Identification of Wheat Cultivars by Reversed-Phase High-Performance Liquid Chromatography of Storage Proteins[1]

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ABSTRACT

Wheat storage proteins comprised of the gliadins and high and low molecular weight glutenins were resolved by reversed-phase high-performance liquid chromatography (RP-HPLC). Resultant chromatograms or protein "fingerprints" were used to identify 18 registered and one nonregistered Canadian and eight nonregistered American spring wheat cultivars. Six commercially available HPLC columns differing in column dimensions, hydrocarbon chain length, and particle and pore size were evaluated, and the effect of column-to-column variability and column stability on resolution and selectivity are discussed. The effect of column temperature on the separation of storage proteins was studied, and this was assessed in relation to quantitation of chromatograms and the cultivar identification process. Reproducibility of peak areas is reported. On a qualitative basis, all registered cultivars were distinguishable from nonregistered cultivars. Quantitation of chromatograms derived from RP-HPLC analysis of ground grain extracts of admixtures of a registered and nonregistered cultivar was carried out. Highly significant linear correlations were obtained when peak areas or relative peak areas were plotted against cultivar concentration. This provided a basis for quantitative computer analysis of admixtures.

Since Bietz (1983) first proposed that reversed-phase high-performance liquid chromatography (RP-HPLC) of storage proteins could be used for the identification of cereal cultivars, several reports have shown that this technique is applicable to the identification of wheat (Burnouf et al 1983, Bietz et al 1984, Bietz and Cobb 1985), barley (Marchylo and Kruger 1984, Wingad et al 1986, Allison and Bain 1986), barley malt (Marchylo and Kruger 1985), oats (Lookhart 1985), and maize (Paulis and Bietz 1986, Smith and Smith 1986) cultivars. RP-HPLC exhibits excellent resolution and reproducibility (Bietz 1983, 1985; Marchylo and Kruger 1984, 1985), but the most attractive features of the technique are its quantitative and computer capabilities. For example, two Canadian barley cultivars indistinguishable on the basis of acidic PAGE analysis (Marchylo and LaBerge 1981) were distinguishable by quantitative analysis of RP-HPLC chromatograms (Marchylo and Kruger 1984). In addition, Bietz and Cobb (1985) provided preliminary evidence that quantitative computer analysis of RP-HPLC gliadin chromatograms may present a means for determining the proportions of wheat cultivars in a mixture. This capability would provide significant time savings in a routine cultivar identification program because it would obviate the need for tedious single kernel analysis.

The purpose of this study was to apply RP-HPLC to the identification of 19 Canadian and eight American wheat cultivars. Extraction and operating conditions suitable for the analysis of storage proteins present in this diverse group of cultivars were investigated. Column performance for a variety of commercially available RP-HPLC columns was evaluated over various times. Preliminary work on quantitative analyses of wheat admixtures also is reported along with a discussion of practical considerations associated with cultivar identification by RP-HPLC.

MATERIALS AND METHODS

Chemicals and Reagents

Sequanal-grade trifluoroacetic acid and 1-propanol were obtained from Pierce Chemical Co. (Rockford, IL); dithiothreitol, 4-vinylpyridine, and tris(hydroxymethyl)aminomethane (Tris; reagent grade) were from Sigma Chemical Co. (St. Louis, MO); and acetonitrile (HPLC grade) with an ultraviolet cutoff wavelength of 190 nm was from Fisher Scientific (Fair Lawn, NJ). HPLC-grade deionized water prepared with a Barnstead Nanopure II water purification system (Boston, MA) was employed in all solutions.

Wheat Samples

Twenty-seven spring wheat cultivars were analyzed in this study. These included the following 18 registered spring wheat cultivars (Seeds Act 1986): Benito, Canuck, Chinook, Columbus, Garnet, Katepwa, Leader, Manitou, Marquis, Napayo, Neepawa, Park, Pembina, Saunders, Selkirk, Sinton, Thatcher (eligible for Canada Western Red Spring [CWRS] wheat grades), and HY 320 (eligible for Canada Prairie Spring [CPS] wheat grades); one nonregistered spring wheat cultivar, Chester; and eight nonregistered spring wheat cultivars from the United States: Butte, Era, Len, Marshall, Oslo, Solar, Waldron, and Wheaton. Pure seed maintained by plant breeders was used in all cases.

Sample Preparation and Extraction Procedures

Samples of wheat cultivars (25 g) were ground in a Udy cyclone sample mill equipped with a 1-mm sieve. Ground grain was mixed thoroughly before extraction.

Wheat proteins were extracted from ground grain (0.1 g) with 600 μl of the appropriate extracting solution (50% 1-propanol containing 1% DTT, unless stated otherwise) in a 1.5-ml microcentrifuge tube. The mixture was vortexed and then placed in a 60° C water bath for 30 min with vortexing at 10 min intervals during the extraction period. Extracts were centrifuged in an Eppendorf microcentrifuge model 5414 (Brinkmann Instruments Inc., Westbury, NY) at 15,600 × g. Supernatant was filtered through Millex HV4 0.45-μm filters (Millipore Ltd., Mississauga, ON) prior to RP-HPLC analysis of 15-μl aliquots. Wheat proteins were alkylated by mixing 100 μl of extract with 100 μl of 50% 1-propanol containing 0.082M Tris-HCl, pH 8.6, and 0.14M 4-vinylpyridine. The mixture then was incubated at 60° C for 15 min prior to RP-HPLC analysis of 30-μl aliquots.

Preparation and Analysis of Neepawa-Len Admixtures

Samples (1 kg) of Neepawa and Len were ground in a Udy cyclone sample mill and admixtures (100 g) of Neepawa with increasing amounts of Len (0–5, 10, 15, 20, 25, 50, 75, and 100%) were prepared on a weight basis. This stock was mixed thoroughly and 1.0-g subsamples were weighed into centrifuge tubes (50 ml) and mixed with 6 ml of extracting solvent. Protein was extracted as noted for the 0.1-g samples with the exception that the extract mixture was centrifuged at 27,500 × g. Aliquots (15 μl) of supernatant were injected for RP-HPLC analysis.

