crops are available, mainly for canola and maize. There is no suggestion that the current research of the VAW CRC should develop such tests, but the procedures being developed for this scenario should provide pointers for ongoing test development for variety and quality attributes.

Near-Infrared Spectroscopy (NIR)

NIR analysis has revolutionised the determination of protein content, moisture, hardness and oil content for grains in recent decades. In addition, claims are being made for the analysis of

many other aspects of grain quality, including the determination of wheat grade (e.g. Williams, 2001). Further extension of the analytical capability of NIR is an attractive prospect, given the ability of the method to provide immediate results and the almost universal availability of NIR equipment at grain-receival sites and in cereal laboratories. Nevertheless, the possibility of NIR to provide distinction between individual varieties is a distant, perhaps unlikely, prospect at present, with no evident research leads on what avenues to follow with this aim. This view of the potential of NIR was also expressed in discussions with the staff of the Grains Centre for NIR (housed in the BRI Australia building).

DNA analysis for variety identification

Analysis at the DNA level is clearly the ultimate level at which to analyse for genotype, with no possibility for interference from growth conditions. New technologies at the genome level now offer the possibility of conducting varietal identification by DNA analysis efficiently and economically. These new approaches involve the choice of specific markers with the potential to provide adequate distinction (Langridge *et al.*, 2001; Harker *et al.*, 2001). Protein markers are becoming identified for those genes for which microsatellite-based PCR methods are available (Preston *et al.*, 1999). High throughput micro-plate colorimetric assays are under development to provide routine identification based on these genes (Gale *et al.*, 2001). However, the extraction of DNA from grain is still too complex an operation for rapid on-the-spot analysis. Nevertheless, analysis of DNA is a strong contender for the role of efficient analysis of many samples at a central laboratory (see Appendix 2), or perhaps even at a regional laboratory if methods can be sufficiently simplified.

A DIVERSITY OF METHODS BASED ON PROTEIN COMPOSITION

Gel electrophoresis

For some decades, the routine method of identifying wheat varieties in the laboratory has been cathodic polyacrylamide gel electrophoresis (PAGE) at pH 3 based on gliadin-protein composition. This approach to identification is so well established that it is enshrined in the standard methods of several scientific bodies, especially the International Association for Cereal Science and Technology (ICC), the International Seed Testing Association, and the Royal Australian Chemical Institute. The gels may be polymerised in the laboratory. Alternatively, pre-cast commercially-available gels may be used, providing convenience, speed, and reproducibility, whilst avoiding the hazards of handling acrylamide monomer.

The main alternative to pH-3 gel electrophoresis of gliadin proteins is the use of sodium dodecyl sulfate (SDS) for both extraction and electrophoresis stages, providing SDS-PAGE patterns that contain the full range of flour polypeptides, as a result of the rupture of disulfide bonds. These patterns have the high-molecular-weight (HMW) subunits of glutenin separated as the largest polypeptides at the top of the patterns. Consequent knowledge of the allelic composition of the HMW subunits (*Glu-1* alleles) can provide valuable information about genetic aspects of dough strength. However, the SDS gel patterns are complex in the lower regions, often making it difficult to make clear-cut distinctions between similar varieties.

A more complex extraction procedure can be used to obtain patterns for both the HMW and the low-molecular-weight (LMW) subunits of glutenin. Consequent determination of both HMW subunits (*Glu-1* alleles) and LMW subunits (*Glu-3* alleles) provides further information about dough properties, but the interpretation of the patterns requires extensive experience. On the other hand, if pattern matching is used to compare patterns, without

regard for quality prediction, SDS-PAGE can provide useful distinction. The extent of such distinction can be predicted approximately by reference to the allelic composition of the respective varieties provided in the CRC Report on about 120 Australian varieties (Wrigley *et al.*, 2001).

Conventional PAGE procedures require up to 24 hours for results of identification to be obtained (Table 3). In addition, interpretation must initially involve subjective examination of the patterns. Automated interpretation and recording of patterns involve optical scanning or image analysis.

A rapid-analysis method in the family of PAGE procedures involves the use of pre-cast micro-gels that provide identification in less than one hour (Table 3) (Bekes *et al.*, 1991). Such gels are likely to be available from Gradipore (Sydney) in 2002, and their suitability for regional lab use should be investigated. **(Recommendation #2d)**

As Table 3 shows, PAGE methods offer the great advantage of low capital costs for the equipment (if computer-based interpretation is not required), but the methods are labour-intensive, and a degree of skill is required.

