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Measurement of Gliadin and Glutenin Content of Flour by NIR Spectroscopy

I. J. Wesley*†, O. Larroque‡, B. G. Osborne†, N. Azudin§**, H. Allen¶ and J. H. Skerritt‡‖

‡*CSIRO Plant Industry, P.O. Box 7, North Ryde, NSW 1670, Australia;* †*BRI Australia Ltd., P.O. Box 7, North Ryde, NSW 1670, Australia;* §*Agrifood Technology, P.O. Box 728, Werribee, Vic 3030, Australia;* ¶*NSW Agriculture, PMB Wagga Wagga, NSW 2650, Australia*

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ABSTRACT

Traditional NIR calibration methods rely on assembling a calibration set of samples and using procedures such as multiple linear regression or partial least squares to develop the calibration. The problem with this methodology is to assemble a calibration set which maximises the diversity of samples represented whilst minimising the intercorrelations between constituents, particularly total protein content and moisture content. The application of NIR measurements of grain has moved beyond simply measuring protein and moisture content. There is now considerable interest in using NIR to measure a range of quality parameters such as Extensograph extensibility and maximum resistance. These parameters are not themselves represented in the NIR spectrum, but are a direct result of the protein composition of the sample. Consequently, a method for predicting the protein composition would be useful. In this paper, we present the results of a comparison of a curve fitting methodology and the more usual partial least squares curve fitting of the component protein spectra, using samples obtained from a wheat breeders' trial. Gliadin and glutenin contents were measured by SE-HPLC and used to develop a partial least squares calibration and the results compared with a curve-fitting methodology. For the situation examined here, the curve fitting methodology did not perform as well as partial least squares calibration. For glutenin, SEP = 0·65 for the curve fitting compared to SECV = 0·38 for a traditional PLS calibration. However, the results from the curve-fitting are independent of the total protein content and show sufficient discrimination for potential use in sample protein ranking.

Keywords: NIR, near infrared, gliadin, glutenin, curve fitting, ranking protein components.

INTRODUCTION

Although total protein content is the primary factor in determining the end use of wheat, there is often a need to measure properties that are indicative of the protein quality. Measurements such as those obtained from the Extensograph, Mixograph and Farinograph are widely used to assess the quality of wheat flour, both by end-users and by plant breeders. These measurements require relatively large amounts of material (although small-scale measurements are available) and are time consuming making them unsuitable for use in the early stages of breeding programmes and at grain receival stations.

There are a number of reports on the use of Near Infrared (NIR) spectroscopy to predict quality parameters. Osborne[1] investigated NIR for predicting SDS sedimentation volume as a

ABBREVIATIONS USED: PLS = Partial Least Squares; NIR = near infrared; HPLC = high performance liquid chromatography; SDS = sodium dodecyl sulphate; PAGE = polyacrylamide gel electrophoresis; SEP-standard error of prediction; SECV = standard error of cross validation.

* *Corresponding author.*

‖ *Present address: Australian Centre for International Agricultural Research, GPO Box 1571, Canberra, ACT 2601, Australia.*

** *Present address: AWB Research Ltd., GPO Box 4562, Melbourne, Vic 3001, Australia.*

measurement of protein quality and concluded that there was not a significant correlation with the NIR spectral data when the contribution of protein content to the calibration was removed. Rubenthaler and Pomeranz[2] studied water absorption, mixing time and loaf volume, achieving reasonable predictions for these characteristics and suggested that NIR was indeed measuring more than simply total protein content. Williams *et al.*[3] and Pawlinsky *et al.*[4] investigated a wide range of quality parameters with a view to developing whole grain NIR calibrations. Delwich and Weaver[5] noted that the ability of NIR to robustly predict parameters such as dough mixing time, mixing tolerance and overall bake score was low due to the complexity of the interactions between protein, starch and lipid.