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RP-HPLC

A Waters HPLC and Waters 840 data and chromatography control station (Waters Associates, Inc., Milford, MA) were used, consisting of model 6000 and model 501 pumps (acetonitrile and water, respectively), a Wisp 710B sample injector, a 490 programmable multiwavelength detector, a temperature control module, systems interface module, Dec Professional 380 computer with a 33-megabyte hard disk and LC Multisystem version 3.0 software, an LA210 letter printer and LVP16 plotter. Analyses were carried out routinely with a Supelcosil LC-3O8 reversed-phase HPLC column (C_8, 300Å pore size, 5 μm particle size, 5 cm × 4.6 mm i.d. (Supelco Inc., Bellefont, PA) preceded by a guard column of the same packing material (Supelguard LC-308, 2 cm × 4.6 mm i.d.). The following columns were also evaluated: a) Supelcosil LC-308 (C_8, 300Å, 5 μm, 25 cm × 4.6 mm i.d.); b) Supelcosil LC-304 (C_4, 300Å, 5 μm, 5 cm × 4.6 mm i.d.); c) SynChropak RP-8 (C_8, 300Å, 6.5 μm, 10 cm × 4.1 mm i.d.); (SynChrom Inc., Linden, IN); d) Vydac 228TP (C_8, 300Å, 5 μm, 15 cm × 4.6 mm i.d. (Hesper, CA); e) MPLC Aquapore RP-300 (C_8, 300Å, 10 μm, 10 cm × 4.6 mm i.d.); (C_8, 300Å, 7 μm, 1.5 cm × 3.2 mm i.d., Brownlee Laboratories, Santa Clara, CA); f) μBondapak C_{18} (50–300Å, 10 μm (irregular), 30 cm x 3.9 mm i.d. (Waters). A 0.5-μm precolumn filter (Upchurch Scientific, Oak Harbor, WA) was inserted before all columns or guard columns and filters were changed daily. Solvents A and B consisted of water and acetonitrile, respectively, each containing 0.1% trifluoroacetic acid. The water was changed daily to prevent contamination from bacterial growth, and both solvents were sparged continuously with helium. Proteins were eluted in 120 or 20 min using gradients described in Table I. Eluted proteins were monitored at 210 nm and 0.5 or 1.0 absorbance units full scale (120- or 20-min analyses, respectively), and chromatograms were stored and analyzed by the Waters 840 system as described previously (Marchylo et al 1986).

Temperature Study

Proteins extracted from Neepawa were separated (120-min analysis) using a 5-cm Supelcosil C_8 column and guard column maintained at temperatures of 30, 40, 50, 60, and 70° C. Following completion of analyses at 70° C, column temperature was lowered to 60 and then 50° C for further analysis of Neepawa extracts. The column was allowed to equilibrate at each temperature for at least 2 hr prior to the first analysis, and at least six analyses were carried out at each temperature.

RESULTS AND DISCUSSION

Column Evaluation

Previous evaluation (Kruger and Marchylo 1985) of three commercially available RP-HPLC columns indicated that an Aquapore RP-300 column (C_8, 10 cm × 4.6 mm i.d.) provided the greatest versatility for resolution of wheat proteins. Continued analysis with this column system, however, has shown that significant variations in resolution and retention times of wheat protein peaks are observed among columns. Because a high degree of reproducibility of protein chromatograms is a prime requirement for any cultivar identification technique, a further evaluation of an additional six commercially available columns was undertaken to determine if a more suitable column was available. Resolution and selectivity of columns were compared using identical gradient conditions (Table I) chosen to provide optimum resolution of proteins. For comparative purposes, analytical columns were not preceded by guard columns because these were not available in all cases. Analysis of resultant chromatograms (Fig. 1A–F) indicated that the maximum number of peaks resolved was achieved with the 25-cm Supelcosil (C_8) column (60 peaks), followed by the 5-cm Supelcosil (C_8) column (55), the 15-cm Vydac (C_8) column (51), the 5-cm Supelcosil (C_4) column (42), the 10-cm SynChropak (C_8) column (40), and the 30 cm Waters μBondapak (C_{18}) column (36). As noted previously for other columns (Bietz 1985, Marchylo and Kruger 1985, Kruger and Marchylo 1985), selectivity of these columns was comparable as indicated by a basic similarity in protein chromatograms. Resolution achieved by the 5-cm Supelco (C_8) column was exceptionally good for a short column and almost approached that of the 25-cm Supelcosil (C_8) column. This is in agreement with reports suggesting that reversed-phase columns separate more on the basis of a selective desorption process (Regnier 1983). Because of the excellent resolution exhibited by the 5-cm Supelcosil, and because of the versatility associated with reduced column length (i.e., increased flow rates, shorter running times) as well as cost considerations, evaluation of this column was continued. Because the 25-cm Supelcosil column exhibited somewhat better resolution than the 5-cm column, two 5-cm columns were joined in tandem to determine if this would improve resolution (Fig. 1G). Only slight improvements in resolution were observed, as illustrated for peaks designated by arrows.

As noted in previous studies (Marchylo and Kruger 1985, Kruger and Marchylo 1985), the use of guard columns is recommended to increase column lifetime when using the extractants described in this paper. As illustrated in Figure 1H, addition of a guard column had little effect on resolution; however, retention times were increased slightly because of the increase in total column void volume. Although guard columns are recommended for analysis of wheat proteins, they are not individually tested by manufacturers. Therefore, when new guard columns are inserted, analysis of a standard protein extract (in this case, a Neepawa extract) is essential in order to ensure that resolution and selectivity are not impaired or altered.

An evaluation of column-to-column variability for the 5-cm Supelcosil was performed using three columns obtained from different lots (Fig. 2A–C). Although chromatograms obtained with these columns again showed a basic similarity, significant differences were observed in peak retention times, resolution, and selectivity. However, columns obtained from the same lot (Fig. 2C,D) exhibited nearly identical chromatograms. Thus, to minimize column-to-column variations in protein chromatographic elution profiles, columns should be selected from the same lot.

