# Identification of Canadian Barley Cultivars by Reversed-Phase High-Performance Liquid Chromatography[1,2]

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ABSTRACT

The potential of reversed-phase high-performance liquid chromatography (RP-HPLC) was assessed for identification of Canadian barley cultivars. An RP-HPLC system previously described by Bietz was adapted to resolve components of the barley prolamin (hordein) protein fraction. Sample preparation and chromatographic conditions were optimized. A significantly larger number of hordein components were resolved by RP-HPLC as compared to electrophoretic techniques. Elution profiles were reproducible. Analysis of 12 Canadian cultivars showed that 10 could be distinguished easily on the basis of elution profiles. The two remaining cultivars showed identical elution profiles, but they could be distinguished by reproducible quantitative differences in the proportions of C and B hordeins. An environmental study demonstrated that elution profile was independent of growth location, year of growth, and protein content. However, a statistically significant interaction between year and location of growth and the proportion of C and B hordeins was evident. However, this interaction did not appear large enough to influence the distinguishability of cultivars based on differences in the C and B hordein proportions. RP-HPLC has considerable potential to serve as a barley cultivar identification technique.

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In recent years, extensive research has been done in the application of high-performance liquid chromatography (HPLC) for analysis and purification of proteins and peptides. Proteins can be separated on the basis of size by high-performance size exclusion chromatography (HPSEC), ionic charge by high-performance ion-exchange chromatography (HPIEC), and hydrophobicity by reversed-phase high-performance liquid chromatography (RP-HPLC) (Regnier and Gooding 1980). Most of this research has been directed toward human and animal proteins, and only recently has HPLC been applied to cereal proteins (Bietz 1983). In particular, Bietz found that RP-HPLC showed excellent resolution of proteins from wheat and corn.

In this laboratory, barley cultivars are routinely identified by electrophoretic techniques such as acidic polyacrylamide gel electrophoresis (Marchylo and LaBerge 1980, 1981) or sodium dodecyl sulfate gradient-gel electrophoresis (*unpublished results*). However, a significant number of Canadian barley cultivars are indistinguishable by these electrophoretic techniques. Therefore, the present study was undertaken to explore the potential of RP-HPLC for complementing electrophoretic methods as a barley cultivar identification technique. The procedures described by Bietz (1983) for separation of wheat and corn proteins were adapted to the separation of hordein proteins from two- and six-rowed barley cultivars grown in Canada.

## MATERIALS AND METHODS

### Chemicals and Reagents

All chemicals used in this study were reagent grade, unless noted otherwise. Acetonitrile (HPLC grade) with an ultraviolet cutoff wavelength of 190 nm was obtained from Fisher Scientific (Fair Lawn, NJ). Trifluoracetic acid (Sequanol grade) was obtained from Pierce Chemical Co. (Rockford, IL). Water was purified initially by passing deionized water through a Millipore Super-Q system (Millipore Corp., Bedford, MA), which contained two 0.8-$\mu$m filters, a charcoal filter, and a deionizing filter. Subsequently, it was purified further by passage through a Millipore type HA 0.45-$\mu$m filter.

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[2]Presented at the AACC 68th Annual Meeting, Kansas City, MO, October 1983.



### Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) Apparatus

The equipment used was a Waters model ALC/GPC HPLC (Waters Associates, Milford, MA) consisting of two solvent reservoirs, Wisp automatic sample injector, models 6000A and M45 solvent-delivery systems controlled by a model 660 solvent programmer, and a model 450 variable-wavelength detector attached to a Spectra-Physics SP4000 computing integrator with digital interface and printer plotter (Technical Marketing Association, Mississauga, Ont., Canada).

A 25 × 0.41-cm Synchropak RP-P column (reversed-phase [$C_{18}$] support, 10-$\mu$m particle diameter, 300-Å pore diameter, Regis Chemical Co., Morton Grove, IL) preceded by a 0.5-$\mu$m precolumn filter (Upchurch Scientific, Oak Harbor, WA) was used. The precolumn filter was changed when column pressure increased by 200–300 lb. Solvents A and B consisted of 15 and 80% acetonitrile, respectively, and 0.1% trifluoroacetic acid. Solvents were placed in volumetric flasks that were sealed to minimize evaporation. As suggested by Brown et al (1981), the solvents were sparged continuously with helium to prevent baseline drift. Elution of proteins was monitored at 210 nm. Optimized running conditions, used throughout the remainder of this study, consisted of a linear gradient extending from 25 to 60% B with a run time of 105 min at a flow rate of 1 ml/min. Following each run, the column was equilibrated with starting solvent for 15 min. After approximately 30 runs, a linear gradient extending from 0 to 100% B was passed through the column to remove adsorbed proteins.

### Barley Samples

The two- and six-rowed barley cultivars used throughout this study were selected from pure seed maintained by plant breeders.

