Seed quality measurements by RP-HPLC

K. H. Sutton

Crop and Food Research, Private Bag 4704, Christchurch, New Zealand.

Abstract

Reversed-phase high-performance liquid chromatography (RP-HPLC) provides a method for rapid and accurate analysis of protein samples derived from seed and grain or their products. When compared to gel electrophoresis, HPLC has both advantages and disadvantages. Advantages include automated analysis and increased sensitivity and reproducibility, whilst disadvantages include high capital cost and the limitation of running samples singly. RP-HPLC can be used qualitatively and quantitatively to measure protein groups which are responsible for the quality characteristics of the sample. In the case of bread wheat, RP-HPLC can be used to measure the quantity of the protein group known as the high-molecular-weight glutenins (HMW-Glu). This result can then be used to predict the baking quality of the flour. Similarly, the qualitative study of wheat gliadin proteins can be used as a means of cultivar identification. RP-HPLC techniques for protein analysis are sufficiently sensitive to allow analysis to be performed on the single grain or even fractions of grains.

Additional key words: *flour, grain, protein, wheat.*

Abbreviations used in text: DDT = dithiothreitol, HMW = high molecular weight, LMW = low molecular weight, PAGE = polyacryamide gel electrophoresis, RP-HPLC = reversed-phase high performance liquid chromatography, SDS = sodium dodecyl sulphate, TFA = trifluoracetic acid, NIR = near infrared.

Introduction

This paper is intended as a review of what has been achieved in the field of cereal protein HPLC, especially in our laboratory. RP-HPLC provides a method for rapid and accurate analysis of large numbers of samples of protein. The technique separates protein components on the basis of their surface hydrophobicity (hate-of-water). Proteins, however, do not exhibit the same chromatographic behaviour as small organic molecules. The most rigorous description of protein RP-HPLC behaviour is the thermodynamic model of Horváth *et al.* (1976) however, more recent theories such as the solvent displacement model of Geng and Regnier (1984) have merit. The reader is referred to these papers for more in-depth treatments.

Although silica based RP-HPLC techniques have existed for some time, it is only in recent times that proteins could be examined. This was primarily due to the delay in availability of wide-pore (300Å) silicas which allow adsorption of the proteins into the silica surface. Cereal proteins were first studied by Bietz (1983). This study was closely followed by the use of RP-HPLC of wheat aqueous alcohol soluble proteins (gliadins) as a qualitative method for determining wheat variety (Bietz *et al.*, 1984). This work has been followed up by using the technique to discriminate varieties of barley (Marchylo and Kruger, 1984), oats (Lookhart and Pomeranz, 1985; Lookhart, 1985) and rice (Lookhart *et al.*, 1987, Hussain *et al.*, 1989; Huebner *et al.*, 1990). More recent studies have concentrated on attempting to relate wheat flour quality to RP-HPLC patterns of glutenin proteins (Huebner and Bietz, 1985; Kruger *et al.*, 1988; Wieser *et al.*, 1989; Sutton *et al.*, 1989; Sutton, 1991).

Most of the workers involved in this field, apart from ourselves, have concentrated on relating HPLC peak area ratios to baking performance rather than adopting a quantitative approach. When I began investigating this area of cereal chromatography it was decided to take a truly quantitative viewpoint. This has shown to be most important. Although I will be discussing mainly wheat, any comments could be equally applied to other grain or cereal types.

Methods

The methods used for protein extraction and analysis are well documented in Sutton *et al.* (1989) and Sutton (1991). Interested readers are referred to these and other

references in the Introduction for more detailed information. The following is a brief summary of the methods used in our laboratory.

RP-HPLC

In general, proteins are separated by reversed-phase chromatography using wide-pore C8 or C18 derivatized silica columns. It is most common to elute the proteins with solvent gradients of water and acetonitrile, both containing low levels (typically 0.1% v/v or less) of TFA. Gradient run times are typically 30-60 min. Proteins are detected by ultraviolet absorption, usually at a wavelength of 210 nm. A well equipped HPLC system will be automated by the inclusion of an autosampling device and a data capture system. The system used in our laboratory is described fully in Sutton *et al.* (1989).