Capillary electrophoresis (CE)

During the past decade, capillary electrophoresis has developed as an alternative to gel electrophoresis, with similar or better resolution of zones (Bean and Lookhart, 2000; Siriamornpun *et al.*, 2001c). The use of CE for variety identification has generally involved the analysis of gliadin composition. The basis of fractionation by CE appears to differ from that of PAGE methods, so CE promises to provide different distinctions between varieties compared to PAGE. Comparative analyses in different laboratories with different brands of CE equipment have established that similar procedures can be applied in different situations (Siriamornpun *et al.*, 2001a). CE offers the advantages of speed, automatic loading and instant interpretation of results (Table 3). The latest advances in methodology offer an analysis time of less than ten minutes, with little longer required for regeneration before application of the next sample. These research advances provide a good basis for ongoing refinement of analysis by CE. **(Recommendation #2b.)**

Colin Wrigley and Ian Batey have had experience with CE, Colin as one of the PhD advisors for Ms Siriamornpun at the University of NSW (with Prof. Michael Wootton and Dr Ferens Bekes) (Siriamornpun *et al.*, 2001a-d). Both Ian and Colin have ongoing interaction with the UNSW group, with access to the BioRad CE equipment there. They also have access to the Beckman CE unit at Food Science Australia, Werribee. The latest Beckman CE equipment has been purchased by Sydney University jointly with the VAW CRC, for methods suited for breeding use to be developed in Project 1.1.1, for later use in routine analyses for the Narrabri breeding program. The development of applications of CE suited to breeding are thus important aims of the project. **(Recommendations #2a and #3.)**

The foremost laboratory for methods of variety identification using CE (also RP-HPLC) is that of George Lookhart and Scott Bean at the USDA laboratories in Manhattan, Kansas. Formal collaboration with them is an integral part of Project 1.1.1 in the VAW CRC. Funding is included in Project 1.1.1 (2001/02) for a brief visit to Food Science, North Ryde, by Dr Scott Bean. This visit has been deferred until Project 1.1.1 is established as an on-going project in a laboratory with appropriate equipment. In addition, Beckman CE

equipment is in use in the laboratory of Prof Khalil Khan, Fargo, USA), who has agreed to share development information with the VAW CRC staff. He has recently published a method for CE analysis of glutenin-subunit composition (Zu and Khan, 2001).

Table 3. The comparative effectiveness of routine methods of variety identification based on protein analysis. Adapted from Wrigley and Bekes (2002).

FACTORS	PAGE	PRE-CAST MICROGELS	RP-HPLC	CE
Prepare gel or column	60 min	1 min	10 min	2 min
Extract sample	20 min	10 min	20 min	20 min
Sample run time	240 min	10 min	30 min	10 min
Protein visualisation	Overnight	10 min	Instant	Instant
Data interpretation	>10 min	>10 min	Instant	Instant
Through-put in 24 hours	20/gel	10/gel	30	100
Health risk to operators	Moderate	Low	Low-medium	Low
Costs – equipment	Low	Low	High	High
Costs – consumables	Low	Medium	Medium	Medium
Costs – labour	High	Moderate	Low	Low

Reports of the use of CE for variety identification have so far involved the use of sophisticated equipment that has been of considerable size and cost, warranting a place in a central or regional laboratory (Table 2). However, CE offers the further promise that it can be moved out of the laboratory with the emergence of small, portable equipment that would provide the ideal combination of convenience, speed of analysis and good resolution, together with portability, and consequently on-site use. **(Recommendation #2c.)** A major company involved in the development and manufacture of this CE equipment is CE Resources, in Singapore. Their main focus has so far been on the analysis of pharmaceuticals, but they are now progressing to protein analysis, and they have expressed the interest in collaborating with the VAW-CRC, together with Dr Lookhart. This further advantage of CE does not appear to exist for parallel methods, such as RP-HPLC, which has the attendant additional of using hazardous and expensive solvents that are not required for CE (Table 3).

