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PROJECT REPORT

Identification of Wheat Varieties and Glutenin Subunits:
Capillary Electrophoresis and Computer-Assisted Interpretation of Results
(Report for FIG Project)

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VAW CRC Project Report

Identification of Wheat Varieties and Glutenin Subunits:
Capillary Electrophoresis and Computer-Assisted Interpretation of Results

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SUMMARY
This manual provides detailed instructions on how to analyse grain-protein to determine wheat variety and glutenin-subunit composition (quantitatively) by capillary electrophoresis, using either the Agilent Lab-on-a-chip or the Beckman MDQ equipment. In addition, software is described to provide automatic interpretation of results.

CONTENTS

INTRODUCTION
Declaration of variety has long been an integral part of the grading of Australian wheat for quality purposes, due to the emphasis in breeding on selecting new varieties for appropriate quality attributes. More recently, Plant Breeders' Rights provide a further emphasis on the need for more efficient methods of verifying the identity of grain at receival (Cornish et al., 2005). Ideally, this analysis is performed before the grain is binned, to ensure that a premium grade is not contaminated with an incorrectly declared load.

A new opportunity for obtaining variety information quickly and beyond the laboratory is now provided by the Lab-on-a-chip concept in equipment for analysing protein composition (Figure 1). The procedure provides the protein composition of a grain extract in a one-minute analysis, giving a digital output that is ready for matching against a library of patterns for authentic variety samples. The heart of the equipment is the "chip", about 5 cm square, which accepts ten sample extracts plus reagents. Although each analysis is very fast, it takes about an hour for the grinding, extraction and analysis of a 'chip-full' of ten samples (Uthayakumaran et al., 2004a, 2004b, 2005).

The Lab-on-a-chip system uses the capillary electrophoresis principle for analysing protein composition. The full set of polypeptides is used for variety identification, using a single extraction with a combination of detergent (sodium dodecyl sulfate, SDS) and dithiothreitol (DTT), which breaks the inter-chain disulfide bonds of glutenin. Alternatively, a more complex extraction procedure involves prior removal of non-glutenin proteins, so that only glutenin-subunit composition is provided, both subunits of high- and low-molecular-weight (HMW and LMW, respectively). This latter information is a basis for predicting dough properties, using the rankings of the respective subunits. The Lab-on-a-chip system is especially suited to on-the-spot applications, such as the regional laboratory, or even for use at the grain-receival station.
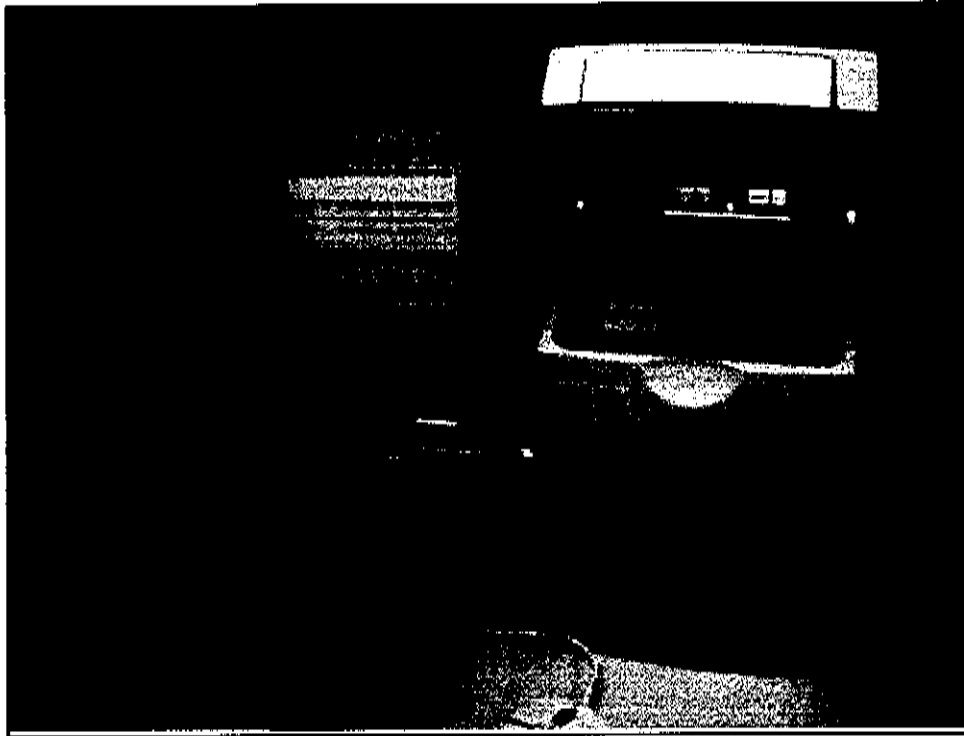
Figure 1. The Agilent 2100 Bioanalyzer. The analysis module
is shown at right. The chip appears at front right of the module.

Capillary electrophoresis is also used in the Beckman MDQ equipment, which offers the distinct advantage of automatic loading of many samples tp permit continuous analysis, even over-night. Capillary electrophoresis in the Beckman MDQ is charge-based, whereas, the Lab-on-a-chip system uses size-based capillary electrophoresis. The variety identification procedure for the Beckman MDQ is based on gliadin composition. The Beckman MDQ procedure for glutenin-subunit composition also provides charge-based fractionation. The Beckman equipment is suited to a central laboratory, taking advantage of the automatic loading facility.

Both types of equipment provide data-output suited to automatic (immediate) interpretation. The procedures for doing so, and for pre-calibration, are described.

A. USE OF THE AGILENT 2100 LAB-ON-A-CHIP BIOANALYZER FOR VARIETY IDENTIFICATION

The Lab-on-a-chip equipment appears to provide the best system so far developed to provide on-the-spot identification of wheat variety with objective interpretation of results, with the ability to distinguish between most Australian wheat varieties. This report also describes the ancillary equipment needed for performing Lab-on-a-chip analysis of variety (also of glutenin-subunit composition) in a setting such as the receival station. Electricity (240 volts) is required for the operation, but no other utilities are necessary.

MATERIALS AND EQUIPMENT
Sources of authentic samples
Authentic grain samples are essential as a basis for performing analyses to provide standard profiles of protein composition to go into the computer program for comparison with profiles from samples being tested. Preferably, these authentic samples should come from the original breeder sample, lodged with the Australian Winter Cereals Collection in Tamworth, NSW. A source of secondary standards is the foundation seed provided for seed propagation.