Sample preparation

Gliadin samples are prepared by direct extraction of the flour sample with 70% (v/v) aqueous ethanol whilst glutenin samples are prepared by firstly pre-extracting the non-glutenin protein (Huebner and Bietz, 1985). The albumins and globulins are removed by extraction with dilute saline solutions then the gliadins are removed by extraction with 70% (v/v) aqueous ethanol. Glutenin subunits are then extracted with a buffered solution containing 2% SDS and 1% DTT. This mixture reduces the S-S bonds in the glutenin and makes it extractable.

HMW-glutenin subunits can be selectively precipitated using the method of Sutton (1991). This involves extracting the flour with 50% propan-1-ol containing 1% DTT then adding propanol to the clarified extract in order to give a final propanol concentration of 60%. The white precipitate obtained is greater than 95% pure HMW-glutenin. Reduction with a buffered urea solution containing DTT and subsequent alkylation with 4-vinylpyridine gives a mixture which can be separated into individual subunits using HPLC.

Results and Discussion

HPLC compared to PAGE

HPLC has advantages when compared to PAGE in that it can be automated, has very good reproducibility and is a quantitative technique. HPLC's drawbacks include the limitation imposed by running samples singly, the absence of a correlation with PAGE mobilities (that is, there is no direct connection between hydrophobicity and molecular weight) and a high capital cost. By comparison, PAGE is cheap to set up and run, gives clear identification of protein subunits and can run many samples at once. However, quantitation of gels is difficult, reproducibility is usually poor and the system cannot be easily automated.

RP-HPLC of alcohol extractable proteins

Chromatograms of cereal alcohol extractable proteins are primarily used to identify cultivars unequivocally. This was first demonstrated by Bietz *et al.* (1984). RP-HPLC is now well established for this purpose (Marchylo *et al.*, 1988) and has, to some extent, supplanted acid-PAGE as the recognised method of cultivar identification for cereals, although the latter technique is still the officially recognised one. Figure 1 illustrates the chromatograms of alcohol extractable proteins obtained from wheat cultivars Otane and Oroua. It can be seen from Figure 1 that the chromatograms are very similar, however, the regions between elution times 39 and 41 mins are sufficiently different as to allow discrimination

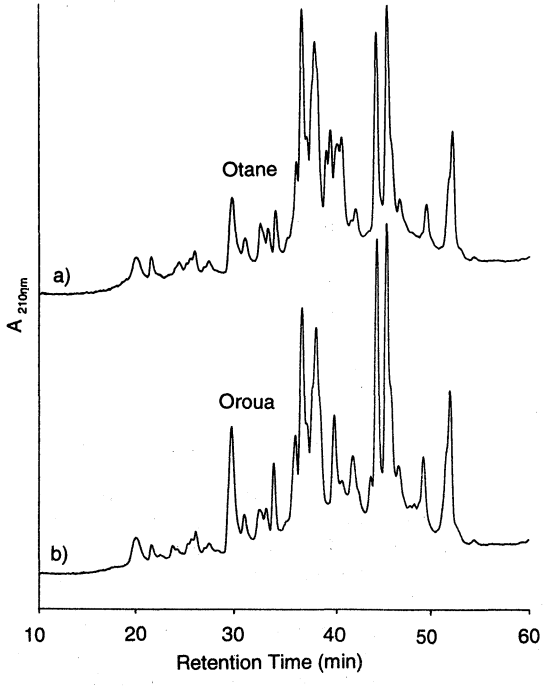

Figure 1. RP-HPLC separations of 70% alcohol extractable proteins from bread wheat cultivar Otane (a) and bread wheat cultivar Oroua (b) from Hay and Sutton (1990).

of the two varieties. It has been demonstrated (Hay and Sutton, 1990) that all New Zealand varieties of bread wheat, durum wheat and rye can be discriminated using RP-HPLC. Currently, the most rapid method of cultivar identification from HPLC chromatograms is still 'by eye'. Some workers overseas (Scanlon *et al.*, 1989) have reported some success using computers to identify varieties, however, further attempts to refine this process have been hampered by sample variation, especially between seasons, and column variations leading to retention time drift and peak misidentification.