This type of NIR calibration (essentially a 'Black Box' approach) suffers from a number of deficiencies. Firstly, there is the requirement to assemble a large sample set, complete with laboratory analysis. This can be time-consuming and there is a real risk of the physical and chemical properties of the samples changing significantly between the time of recording the spectra and completing the reference measurements. A second problem is the relationship between the material used in the physical test and the material used to obtain the NIR spectrum. Many of the properties of interest are not properties of the grain or flour. For example, the extensibility measurement is made on a piece of dough prepared under specified conditions. Although its properties are undoubtedly related to the chemical composition of the flour and grain, it is not the same material. In many cases, there is a high correlation between the quality parameters especially dough extensibility and total protein content. The nature of traditional NIR calibration methodologies gives much greater weight to the larger absorbances in the NIR spectrum and thus biases any prediction towards outcomes that favour this weighting. Consequently, the unwary user can inadvertently derive a poor protein calibration. A third point to consider is that the physical properties of interest do not themselves have NIR spectra. It is the individual chemical structures in a wheat grain or a flour that give rise to the absorption features on which NIR measurements are based. Unfortunately, the exact nature of the relationship between chemical composition and dough rheological properties is not known. It is known that total protein content is an important factor, as is the protein composition which is often described in terms of the gliadin and glutenin content and the ratio of high molecular weight to low molecular weight glutenin subunits. It is the relative amounts of these components that determine the performance of a flour at a given protein content. Hence, an alternative to a 'Black-Box' calibration based on physical properties is to independently measure the composition of the protein and to use these values to calculate the likely performance of the flour under varied conditions.

Measurements of protein composition are made by HPLC methods that, despite their effectiveness, are time-consuming and thus unattractive for situations where speed of analysis or the number of analyses required are the deciding factors. Size Exclusion (SE-) HPLC separates protein extracts, usually in unreduced form, based on their molecular sizes. A typical SE-HPLC chromatogram and SDS-PAGE analyses of fractions collected from each sector is shown in Figure 1(a,b). Three main size classes are obtained from total protein extracts, mainly consisting of glutenins (M_r = millions – 20 million, peak 1 in Figure 1(a)], gliadins [M_r = 30 000–70 000, peak 2 in Figure 1(a)] and albumins + globulins (M_r = 20 000–30 000, peak 3 in Figure 1(a)], respectively, although some of the albumins and globulins may elute with the gliadins in the second peak.

In a recent paper[6], Delwiche *et al.* showed the potential of NIR to measure gliadin and glutenin content of whole wheat beyond what would be expected by a simple correlation with total protein content. The methodology used by Delwiche *et al.*[6] still leaves open the question of how to develop an NIR calibration that gives results independent of protein content. The obvious method, simply correcting the laboratory or predicted values to a given protein content, is not valid as there is no reason to assume that the relationship between the protein composition and the total protein content will be the same for all samples. In a PLS NIR calibration, the laboratory reference data is regressed onto the NIR spectral data. Thus, if the effect of a particular component is to be removed, then it should be removed from the spectral (x-axis) data. This can be done easily in some cases by removing particular wavelengths from the calibration. However, in the case of measuring gliadin and glutenin, the effect we wish to remove is that of total protein, and yet we wish to measure the content of a protein component. Clearly, removing spectral regions with a high correlation to total