Effect of Column Temperature

Previous studies showed that increasing column temperature can affect significantly elution profiles of gliadins (Bietz and Cobb 1985) and hordeins (Marchylo and Kruger 1985). Elevated column temperatures resulted in improved resolution of gliadins, and it was recommended that column temperatures of approximately 70° C be used (Bietz and Cobb 1985). For this reason, the effects of column temperature on the resolution of the gliadin and glutenin proteins, which both were extracted by solvents employed in this study, were assessed (Fig. 3). Results of previous studies (Marchylo and Kruger 1985, Bietz and Cobb 1985) were confirmed in that there were significant shifts in relative retention times with increasing temperature. For example, peak A (Fig. 3) increased in retention time, whereas peak D showed a significant decrease in retention time. Resolution improved progressively with increasing

TABLE I
Gradient Programs for Separation of Storage Proteins by Reversed-Phase High-Performance Liquid Chromatography

Time of Gradient Segments (min)	Gradient Composition (% acetonitrile)		Gradient Type
	Start	End	
120 min analysis (flow rate = 1 ml/min)			
0.0– 3.0	24	24	isocratic
3.0–105.0	24	48	linear
105.0–108.0	48	48	isocratic
108.0–109.0	48	24	linear
109.0–120.0	24	24	isocratic
20 min analysis (flow rate = 2 ml/min)			
0.0– 1.0	25	25	isocratic
1.0–10.0	25	52	linear
10.0–11.0	52	52	isocratic
11.0–11.5	52	25	linear
11.5–20.0	25	25	isocratic

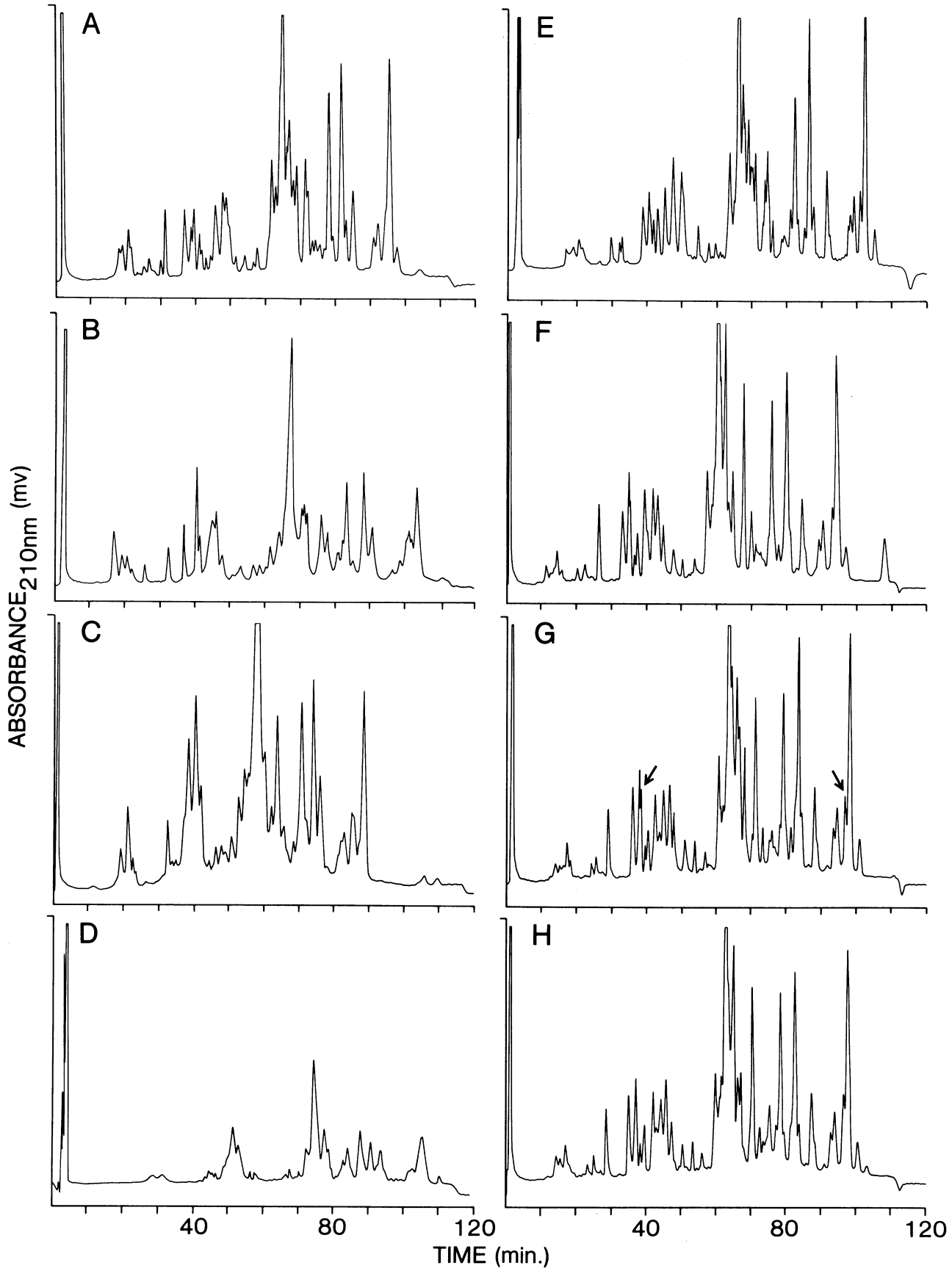

Fig. 1. Reversed-phase high-performance liquid chromatographic separations of storage proteins, extracted from Neepawa, using the following columns maintained at 30° C: **A,** Vydac 228TP (C_8, 15 cm × 4.6 mm i.d.); **B,** SynChropak RP-8 (C_8, 10 cm × 4.1 mm i.d.); **C,** Supelcosil LC-304 (C_4, 5 cm × 4.6 mm i.d.); **D,** μBondapak (C_{18}, 30 cm × 3.9 mm i.d.); **E,** Supelcosil LC-308 (C_8, 25 cm × 4.6 mm i.d.); **F,** Supelcosil LC-308 (C_8, 5 cm × 4.6 mm i.d.); **G,** Supelcosil LC-308 (C_8, 2 × 5 cm × 4.6 mm i.d. in tandem); **H,** Supelguard LC-308 (C_8, 2 cm × 4.6 mm i.d. + Supelcosil LC-308 (C_8, 5 cm × 4.6 mm i.d.).