Different cereal types have markedly different RP-HPLC patterns for their alcohol extractable proteins. This can be put to practical use in determining the degree of contamination of one cereal type in another (McCarthy *et al.*, 1990). Recently, work in our laboratory has demonstrated that the elution time of late eluting alcohol extractable proteins were sufficiently different between bread wheats and rye (Sutton and Hay, 1990). Figure 2 illustrates the chromatogram of alcohol extractable proteins from the bread wheat variety Konini and the rye variety Rahu. It can be seen from Figure 2 that the peak eluting at 50.2 min in the rye chromatogram (labelled A) is absent in the wheat chromatogram. This makes the peak ideal as a calibration peak and, indeed, a highly linear calibration was found for this peak area versus rye composition. There appears to be no reason why this technique cannot be applied to other cereal types, or indeed, to any other seed type.

RP-HPLC of glutenin proteins

The glutenin proteins have long been thought to be responsible for dough strength and baking performance in bread wheats. Glutenin strength is also an important parameter in determining the suitability of durum wheats for pasta manufacture.

The question of predicting bread wheat baking performance by studying the glutenin proteins using the HPLC was one which I decided must be approached in a truly quantitative manner. The study itself was carried out on 147 samples grown during 1986 as part of the Autumn wheat breeding programme at Lincoln (Sutton *et al.*, 1989). The samples were milled and bake tested as for the normal breeders trial tests and it was found that they had extremely wide range of baking performance with bake scores ranging form 16 to 36 (Swallow and Baruch, 1986). A sequential extraction procedure was developed to first remove, from a flour sample of known weight, the aqueous extractable albumin and globulin proteins and then to remove the alcohol extractable gliadin proteins. The glutenins could then be solubilized by adding a known volume of a solution of a detergent and a reducing agent to the pre-extracted pellet. This quantitative approach meant that we could regard the proteins like any other chemical for analytical HPLC purposes.

Figure 3 illustrates a typical glutenin subunit chromatogram (Sutton *et al.*, 1989). Peak height and area data for every peak in each of the 147 chromatograms were entered into a statistical computer program and a multiple linear regression analysis was performed. The results of this analysis produced a model for predicting loaf volume (in our test baking procedure) by measuring the peak areas of the two peaks eluting at 33.8 and 34.4 mins (labelled A and B respectively and containing, as determined by SDS-PAGE, the HMW subunits of glutenin). This equation is outlined in Table 1. The model was capable of discriminating between

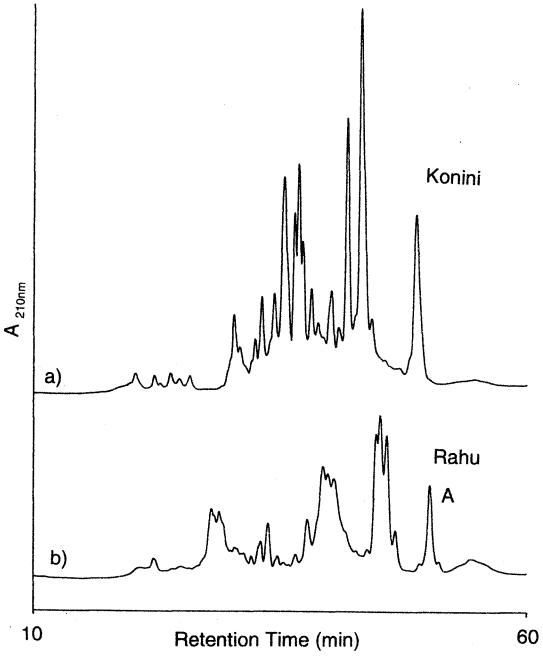

Figure 2. RP-HPLC separations of 70% alcohol extractable proteins from bread wheat cultivar Konini (a) and rye cultivar Rahu (b). The peak at retention time 50.2 min in the Rahu sample (peak A) can be used to calibrate for rye content in a mixture (from Sutton and Hay, 1990).