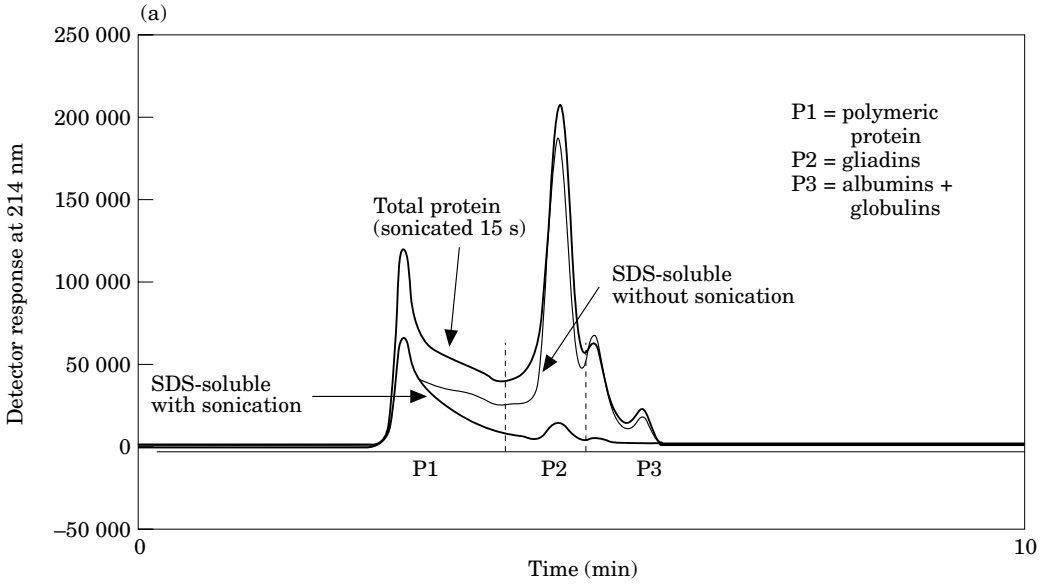


Figure 1 Legend on following page.

protein content is going to have an adverse effect on a calibration for gliadin and glutenin content.

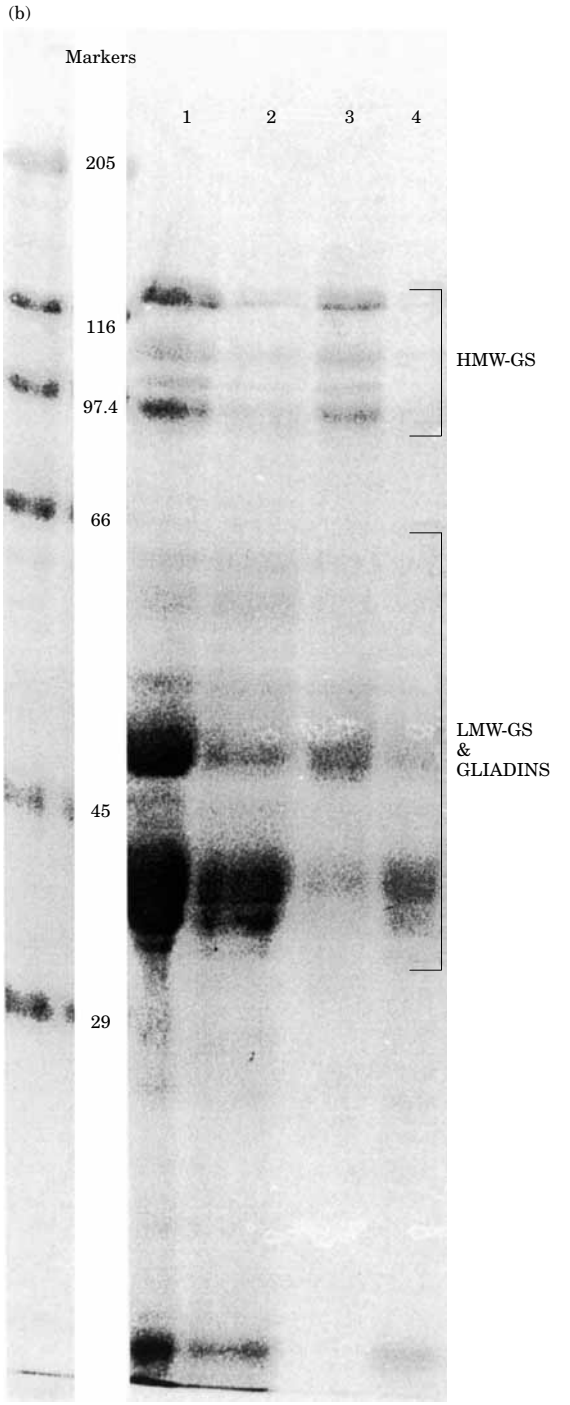
In essence, the problem is to develop a calibration for protein composition that is independent of the protein content. Whilst there is a general trend for the gliadin and glutenin content to be correlated with total protein content, there is no reason to assume that the specific mathematical relationship found in a calibration set is valid for all possible samples. Therefore, a calibration must truly measure the amounts of the individual components present.