Requirements for sample extraction

- <u>Grinder</u> – any simple type is suitable (electrically operated), preferably taking a small-sized sample of grain (say, 10 – 50 grams). Fine grinding is not essential.
- <u>Balance</u> – capable of weighing about 30 mg ground grain into a sample tube.
- <u>Extraction reagent</u> – 1% sodium dodecyl sulfate (SDS) together with 1% dithio threitol (DTT). A stock solution of 1% SDS can be kept almost indefinitely at room temperature (1.0 gram SDS in water – distilled or demineralised – made to 100 mL). A fresh reagent should be made from this daily, by adding 100mg DTT to 10 mL of the 1% SDS solution.
- <u>Vortex mixer</u> or a <u>Thermomixer</u> – useful for quickly suspending the wheatmeal in the extraction solution. It is not essential; mixing can be done by hand shaking.
- <u>Centrifuge and sample tubes</u> – generally a multi-place small bench-top model that takes "Eppendorf-type" plastic tubes.
- <u>Water bath</u> – to heat extracts + Agilent reagents at 100 °C for a few minutes
- <u>Pipettes</u> – capable of dispensing up to 1 mL of extraction solution. Preparation of extracts for the Lab-chip requires the use of an automatic pipette that can dispense about 20 µL.
- <u>Lab-on-a-chip equipment</u> – Agilent bioanalyzer model 2100, illustrated in Figure 1. (Recently, alternative equipment has become available, marketed by BioRad, USA.) The Agilent equipment has a "footprint" on the bench of 15 X 40 cm. A laptop computer is placed beside it, but this could alternatively be some other form of computer.
- <u>Protein chips and reagents</u> – Agilent provides a packet of 25 LabChips (type Protein 200+), together with reagents.

Likely costs (in Australian dollars)

- <u>Grinder</u> (Mortar and pestle) – $30
- <u>Balance</u> – $2,700
- <u>Extraction reagent</u> – sodium dodecyl sulfate - $170   Dithio threitol - $170
- <u>Vortex mixer</u> – $320
- <u>Thermomixer</u> - $3,000
- <u>Centrifuge and sample tubes</u> – $2,200
- <u>Water bath</u> – $1,055
- <u>Pipettes</u> – to dispense extraction solution (1,000 µL) and automatic pipette (20 µL) to prepare extracts for the Lab-chip with relevant pipette tips and boxes - $800
- <u>Lab-on-a-chip equipment</u> – Agilent bioanalyzer model 2100 - $39,000
- <u>Protein chips</u> – packet of 25 LabChips + reagents is $800-$900, equivalent to 2,500 tests
- <u>Other relevant items</u> (Beakers (10, 100 and 250 mL), Timer, Spatula) - $50

PROCEDURE

1. **Grind** a sample of grain, noting identification details for grain and wholemeal containers. The procedure is equally valid for flour samples. Alternatively, it may be necessary to crush and extract single grains individually – see comments below on interpreting results for samples containing a mixture of varieties. It is usual to include one or a few authentic samples relevant to those being tested.

2. **Weigh** out 20 ± 2 mg into a small plastic tube. Add 0.3 mL extraction solution (1% SDS + 1% DTT), and agitate vigorously, preferably by vortex mixing, for a few seconds. Shake by hand occasionally during 3 minutes' standing in a heating bath at 65°C or use the thermomixer at 65°C. The actual weights and volumes are important only to ensure that there is a constant ratio of wheatmeal to extracting solution, namely, 20 mg + 0.3 mL. It is usual to preform extractions in multiples of ten, to suit the ten-place capacity of the lab-chips.

3. **Centrifuge** the tubes, to provide a clear supernatant for application to the LabChip. In the absence of a centrifuge, it may be possible to stand the tubes for about 10 minutes, to allow the wheatmeal particles to settle. However, this is a less desirable option.

4. **Mix** 4 μL of clarified extract with 2 μL of Agilent sample buffer, and heat for 3 or 4 minutes at 100°C in a boiling water bath. Add 84 μL water to each, mix, and load extracts into the respective sample positions of the LabChip, recording sample identities. Also load the "protein ladder" reagent, mixed with Agilent sample buffer as specified by the Agilent manual.

5. **Switch on** the equipment and control running conditions from the software.

6. **Interpret** the results of identification using the PatMatch program. Some typical results are shown in Figure 2 (showing the elution profiles) and Figure 3 (with profiles altered to simulated gel-electrophoresis patterns, which may be more suitable for comparison purposes).
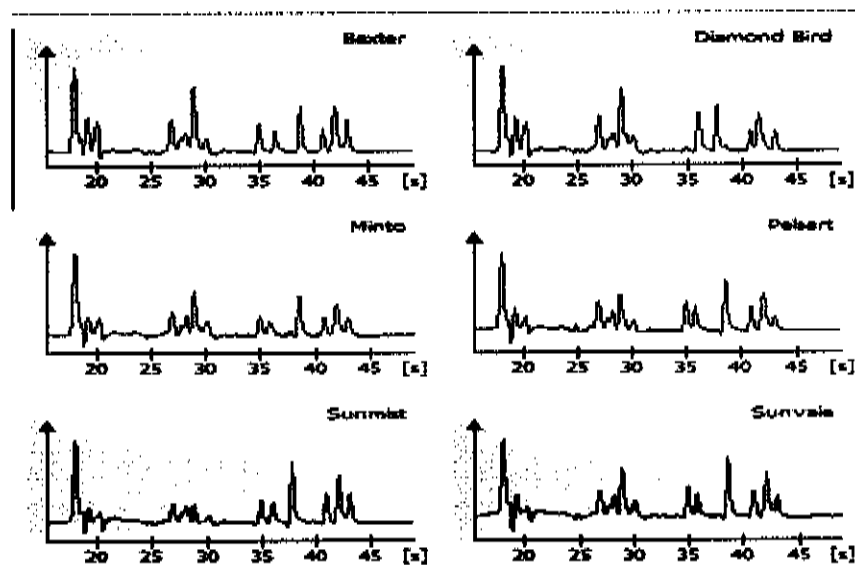

Figure 2. Elution profiles from Lab-on-a-chip analysis of SDS-DTT extracts of wheatmeal samples for six Australian varieties. The horizontal axis shows seconds of elution rate.