temperature in some regions of chromatograms such as region C, where a doublet observed at 30° C was resolved into a triplet at 70° C. In other regions such as region B, however, little improvement in resolution was observed with increasing temperature until 70° C was reached. Much of the improved

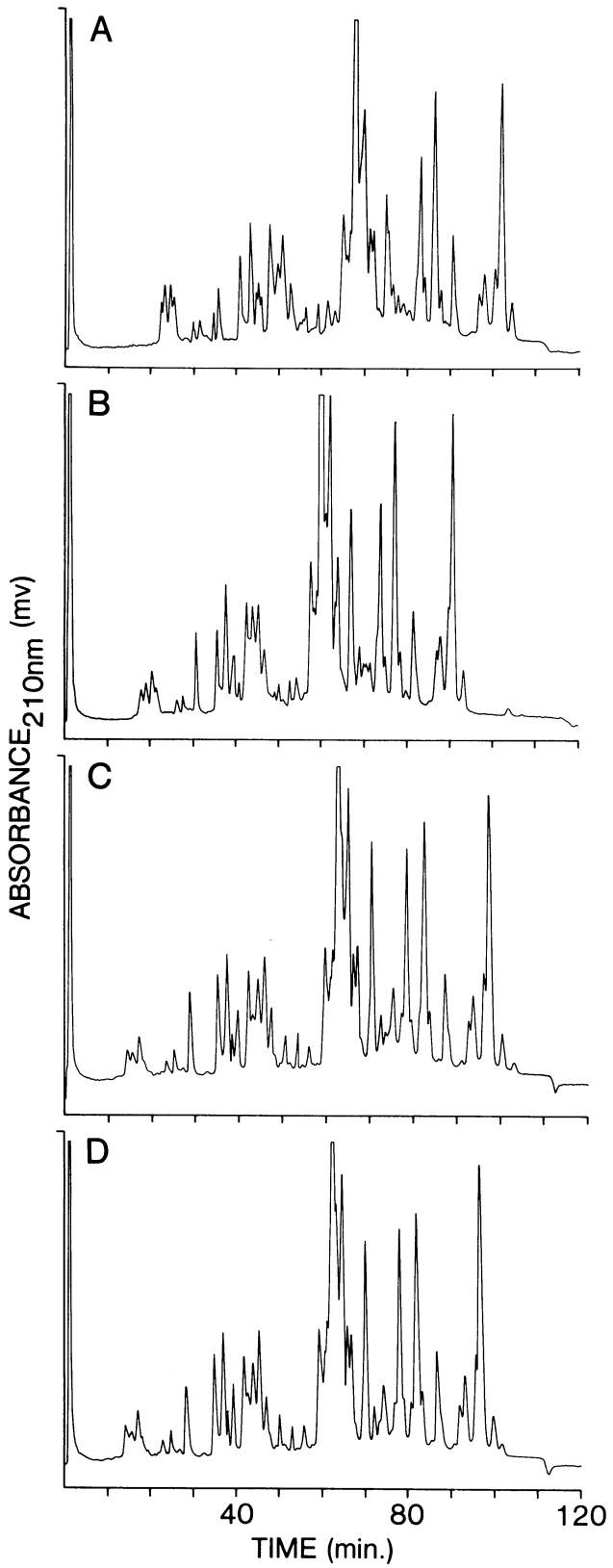

Fig. 2. Reversed-phase high-performance liquid chromatographic separation of storage proteins, extracted from Neepawa, using Supelcosil LC-308 (C_8, 5 cm × 4.6 mm i.d.) columns maintained at 30° C with the following column numbers: **A,** 108087; **B,** 108093; **C,** 108142; **D,** 108117.

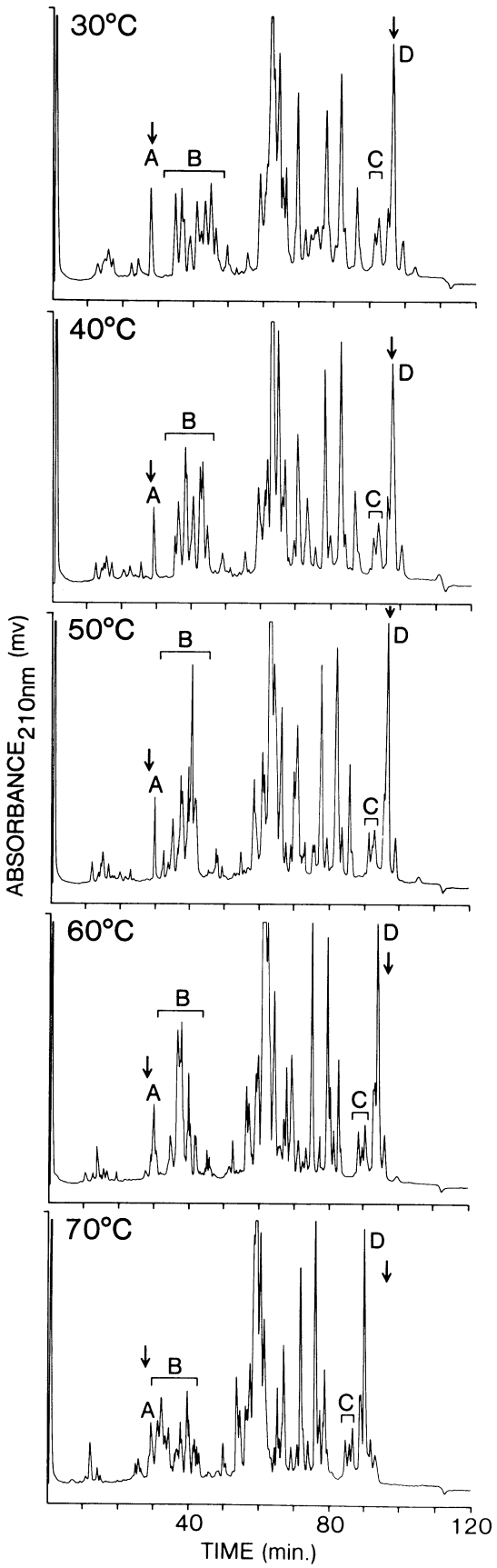

Fig. 3. The effect of column temperature on the reversed-phase high-performance liquid chromatographic separation of Neepawa storage proteins, using a Supelcosil LC-308 column (C_8, 5 cm × 4.6 mm i.d.) which also was employed in all subsequent figures. Arrows denote position of peaks at 30° C.