### Extraction Studies

The basic solvent used to extract hordein proteins consisted of 50% 1-propanol containing a reducing agent. Three reducing agents, dithiothreitol, mercaptoethanol, and monothioglycerol, at concentrations of 0.5, 1.0, 2.0, and 5.0% (w/v or v/v) were tested for extraction periods of 30, 60, and 120 min.

Ground barley was prepared in a Udy Cyclone sample mill equipped with a 1-mm sieve, unless otherwise noted.

Hordein proteins were extracted from ground grain, for the two-rowed cultivar Betzes, by placing 0.1 g of ground grain with 400 $\mu$l of the appropriate extracting solution in a 1.5-ml micro test tube (Bio-Rad Laboratories, Richmond, CA). The mixture was vortexed and then placed in a 60° C water bath for the appropriate extraction period. The mixture was vortexed at 10-min intervals during the extraction period. Extracts then were centrifuged in an Eppendorf Microcentrifuge model 5413 (Brinkmann Instruments

Inc., Westbury, NY) at 8,800 × *g*. After centrifugation, supernatant was passed through a Millipore type FH 0.5-μm filter, and the filtrate was placed in sample vials for HPLC analysis.

Optimum extraction conditions were determined to be 30 min extraction at 60° C using an extraction solvent consisting of 50% l-propanol containing 1% dithiothreitol. Subsequent extractions were performed using these optimized conditions.

### Alkylation of Hordein Proteins

Hordein proteins extracted under optimum conditions from ground Betzes were alkylated as described previously (Marchylo and LaBerge 1980) with some modifications. Extracts (100 μl) were mixed with varying concentrations (0.1–0.5*M* and 1.0*M*) of iodoacetamide (100 μl) dissolved in 50% l-propanol and then were incubated at 60° C for 15 min.

### Preparation of Single Kernel Extracts

Single kernels were ground to a powder in a mortar with a pestle. Ground single kernels then were extracted with 250 μl of extracting solvent as described for ground grain using optimized conditions.

### Sodium Dodecyl Sulfate (SDS) Gradient-Gel Electrophoresis

Hordein protein was extracted from the cultivar Betzes using optimized conditions for ground grain. The extract (2 × 100 μl) was analyzed by RP-HPLC, and eluent fractions were collected. The fractions were reduced to about 1 ml with an SC-3 Sample Concentrator (Techne [Cambridge Ltd.], Cambridge, England). The concentrate was freeze-dried and then resolubilized in electrophoresis buffer. Hordein proteins, present in each fraction,

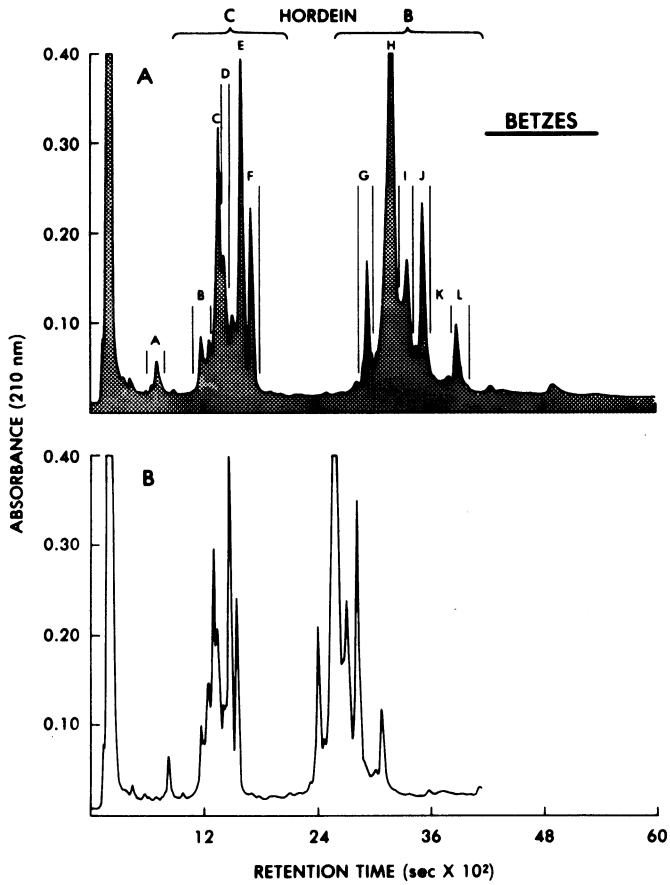


**Fig. 1.** The effect of run time on resolution of hordein proteins by reversed-phase high-performance liquid chromatography. **A,** 105 min, **B,** 70 min. A–L represent eluent fractions collected for electrophoretic analysis. Extracts were prepared from ground grain, using optimum extraction conditions. Column elution conditions consisted of a linear gradient extending from 25 to 60% B at a flow rate of 1 ml/min. Column eluent was monitored at 210 nm with a detector sensitivity of 0.4 absorbance units full-scale.