INTERPRETATION OF RESULTS

Various questions may be asked in relation to interpreting the identification results. These form the basis of developing the most appropriate strategy for conducting identification, depending on the details of the situation.

1. Is the sample true-to-declaration? This is the most common question. It generally involves visual examination of the pattern for the test sample, comparing it with the authentic pattern of the declared variety (preferably analysed beside the test sample). It is thus evident whether or not they are essentially similar. Differences may be due to incorrect declaration of variety (see item 3 below) or to significant admixture with other varieties (see item 4, below).

The use of PatMatch is valuable in this case, to provide a quantitative estimate of degree of difference. When the tested and authentic samples are compared, a similarity value of less than 80% would provide evidence that the sample is questionable (if not in identity at least in purity of composition).
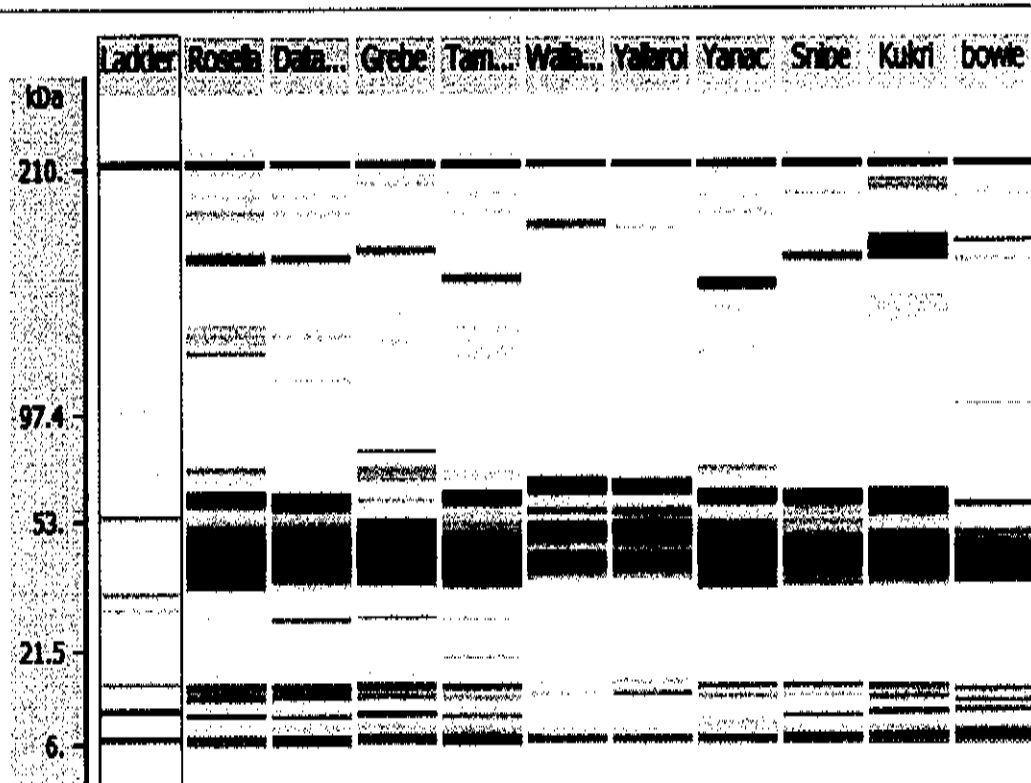
Figure 3. Simulated gel-electrophoresis patterns from Lab-on-a-chip analysis of SDS-DTT extracts of wheatmeal samples for ten Australian varieties. The HMW subunits of glutenin appear at the top of the patterns. The pattern at the extreme left is the "protein ladder", providing a calibration of apparent molecular weights for sample proteins.
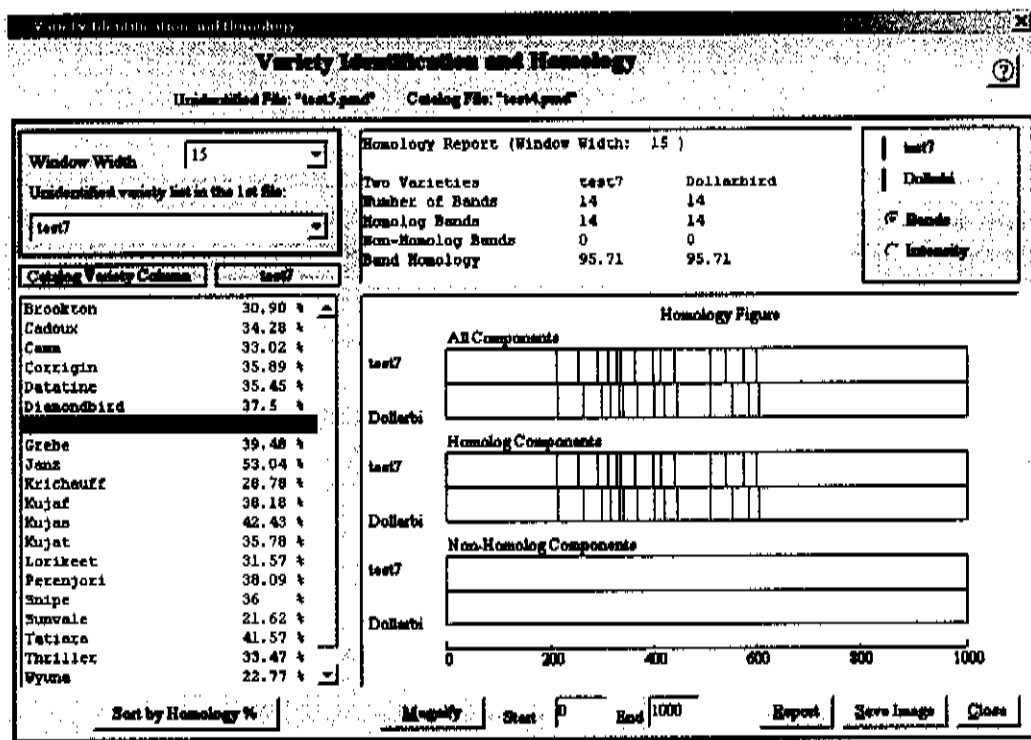

Figure 4. PatMatch computer screen from comparing a test sample with the library of Australian varieties. The list at left shows the similarities (as %) of the test sample to authentic samples of the most similar varieties. These results indicate that the test sample is Dollarbird.