resolution at 70° C, however, was the result of peaks separating into a number of closely spaced doublets or triplets.

An overall improvement in resolution at lower column temperatures was observed when columns were conditioned first at a higher temperature. For example, a comparison of protein profiles obtained at 50° C before (Fig. 4A) and after (Fig. 4B) column equilibration at 70° C (2 hr) indicated substantial improvement in resolution of peaks in regions 2, 3, and 4. In a few cases, such as in region 1, resolution of some peaks deteriorated. It would appear then that conditioning of a column at 70° C results in changes in column characteristics that beneficially affect overall resolution or apparent selectivity for subsequent analyses at lower temperatures. Thus, a column conditioning period at elevated temperatures of 70° C is recommended prior to analysis at lower temperatures.

Because resolution and apparent selectivity changed with temperature, 10 cultivars were analyzed to determine if one temperature was more suitable for distinguishing among cultivars. Although more peaks were resolved at 70° C, the complexity of chromatograms hampered cultivar identification. For example, Neepawa and Solar exhibited very similar chromatograms, and as shown in Figure 5, Neepawa was distinguishable from Solar primarily on the basis of one peak (identified by an arrow) that is present in Neepawa and absent in Solar. Resolution of this peak at 70° C was inferior to that observed at lower temperatures of 50 and 30° C. At 70° C, this peak overlapped adjacent peaks, whereas the peak of interest was most completely separated from adjacent peaks at 30° C. Because this peak was useful for quantitative analysis of admixtures, it would appear that lower temperatures of 50 or 30° C, in this particular case, would be more appropriate. Similar results were observed for all cultivars. These results suggest that one set temperature may not be suitable for the identification of all cultivars and as noted previously (Marchylo and Kruger 1985), temperature effects may be used as an additional tool to assist in the identification of closely related cultivars.

TABLE II
Reproducibility of Peak Areas Resolved by Reversed-Phase High-Performance Liquid Chromatography Analysis of Neepawa Extracts (120-min analysis time)[a]

Chromatogram Segment[b]	Mean area $\bar{x}$ ($\times 10^7$)	Standard Deviation Sx ($\times 10^7$)	Coefficient of Variation (%)
Column temperature = 30° C (n = 10)			
A	4.01	0.07	1.69
B	12.65	0.11	0.87
C	1.56	0.03	1.81
D	24.37	0.18	0.73
E	20.06	0.18	0.87
F	10.82	0.13	1.17
		Mean	1.19
Column temperature = 50° C (n = 9)			
A	2.21	0.14	6.34
B	13.76	0.26	1.86
C	3.43	0.09	2.53
D	25.65	0.41	1.60
E	20.50	0.43	2.08
F	10.49	0.18	1.74
		Mean	2.69
Column temperature = 70° C (n = 9)			
A	1.02	0.03	2.63
B	13.90	0.14	1.02
C	2.07	0.03	1.40
D	27.88	0.52	1.87
E	18.48	0.35	1.92
F	10.37	0.19	1.80
		Mean	1.77

[a] Gradient conditions as in Table I.
[b] Chromatograms were divided into segments as illustrated in Figure 7 for 50° C. Chromatograms at 30 and 70° C were segmented in a comparable manner (results not shown).

TABLE III
Reproducibility of Peak Areas Resolved by Reversed-Phase High-Performance Liquid Chromatography Analysis of Neepawa Extracts (20-min analysis time)[a]

Chromatogram Segment[b]	Mean Area[c] $\bar{x}$ ($\times 10^7$)	Standard Deviation Sx ($\times 10^7$)	Coefficient of Variation (%)
Column temperature = 30° C			
A	2.40	0.04	1.82
B	6.18	0.02	1.27
C	0.68	0.02	2.84
D	11.64	0.11	0.91
E	9.65	0.11	1.16
F	4.93	0.14	2.85
		Mean	1.74
Column temperature = 50° C			
A	0.39	0.02	4.16
B	3.31	0.01	0.67
C	0.39	0.01	1.89
D	5.47	0.03	0.52
E	4.71	0.03	0.58
F	2.64	0.02	0.69
		Mean	1.42

[a] Gradient conditions as in Table I.
[b] Chromatograms were segmented as illustrated in Figure 8 for 50° C analyses.
[c] n = 10.

