Effect of Environment on Wheat Storage Proteins as Determined by Quantitative Reversed-Phase High-Performance Liquid Chromatography[1]

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ABSTRACT

Reversed-phase high-performance liquid chromatography (RP-HPLC) was used to quantify variations in extracted wheat proteins (gliadins and glutenins) resulting from Canadian prairie environmental influence. The wheat cultivars Neepawa and HY 320 were studied and, qualitatively, environment did not influence protein chromatograms (gliadins and glutenins). Quantitatively, small but statistically significant influences were observed. These influences could contribute errors in automated varietal identification procedures that would affect the estimation of proportions of varieties present in small amounts. This, in turn, could affect grade assignment. Quantitative analysis of sequentially extracted gliadins and glutenins (including high molecular weight glutenin subunits) indicated that the relative proportions of extracted proteins could be influenced by environment. This could affect RP-HPLC wheat quality prediction.

The application of reversed-phase high-performance liquid chromatography (RP-HPLC) to the identification of wheat cultivars is well documented (Bietz et al 1984, Cressey 1987, Marchylo et al 1988). The analysis of gliadins and glutenins by RP-HPLC also may be used for predicting wheat quality (Huebner and Bietz 1985, 1986; Kruger et al 1988; Marchylo et al 1989; Huebner 1989). Quantitation of individual or groups of storage protein components is an important facet of the RP-HPLC analysis procedure in both of these applications. Consequently, variation in the relative contributions of individual or groups of peaks due to environmental influence could affect cultivar identification or quality prediction procedures.

In a preliminary study (Kruger and Marchylo 1985), using the cultivar Neepawa, it was found that environment did not influence qualitatively the RP-HPLC elution profiles of gliadins and glutenins. Differences in relative peak heights were observed, however, in elution profiles of Neepawa samples grown under different environmental conditions. This suggested that environmental conditions could affect quantitatively the relative proportions of some storage protein components. More recently, it was shown that wheats grown under different sulfur fertilization levels exhibit major quantitative variations in gliadin components and that sig-

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nificant quantitative variation also exists for gliadins from different locations (Huebner and Bietz 1988). Quantitative variations in storage proteins due to environment as assessed by RP-HPLC also have been reported for barley (Marchylo and Kruger 1984, Griffiths 1987) and maize (Smith and Smith 1986). In the case of barley, the magnitude of the environmental influence appeared to be substantially different for the Canadian prairie environment (Marchylo and Kruger 1984) as contrasted to the environment at the Scottish Crop Research Institute (Griffiths 1987).

The purpose of this study was to use RP-HPLC to quantify variations in storage protein composition (gliadins and glutenins) resulting from Canadian prairie environmental influence. We examined the wheat cultivars Neepawa (eligible for Canada Western Red Spring [CWRS] wheat grades) and HY 320 (eligible for the Canada Prairie Spring [CPS] wheat grades until the end of the 1989–1990 crop year). Implications of variations in protein composition upon wheat varietal identification or quality assessment by RP-HPLC are discussed.

MATERIALS AND METHODS

Wheat Samples

Neepawa samples were obtained from four crop years, 1983–1985 and 1987, as part of the Central Bread Wheat Cooperative Test program. Samples were selected from seven sites in Western Canada comprised of Saskatoon, Indian Head, Regina, Melfort, Portage La Prairie, Brandon, and Morden for the 1983–1985 crop years, and from nine sites comprised of Saskatoon, Regina, Kernen, Acme, Stewart, Scott, Watrous, Glenlea, and Teulon for the 1987 crop year. HY 320 wheat samples were obtained from the same test program for one crop year, 1987, and were comprised of samples from the nine sites listed above for Neepawa (1987) plus Ellerslie, Portage La Prairie, and Winnipeg. An additional sample, harvested early at Glenlea, was included to give a total of 13 samples for HY 320.