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

The use of RP-HPLC is longer established than CE, so it has been endorsed for routine use by the development of some standard methods of analysis. RP-HPLC has been applied to the analysis of gliadin composition, and also of glutenin subunits (Larroque *et al.*, 2001). For this reason, the method has also been used as a research tool for determining aspects of composition that relate to dough properties, such as the presence of specific HMW-glutenin subunits and the ratio of HMW to LMW glutenin subunits. It shares some of the advantages of CE, namely, automatic loading and instant interpretation of results (Table 3). One of the two HPLC units from the QW CRC is in use in the Food Science Laboratory at North Ryde. It is proposed that this method should be studied, in parallel with CE methodology, as an established 'bench-mark', which must be exceeded in CE studies, for CE to be considered worthy of ongoing development.

Mass Spectrometry (MS)

Mass spectrometry is another alternative to be considered in the range of alternatives for analysis of grain-protein composition. MS equipment is very expensive, but 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 38KD. 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.

The role for this approach to variety identification lies clearly in a central laboratory (Table 2). The VAW CRC has direct access to this equipment in its formal agreement with the Australian Proteome Analysis Facility (APAF). It is therefore proposed that MS should be applied to a suitable range of Australian wheat varieties, using the methods already published, to verify these reports with respect to Australian requirements. Arrangements have been agreed for this approach to be tested at the Australian Proteome Analysis Facility at Macquarie University, as part of Project 3.1.3. **(Recommendation #2e.)** Eventually, this approach to the identification of large numbers of samples may warrant the use of robotics for sample handling, thus to reduce the major expense of labour. In the meantime, it is a possible method for application at a central facility. The future holds the (rumoured) prospect of smaller and cheaper MS equipment that may permit the more general use of this approach to variety identification.

IMPLEMENTATION OF RESEARCH FINDINGS

A priority of the project is to conduct trials of procedures, as there is progressive achievement in developing methods of protein analysis suitable for practical implementation. **(Recommendation #5.)**

There are good opportunities for obtaining practical advice as this stage is approached, and then for the actual trials to operate, given the direct involvement of VAW CRC partners, such as GrainCorp and Departments of Agriculture. Advice on implementation will also be available from C-Qentec, the CRC's partner company in diagnostics. Also relevant to

trialling and implementation is the project's association with Nadine Morgan (WA Department of Agriculture), involved in developing practical methods of variety identification to monitor PBR for registered varieties.

QUALITY-TYPE ANALYSIS

Given that a primary aim of variety identification is often to ensure that the grain under analysis is suited to a specific quality class, a satisfactory approach may involve determination of key quality attributes in preference to the identification of variety. This approach promises to provide the important advantage over variety identification that it should take into account the effects of growth environment, in addition to the genetic potential that is indicated by variety.

The application of this approach, using the analysis of protein composition, requires a knowledge of marker proteins whose presence would indicate sufficient information about quality. Progressive advances in cereal chemistry are providing such information, but much more research is still needed. Table 1 lists several candidate proteins that would serve this purpose, together with methods originally devised for their analysis. Notably, the methods involved are suited to the research laboratory, rather than to routine analysis such as would suit any of the scenarios of Table 2. There is thus the need to develop this approach by simplifying the analytical methods.

The most obvious candidate for this approach is the analysis of the subunits of glutenin, which may be provided by routine SDS-PAGE. Nevertheless, the next step of determining the identities of the specific subunits present in the SDS-PAGE patterns is considerably more difficult than merely matching the patterns to assess similarity or otherwise. Nevertheless, this difficulty can be overcome by the development of software programs to determine subunit composition and also to quantify the overall ratio of HMW to LMW subunits.

A broader approach to the identification of marker proteins has been undertaken in parallel with a National Wheat Molecular Marker Program (Cornish *et al.*, 2001), thereby complementing the DNA approach with protein studies. In one of these studies, capillary electrophoresis was applied to the gliadin proteins from 172 doubled-haploid lines from a cross between the varieties Halberd and Cranbrook, which have contrasting dough properties. The results were interpreted with the *PatMatch* program (Siriamornpun *et al.*, 2001d). A high degree of correlation was demonstrated between dough extensibility and the constitution of the gliadins coded by the *Gli-2* loci of the Group-6 chromosomes. There is thus good promise for the production of protein markers by pursuing this approach further. The consequent identification of more quality markers would provide valuable input for the immuno-assay project (1.1.2), provided antibodies of sufficient specificity and avidity can be produced.