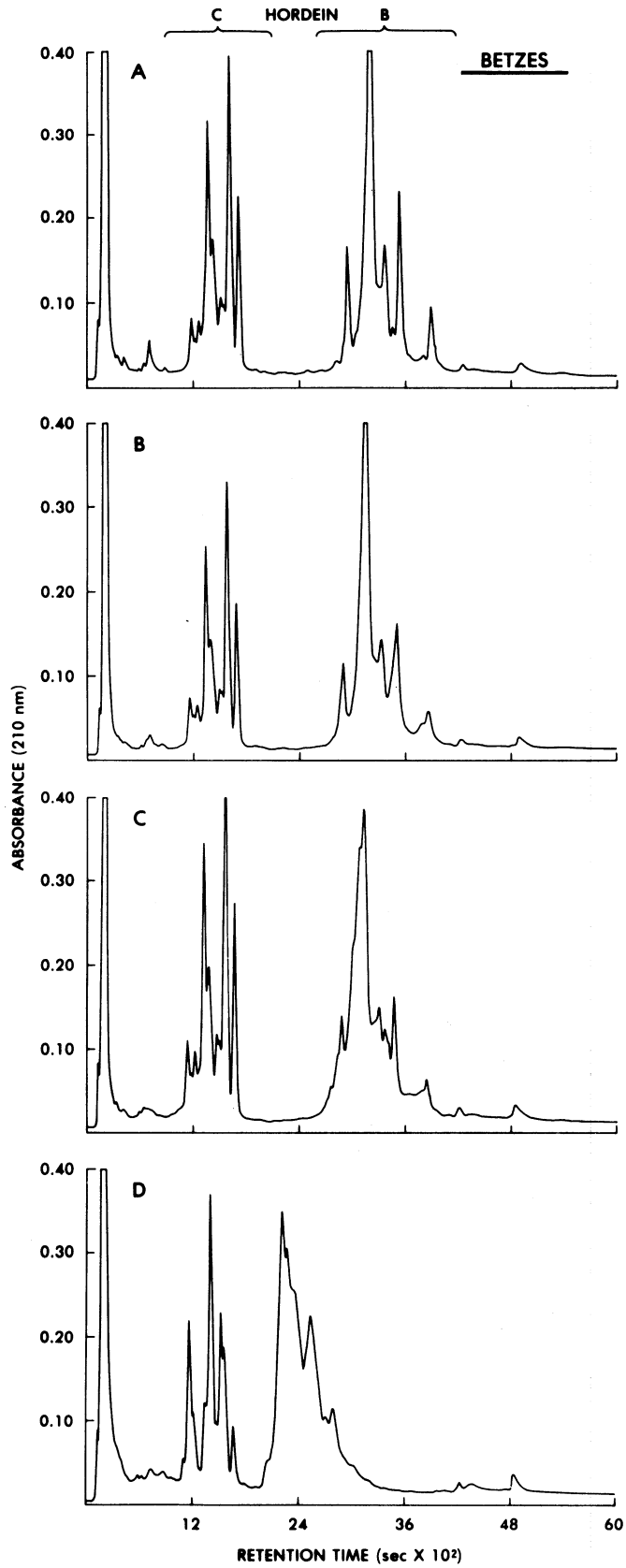


**Fig. 2.** The effect of reducing agent and alkylation on resolution of hordein proteins by reversed-phase high-performance liquid chromatography. **A,** dithiothreitol, **B,** mercaptoethanol, **C,** monothioglycerol. The following quantities were used to prepare the extracts: 0.1 g of ground grain + 400 μl of 50% l-propanol containing 1% reducing agent. The extraction mixture was incubated for 30 min at 60° C. **D,** alkylated extract. The extract was prepared using dithiothreitol (as described in **A**), then was alkylated with 0.5*M* iodoacetamide.

were resolved by sodium dodecyl sulfate (SDS) gradient-gel electrophoresis (11–17% acrylamide gradient) performed in a Bio-Rad Protean vertical electrophoresis cell. The hordeins then were stained by a rapid ultrasensitive silver-based color stain (*unpublished data*).

### Effect of the Environment

The hordein protein composition of the six-rowed cultivar Bonanza was analyzed by RP-HPLC for samples grown during the 1981 and 1982 crop years at seven locations in western Canada. Growth locations were Winnipeg, Brandon, Indian Head, Saskatoon, Regina, Lethbridge, and Calmar. Protein content of these samples was determined by a standard Kjeldahl procedure. One sample from each location and year was ground in a coffee mill. Hordein protein extracts then were prepared in duplicate, using the optimized extraction conditions for ground grain.