One of the problems with developing calibrations for use in plant breeding programs is that the material to be predicted will be unusual – i.e. it is unlikely to be represented well in any calibration set. This is because calibration sets are usually based on material which is the result of several generations of selection. At this point, there has already been quite significant selection to remove material that is clearly undesirable. However, it is precisely the unusual material that the breeder wishes to identify at an early generation by NIR methods. A recent paper by Wesley *et al.*[8] offers an alternative to traditional NIR calibration methods based on curve fitting the spectra of the pure components. This method is based on identification of regions in the NIR spectrum that are unique to the components of interest, in this case gliadin, glutenin and starch. Initially, the NIR spectra for each of the pure components are determined by performing a least squares deconvolution on the spectra of a set of mixtures of known composition. Subsequently, a simple least squares fit is then used to determine the proportion of each spectral component required to reconstruct the spectrum of the target. These proportions can be directly translated to concentrations of the component present. In the present paper, we compare the Curve-Fitting methodology with the more usual Partial Least Squares (PLS) calibration on a set of samples of wide range of physical properties and cultivars and grown at a number of sites in Australia.

EXPERIMENTAL

Samples

Flour samples (total = 78) were obtained from two sources. Forty-eight samples were obtained from a Stage 3 Breeders' trial co-ordinated by NSW Agriculture, Wagga Wagga, NSW Australia. The material in this set represents a broad range of advanced breeders' lines and released varieties from breeding programs at Wagga Wagga (NSW), Temora (NSW), Narrabri (NSW) and Toowoomba (Queensland). The samples were grown at two sites (Wagga Wagga and Condobolin (NSW)) planted early in the growing season and mid-season. Each sample was a composite of three replicates grown in the field so as to eliminate field variation. Thirty



1: Total protein extract. Peak 1. Glutenins
2: SDS-soluble without sonication. Peak 1. Glutenins
3: SDS-soluble with sonication. Peak 1. Glutenins
4: Total protein extract. Peak 2. Gliadins

Figure 1 (a) SE-HPLC chromatogram of the proteins of wheat flour (unreduced) and (b) SDS-PAGE gel of extracted component proteins (reduced).

samples were obtained from Agrifood Technology, Werribee, Victoria, Australia. This set consists of 14 varieties representing Australian wheats with a range of physical properties. The samples were collected from four sites (Horsham (Vic), Moree (NSW), Yeelanna (SA) and Wongan Hills (WA)). At each site, all of the varieties were grown in plots in the same field. In addition, the grain for the whole trial came from the same source for a given variety. The only difference between samples from a particular site is varietal and for a given variety, the only difference in environmental.

A further set of samples (total = 100) were grown in 1997 and in 1998 at Roseworthy, SA. The samples were selected from a range of wheat lines with the aim of maximising the physical and chemical properties of the sample set.

SE-HPLC Measurements of Gliadin and Glutenin Content

Different wheat storage protein extracts were subjected to SE-HPLC using a Beckman System Gold HPLC (Beckman Instruments, Inc., Fullerton, CA, U.S.A.), configured with two model 126 Pumps, a model 166 Detector and a model 507E Autosampler. Integration of chromatograms was performed using Beckman Gold Nouveau v1.5. Analyses were performed on total protein[9], 'extractable' and 'unextractable' polymeric protein[10]. For total protein analysis, 10 mg of flour was extracted with 1 mL 0·5 % SDS- 0·05 M phosphate buffer (pH 6·9), subjected to 15 s sonication (sonifier set at output 5, Branson Sonic Power Company, Danbury, CT, U.S.A.), centrifuged at 17 000 × ***g*** for 15 min (Jouan A-14 microcentrifuge, Societe Jouan, Saint Herblain, France) and filtered through a 0·45 µm polyvinylidene fluoride filter (Gelman Sciences Inc., Ann Arbor, MI, U.S.A.). Aliquots of 20 µL were injected into a Phenomenex Biosep SEC-4000 column (Phenomenex, Torrance, CA, U.S.A.) running at room temperature with an eluant consisting of 50% acetonitrile (190 grade, Ajax Laboratory Chemicals, Auburn, NSW, Australia) and 50% high purity water (Millipore Water Purification System, Millipore Corp., Bedford, MA, U.S.A.) both containing 0·05% trifluoroacetic acid (sequanal grade, Pierce, Rockford, IL, U.S.A.) according to Batey *et al.*[9] A running time of 10 min, based on a flow rate of 2 mL/min, was used according to Larroque *et al.*[11] Eluted protein was detected at 214 nm.