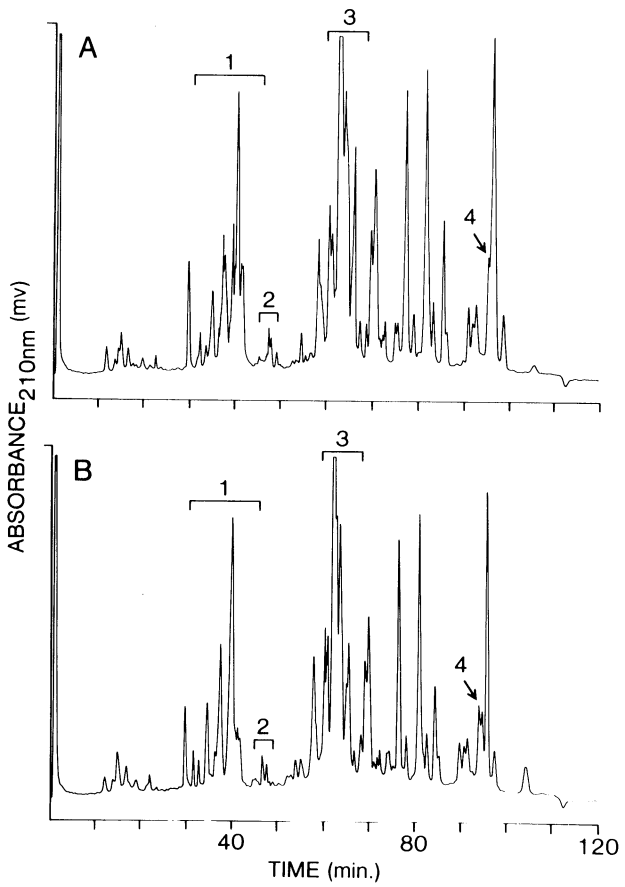

Fig. 4. The effect of high-temperature column conditioning on the separation of Neepawa storage proteins. Separation at 50° C before **A**, conditioning at 70° C; **B**, following conditioning at 70° C using a Supelcosil LC-308 column (C_8, 5 cm × 4.6 mm i.d.).

Reproducibility of Peak Retention Times and Areas

Reproducibility of peak retention times and areas were assessed using the 5-cm Supelcosil C_8 and guard column for analysis times of 120 min and 20 min (Table I) in conjunction with column temperatures of 30, 50, and 70° C. For retention time analysis, six peaks were selected within replicate Neepawa chromatograms (n = 10, using one extract) analyzed during a 24-hr period for each temperature and analysis time. Reproducibility of retention times was comparable at each temperature and average coefficients of variation (CV) of 0.20% and 0.13% were obtained for 120- and 20-min analyses, respectively. These results are in agreement with previous reports for gliadins (Bietz and Cobb 1985) and hordeins (Marchylo and Kruger 1985). To determine reproducibility of integrated peak areas, Neepawa chromatograms were divided into six segments as noted in Tables II and III (and subsequently in Figs. 7 and 8). Chromatogram segments were integrated using the "scanner" mode of the Waters LC Multisystem version 3.0 software. Areas were quantified using a baseline projected from a predetermined position at the beginning of each chromatogram. Statistical analyses of integrated chromatogram segments (Tables II and III) indicated that peak areas were more variable than peak retention times. With the exception of segment A, reproducibility was similar among analysis times and temperatures, and an overall mean CV of 1.78% was obtained. The reproducibility of chromatogram segment A decreased at elevated temperatures of 50 and 70° C, and total integrated areas decreased with increasing

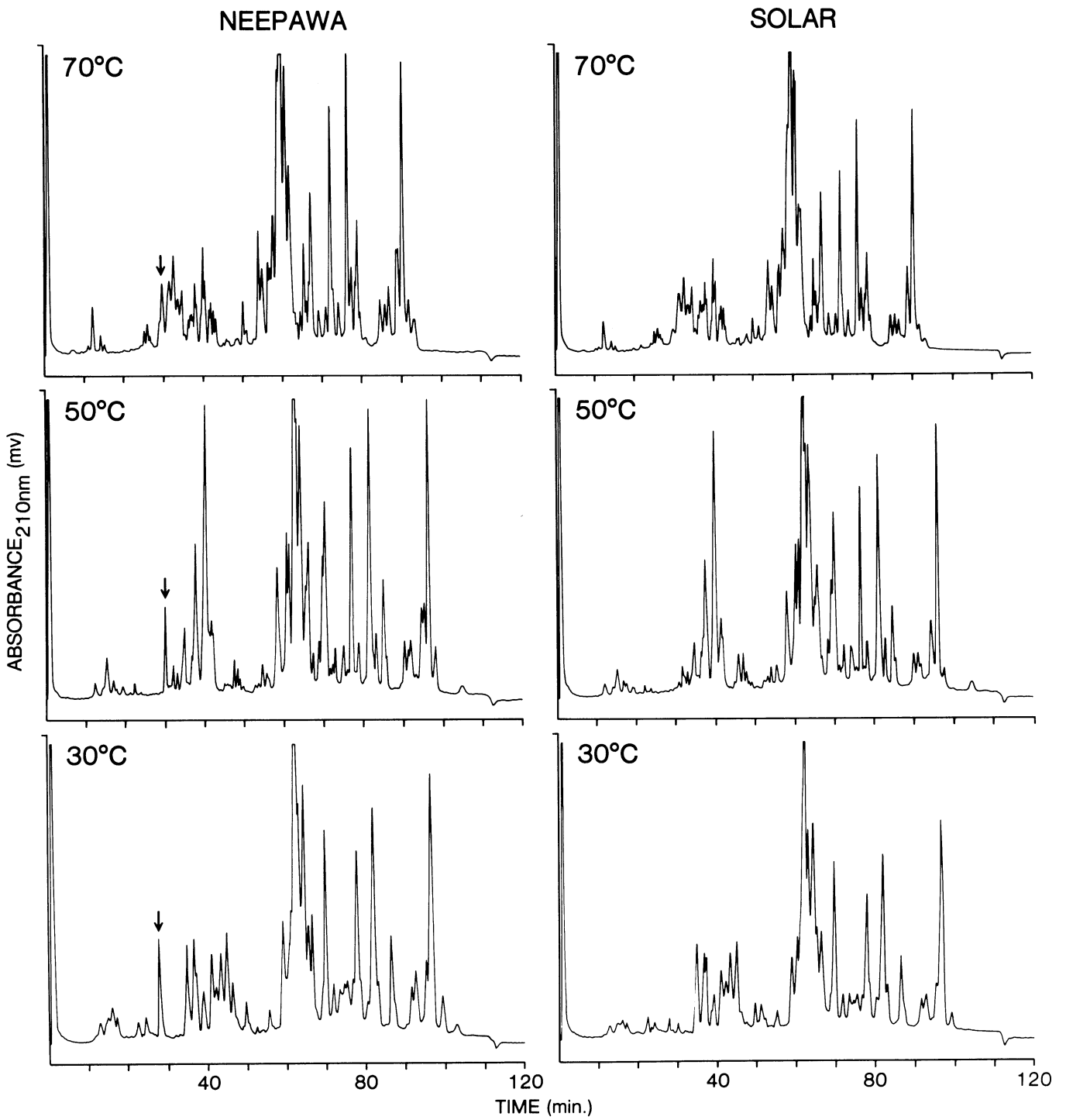

Fig. 5. The effect of column temperature on the distinguishability of wheat cultivars exhibiting similar storage proteins separated by reversed-phase high-performance liquid chromatography.

temperature (Tables II and III). This may indicate that a portion of the segment A proteins is not binding to the column and is eluting in the frontal peak.