## RESULTS AND DISCUSSION

### Chromatographic Conditions

Chromatographic conditions for elution of hordein proteins were optimized according to the procedure of Bietz (1983). A linear gradient, with 15 and 80% acetonitrile containing 0.1% trifluoroacetic acid as solvents A and B, respectively, extending from 25 to 60% B, eluted all hordein proteins. As evident in Fig. 1A and B, the range of percent B could have been decreased further to increase resolution; however, room at the beginning and end of the gradient was maintained to accommodate any cultivars with earlier- or later-eluting proteins. A running time of 105 min at a flow rate of 1 ml/min (gradient slope, 0.33% B/min) was chosen as optimum for resolution of hordein proteins (Fig. 1A). A shorter run time of 70 min (Fig. 1B) resulted in a slight decrease in resolution, as indicated by the loss of some minor peaks. Resolving power decreased significantly for shorter run times.

Injection volumes were varied according to the amount of protein extracted for different cultivars. Typically, 20–40-$\mu$l injections were used, with no improvement in resolution evident for smaller injection volumes.

### Extraction Conditions

1-Propanol (50%) was the alcohol chosen to extract hordein proteins because of its demonstrated extraction efficiency (Shewry et al 1980). Reducing agent was included in the extracting solvent for the efficient extraction primarily of the B and D hordeins (Shewry et al 1980, Miflin et al 1983). Reducing agents such as mercaptoethanol (Shewry et al 1980) or monothioglycerol (Marchylo and LaBerge 1980) previously have been used to aid in the extraction of hordein protein. A series of extractions performed at 60° C using dithiothreitol (DTT), mercaptoethanol (ME), and monothioglycerol (MTG) (0.5, 1.0, 2.0, 5.0%; w/v or v/v) for extraction times of 30, 60, and 120 min indicated that extraction with 1% DTT for 30 min yielded the best chromatograms following RP-HPLC. As shown in Fig. 2, for comparable concentrations of reducing agent (1%) and extraction time (30 min) chromatograms of extracts containing DTT (Fig. 2A) exhibited the sharpest peaks and best resolution primarily within the B hordeins. Increasing extraction time or DTT concentration did not increase the number of proteins extracted and did not improve resolution. A lower DTT concentration of 0.5% exhibited a slight decrease in resolution. Band spreading for B hordein peaks concomitant with decreased resolution of minor peaks was apparent for extracts containing ME (Fig. 2B). Increasing the ME concentration or extraction time did not improve band spreading or resolution and did not increase the number of proteins extracted. Severe band spreading and poor resolution of B hordeins were shown for extracts containing MTG (Fig. 2C). Band spreading and resolution did improve appreciably with increasing MTG concentration and extraction time. However, even after 2 hr of extraction with 5% MTG, inferior resolution was evident in comparison to DTT results. Changes in the peak pattern within the B hordein fraction with increasing extraction time and concentration suggested that the B hordeins were incompletely reduced.

In all cases, resolution of C hordeins was comparable, which probably reflects the low content of sulfur amino acids and easy extractability of this protein.

Alkylation of hordein proteins with iodoacetamide following reduction was performed to prevent reoxidation. As shown in Fig. 2D, this procedure had a deleterious effect on resolution and significantly changed the elution profile of both C and B hordeins. Increasing the concentration of iodoacetamide (0.1–1.0 *M*) resulted in progressive deterioration of both C and B hordein resolution. In addition, retention times progressively decreased with increasing iodoacetamide concentration. Hordein proteins, therefore, were not alkylated before RP-HPLC. The stability of unalkylated reduced hordeins was determined by successively analyzing aliquots of an extract (cultivar Betzes) over three days. Reproducible elution profiles obtained indicated that reoxidation of the unalkylated reduced hordein was not taking place. However, elution profiles for extracts exposed to the air for extended periods showed changes in B hordein components, which was suggestive of reoxidation.

### Reproducibility of Retention Times

Separate extracts of the two-rowed cultivar Betzes and six-rowed cultivar Bonanza were analyzed over several days to determine reproducibility of the RP-HPLC technique within and between days. The average coefficient of variation (CV) in retention time for a set of runs performed within one day (Table I) were comparable to the results reported by Bietz (1983) for wheat gliadins. However, the average CV obtained for runs performed over a number of days was higher (Table II). This increase in CV could in part be due to column temperature variations (the column was not thermal-regulated) or to slight day-to-day variations in buffer composition. Retention times of peaks typically differed by about 7–12 sec between successive runs; however, some drift in retention times with time was observed. Control of acetonitrile evaporation from solvent buffers minimized this problem. In general, reproducibility of retention times is more than acceptable for cultivar identification purposes.