Three main peaks were determined: peak 1, consisting of polymeric protein (mainly glutenins); peak 2, made up of monomeric gliadins and peak 3, composed of a mixture of monomeric protein: albumins and globulins [Fig. 1(a)]. A SDS-PAGE pattern of reduced fractions collected from the three main peaks is shown in Figure 1(b) (lanes 1, 4 and 5 respectively).

For 'extractable' and 'unextractable' polymeric protein analysis, 10 mg of flour was extracted for 10 min with 1 mL 0·5% sodium docecyl sulphate, 0·05 M phosphate buffer (pH 6·9) in a shaker. The supernatant obtained after centrifugation (17 000 × ***g*** for 15 min) was considered as SDS-soluble (or 'extractable') polymeric protein and was filtered into vials for HPLC analysis. The remaining pellet was resuspended in the same extraction buffer and sonicated for 30 s. Samples were then centrifuged at 17 000 × ***g*** for 15 min, and the supernatant, considered as SDS-soluble polymeric protein 'with sonication' (or 'unextractable'), filtered into vials for SE-HPLC analysis in the same conditions as reported for total protein. Figure 1(a) shows the profiles for both 'extractable' (arrowed as 'SDS-soluble without sonication') and 'unextractable' (arrowed as 'SDS-soluble with sonication') chromatograms. SDS-PAGE patterns of reduced fractions collected from the peak 1 of each of these two profiles are shown in Figure 1(b) (lanes 2 'extractable' and 3 'unextractable').

A ratio ((area P1 unextractable polymeric protein * (area P1 extractable + area P1 unextractable polymeric protein)$^{-1}$)*100 was used determine the molecular size distribution of polymeric protein. This parameter, known as the percentage of unextractable polymeric protein (%UPP), has been consistently linked to wheat quality.

NIR Measurements

NIR Spectra were recorded using a NIR Systems 6500 scanning monochromator spectrometer fitted with a sample transport accessory (Foss NIRSystems Inc., Silver Spring, MD, U.S.A.). Spectra were recorded in reflectance mode using an ISI ring cup. Spectral data were recorded from 400 to 2498 nm at 2 nm intervals and saved as the average of 32 scans for each sample. A four-point Fourier smoothing was applied to the data during collection.

NIR Calibrations

Calibrations were developed using ISI3 version 4 software (Foss NIRSystems Inc., Silver Spring, MD, U.S.A.) over the range 1100–2498 nm, the range (covered by the near infrared reflection detector on the instrument) which is the focus of this paper. The region 800–1100 nm is generally only applicable to transmission spectroscopy where the long pathlengths give rise to reasonable log (1/T) values. In reflectance, the log (1/R) values in this region are very small and have poor resolving power for reconstruction and calibration. All spectra were scatter corrected using Standard Normal Variance and Detrend prior to calibration[12]. A second derivative calculated over a 8-point (16 nm) gap and an 8-point (16 nm) smooth was applied. Calibrations were developed using a modified Partial Least Squares algorithm and a one-out cross validation strategy with no outlier elimination. Curve fitting was performed as described by Wesley *et al.*[8] using GRAMS32 v5 (Galactic Industries Corporation, Salem, NH, U.S.A.). An alternative method for calculating derivatives, a stabilised second derivative[13] calculated using a 9-point template with an even grid spacing of 2 nm, was used.

RESULTS AND DISCUSSION

When considering the performance of NIR calibrations, it is useful to establish the typical error on the laboratory reference method. SE-HPLC is a well-established technique and recent advances in instrument design, computerisation and column performance mean that the SE-HPLC measurement itself is highly repeatable. Most of the errors lie in the preparation of the samples, particularly the sonication step, and in the interpretation of the SE-HPLC data. Sonication is used to solubilise the largest protein molecules by breaking them into lower molecular weight fractions. The extent to which this is repeatable is dependent largely on the technical skill of the operator. In order to investigate these effects, a repeatability trial was performed using one flour. Six separate extracts were prepared and each extract injected five times. Analysis of Variance showed that most of the error was associated with the sample extraction, so comparing extractions, the standard deviation of difference is 0·08% (total flour basis). Another source of error is the integration of the SE-HPLC trace. The SE-HPLC trace of the extracted protein consists of three peaks [Fig. 1(a)]. The first rep-

resents polymeric material, while the second and third are monomeric material. However, as we are actually measuring the size of the protein molecules, which is a continuum, there is no clear differentiation between the peaks. Other errors include assumptions that 100% of the protein is extracted and that all glutenin is in Peak 1 and all gliadin is in Peak 2. In the final analysis, we can expect the SE-HPLC data to have a standard deviation of differences of 0·1% total flour basis, and it is this value which is crucial to understanding the performance of the NIR methods. This is perfectly acceptable given the range of values in the calibration set, giving a range/error ratio of approximately 20.