wheats of poor, medium and high baking quality, as defined by the bake test procedure. Figure 4 illustrates the predicted versus observed loaf volume results for the 147 samples. It can be seen from Figure 4 that the methods has very good predictive power.

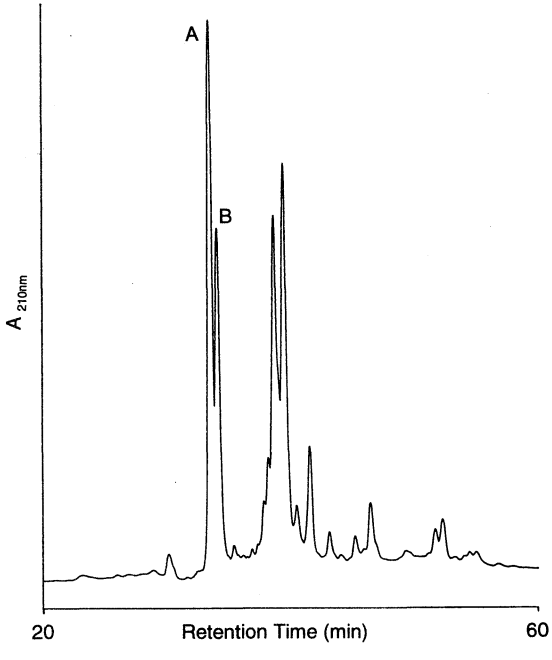

Figure 3. RP-HPLC separation of bread wheat glutenin subunits solubilized in 2% SDS/ 1% DTT/0.1M Tris buffer at pH 6.8. The peaks eluting at retention times 33.8 and 34.4 min, which are highly correlated with loaf volume are labelled A and B respectively.

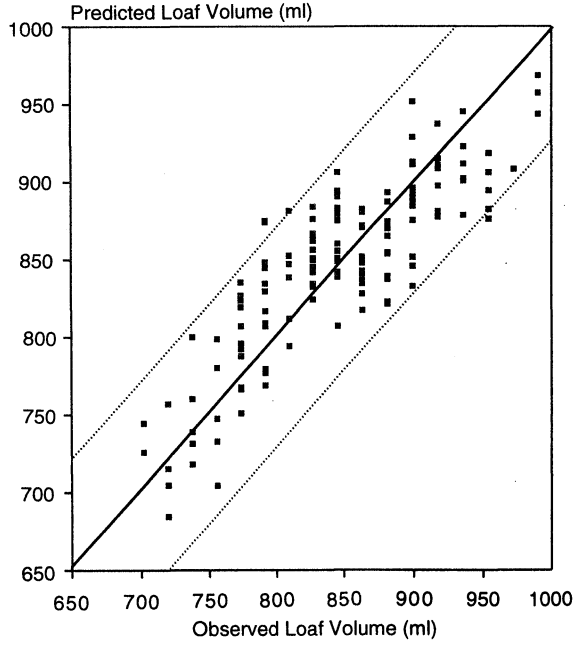

Figure 4: Scatter plot of experimental versus predicted loaf volume for 147 Autumn wheats grown at Lincoln in 1986. The prediction model uses the peak area formula in Table 1 (Sutton *et al.*, 1988). The 1:1 correspondence line is shown solid whilst the 95% prediction limits (shown in Table 1) are shown broken.

Table 1. Results of multiple linear regression of RP-HPLC peak data, NIR protein data and bake test loaf volume data.