Column Stability

In the short term, wheat protein chromatograms or "fingerprints" were highly reproducible relative to peak retention times and overall protein peak patterns. Changes in these parameters were observed, however, with prolonged use. As illustrated in Figure 6A and B, after 700 analyses with a 5-cm Supelcosil C_8 column at 30° C, the retention time of peak 1 increased from 24.67 to 28.13 min, whereas that of peak 2 increased from 100.27 to 102.40 min. In addition, changes in apparent selectivity and/or losses in resolution were observed in regions A–D. Using another 5-cm Supelcosil C_8 column maintained at 50° C, changes in retention time were not as pronounced. As seen in Figure 6C and D, after 500 analyses, retention time of peak 1 increased from 15.47 to 16.13 min, whereas that of peak 2 increased from 96.13 to 96.53 min. A loss in resolution also was observed in regions A–D, but changes in apparent selectivity were minimal as contrasted to changes seen at 30° C. Column cleaning procedures, described by Bietz (1985), were employed at regular intervals in both instances and when necessary, guard columns were replaced. Observed changes may be caused by the binding of nonproteinaceous material to columns, to changes in carbon loading due to acid hydrolysis of bound C_8 hydrocarbon chains (Glajch et al 1987) or, alternately, to column degradation at high temperatues (> 50° C) after long-term use. Similar changes also were observed for Aquapore and SynChropak columns maintained at 30° C.

Column stability is a major consideration because significant changes as observed in Figure 6A and B would seriously hamper cultivar identification. Although slight deviations in retention time could be compensated for by normalization of chromatograms, major changes in apparent selectivity or resolution would be difficult if not impossible to correct.

Effect of Alkylation

Wheat proteins initially were extracted using 50% 1-propanol containing 4% DTT and 1% acetic acid (Kruger and Marchylo 1985) but some variability in peaks eluting between about 45 and 60 min occasionally was observed in replicate analyses. This variability was attributed to incomplete reduction of proteins under acidic conditions (1% acetic acid). In the absence of acetic acid, 1% DTT was sufficient to ensure complete reduction of proteins. Wheat proteins were extracted routinely, therefore, with 50% 1-propanol containing 1% DTT in order to improve reproducibility and decrease extraction cost. Extractability was only marginally less when 1% acetic acid was omitted (Byers et al 1983).

It has been shown (Burnouf and Bietz 1984) that resolution of glutenin proteins is improved by alkylation. Because glutenins in addition to gliadins are extracted in the solvent employed in this study, extracts were alkylated to determine if improved resolution suitable for cultivar identification was achieved. Significant changes in elution profiles were obtained for alkylated proteins analyzed at column temperatures of 30, 50, and 70° C (results not shown). At all temperatures, alkylated proteins eluted earlier, indicating decreased surface hydrophobicity, and were compressed into a smaller region of the chromatogram. Modification of