Chemicals and Reagents

Sequanol grade trifluoroacetic acid and 1-propanol were supplied by Pierce Chemical Co. (Rockford, IL); dithiothreitol (DTT) and acetonitrile (HPLC grade) were from Fisher Scientific (Fairlawn, NJ). HPLC grade deionized water, prepared with a Barnstead Nanopure II water purification system (Boston, MA), was employed in all solutions.

Protein Determination

Protein contents of grain samples were determined in duplicate by Kjeldahl analysis (Williams 1973).

Sample Preparation and Extraction Procedures

Wheat samples (50 g) were ground in a Udy cyclone sample mill equipped with a 1-mm sieve. Ground grain was mixed thoroughly before extraction.

Wheat proteins, comprised primarily of gliadins and glutenins, were extracted from ground grain (1 g) with continuous stirring for 30 min at 60°C using 6.0 ml of 50% 1-propanol containing 1% DTT. With this procedure, only trace levels of albumins and globulins were extracted as seen by RP-HPLC analysis (*unpublished results*). Extracts then were centrifuged at 20,000 × *g* for 10 min and supernatants were filtered through Millex HV4 0.45-μm filters (Millipore Ltd., Mississauga, ON) prior to RP-HPLC analysis. All samples were extracted in duplicate.

Sequential Extraction and Precipitation of High Molecular Weight Glutenin Subunits

Samples of ground grain (1 g) were extracted sequentially as follows: 0.5*M* NaCl (1 × 6 ml, 1 × 3 ml), H_2O (1 × 6 ml), 50% (v/v) 1-propanol (1 × 6 ml, 1 × 3 ml), 50% (v/v) 1-propanol containing 1% (w/v) DTT (1 × 6 ml, 1 × 3 ml), 50% (v/v) 1-propanol containing 4% (w/v) DTT, and 1% (v/v) glacial acetic acid (1 × 6 ml). Extractions were carried out at 60°C, except for salt extractions which were carried out at 4°C to minimize proteolytic degradation. The water wash, which was conducted at room temperature, was inserted to remove traces of salt. Extraction time in all cases was 30 min with continuous stirring followed by centrifugation for 10 min at 20,000 × *g*. First and second extracts were pooled proportionately (2:1) prior to RP-HPLC analysis, and the pooled 50% (v/v) 1-propanol extract was reduced before precipitation to facilitate RP-HPLC analysis. Precipitation of high molecular weight (HMW) glutenin was performed by the addition of 1-propanol containing 1% (w/v) DTT to bring the final 1-propanol concentration to 65% (v/v). Following precipitation and centrifugation, the precipitate was resuspended at 60°C in 50% (v/v) 1-propanol buffered to pH 7.5 with 0.082*M* Tris-HCl and containing 2*M* urea and 1% (w/v) DTT. The resuspended protein then was alkylated with 71% (v/v) 4-vinylpyridine in 50% 1-propanol at 60°C just prior to HPLC analysis. Details of the procedure are as previously described (Marchylo et al 1989). All samples were extracted in triplicate.

RP-HPLC

A Waters HPLC and Waters 840 data and chromatography control station (Waters Associates, Inc., Milford, MA) in conjunction with a Supelcosil LC-308 RP-HPLC column (C_8, 300 Å pore size, 5 μm particle size, 5 cm × 4.6 mm i.d. [Supelco Inc., Bellefont, PA]) were used for quantitative analysis of extracted storage proteins essentially as described before (Marchylo and Kruger 1988, Marchylo et al 1989).

RESULTS AND DISCUSSION

Environmental Influence on Variety Identification

At the present time, the method most widely used for routine varietal identification of wheat samples is electrophoresis. For example, at the Canadian Grain Commission, acid polyacrylamide gel electrophoresis (Tkachuk and Mellish 1980) is used to routinely identify car lot and cargo samples as well as submitted producer and appeal samples on behalf of the Grain Inspection Division. Although this procedure is very useful as a supplement to visual identification, it is a time-consuming and labor-intensive procedure because single kernels must be analyzed in order to determine the proportions of varieties in a mixed sample. Conversely, because of the computerized and quantitative nature of RP-HPLC, it may be possible to use this technique in a system designed to automatically identify the varieties present in a mixture based on the analysis of a single ground bulk sample (Bietz and Cobb 1985, Marchylo et al 1988). In order for such a system to work, however, the qualitative and quantitative effect of environment on the gliadin and glutenin composition of wheat varieties must be minimal.