There is no assurance that the procedure will provide distinctive profiles to distinguish clearly between all varieties that may be presented, even though a survey of over forty commonly grown varieties could be distinguished. It is thus possible that a different procedure of identification may be needed for some combinations of varieties.

2. Is it this or that variety? A common question arises when a grain sample has become confused between one or a few varieties, e.g., rain washed the chalked name off the seed bin. This case is similar to that considered above (item 1). It should be resolved by side-by-side analysis of the test sample with authentic samples of the suspected varieties.

3. What is it? This question arises especially if the result of item 1 above is that the sample has been declared incorrectly. The question is difficult to answer if there is no background information available to suggest what authentic samples should be analysed beside the test sample. Resolution of this question is the most valuable feature of the PatMatch program, but even so there is the limitation of the range of profiles of authentic samples that are contained in the PatMatch library.

Interrogation of PatMatch provides the screen shown in Figure 4. At left, there is the result that the test sample showed a similarity of 96% to Dollarbird, and progressively less similarity to a range of other varieties. This analysis assumes that the test sample is homogeneous, and not a mixture of varieties. The absence of a close fairly match may indicate that the test sample is heterogeneous (unless, of course, it was a single grain). Alternatively, the test sample may be a variety that is not represented in the library of varieties provided in PatMatch.

4. Is it pure/homogeneous? Analysis of heterogeneous samples is very difficult by any method of identification. The main approach to resolving this question is the analysis of the sample one grain at a time. This tedious procedure is only worthwhile if there is a proper appreciation of the statistical implications. Table 1 illustrates the poor statistical value of analysing only a few single grains. For example, if only five grains were analysed from a sample, and if one of these was found to be different in identity, the confidence limits for this apparent 20% contamination are 0% to 72%, that is, this different grain may be the only one in the sample, or there is a 95% probability that it represents a contamination level of less than 72%. Such estimates involve reference to tables of confidence limits (e.g., Lentner, 1982).

Table 1. Statistics of single-grain analysis, shown as confidence limits using a 95% probably level

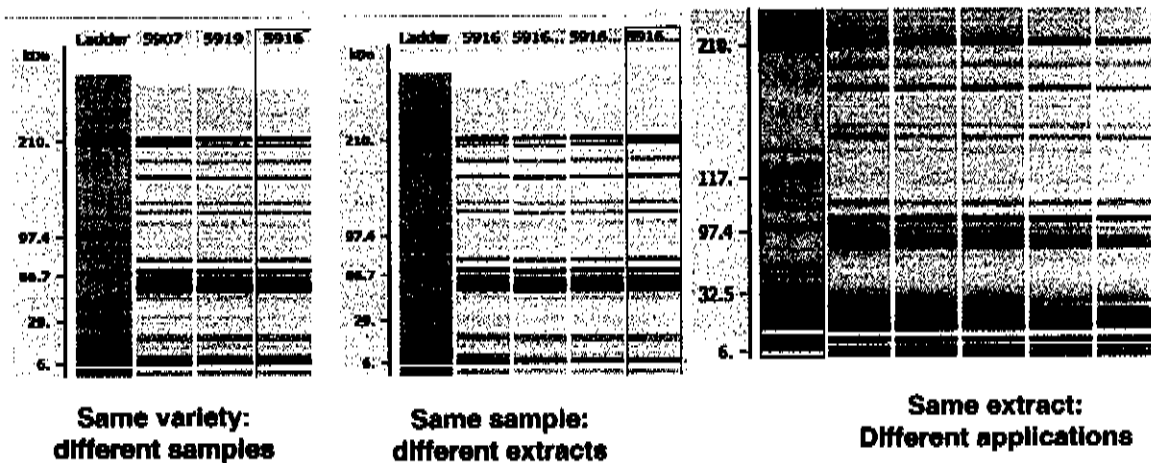| Proportion found (%)	Confidence limits (%), depending on number of grains examined				
	20 Grains	50 Grains	100 Grains	200 Grains	500 Grains
1	–	–	0 – 5	0 – 3	0 – 2
2	–	0 – 10	0 – 7	0 – 4	1 – 3
3	–	–	1 – 8	1 – 5	2 – 4
4	–	1 – 13	1 – 10	2 – 7	2 – 6
· 5	0 – 25	–	2 – 11	2 – 9	3 – 7
6 ·	–	1 – 16	2 – 12	3 – 10	4 – 8
8	–	2 – 19	3 – 13	4 – 12	6 – 10
10	1 – 32	3 – 22	5 – 17	6 – 14	7 – 13
20	6 – 44	10 – 34	13 – 29	15 – 25	17 – 23
30	12 – 54	18 – 45	21 – 40	24 – 36	26 – 34
40	19 – 64	26 – 55	30 – 50	33 – 47	36 – 44
50	27 – 73	35 – 65	40 – 60	43 – 57	46 – 54

Figure 5. Representations of patterns produced to evaluate the reproducibility of Lab-on-a-chip analysis of SDS-DTT extracts of wheatmeal (variety Rosella, in this case).

REPRODUCIBILITY OF RESULTS

What level of similarity is due to lack of reproducibility or to differences in season or growth conditions? Results from the Lab-on-a-chip have proved to be very good in its laboratory use. (Deployment beyond the laboratory is still being assessed for all aspects of reliability.) The similarities of patterns are shown in Figure 5 for the same extract analysed many times, and also for different extracts of the same sample with repeated analyses. The analysis of protein composition is general has been found to be very reliable for the purposes of variety identification, as the positions are stable for the protein bands (shown in Figure 3) or for peaks in the elution profiles (of Figure 2).

Differences in the protein content of the samples are likely to alter the overall intensity of the patterns, but not the overall arrangement of the bands (peaks). For this reason, it may even be necessary to increase the sample-to-extractant ratio for low-protein samples (and reduce it for high-protein samples). Variations in band intensity (or in peak height) may occur to a minor extent, due to fluctuations in growth conditions. For this reason, the PatMatch program is designed to concentrate mainly on the positions of the bands, taking intensities into account as a secondary feature of the patterns.