Variable	Regression equation[a] (Loaf volume (ml) =)	Adj-rsq[b]	C.V.[c] (%)	95% c.l[d] (ml)
Peak area	616 + 0.0130A + 0.0064B	0.72	4.1	70
NIR protein	405 + 36.3Protein	0.69	4.4	74

[a] Symbols A and B refer to the peak areas of the two peaks with retention times 33.8 and 34.4 min respectively. Protein refers to the percentage total protein as determined by NIR.
[b] Coefficient of Determination
[c] Coefficient of Variation
[d] The 95% confidence limit for a prediction.

Although the results in Table 1 would seem to indicate that the total protein, as determined by NIR, can be used to satisfactorily predict baking performance, there were many glaring exceptions. The most notable of these was demonstrated when a sample of Hilgendorf was examined. Hilgendorf typically contains around 12% protein, much higher than Otane, although its baking performance is very poor. HPLC analysis reveals a glutenin profile which contains very little material in peaks A and B (that is, very little HMW-glutenin). The HPLC method thus predicts a low baking performance for Hilgendorf which fits the observed results.

The original 147 samples were derived from a single site in a single season. In order to be of use as a breeding tool the technique had to be more widely applicable. That is, applicable over many seasons and locations and be independent of mill type. The follow-up study was more ambitious in scope taking in another 234 samples (Sutton *et al.*, 1990). These samples came from various countries, were grown in different conditions and years and were milled on different mill types. Rye 1B/1R translocated varieties were also tested. Using the prediction equations developed in the first study (Table 1) baking performance results were predicted from HMW-glutenin peak areas. Figure 5 represents a plot of predicted versus observed loaf volumes for these samples. It was shown that although the model had lost some of its 'robustness' by including such a wide variety of samples, it still gave better baking performance predictions than using NIR protein values (data not shown). Specifically, the model could still differentiate poor from high quality wheats even after including all of the new samples.

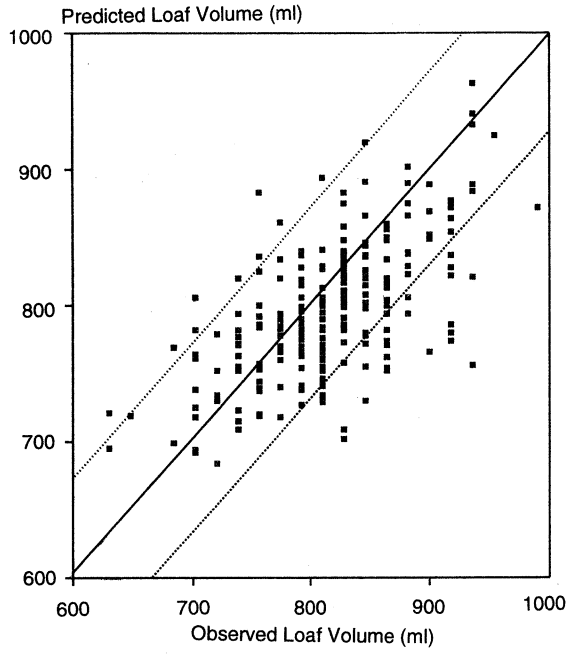

Figure 5. Scatter plot of experimental versus predicted loaf volume for 234 wheats of diverse origin as described in Sutton *et al.* (1990). The 1:1 correspondence line is shown solid whilst the 95% prediction limits (shown in Table 1) are shown broken.

Separation and assignment of HMW-glutenin subunits using RP-HPLC

The discussion in the previous section concerned HMW-glutenin subunits which had been extracted using an SDS buffer solution. When this type of buffer solution is used, SDS coordinates strongly to the different protein subunits and causes their hydrophobicities to be very similar. Although this effect does not hamper the method for predicting baking performance outlined above, it makes the method of no use for quantitation of individual HMW-glutenin subunits. The method which I developed (Sutton, 1991) was a logical extension of the one published by Marchylo *et al.* (1989). The revised method, using a selective extraction/precipitation step, gave an almost quantitative yield of highly pure (>95%) HMW-glutenin subunits. The highest level impurity was a very low molecular weight protein: probably a non-saline extractable globulin.