Qualitatively, samples grown at different locations exhibited the same characteristic protein "fingerprints" as illustrated in Figure 1 for Neepawa and HY 320 samples of widely different protein contents. This corroborates previous work (Kruger and Marchylo 1984) and meets the necessary criteria for an automatic variety identification procedure. Some differences in relative peak heights were evident, however, among chromatograms of samples from different environments. Examples of peaks affected by environment are pointed out by arrows in Neepawa (Fig. 1A and B) and HY 320 (Fig. 1C and D) chromatograms. In the case of some minor peaks, as exemplified by peaks in Neepawa area 3 (Fig. 1A and B), this variation resulted in an apparent change in protein fingerprint, because a relative increase in one peak obscured an adjacent peak. Although these changes were minor, they could lead to problems in distinguishing among varieties with similar protein fingerprints such as Neepawa and Marshall (Marchylo et al 1988).

Analysis of Extracted Storage Protein

The relationship between total protein and extracted storage protein area will be a required part of any automated RP-HPLC varietal identification procedure based on the analysis of a single ground bulk sample. This is necessary because of the use of catalogued varieties of known protein contents for comparison and quantitation as described previously (Bietz and Cobb 1985,

Marchylo et al 1988). Since the protein content of unknown samples will not necessarily be equal to those of catalogued reference varieties and since the extracted protein fraction (gliadins and glutenins) and not total protein is used in RP-HPLC, an understanding of the relationship between total protein and extracted protein is essential.

Scatter diagrams of extracted storage protein (i.e., extracted gliadins and glutenins) chromatogram area versus total protein content are shown in Figure 2 for Neepawa and HY 320, respectively. Statistical regression analysis indicated that total extracted protein chromatographic area was highly positively correlated (r^2 values equal to or better than 0.90) with total protein content. The observed linear relationship between total chromatogram area and total protein was comparable for Neepawa and HY 320, there being no statistical difference between regression lines at the 5% level of significance. In the case of Neepawa, statistical comparison of the regression lines obtained for each of the four years tested failed to detect any differences between years at the 5% level of significance.

Scatter of points around the regression lines, due to environmental influence, could result in considerable errors in accurate quantitative estimation of the relative proportions of varieties in a mixture. Such deviations were observed for two HY 320 samples from geographically distinct sites, Regina and Glenlea, as shown by the circled points in Figure 2. These samples had total protein levels of 13.1 and 14.0% (±0.1% [Williams 1975]), respectively, yet they exhibited equivalent extracted storage protein areas at the 95% significance level as determined by a two-sample t test for a difference in means following six replicate HPLC analyses. These points were chosen to illustrate how similar chromatographic areas for extracted storage protein can result from wheat samples differing in protein content. Similarly, the corollary of this is that extractable protein (i.e., total chromatogram area) also may vary for wheat samples of comparable protein content. Deviations such as this could be especially critical when analyzing samples containing small amounts of nonprescribed varieties since this could affect grade assignment.