### SDS Gradient-Gel Electrophoresis Characterization of Hordein Proteins Separated by RP-HPLC

Fractions as indicated in Fig. 1A were collected and analyzed by SDS gradient-gel electrophoresis to determine the hordein protein

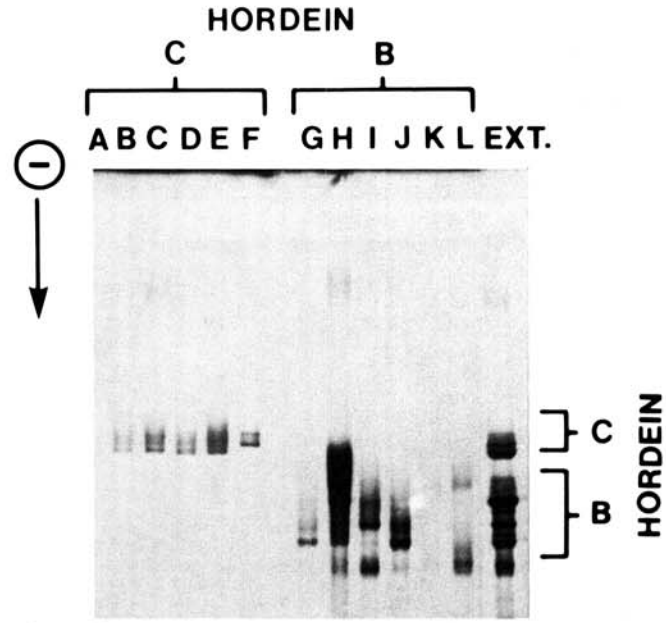


**Fig. 3.** Electrophoregram of hordein proteins (from Betzes barley) separated by reversed-phase high-performance liquid chromatography. Wells A–L correspond to collected fractions indicated in Fig. 1A. Ext. represents the initial extract before RP-HPLC analysis. Fractions were collected, prepared, and analyzed by sodium dodecyl sulfate gradient-gel electrophoresis.

composition of peaks eluted by RP-HPLC. As shown in the resulting electrophoregram (Fig. 3), all hordein components present in the original extract were eluted by RP-HPLC. The first group of peaks resolved (fractions A–F) were composed of C hordeins, whereas the second group (fractions G–L) represented the B hordeins. Resolution of these two groups was excellent with no overlap. Individual fractions all contained more than one protein component, although there was little overlap between adjacent peaks (eg, fractions I,J). The separation of hordein proteins by RP-HPLC within the C and B groups differed significantly from that exhibited by SDS gradient-gel electrophoresis, which separates proteins on a molecular weight basis. This behavior is consistent with results reported by Bietz (1983), which showed that separation of gliadin or zein proteins by RP-HPLC differed markedly from separations primarily based on charge.

**Cultivar Analyses**

The hordein protein compositions of 12 six- and two-rowed barley cultivars were analyzed by RP-HPLC to determine whether elution profiles could be used to distinguish among cultivars (Figs. 1A, 4, 5). Analyses were performed on ground grain samples as well as on single kernels. Elution profiles were qualitatively the same in both cases. With the exception of the two-rowed cultivars Fairfield and Fergus, all cultivars were distinguished easily on the basis of elution profiles. Subsequently, quantitative analysis of elution profiles for Fairfield and Fergus showed that the relative proportions of C and B hordein differed between cultivars. Reproducibility of the C/B ratio then was determined. Ten ground grain extracts of each cultivar were analyzed over the course of several days, and the C/B ratio was determined from each elution profile. Data were analyzed statistically with an unpaired $t$ test in which variances are assumed equal but unknown. A C/B ratio of