The approach of identifying quality type is especially well suited to the identification of varieties of extreme quality type, such as ingredient wheats, where a specific protein (or group of proteins) has been modified to produce the desired result (Table 1). Such is the case with waxy wheats. The approach may also apply to genetically modified wheats, where a marker protein can provide distinction, such as is possible with Roundup Ready canola and StarLink corn, using immuno-assays.

GRAIN SAMPLING AND STATISTICS

Finally, it is important to emphasise that, irrespective of the accuracy of the analytical procedures in use, the reliability of the results is determined firstly by the sampling procedure. It is critically important that the sample taken for analysis is representative of the whole load of grain.

Interactive with the sampling strategy is the need for an assessment of the homogeneity (or otherwise) of the whole consignment of grain. Analysis of a single grain from the consignment is no indication of the identity of the remainder of the consignment, as it may be the only grain of that variety present. Analysis of a milled sample provides a much better indication of identity if only one analysis is to be undertaken initially. However, the result for the milled sample must be examined for the possibility that, in so averaging the composition of many grains, there are multiple patterns for the mix of varieties present.

The only satisfactory approach to the analysis of a heterogeneous consignment is to perform grain-by-grain analysis, which is a tedious and expensive task. The results must then be analysed statistically to determine confidence limits, as described by Wrigley and Batey (1995). For example, two grains of durum wheat identified in a total of 50 grains from a bread wheat sample would indicate confidence limits of 1-13%, even though these two durum grains represent a contamination of only 4% by this analysis. Statistically, this means that the original sample is 95% likely to contain between 1% and 13% durum wheat. Conversely, up to 100 grains must be analysed individually to be 95% sure of detecting one grain present at a level of 5%.

FUNDING OF ONGOING RESEARCH IN PROJECT 1.1.1

Proposals were invited by the GRDC for funding two Post-Doctoral Fellows, with support, for three-year periods. These proposals, submitted in January, 2002, covered the identification of variety and of quality type, involving much of the content of this report. As these proposals appear to have been unsuccessful, funding is requested from the VAW CRC **(Recommendation #6)**, with scope adjusted to the level of support.

POSSIBILITIES FOR ROYALTY GENERATION AND VALUE ADDITION

It cannot be foreseen that this project has the potential to generate royalty income for the VAW CRC in the way that this is expected for the immuno-assay project. Royalties might be negotiated if mass spectroscopy develops as a means for the efficient identification of large numbers of samples. Furthermore, the interaction with CE Resources (Singapore) may provide an opportunity for royalty generation, if the input of the VAW CRC is considered to warrant this. Otherwise, the project is likely to generate methods for the industry to apply in equipment that they would purchase, with profits going to the manufacturers of the equipment.

One possibility for income generation would be to set up a laboratory to conduct variety identification as a service. At least one bulk-handling authority (CBH Perth) expressed a desire to be able to send samples off to a lab for overnight testing rather than conduct their own testing in-house.

Variety identification, as a business venture, would need ongoing research to keep up with changes in varieties, to expand into other cereal species (especially barley), and to evaluate new methods. Such a laboratory should be self-funding for its own research, as well as producing additional revenue for the CRC.

However, most monetary value should be earned by the Australian wheat industry as it takes the opportunity to capitalise on the methods developed, and thereby, to add value to wheat that is produced and processed by ensuring correct quality type and/or variety.

Section B. The Analysis of Protein-Composition: Practice

Project Plans:-

[These plans are based on the original plan for two Post Doctoral Fellows to be provided, with resources. First-priority items are indicated by an asterisk (*).]

A. INFORMATION MANAGEMENT

1. **Assemble data on Australian wheat varieties** (already started in CRC Report #48) * Ditto later for overseas sets if methods are to be marketed internationally.

2. **Choose subsets of varieties with special need** (already started in parallel with Project 1.1.2, interaction with GrainCorp and Nadine Morgan, WA Ag. Soft wheat set analysed for gliadins by CE) *

3. **Up-grade and up-date *WhatWheat* software to manage results** (devised by Batey, and already set up for older Australian varieties) *

4. **Up-grade and up-date *PatMatch* software to sort results** (devised by Bekes, and suited for HPLC) *

5. **Assemble sets of authentic samples** (grains, wheatmeal, flour, bran/germ) *

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** (Adapt existing method. Apply to sub-sets. Sth NSW soft varieties already started, providing good distinctions.) *

2. **Glutenin subunit composition** (Adapt published method. Apply to sub-sets.)