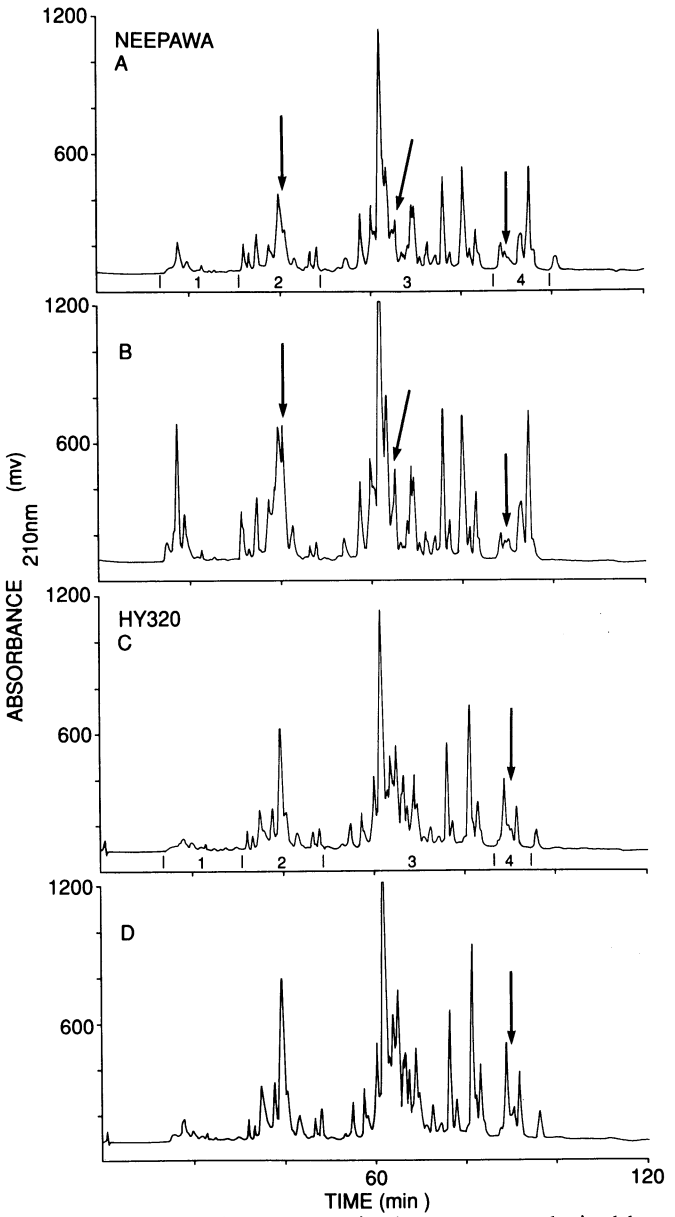

Fig. 1. Comparison of storage protein chromatograms obtained by reversed-phase high-performance liquid chromatographic analysis of wheat samples grown at different sites in Western Canada and exhibiting different protein contents. **A,** Neepawa grown at Indian Head in 1985 with a protein content of 11.8% (13.5% moisture basis [mb]); **B,** Neepawa grown at Acme in 1987 with a protein content of 17.0% (13.5% mb); **C,** HY 320 grown at Ellerslie in 1987 with a protein content of 11.5% (13.5% mb); **D,** HY 320 grown at Scott in 1987 with a protein content of 14.1% (13.5% mb). Arrows denote some peaks that appear to be influenced by environment and chromatograms A and C are subdivided into area segments 1–4 for quantitative analysis.

Quantitative Effect of Environment on Storage Protein Composition

Chromatograms were divided into four area segments (Fig.