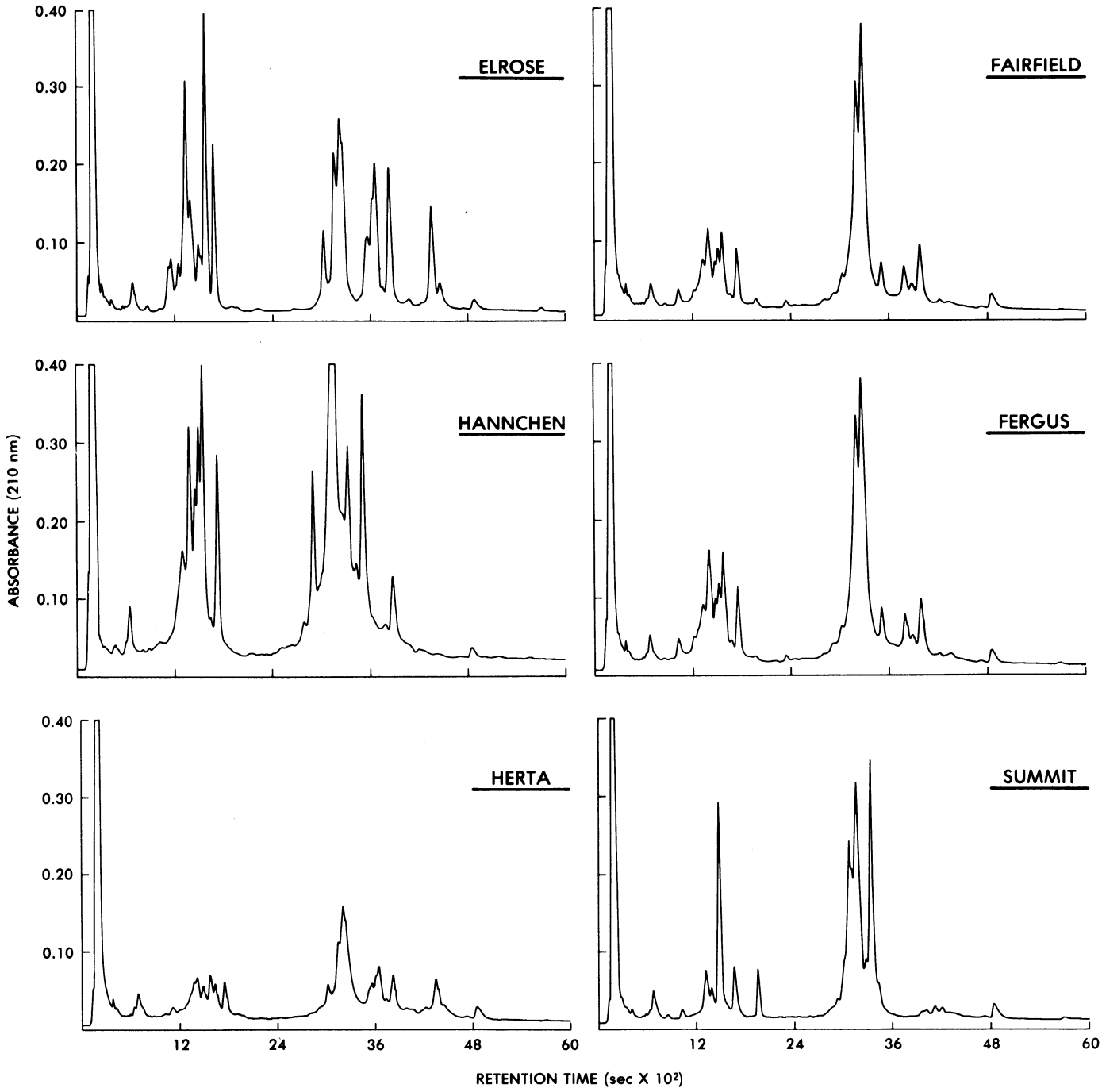


**Fig. 4.** Reversed-phase high-performance liquid chromatography separations of hordein proteins extracted from two-rowed Canadian barley cultivars. Optimum extraction conditions for ground grain and optimum chromatographic conditions were used.

$0.395 \pm 0.033$ ($\bar{x} \pm s$) was obtained for Fairfield as compared to 0.529 $\pm$ 0.038 for Fergus. The observed value of $t$ was 8.394 with 18 degrees of freedom and significance probability of 0.0000. In other words, the difference in the C/B ratio between cultivars was highly significant. Thus, it would appear that two cultivars with identical elution profiles can be distinguished by using the quantitative capabilities of RP-HPLC.

**Environmental Study**

The extent of interaction of environment with the hordein elution profile must be determined to correctly assess the usefulness of RP-HPLC as a barley cultivar identification technique. Samples of the six-rowed cultivar Bonanza, grown at seven locations in western Canada during two crop years, were analyzed by RP-HPLC to ascertain the extent of environmental interaction. Protein content of these samples ranged between 13.5 and 16.4% (dry weight basis).

Qualitatively, the hordein elution profiles for all samples were identical, within the limits of reproducibility of retention times, to the Bonanza profile illustrated in Fig. 5. Thus hordein elution profiles are not affected qualitatively by location of growth, year of growth, or protein content.

Quantitatively, statistical analysis of the 28 samples analyzed yielded a C/B ratio of $0.734 \pm 0.047$ ($\bar{x} \pm s$). There was no observed interaction between protein content and this ratio. Further statistical analysis failed to detect an interaction between location and year of growth, but there was evidence of a year and a location effect on the C/B ratio.

The influence of environment on the C/B ratio was determined by using a two-factor completely randomized design with two replications, where factor A is year of growth and factor B is location of growth. A fixed-effects model was assumed appropriate. A summary of the statistical analysis is shown in Table III. The levels of significance ($\alpha$) for the year and location interactions were 0.025 in both cases. This observed effect of environment on the C/B ratio may be due to a variable supply of S relative to N between growth locations and years. Shewry et al (1983) recently reported evidence that showed that a variable supply of S relative to N could alter the balance of the endosperm storage proteins (ie, the hordeins). Even though the experiments