The calibration results are shown in Table I and illustrated in Figure 2. Superficially, the PLS calibrations look marginally better than the Curve Fitting results, although neither approaches the repeatability error of the SE-HPLC method. However, the extremely good performance of the PLS calibrations is quite likely due to a high intercorrelation with total protein content (Table II). This correlation arises because of the necessity of using gliadin and glutenin contents quoted on a total flour basis. This will inevitably invoke a high correlation with protein unless the sample set has been designed to specifically avoid such problems. Given the high correlation between protein and the gliadin and glutenin, and the well known ability of NIR to predict total protein content, the high R^2 and low SECV values for the gliadin and glutenin predicted by a PLS calibration is not surprising.

The correlation analysis shows the extent to which a PLS calibration for gliadin and glutenin can be dependent on the total protein content. Given that all likely calibration sets will include a wide range of cultivars, growing sites, etc and therefore a wide range of total protein content, it is extremely difficult to develop a calibration which measures gliadin and glutenin content independently of total protein content. As can be seen from Table I, the results compare very favourably with those obtained from a PLS methodology, using R^2 as a criteria. The more important criterion is the SEP and the more important constituent is glutenin, which is the major contributor to dough quality[14]. On this basis, the error on both measurements ($\pm 0{\cdot}76$ for PLS and $\pm 1{\cdot}3$ for Curve-Fit) is unacceptably large and at best this would allow the samples to be classified on the basis of either high or low glutenin content. This indicates that PLS is in fact doing no better. However, working with such coarse estimates is acceptable in most plant breeding situations where a simple cut-off is applied. The other obvious problem with the Curve-Fit is the large bias between the actual and predicted values (Fig. 2). This is caused by the extreme simplicity of the model. There is a considerable amount of material (water, lipid, water-soluble proteins) that is not represented in the three-component model. This material should be included so that the spectral reconstruction is more relevant. The easiest method of including this material is to calculate a residual spectrum using spectra with known gliadin, glutenin and starch content. The application of the method for determining the residual spectrum must take account of and be stratified with respect to the physical (whole grain, ground grain or flour) and varietal nature of the samples.

The bias between predicted and actual values is not important as long as the method reliably predicts the ranking of samples. In order to test this, a further two sets of 100 samples were analysed. Both sets contained the same material, but grown in different seasons (1997 and 1998). The results are shown in Figure 3 and Table III. The lines on the figures represent the boundaries when each year's data is divided into three. The boundaries were calculated as:

$$\text{Lower boundary} = \text{median} - \text{standard deviation}$$
$$\text{Upper boundary} = \text{median} + \text{standard deviation}$$

If the values are approximately normally distributed, then 1 standard deviation from the median represents approximately 66% of the range. The use of the median rather than the mean allows for skewed populations where a few samples may significantly alter the mean value. In these cases, using the median value simply means that some unwanted samples may be retained. However, no desirable samples will be eliminated. In the case described here, there are no samples that are ranked in the top 16% in one year and in the bottom 16% in the other. There are some samples that are ranked in the bottom and middle. In these cases, most are ranked lower than the median value. Only 3 samples (labelled □) are ranked low one year and above the median value in the other year. A similar result is found for the samples ranked in the top 16% in one year and in the middle in the other year. A Spearman Rank Correlation test was performed on the sample rankings