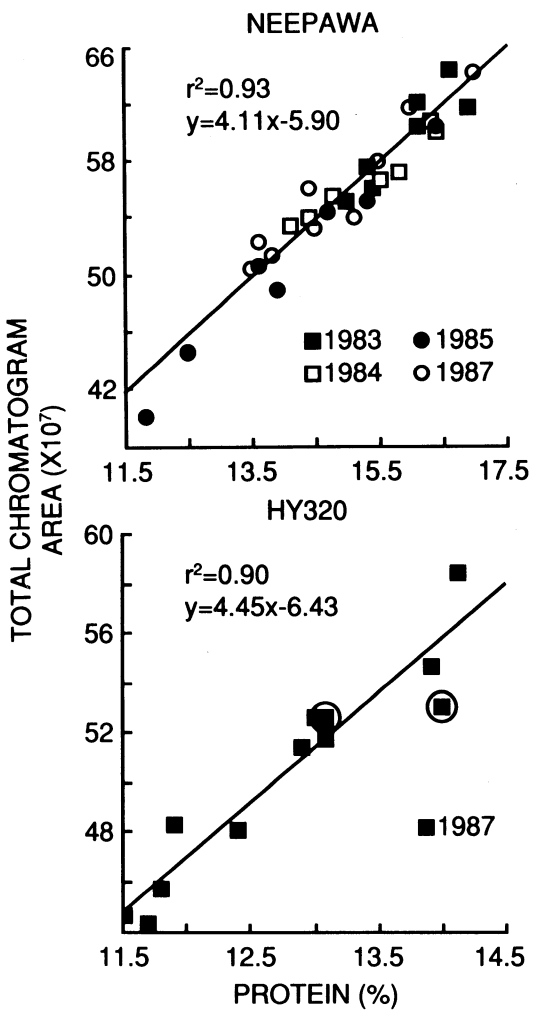

Fig. 2. Relationship between wheat protein content and extracted storage protein (gliadins and glutenins) content as total chromatogram area by reversed-phase high-performance liquid chromatography analysis of 50% 1-propanol + 1% DTT extracts of Neepawa and HY 320 wheat samples grown at different sites in Western Canada during 1983–1985 and/or 1987. Circled squares for HY 320 highlight two samples differing in total protein content by almost 1% but exhibiting comparable storage protein areas.

1A and C) and were expressed as a percentage of total chromatogram area. The area segments generally were divided at exactly the same retention times; however, in some instances, these times were varied slightly to accommodate minor changes in retention times due to column variability. Previous work (Huebner and Bietz 1988, Kruger et al 1988, Marchylo et al 1989) has shown that group 1 proteins consisted primarily of ω-gliadins; group 2 contained both the HMW glutenin subunits as well as some ω-gliadins; and groups 3 and 4 were comprised of α-, β-, and γ-gliadins and low molecular weight (LMW) glutenin subunits. As shown in Table I for both Neepawa and HY 320, the variability of the individual area segments was evident as indicated by differing coefficients of variation (CV %). Area segment 1, i.e. the ω-gliadins, showed the greatest variability for both varieties. This may be observed visually in chromatograms of Neepawa samples from Indian Head and Acme (Fig. 1A and B), where the relative contribution of the group 1 proteins (ω-gliadins) is obviously higher for the Acme sample. This would be in agreement with the results reported by Huebner and Bietz (1988) for some wheat varieties grown in the northern United States. In all cases the CVs for the area segments were greater than the average CVs for duplicates. To determine if this variability was due to environmental influence, a two-factor (year and location) completely randomized design analysis of variance (ANOVA) was performed on the Neepawa data for the years 1983–1985 and 1987 and for the locations noted in Materials and Methods. All chromatogram segment areas (as % of total area) displayed a statistically significant ($\alpha = 0.025$) environmental effect of year and location. A one-way (location) ANOVA was performed on the HY 320 data for 12 locations noted in Materials and Methods, since only samples grown in 1987 were analyzed and a very significant ($\alpha = 0.005$) location effect also was obtained for all segment areas. Although not determined in this study, consideration should be given to the possibility that individual peaks could exhibit far greater variation, which might be masked within each of these groups. Finally, it is of note that although these results are indicative of a statistically significant environmental influence under Canadian prairie environmental conditions, the overall magnitude of the interaction is not large as shown by the relatively low CVs in Table I. This is in keeping with previous reports for barley (Marchylo and Kruger 1984) and maize (Smith and Smith 1986).

These results suggest that the quantitative automatic determination by RP-HPLC of varieties present in a mixture (Bietz and Cobb 1985, Marchylo et al 1988) may be influenced by environment. The magnitude of the interaction may not be large but could introduce considerable error into the quantitative estimation of the relative proportion of varieties present in small quantities in a sample.