THE PROBLEM OF BIOTYPES WITHIN A VARIETY

Some varieties are represented by more than one pattern of bands; i.e., different grains from an authentic sample give two (or even more) different patterns, each being truly representative of the variety. This occurs because the breeder may have intentionally combined a few distinct lines from the same cross, these lines being indistinguishable at the time of doing so. 'Biotypes' is the term used to describe these lines that are distinguishable by a method such as the analysis of protein composition.

The presence of such biotypes in a variety obviously complicates the task of identification. It means that the analysis of a wheatmeal or flour sample provides a combination of the patterns for the biotypes, each being represented in the proportion that it is present in the sample. If these proportions are constant for the variety, then the resulting pattern is used to represent the variety for comparisons involving the analysis of ground samples. For single-grain analyses, it is essential use authentic samples of the individual biotypes.

The prevalence of multiple biotypes within Australian wheat varieties depends on the procedures used for identification. The risk of biotype complications is greater for methods that provide greater degrees of discrimination. For the Lab-on-a-chip method, biotypes have been detected in some varieties. Naming is sometimes handled by adding a letter (A, B, ...) after the variety name for each biotype.

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B. USE OF THE AGILENT 2100 LAB-ON-A-CHIP BIOANALYZER FOR ANALYSIS OF GLUTENIN-SUBUNIT COMPOSITION

The procedure described above provides the patterns for the high-molecular-weight glutenin subunits (HMW-GS) in the top of the simulated gel patterns (Figure 3) and also in the right third of the elution profiles (Figure 2). The composition of these proteins provides a valuable basis for predicting the genetic aspects of dough strength. It may thus be worthwhile to determine HMW-subunit composition, in addition to variety. Of course, if the variety is known, then the HMW-subunit composition is also known, by reference to tables of quality-related gene, such as those provided by the Wheat CRC (Wrigley et al., 2001; Cornish et al., 2002; Cornish et al., 2005). Testing of breeders' lines is a major application of analysing HMW-subunit composition.

Ideally, analysis of glutenin-subunit composition should include the low-molecular-weight (LMW) subunits as well as the HMW subunits. In the patterns for the SDS-DTT extracts (Figures 2 and 3), the LMW glutenin subunits (LMW-GS) are masked by gliadin proteins, so it is necessary to perform a preliminary extraction to remove gliadins prior to extracting glutenin subunits. The procedure of double extraction is described below. It produces patterns such as those shown in Figure 6. The HMW subunits (in the upper half of the patterns) have an agreed numbering system, but this is not so for the LMW subunits (in the lower half of the patterns). The LMW subunits are indicated only as alleles (by letters – a, b, c, etc), each letter indicating a group of protein bands.

PROCEDURE FOR GLUTENIN-SUBUNIT ANALYSIS

The procedure is the same as for variety identification (above), except that the extraction procedure involves the added step of preliminary removal of gliadin proteins with dimethyl sulfoxide (DMSO) as follows.

1. **Grind** a sample of grain. Alternatively, use a flour sample.

2. **Weigh** out 20 ± 2 mg into a small plastic tube. Add 1 mL dimethyl sulfoxide (DMSO). Mix immediately by vortexing.

3. **Centrifuge** the tubes and discard the supernatant. Repeat the extraction with 1mL 50% aqueous propan-1-ol.

4. **Add** 0.3 mL extraction solution (1% SDS + 1% DTT), and agitate vigorously, preferably by vortex mixing, for a few seconds. Shake by hand occasionally during 3 minutes' standing in a heating bath at 65°C or use a thermomixer. Centrifuge, to provide clear supernatants.

5. **Mix** 4 µL of clarified extract with 2 µL of Agilent sample buffer, and heat for 3 or 4 minutes at 100°C in a boiling water bath. Add 84 µL water to each, mix, and load extracts into the respective sample positions of the LabChip, recording sample identities. Also load the "protein ladder" reagent, mixed with Agilent sample buffer as specified by the Agilent manual.

6. **Switch on** the equipment and control running conditions from the software.

7. **Interpret** the results of identification using the PatMatch program. Some typical results are shown in Figure 6 simulated as gel-electrophoresis patterns. The PatMatch program can provide identification of the HMW-subunit numbers and also estimates of their quantities individually on the basis of peak areas.

The inclusion of authentic samples and multi-null lines lacking specific glutenin subunits has enabled us to assign numbers to the protein bands obtained by the Lab-on-a-chip technique. We have now identified all the common HMW-GS present in wheat and this is given in Figure 6. The apparent molecular sizes of these HMW-GS obtained on the lab-on-a-chip is given in Table 2.

Table 2. Apparent sizes of these HMW-GS obtained by Lab-on-a-chip analysis

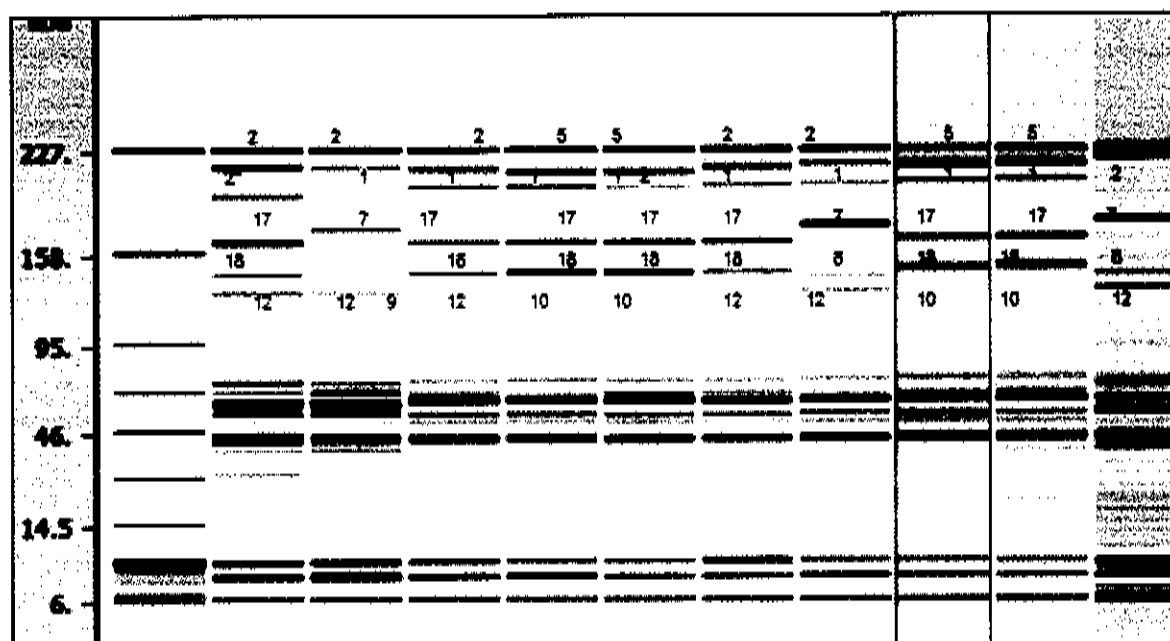| HMW-GS	Apparent size, kDa
Glu-A1	
1	199
2*	193
Glu-B1	
7	170
8	138.5
9	130
17	162
18	141
13	175
16	149
20a	161
20b	151
Glu-D1	
2	212
12	128
5	210
10	144