The precipitated HMW-glutenin is prepared for HPLC by reducing it with DTT in a urea/propanol buffer and then alkylating the thiol groups produced with 4-vinylpyridine. This procedure gives the pyridylethylated (PE) derivatives of the HMW-glutenin subunits. A shallow acetonitrile/water gradient is sufficient to completely resolve the subunits in any variety. Figure 6 illustrates some of the typical RP-HPLC patterns obtained. The six varieties shown in Figure 6 contain all of the subunits found in New Zealand wheats. Table 2 outlines the retention times for all of the varieties studied, using the HPLC system in our laboratory. Subunit assignments were made using SDS-PAGE. Peaks collected from the HPLC were dried and run on gels against whole protein extracts. All assignments

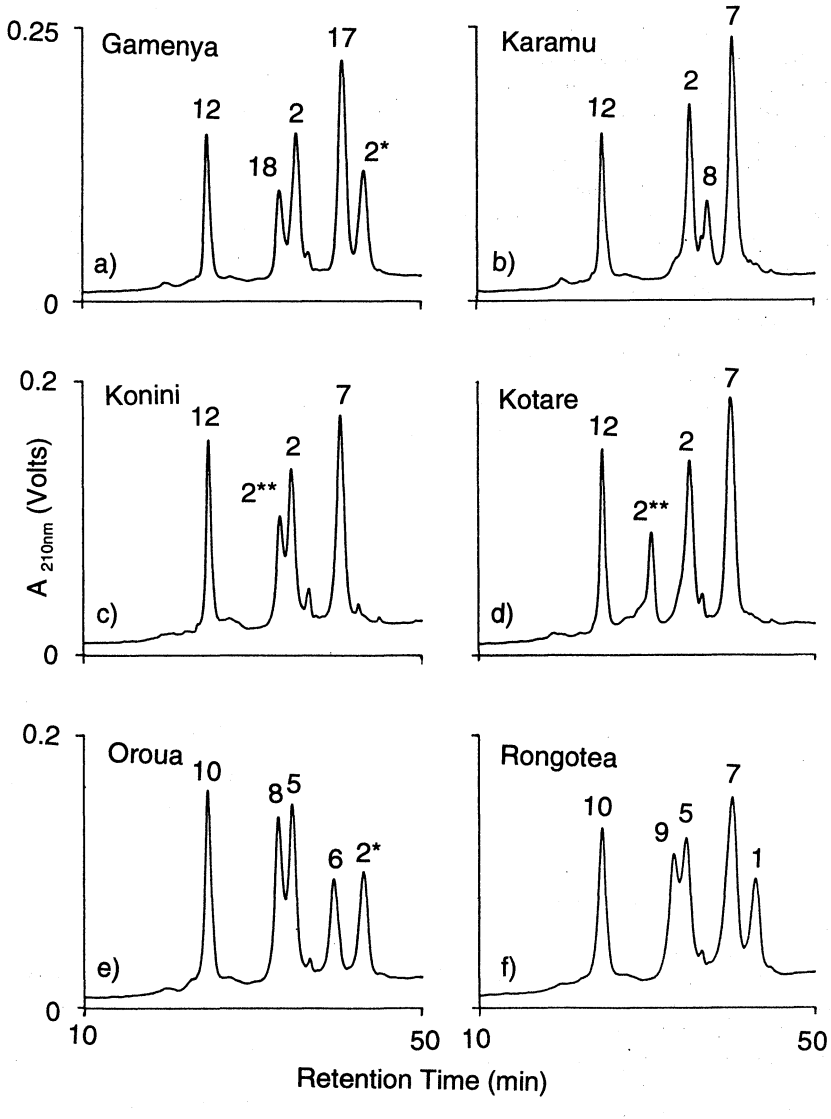

Figure 6. RP-HPLC chromatograms of alkylated HMW-glutenin subunits for wheat cultivars Gamenya (a), Karamu (b), Konini (c), Kotare (d), Oroua (e) and Rongotea (f). Numbers indicate subunits assigned by SDS-PAGE (refer Table 2).