3. **Glu:Gli ratio; HMW:LMW ratio.** (Required initially for quality segregation in breeding initially.) *

4. **Protein markers** (Use existing methods for known markers. Develop improved methods for existing and new markers from proteome research, from the literature and by analysis of NWMMP samples.) *

C. ROUTINE ANALYSIS METHODS

1. **Develop simplified extraction methods** (for best markers, based on research-scale results.) *

2. **Adapt research-scale CE methods to small-scale CE unit** (Collaboration with CE Resources, Singapore.) *

3. **Field-test simplified CE systems** (Interaction with GrainCorp, Ag WA – Nadine Morgan)

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

5. **Trial Gradipore MicroGels** (<60 min total time) *

6. **Trial mass spectroscopy for identification** (Adapt published method, using gliadins, at APAF) *

D. MARKER IDENTIFICATION

1. **Characterize distinguishing peaks from CE and RP-HPLC** (use APAF resources) *

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

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

4. **Determine protein composition of suitable sets of genotypes** (to identify quality markers, such as further sets of National Molecular Marker Wheats)

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APPENDIX 1.
Grain Receival Conditions and Testing in the 2001/02 Harvest

A. Visit to Northern NSW Receival Stations by Ian Batey in November, 2001.

Following the Variety Identification Workshop in August, Jodi MacLean of GrainCorp, Narrabri, issued an invitation for a visit during the harvest to see the conditions under which any variety identification test would be applied. This invitation was accepted and Ian Batey visited the GrainCorp office and several receival sites on November 14.

Sites visited were:

Culgoora – small site, basic facilities and no water
Wee Waa – larger 24-hour site
Burren Junction – 24-hour site
Cryon – 24-hour site
Walgett – 24-hour operation, new site but just a larger old style
Moree – newer style, larger and with better layout to improve efficiency of operation in the shed
Gurley – 24-hour site
Bellata – 24-hour site
Edgeroi – 24-hour site

In addition, the GrainCorp laboratories at Narrabri were visited. This laboratory is both a regional lab for the surrounding districts, and GrainCorp's central facility. Other regional labs are located at Temora, Parkes, Newcastle and Pt Kembla in NSW, and Mooray, Geelong and Portland in Victoria.

Regional labs perform basic quality testing on local samples (labs at export terminals test loads for shipping). The central lab checks samples from regional centres, and also conducts research into test methods. Methods need to be applied to durum, barley and oil seeds, as well as for wheat.

On receival, all testing is done on whole grain. Currently there is no grinding done unless Falling Number is required. Loads are sampled, tested by NIR (protein and moisture), tested for screenings and, if necessary, ground and tested for Falling Number.

There was a mixed response when discussing the potential for an on-the-spot test for variety and/or quality. Most comments indicated that there is already enough to do in the time available, and at one site (Culgoora) the lack of facilities would hamper any further testing. At another site, WheatRite had been trialled in a previous year, and they had experienced problems in making the test work under the ambient conditions. The operator at one site,

however, was keen on the idea as he had just sent a sample away to Narrabri for variety testing, having felt that its visual appearance did not quite match what he was used to for that particular variety. A quick test may have solved his problem on the spot, and prevented potential contamination of a grade by an unsuitable variety.

All samples for variety testing are presently sent away to Werribee. Jodi thought that it was unlikely that they would set up facilities in Narrabri in the short to medium term. In general, compliance was good, and most samples tested were indeed the stated variety. Samples are tested at random (to monitor compliance), when requested by AWB or when there is reason to suspect a mis-declaration of variety. GrainCorp is aware of the likely need for testing to determine end use royalties but have yet to determine how this will be done.

In conclusion, it is hard to see on-the-spot testing being acceptable. This is not so much an issue of speed as one of limitations in available facilities and operator skills. In terms of speed, the sample stand is NOT the limiting factor. The queue for dumping into the appropriate stack was usually longer than that at the sample stand.