**TABLE I**
**Reproducibility of Retention Times (within one day) for Reversed-Phase HPLC Separation of Hordein Proteins**

| Peak Number | Mean Retention Time $\bar{x}$ (sec) | Standard Deviation of the Mean $S\bar{x}$ (sec) | Coefficient of Variation (%) | Retention Time (sec) Minimum | Retention Time (sec) Maximum |
|---|---|---|---|---|---|
| | | **Betzes** | | | |
| 1 | 1,198 | 2.67 | 0.55 | 1,187 | 1,207 |
| 2 | 1,621 | 2.00 | 0.30 | 1,611 | 1,623 |
| 3 | 1,726 | 1.61 | 0.23 | 1,719 | 1,731 |
| 4 | 2,981 | 3.91 | 0.32 | 2,963 | 2,989 |
| 5 | 3,597 | 4.18 | 0.28 | 3,579 | 3,605 |
| 6 | 3,954 | 3.89 | 0.24 | 3,939 | 3,965 |
| Average n = 6 | | | 0.32 | | |
| | | **Bonanza** | | | |
| 1 | 1,180 | 2.81 | 0.82 | 1,159 | 1,194 |
| 2 | 1,459 | 1.74 | 0.41 | 1,454 | 1,469 |
| 3 | 1,542 | 1.69 | 0.38 | 1,534 | 1,549 |
| 4 | 1,633 | 1.35 | 0.29 | 1,629 | 1,639 |
| 5 | 1,822 | 2.42 | 0.46 | 1,814 | 1,834 |
| 6 | 3,512 | 3.67 | 0.36 | 3,499 | 3,529 |
| 7 | 4,044 | 3.40 | 0.29 | 4,034 | 4,064 |
| Average n = 12 | | | 0.43 | | |

**TABLE II**
**Reproducibility of Retention Times (day to day) for Reversed-Phase HPLC Separation of Hordein Proteins**

| Peak Number | Mean Retention Time $\bar{x}$ (sec) | Standard Deviation of the Mean $S\bar{x}$ (sec) | Coefficient of Variation (%) | Retention Time (sec) Minimum | Retention Time (sec) Maximum |
|---|---|---|---|---|---|
| | | **Betzes** | | | |
| 1 | 1,187 | 4.59 | 1.68 | 1,147 | 1,215 |
| 2 | 1,608 | 4.04 | 1.10 | 1,575 | 1,627 |
| 3 | 1,716 | 3.87 | 0.98 | 1,705 | 1,727 |
| 4 | 2,972 | 4.26 | 0.62 | 2,927 | 3,004 |
| 5 | 3,588 | 5.09 | 0.62 | 3,539 | 3,629 |
| 6 | 3,952 | 4.59 | 0.51 | 3,909 | 3,994 |
| Average n = 19 | | | 0.92 | | |
| | | **Bonanza** | | | |
| 1 | 1,194 | 3.23 | 1.69 | 1,159 | 1,235 |
| 2 | 1,471 | 3.38 | 1.43 | 1,444 | 1,523 |
| 3 | 1,551 | 3.10 | 1.25 | 1,524 | 1,599 |
| 4 | 1,643 | 3.09 | 1.18 | 1,619 | 1,691 |
| 5 | 1,832 | 3.07 | 1.05 | 1,804 | 1,875 |
| 6 | 3,516 | 3.19 | 0.57 | 3,479 | 3,551 |
| 7 | 4,040 | 2.47 | 0.38 | 4,014 | 4,067 |
| Average n = 39 | | | 1.08 | | |

performed in this study were artificially extreme, the authors did suggest that similar effects could occur in the field.

In view of the observed $t$ statistic (8.394) for comparison of the mean Fairfield and Fergus C/B ratio, the environmental interaction with the C/B ratio, although statistically significant, appears to be of insufficient magnitude to interfere with the distinguishability of these cultivars. However, further analysis of more cultivars sampled over a longer period of time will be necessary to determine more accurately the influence of environmental factors on the C/B ratio.

**TABLE III**
**ANOVA Summary Table**

| Source | DF | SS | MS | F[a] |
|---|---|---|---|---|
| A | 1 | 0.0084 | 0.0084 | 7.498 |
| B | 6 | 0.0245 | 0.0041 | 3.634 |
| AB | 6 | 0.0115 | 0.0019 | 1.695 |
| Error | 14 | 0.0158 | 0.0011 | ... |
| Total | 27 | 0.0602 | ... | ... |

[a] Computed F values assume a fixed-effects model.

## CONCLUSIONS

Reversed-phase HPLC represents an exciting new alternative for separation of hordein proteins, and it appears to be applicable to the identification of Canadian barley cultivars. The many advantages of RP-HPLC already have been discussed by Bietz (1983); however, for the resolution of hordein proteins these advantages are even more apparent. A significantly larger number of hordein components are resolved by RP-HPLC as compared to electrophoretic techniques used in our laboratory. Resolution of a