were unequivocal. Although the HMW-glutenin subunit retention times in Table 2 were self consistent for the most part, there were two anomalies. The retention time of subunit 8 from Chinese Spring and Karamu was different from that in all other New Zealand cultivars containing this subunit and the retention time of subunit

Table 2. RP-HPLC Retention times for alkylated HMW-glutenin subunits[a]

Cultivar	Subunits 1	2	2*	2**	5	6	7	8	9	10	12	17	18
Aotea		35.3		30.7		39.8		33.2			24.9		
Arawa	43.1				34.8		40.1			24.9			
Bounty	43.2	35.1					40.1				24.7		
C.Spring		35.1					40.2	**37.4**[b]			24.9		
Gabo		35.1	43.1								24.7	40.6	33.3
Gamenya		35.2	43.0					33.2			24.7	40.6	33.3
Hilgendorf		35.1		30.7		39.7		**37.3**			24.8		
Karamu		35.2					40.2				24.7		
Konini		35.1		**33.3**			40.2	33.2			24.9		
Kopara	43.1	35.1				39.7		33.0			24.8		
Oroua			43.0		34.7	39.5		33.3		24.8			
Otane		35.0	42.9				39.9				24.7		
Rongotea	43.0			30.7	34.7		40.2	33.2	33.2	24.7			
Takahe		35.1				39.7					24.7		
Tiritea			43.0	30.6	34.6		40.2		33.3	24.7			
Weka					34.5		39.8			24.7			

[a] Retention times as measured using the HPLC system in Sutton (1991).
[b] Proteins highlighted in bold exhibit different retention times than those of other cultivars.

2** from Konini was different from the other New Zealand varieties containing this subunit. Some of these features are evident in Figure 6. Fraction collection of the PE-HMW -glutenin subunits and subsequent SDS-PAGE of the dried fractions showed that the subunit elution order in Chinese Spring and Karamu (12, 2, 8, 7) was different from that in Otane (12, 8, 2, 7). The difference in retention times between subunit 8 in the different varieties suggested that these proteins are different.

Otane has possibly one of the best baking performances of any New Zealand wheat whilst Karamu has a poor baking performance. However, total protein values for the two cultivars are usually similar and the only apparent different in their HMW-glutenin composition is that Otane contains the good quality 2* subunit in addition to the 2, 7, 8 and 12 subunits observed for Karamu. This small difference cannot explain the large difference in baking performance between the two cultivars. It is possible that the difference in subunit 8 may be an important contributor to the baking performance differences between the two cultivars. Interestingly, all other New Zealand cultivars with the same subunit 8 as Otane are of reasonable baking quality, whilst Chinese Spring, which has the same subunit as Karamu, is of poor baking quality.

The baking potential of the cultivar may also depend on the quantity of the HMW-glutenin subunits present. Figure 7 illustrates the HMW-glutenin subunit patterns for Otane and Karamu. It can be seen from Figure 7 that the quantities of each of the subunits 2, 7, 8 and 12 in two cultivars are very different. In particular, the quantity of subunit 7 in Otane is much greater than in Karamu. Quantification of the RP-HPLC peak areas for cultivars Otane and Karamu is presented in Table 3. Significant differences in the relative abundance of subunits in Otane and Karamu are evident with subunit 7 being the most abundant, representing 49.1% in Otane and 40.7% in Karamu of the total HMW-glutenin subunit area. The proportion of subunit 8 was similar in the two cultivars, ignoring the difference in retention times. The combined proportion of subunits 2 and 12 was very different between the cultivars. For Otane, the sum of subunits 2 and 12 represented 28.9% of the HMW-glutenin subunit area, whereas in Karamu this same figure was 50.7%. If one subscribes to the theory that baking performance differences can be explained by the differences in proportion of the various HMW-glutenin subunits then the poor performance of Karamu follows from its high proportion of subunits 2 and 12, associated with poor baking quality (Payne *et al.*, 1987). Alternatively, or in addition, the good baking quality of

Otane could be ascribed to its greater proportion of subunits 7 and 8, associated with good quality, and to the presence of the 2* subunit, associated with good quality.