Figure 6. Lab-on-a-chip capillary electrophoresis of glutenin subunits (as simulated gel-electrophoresis patterns) from two known and eight unknown samples. The HMW subunits are numbered. The LMW subunits appear in the lower half of the patterns. The varieties shown are (left to right): The known varieties are Arrino (lane 2) and Tatiara (lane 3). Lane 1 is the ladder of proteins of known molecular weight (indicated at extreme left).

In certain varieties, subunit 7 is been recognised as been over-expressed, e.g., in Chara, compared to Janz (Figure 7). Its higher quantity makes a significant difference in its contribution to dough strength. It is thus important to recognise the over-expressed version of subunit 7, but this may not be readily observed by conventional gel electrophoresis. The quantitative analysis capability of the Lab-on-a-chip system is valuable to quantify the band/peak representing subunit 7, and thus detect over-expressed version of it (Figure 7).
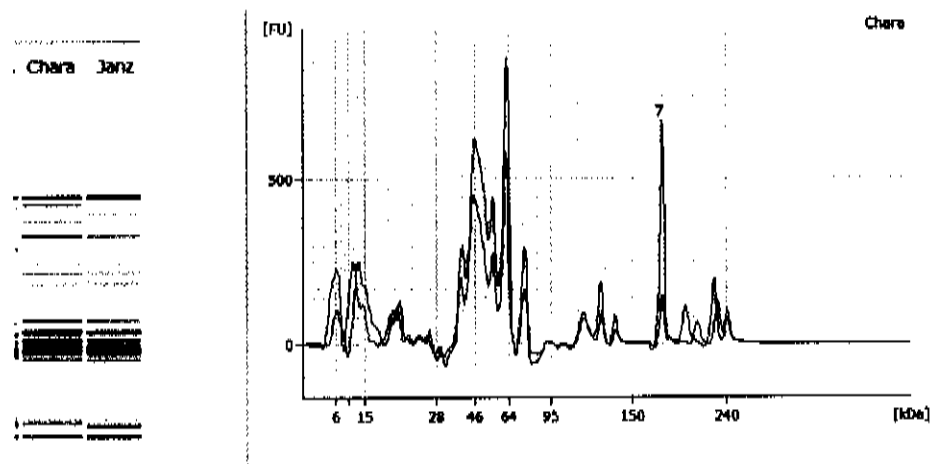

Figure 7. Glutenin-subunit patterns; the gel-simulation patterns appear at left, and the elution profiles on the right. (HMW-GS, upper half of simulated gel patterns, and LMW-GS) contrasting the over-expressed subunit 7 (left, Chara) with the normal subunit 7 (Janz). The area calculated for over-expression by the software is 4 times as much as the normal. Subunit 7 is indicated in the elution profiles, with that of Chara being the one with the very high peak designated "7".

Some of the most common LMW-GS patterns have been identified and these are indicated in the lower half of Figure 8.

Figure 8. Simulated gel-electrophoresis patterns from Lab-on-a-chip analysis of LMW-GS, appearing in the lower half of the patterns, under the HMS subunits. From the left (after the ladder of molecular weight markers), the LMW alleles are in the order of *Glu-3A, Glu-3B and Glu-3D*.

PREDICTION OF DOUGH STRENGTH USING GLUTENIN-SUBUNIT COMPOSITION

For a few decades now, genetic potential for dough properties has been estimated from HMW glutenin subunit composition using the Payne score (Table 3), which allocates scores for the respective subunits (alleles) in each of wheat's three genomes. Further details are provided in Cornish et al. (2005a, 2005b). Prediction of the actual dough properties for specific samples, considering the contributions of both genetic potential and growth conditions, now becomes a possibility with the added features of quantitative analysis of HMW subunits, and also the identification of LMW-subunit composition. This type of approach is outlined Eagles et al. (2002) and by Cornish et al. (2005b). It involves mathematically modelling the dough properties of many wheat samples, using the actual amounts of the glutenin subunits, plus estimates of other aspects of protein composition, such as glutenin:gliadin ratio. The prospect of direct input of the amounts of glutenin subunits, via Lab-on-a-chip analysis, takes this approach to a new level.

Table 3. Scores assigned to individual (or pairs of) HMW glutenin subunits

Score	Chromosome 1A	Chromosome 1B	Chromosome 1D
4	-	-	5+10
3	1; 2*	17+18; 7+8; 7+9[a]; 13+16; 14+15[b]	-
2	-	-	2+12; 3+12
1	Null	7; 6+8; 20[b]	4+12; 2.2+12[c]

Adapted from Cornish et al. (2005b). Subunit scores were deduced by genetic analysis and quoted by Payne et al (1987), except: [a] this subunit combination was upgraded from 2 to 3 after analysing varietal sets; [b] allocated subunits after observing partial *Glu-1* scores of varieties with these non-analyzed subunits and their mixing properties; [c] from Takata (2003)

C. USE OF THE BECKMAN MDQ FOR VARIETY IDENTIFICATION

The first research publications on capillary electrophoresis of wheat proteins appeared in the mid-1990s (e.g., Lookhart and Bean, 1995; Bean and Lookhart, 2000), mainly involving charge-based separations. The first Australian use of capillary electrophoresis for wheat-grain proteins involved the BioRad BioFocus 3000 equipment (Siriamornpun et al., 2001), demonstrating that charge-based capillary electrophoresis of gliadin composition could distinguish 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).

Figure 9. The Beckman Coulter P/ACE™ MDQ Series for capillary electrophoresis.