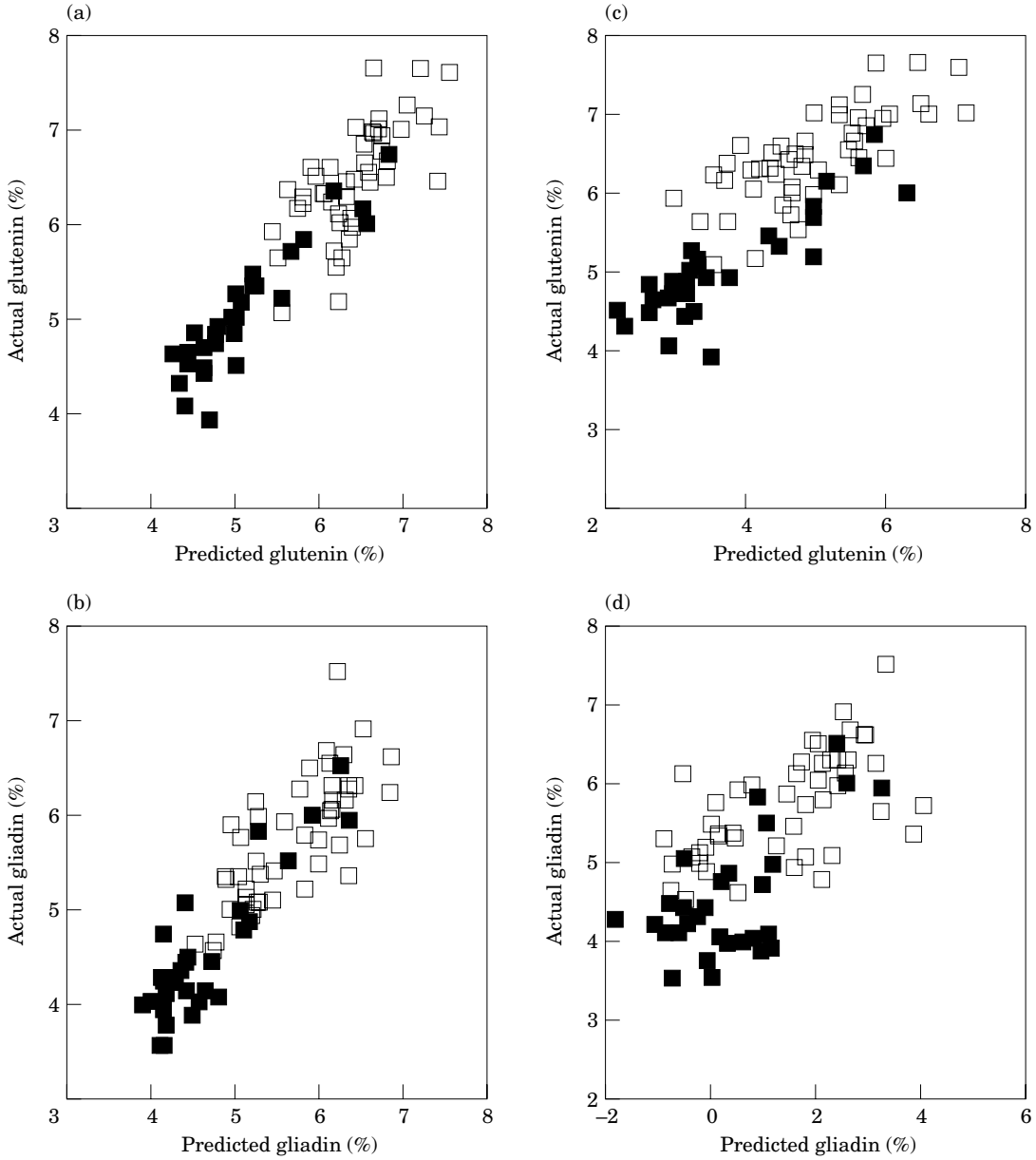


Figure 2 Actual vs Predicted for PLS calibration for glutenin (a) and gliadin (b) and for Curve Fit for glutenin (c) and gliadin (d). (■ = samples from Agrifood technology, □ = samples from NSW Agriculture).

Table I Calibration results

			Curve Fit		PLS		
	Range (%)	s.d.	R^2	SEP	R^2	SECV	Terms
Glutenin	3·92–7·65	0·93	0·71	0·65	0·83	0·38	4
Gliadin	3·55–7·55	0·91	0·46	1·02	0·78	0·43	1

s.d. = standard deviation of laboratory values. R^2 = multiple correlation coefficient between actual and predicted values. SEP = Standard Error of Prediction. SECV = standard error of cross validation. Terms = number of PLS terms in equation.