The absolute quantity of storage proteins varies little between Otane and Karamu, with total chromatogram areas of 420, 260 mV.s and 418, 666 mV.s respectively. However, the total of the HMW-glutenin subunit peak areas are different at 33,196 mV.s and 28,986 mV.s, representing 7.9% and 6.9% of the total storage proteins respectively. The higher absolute quantity and relative proportion of HMW-glutenin subunits observed in Otane agrees with the SDS-glutenin work described in the section above (Sutton *et al.*, 1989) however, these results are at variance with the work of Marchylo *et al.* (1989).

This technique could prove useful to breeders as a more reliable method than SDS-PAGE for screening progeny. It can be used, in conjunction with subunit standards, to identify and quantify individual subunits although further studies are required before the technique attains the predictive ability demonstrated by the SDS-glutenin HPLC method detailed above.

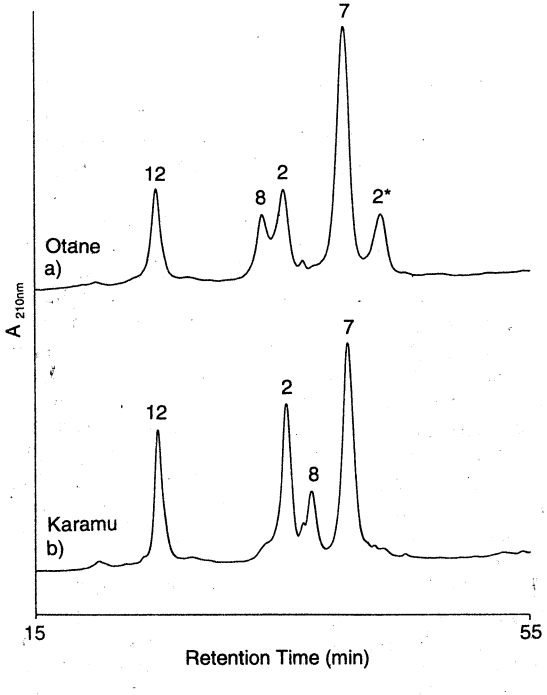

Figure 7. RP-HPLC chromatograms of alkylated HMW-glutenin subunits for cultivars Otane (a) and Karamu (b).

Table 3. Quantitative analysis by RP-HPLC of Otane and Karamu alkylated HMW-glutenin subunits.

Subunit	Retention time (min)	Peak area (mV.s)	Proportion of HMW-glutenin subunit area (%)	Proportion of total storage protein area (%)
Otane				
2	35.0	5207	15.7	1.2
2*	42.9	3745	11.3	0.9
7	39.9	16317	49.1	3.9
8	33.3	3556	10.7	0.9
12	24.7	4371	13.2	1.0
Subtotals		33196		7.9
Total chromatogram peak area = 420,260 mV.s				
Karamu				
2	35.2	9128	31.5	2.2
7	40.2	11790	40.7	2.8
8	37.3	2498	8.6	0.6
12	24.7	5570	19.2	1.3
Subtotals		28986		6.9
Total chromatogram peak area = 418,666mV.s				

Extension of RP-HPLC techniques to other grains and seeds

Although the discussion in this paper relates primarily to wheat, there is no reason why the same type of analysis cannot be applied to other grain or seed types. As long as the physical attributes of the grain or seed can be shown to be related to some physicochemical property of the grain then it should be possible to design an analysis to measure this property. Work currently underway at Crop and Food Research is considering this very type of problem in numerous field crops, especially at the single grain analysis level.

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