Ian Batey

B. Visit to Agrifood Technology, Werribee, Vic., by Colin Wrigley in March, 2002

Colin Wrigley visited Agrifood Technology, Werribee, on March 19th, to discuss the needs of AWB Ltd for variety and quality-type identification. The staff members interviewed were open and eager to discuss methods and interpretation, with questions about principles (e.g. biotypes, sources of standard samples, interpretation and stats) and about practical problems with gels (follow-up with Gradipore has proved useful). They were keen for faster run times, such as we would get with the Micro Gels, and they would appreciate it if we would evaluate Gradipore's Micro Gels and report back to them. They were also interested in the possibilities offered by CE analysis.

During the three months after harvest each year, they receive about 4,500 'audit' samples of wheat for variety identification. They normally crush one grain (between pliers) from each sample for gliadin composition by acidic PAGE. (This presents statistical problems, compared to using a ground sample.) If more detail is needed, they analyse 30 individual grains by gel electrophoresis of gliadins at pH 3. Although all this work comes in over three months, they have a turn-around time of only three days for each sample - so they are still pressed for quick results. They charge about $30 per sample, employing casuals to cope with the extra workload.

They indicated that it would be useful if they could apply identification tests to leaf or to immature grain - using samples taken pre-harvest - to verify identity before actual harvest.

Colin Wrigley

APPENDIX 2. Canadian Research on Variety Identification

Overseas Trip by Colin Wrigley, May, 2001.
Extract from report relevant to variety identification

1. Itinerary and general activities

Visits in Winnipeg to ...

- Grain Research Laboratory, 303 Main Street, Winnipeg, MB

Discussions with Bill Scowcroft, Ken Preston, Susan Stevenson, and other staff, covering their routine and planned methods of variety identification, protein analysis, and other diagnostics testing.

- Agriculture and Agri-Food Canada, (U of Man campus), Dafoe Road, Winnipeg, MB

Discussions with about 12 staff on the range of their cereal research activities, including plans for Surjani's Post-Doc year with them. Main contact was Nancy Ames, in the absence of Odean Lukow. Interaction on plans for Z-arm mixer to be delivered to them.

- Department of Food Science, University of Manitoba, Winnipeg, MB

I was asked to lead a seminar on the nature of baking quality and dough properties with post-graduate staff.
Discussions with Walter Bushuk in planning for 'Bushuk Symposium' to be held in association with AACC meeting, 13 October, 2001. Discussions with Harry Sapirstein on diagnostics, using image analysis.

2. Specific comments, involving diagnostic methods

Variety identification by gel electrophoresis

Routine variety identification is still performed by acidic polyacrylamide gel electrophoresis at the Grain Research Laboratory (GRL). This mainly involves wheat and barley. They make their own gels, which take 30 samples each. They run 60 single grains per grain sample (!!), to get indications of varietal purity. They reckon in this way to detect any contaminant at >5%. They use visual inspection of the gel patterns, having only about two dozen varieties to deal with. They claim that methods of scanning and image analysis are not worth the trouble.

Variety identification by Mass Spectroscopy (MS)

Ken Preston (GRL) has demonstrated that he can use MS of gliadins to distinguish among all of 16 Canadian wheat varieties (all grades represented; grown at 15 sites each). He interacts with the Department of Physics at the University of Manitoba, using MALDI TOF laser, 3 channel, in the range 30 to 38KD. Analysis takes 2 minutes per sample manually, or 15 seconds per sample if automated. The method requires only 10^{-16} mole of sample. He claims that it is likely to be a method of the future in a central lab with robotics for sample handling. He also says that small/cheap equipment is coming for MS, costing only about US$30,000. He has also experimented with MS of tryptic digests of grain proteins. This brings him close to our proteome studies, which impressed him.

See his recent papers in ...

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Variety identification by DNA analysis

Douglas Procunier and Mark Sage (Agriculture and Agri-Food Canada) demonstrated their system for varietal identification of multiple samples based on DNA analysis. They use capillary electrophoresis of SNP markers (single-base changes) in ABI equipment that runs 16 samples simultaneously. It is auto-loading with the potential to handle 1,000 to 6,000 samples per 24 hours. They reckon that it would be suited to a central lab, fed by a robotics system for sample handling. Most impressive was their DNA chip system that is under development. They claimed that the SNP method is so sensitive that it is capable of detecting as little as a 1% contaminant in a ground-grain sample. This is a very important feature (if proven to be true) as it avoids the procedure now in operation at GRL of separate analysis of 60 single seeds to establish varietal purity.