American use of capillary electrophoresis for wheat proteins involved the Beckman Coulter P/ACE™ MDQ Series (Figure 9). This equipment, installed at Food Science Australia in October 2002, provided the first opportunity of testing the Beckman-CE method for wheat proteins in Australia. The preferred method of variety identification with the Beckman has been charge-based capillary electrophoresis of gliadin proteins, thus following the tradition of using the gliadins for identification using gel electrophoresis (Uthayakumaran et al., 2004a).

MATERIALS AND EQUIPMENT

Many of the method details in this section are similar to those for the Agilent Lab-on-a-chip section, except that variety identification recommended for the Beckman involves extraction of gladin proteins.

Sources of authentic samples

Authentic grain samples are essential as a basis for performing analyses to provide standard profiles of protein composition to go into the computer program for comparison with profiles from samples being tested. Preferably, these authentic samples should come from the original breeder sample, lodged with the Australian Winter Cereals Collection in Tamworth, NSW. A source of secondary standards is the foundation seed provided for seed propagation.

Requirements for sample extraction

- Grinder – any simple type is suitable (electrically operated), preferably taking a small-sized sample of grain (say, 10 – 50 grams). Fine grinding is not essential.
- Balance – capable of weighing about 30 mg ground grain into a sample tube.
- Extraction reagent – 30% propan-1-ol (30mL of propan-1-ol in water – distilled or demineralised – made to 100 mL).
- Vortex mixer or a Thermomixer – useful for quickly suspending the wheatmeal in the extraction solution. It is not essential; mixing can be done by hand shaking.
- Centrifuge and sample tubes – generally a multi-place small bench-top model that takes "Eppendorf-type" plastic tubes.
- Pipettes – capable of dispensing up to 1 mL of extraction solution.
- Beckman MDQ equipment and computer
- 50µm i.d. uncoated capillary and vials – Can be bought from beckman.
- Internal marker – Carbonic anhydrase, 1.5mg dissolved in 1 mL 30% propan-1-ol.

PROCEDURE

1. Grind a sample of grain, noting identification details for grain and wholemeal containers. The procedure is equally valid for flour samples. Alternatively, it may be necessary to crush and extract single grains individually – see comments below on interpreting results for samples containing a mixture of varieties. It is usual to include one or a few authentic samples relevant to those being tested.

2. Weigh out 100 ± 2 mg into a small plastic tube. Add 1.0 mL Propan-1-ol), and agitate vigorously, preferably by vortex mixing, for 10 seconds.

3. Add internal marker (30 µL of carbonic anhydrase solution)

4. Centrifuge the tubes, to provide a clear supernatant for application to the LabChip.

5. Filter sample into the sample vial (0.5 mL vial) using a 0.45 µm filter.

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 20 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 (given

below). 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 .

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.

Separation conditions

Optimum separation conditions established for 0.1 M phosphate running buffer is 17 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.

Interpret the results of identification using the PatMatch program. Some typical results are shown in Figure 10 as elution profiles. PatMatch can be used to provide identification of varieties from MDQ gliadin profiles, based on a library of profiles for authentic samples. See comments on interpretation of variety identification following the section on Lab-on-a-chip analysis of variety.
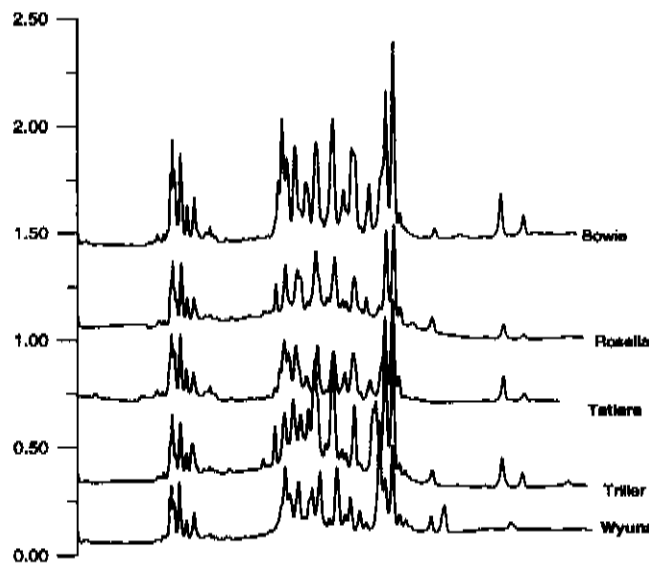

Figure 10. Capillary electrophoresis profiles for gliadin proteins from Australian wheat varieties.

Gliadin fractionation in the he Beckman MDQ has the advantage of providing reasonably fast analysis times, automatic loading, permitting 24-hour operation, instant visualisation of results as a profile of peaks, and thus automatic data capture, providing the potential for automatic interpretation. As for capillary electrophoresis, it uses environmentally 'friendly' reagents, so that there are no significant safety or environmental problems. Operating costs are low; one capillary can be used many times (about 50), and the cost of the capillaries for the method developed is small (about $400 for a 5M roll).

However, the Beckman MDQ equipment is expensive, about Aust $110,000, and only one sample is analysed at a time, restricting the throughput of the method, compared to gel electrophoresis for which many samples are analysed concurrently (but at a slower rate). A significant disadvantage is that reproducibility has been found to be poorer than for the Lab-on-a-chip version of capillary electrophoresis. It has proved more difficult with the MDQ in charge-based mode to provide a range of internal markers to assist in enhancing reproducibility.

D. USE OF THE BECKMAN MDQ FOR ANALYSIS OF HIGH-MOLECULAR-WEIGHT GLUTENIN-SUBUNIT COMPOSITION

The following procedure provides a charge-based separation for the high-molecular-weight (HMW) subunits. Since high- and low-molecular-weight glutenin subunits have the same range of charge, they overlap in mobility if both groups are analysed together by charged-based capillary electropjoresis. Therefore, it is necessary to pre-fractionate HMW subunits from LMW subunits, so that they can be analysed separately (Figure 11).

Size-based separation of high- and low-molecular-weight glutenin subunits is possible in a Beckman capillary electrophoresis. However, this approach has a few disadvantages, such as the need for special buffers (expensive) and for a special type of coated capillaries (also expensive). In addition, the time taken for analysis is more than 45 minutes. Hence, we have developed the charge-based separation of glutenin subunits. Carbonic anhydrase has been used as an internal marker to compare mobilities between samples.