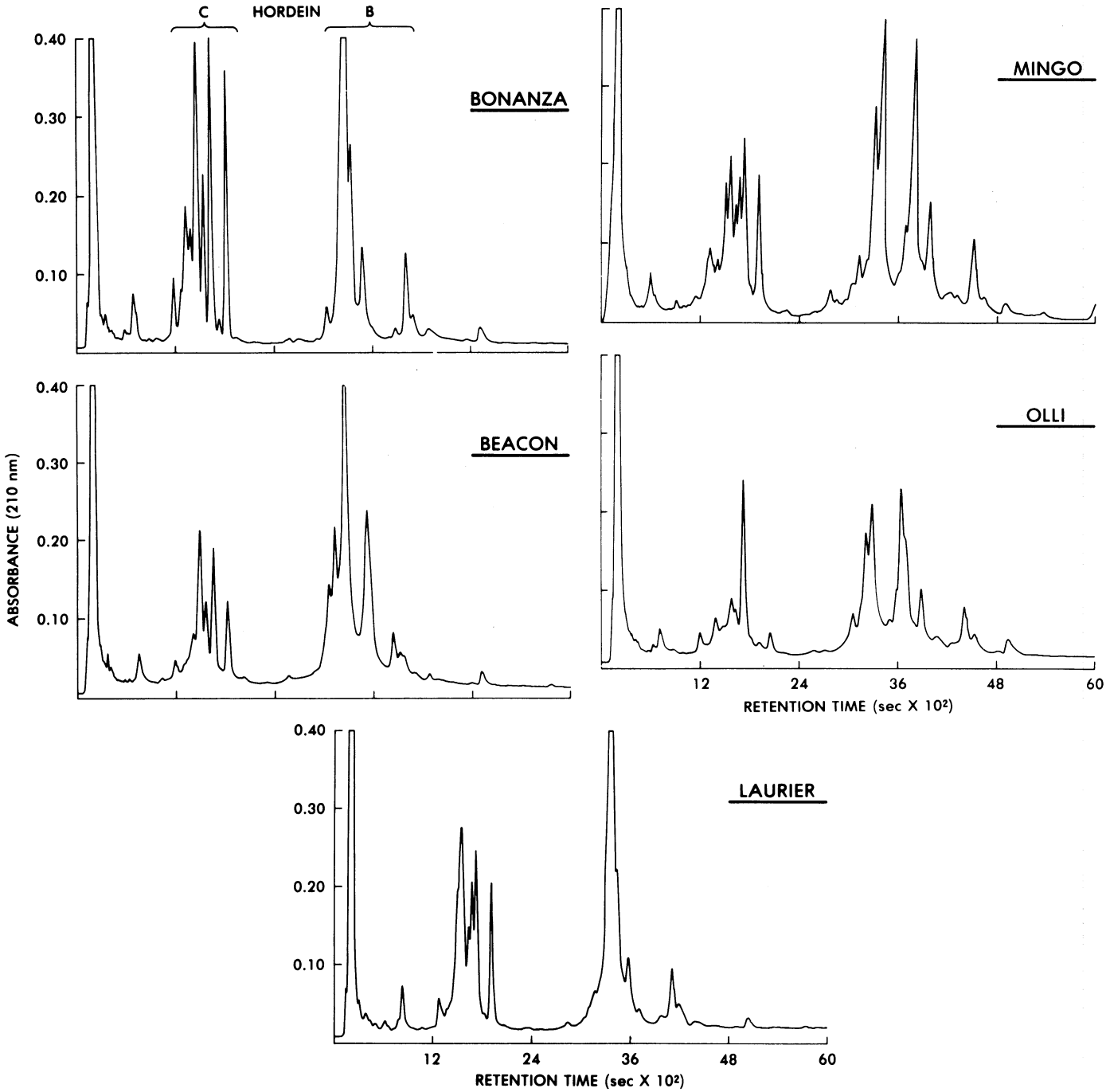


**Fig. 5.** Reversed-phase high-performance liquid chromatography separations of hordein proteins extracted from six-rowed Canadian barley cultivars. Optimum extraction conditions for ground grain and optimum chromatographic conditions were used.

larger number of hordein components presents more possibilities for determining differences between closely related cultivars. The ability of this technique to quantitate individual hordein components also should enhance cultivar identification. After initial setup of the HPLC system, analyses can be easily performed, due to the automated capabilities of the system. This is in contrast to electrophoretic techniques that have considerable "hands-on" time requirements. Substantial run time requirements per sample represent one negative aspect of the RP-HPLC technique. At present, under maximum resolution conditions (ie, 105 min + 15 min of equilibration time per sample), only 12 samples can be processed per day. By comparison, with SDS-gradient gel electrophoresis, up to 40 samples can be processed each day. However, quantitative densitometric analysis of electrophoregrams is time consuming and decreases productivity by about 50% (*unpublished data*). With RP-HPLC, quantitation of the hordein chromatogram is performed within each sample's run time. In addition, maximum resolution may not be necessary for the identification of all samples and substantially shorter run times may be used. This would almost double the output to about 20 samples per day. Development of new columns and HPLC systems within the next few years also could substantially decrease run time requirements for RP-HPLC.

This technique represents a welcome addition to the arsenal of techniques available for identifying Canadian barley cultivars, and further work presently is being undertaken to fully use and assess all its capabilities.


## ACKNOWLEDGMENTS

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