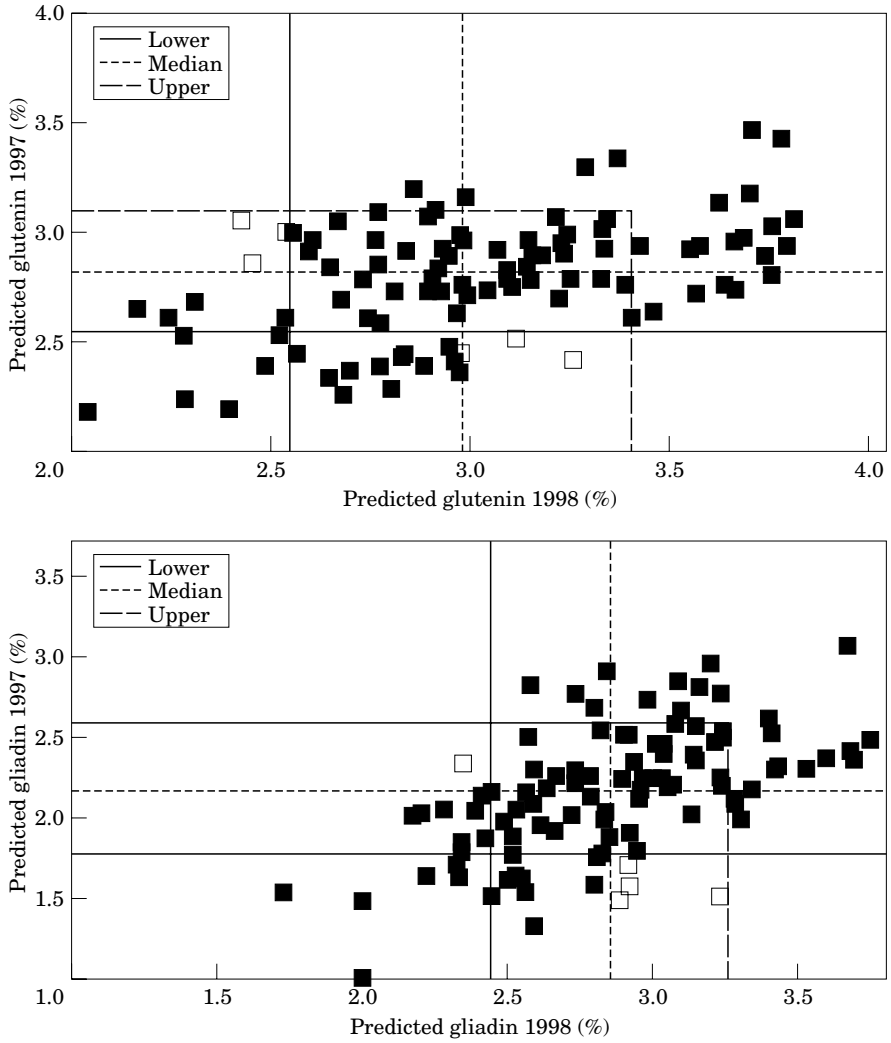


Figure 3 Comparison of prediction of glutenin content and gliadin content by Curve Fitting of samples grown over two consecutive years. □ = samples which are measured low in one year and above the median in the other year.

Table II Intercorrelation of laboratory data

	Gliadin	Glutenin	Total protein
Gliadin	1		
Glutenin	0·72	1	
Total protein	0·92	0·93	1

Table III Spearman Rank Correlation statistics for samples grown in consecutive years

	R^2	SED	Spearman Rank Correlation
Gliadin	0·31	0·38	0·5149[a]
Glutenin	0·26	0·38	0·5819[a]

[a] Significant at $P<0·01$. R^2 is the coefficient of determination for the line of best fit through the data. SED is the Standard Error of Differences (standard deviation of differences between predicted values for each sample).

(top, middle, bottom). The results (Table III) show that the rank correlation is highly significant between the two years and hence the method is correctly predicting the glutenin content.

Although the Curve Fitting method does not predict the 'correct' answer for the gliadin and glutenin content as measured by SE-HPLC, the R^2 values and the rank correlation values for samples grown in separate years do suggest that the method will rank the samples sufficiently for

a discrimination into high, medium and low content for each component. Most importantly, the results are independent of total protein content.

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