Figure 11. Charge-based separation of high- and low-molecular-weight glutenin subunits in the Beckman MDQ. Top - combined, middle - LMW subunits, bottom – HMW subunits.

Sample preparation and analysis

The procedure is the same as for variety identification in the MDQ (above), except that the extraction procedure involves the added step of preliminary removal of gliadin proteins with dimethyl sulfoxide (DMSO) as follows.

1. **Grind** a sample of grain. Alternatively, use a flour sample.

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

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

4. **Vortex** the sample for 10 sec.

5. **Centrifuge** for 10 min at about 14,000 rpm and discard supernatant. Repeat this preliminary extraction two times (a total of three extractions).

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6. 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.

The resulting glutenin extract: The supernatant contains both HMW-GS and LMW-GS. This supernatant 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.

- **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.

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 should be rinsed with 1 M phosphoric acid (filtered through a 0.45 µm filter) for 10 minutes followed by a 20-minute 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.

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.
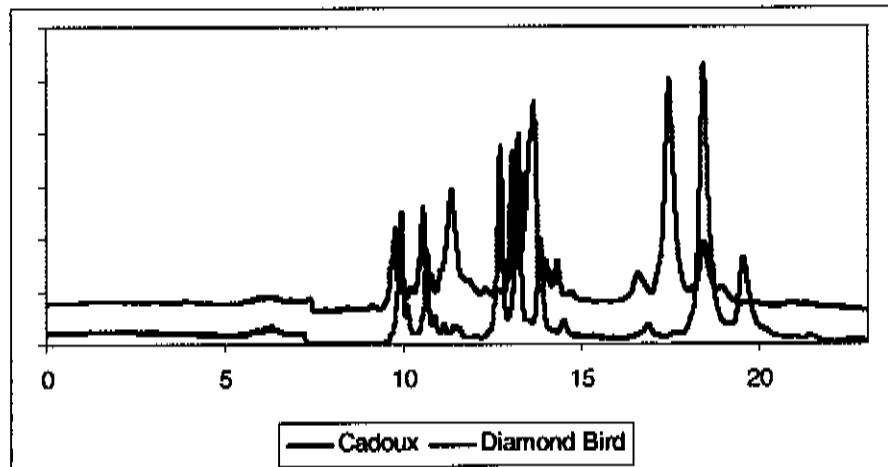

Figure 12. Capillary electrophoretic profiles of HMW-GS of known wheat samples: Diamondbird (upper trace) and Cadoux.

Preparation of 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.
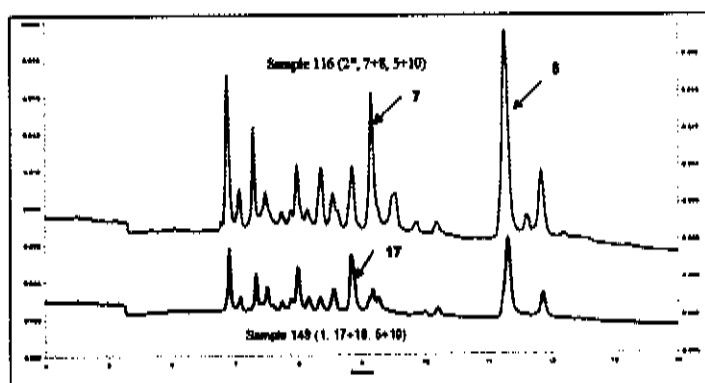

Figure 13. Capillary electrophoretic profiles of HMW-GS of unknown wheat samples
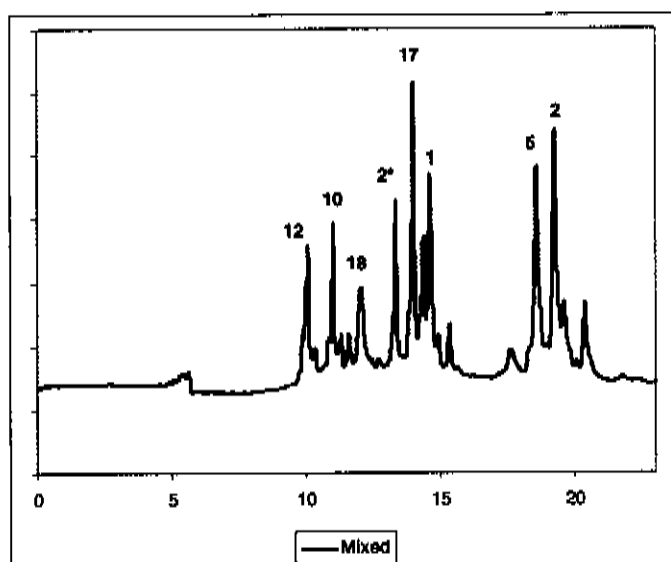

Figure 14. Capillary electrophoretic profiles of some of the common HMW-GS, showing their respective positions in the standard electrophoresis profile. This profile was produced using a mixture of extracts, chosen so as to include this range of subunits.

INTERPRETATION

Compare the CE profiles for test samples with those of genotypes of known subunit composition to determine the subunit composition (Figures 12 and 13). Determination of subunit composition by visual comparison of profiles is difficult. The provision of interpretative software assists in this task. Using this technique, we have identified the positions in the profile of most common HMW-GS (Figure 14), thus allowing for their automatic identification by the PatMatch software.

Computerised interpretation of HMW-GS for Lab-on-a-chip method
Despite the best quality controls in the preparation of the chips for this analysis, it must be recognized that some variation in the mobility of the HMW-GS will occur. It is necessary, therefore, to allow a window for each peak. For the samples we have tested, the relative standard deviation (RSD) has been found to be less than 2%, but even a window of this size creates some overlap between HMW-GS (Table 1). This makes it difficult for automatic identification of individual HMW-

GS. By allowing a generous 5% window, overlap occurs with most sub-units. Accordingly, a 3% window has been chosen to allow for situations worse than the 2% RSD presently experienced.

It is possible, however, to take advantage of the occurrence of certain sub-units in pairs, as in 5 + 10, 2 + 12, 17 + 18, and so forth. As can be seen from the table, sub-unit 2 cannot be distinguished from sub-unit 10 within a 3% window. Sub-unit 10 can be easily distinguished from sub-unit 12, and so the assignment may be made. Where there is still ambiguity, a distinction can be made by the difference in mobility between the pair of sub-units.

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