TABLE I
Statistical Analysis of the Relative Contribution of RP-HPLC[a] Chromatogram Area Segments to Total Extracted Storage Protein Area for Wheat Samples Grown Under Different Environmental Conditions

Area Segment[a] No.	Area Segment as a % of Total Area	Standard Deviation (%)	CV (%)	Average CV of Duplicates (%)
Neepawa[b]				
1	7.0	0.6	9.0	4.8
2	20.5	1.1	5.3	1.2
3	60.5	1.2	2.0	0.5
4	12.1	0.5	4.3	1.1
HY 320[c]				
1	3.7	0.4	10.9	4.5
2	20.1	0.5	2.7	0.5
3	67.5	1.0	1.5	0.4
4	8.7	0.6	7.0	1.1

[a] Reversed-phase high-performance liquid chromatography (RP-HPLC) chromatograms are subdivided into area segments as shown in Figure 1A (Neepawa) and 1B (HY 320).
[b] $n = 30$.
[c] $n = 13$.

TABLE II
Ratios of Selected Protein Segment Areas in Sequential Extracts of Neepawa Wheat Samples Grown in Different Environments

Location	Year	Wheat Protein[a] (%)	HMW(P)/[HMW(PD) + HMW(PDA)][b]	(P)/(PD)[c]
Acme	1987	17.0	0.3	1.7
Saskatoon	1987	13.6	0.3	2.7
Indian Head	1985	11.8	0.4	2.8

[a] Results are averages of duplicates with standard deviations of 0.1% (Williams 1975).
[b] High molecular weight (HMW)(P), HMW(PD), and HMW(PDA) represent the total peak area of HMW glutenin subunits sequentially extracted into 50% 1-propanol, 50% 1-propanol + 1% dithiothreitol (DTT) and 50% 1-propanol + 4% DTT + 1% acetic acid, respectively. Results are means of three separate analyses and the average standard deviation is 0.1 (Marchylo et al 1989).
[c] P and PD represent the total chromatographic area of gliadins and glutenins extracted sequentially into 50% 1-propanol (P) and 50% 1-propanol + 1% DTT (PD), respectively. Results are the mean of three separate analyses and the average standard deviation is 0.2 (Marchylo et al 1989).

Environmental Influence on Quality Prediction by RP-HPLC

RP-HPLC also may be useful for quality prediction purposes (Huebner and Bietz 1985, 1988; Kruger et al 1988; Marchylo et al 1989). For example, the relative extractability of both gliadins and glutenins in a sequential extraction scheme, using solvents including 50% 1-propanol (P), 50% 1-propanol containing 1% DTT (PD), and 50% l-propanol containing 4% DTT and 1% acetic acid (PDA), appeared to be related to dough strength and loaf volume (Marchylo et al 1989). Therefore, to obtain some indication if protein differences resulting from environment can influence the prediction of quality by RP-HPLC, three Neepawa samples grown at different locations and with a wide range of total protein contents (11.8, 13.6, 17.0%) were sequentially extracted; HMW subunits were precipitated and alkylated, and proteins were analyzed quantitatively by RP-HPLC. The amount of HMW glutenin subunits extracted into propanol relative to those extracted into propanol + DTT and propanol + DTT + acetic acid (i.e., HMW [P]/HMW [PD] + HMW [PDA]), previously was shown to increase with decreasing dough strength and loaf volume (Kruger et al 1988; Marchylo et al 1989). In the present study (Table II), however, this ratio was not significantly different at the 95% confidence level, as determined by two sample t tests, for the three Neepawa samples. This suggests that there is little environmental influence. Ratios of total chromatogram area in 50% 1-propanol (gliadins and some glutenins) relative to the total chromatogram area in 50% 1-propanol + 1% DTT (primarily HMW and LMW glutenins) (i.e., P/PD, Table II) also were not significantly different for the lower protein Neepawa samples grown at Saskatoon and Indian Head. However, the high-protein sample grown at Acme exhibited a P/PD ratio that was significantly lower (95% confidence level) than that of the lower protein samples. This ratio, which was shown also to be related inversely to dough strength and loaf volume (Marchylo et al 1989), would predict a higher dough strength and loaf volume potential for the Neepawa Acme sample than for the Saskatoon and Indian Head samples of the same variety. Thus, it appears that environmental variations can significantly influence this ratio, and quality screening by RP-HPLC such as in a plant breeding program could be affected.

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