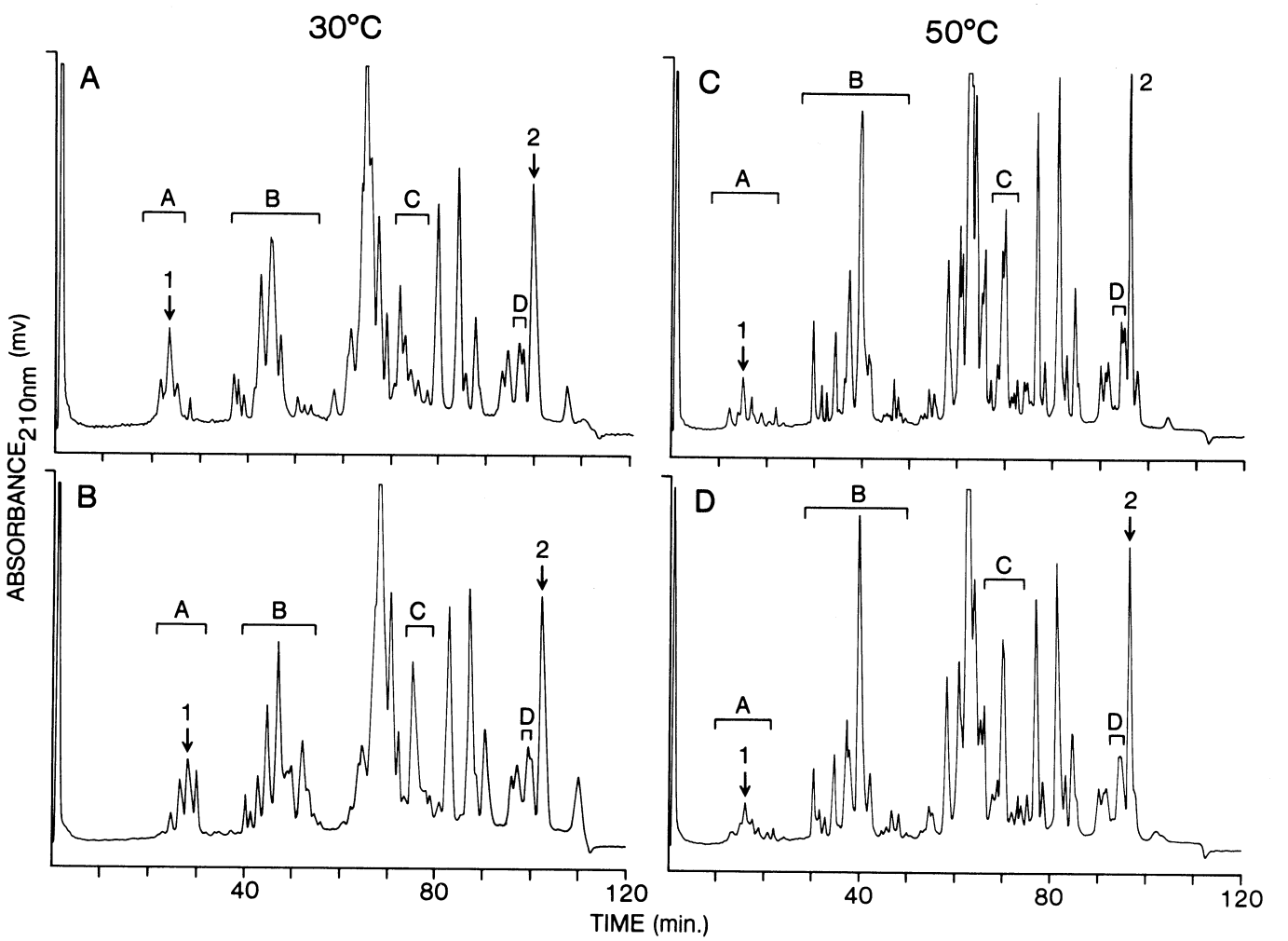

Fig. 6. Changes in the reversed-phase high-performance liquid chromatographic separation of storage proteins, extracted from Neepawa, as a function of column lifetime. **A** and **C**, New columns; **B**, after 700 analyses; **D**, After 500 analyses.

gradient conditions to improve resolution of peaks (i.e., decreasing gradient slope and lowering initial and final acetonitrile concentrations) resulted in unacceptable peak broadening concomitant with minimal improvements in resolution. Comparison of chromatograms derived from unalkylated and alkylated proteins indicated that alkylation was unsuitable for routine cultivar identification purposes.

Cultivar Analysis

Cultivars were analyzed at 30 and 50° C and analysis times of 120 and 20 min. Chromatograms representative of each cultivar were prepared from ground grain rather than single-kernel extracts. Because electrophoretic analyses have shown that many of these cultivars contain biotypes (Kosmolak 1979; Tkachuk and Mellish 1980; Sapirstein and Bushuk 1985; Marchylo 1987), observed chromatograms were composites representative of biotype mixtures. This procedure was used because quantitative analyses of admixtures would be carried out on ground grain extracts. Qualitative comparison of chromatograms was carried out using the "compare plot" mode of the Waters LC Multisystem version 3.0 software. Chromatograms were normalized for visual comparison by scaling the largest peaks to equal heights, because protein levels varied among cultivar samples. Chromatograms then were overlaid in different colors to highlight qualitative differences. It was observed that all cultivars with the exception of the two genetically similar cultivars Neepawa and Katepwa were distinguishable at both 30 and 50° C. Typical chromatograms obtained for 10 of the cultivars analyzed at 50° C are illustrated in Figure 7. The majority of cultivars (e.g., Neepawa and Columbus) exhibited sufficient qualitative differences to easily distinguish them. All registered cultivars were distinguishable from nonregistered cultivars.

One group of six registered cultivars (eligible for top CWRS grades) including Neepawa, Katepwa, Benito, Thatcher (Fig. 7), Manitou, and Napayo (not shown) exhibited very similar chromatograms. However, with the exception of Neepawa and Katepwa, minor qualitative differences in chromatograms enable these cultivars to be distinguished. For example, Neepawa could be differentiated from Benito by a shoulder peak (indicated by arrow) present in Benito and not Neepawa. Similarly, Thatcher chromatograms exhibited characteristic peaks (as indicated by arrows) that distinguished it from the other cultivars having similar chromatograms.

Quantitative analysis of chromatograms also provided further information that assisted in differentiating between Neepawa (Katepwa) and the other four similar cultivars. The ratio of the integrated areas of segment F to total area (segments A–E) at 50° C (Fig. 7) was significantly different among these two groups of cultivars. A ratio of 0.168 ± 0.001 ($\bar{x} \pm$ Sx) with a CV of 0.48% ($n =$ 11) was obtained for Neepawa as contrasted to ratios of 0.150, 0.148, 0.154, and 0.155 for Benito, Thatcher, Manitou, and Napayo, respectively. Katepwa exhibited a ratio of 0.164, which was comparable to that of Neepawa.

Most cultivars also were distinguishable on the basis of qualitative differences in chromatograms obtained by rapid 20-min analysis at 30 and 50° C despite a significant decrease in resolution as illustrated in Figure 8 (50° C) for 10 example cultivars. All registered cultivars were distinguishable from nonregistered cultivars, but loss of resolution made it impossible to distinguish among the six registered cultivars Neepawa, Katepwa, Benito, Thatcher (Fig. 8), Napayo, and Manitou (not shown).

Quantitative Analysis of Admixtures

Two component admixtures were prepared using varying proportions (1–75%) of Len, an American cultivar nonregistered in Canada, mixed with Neepawa, a registered Canadian cultivar eligible for top CWRS wheat grades. These cultivars were chosen for illustrative purposes because they exhibited significantly different protein chromatograms (Fig. 9A and B). This simplified visualization of changes in mixture chromatograms as the proportion of Len increased. Chromatograms obtained by 120- and 20-min analyses at 50 and 30° C were analyzed quantitatively in the "scanner" mode. Examples of mixture chromatograms are illustrated in Figure 9C and D for admixtures containing 25% and 50% Len.

Initially, individual and groups of peaks characteristic of each cultivar were integrated to determine if there was a linear response between peak area and cultivar concentration. Because total chromatogram areas were similar for Neepawa and Len, peak areas were reported relative to total chromatogram area (%) to minimize variations in protein extraction. Highly significant linear correlations were obtained when relative peak areas (%) were plotted versus increasing Len concentration (%) for peaks 1 and 3 ($r^2 = 0.990$ and 0.998, respectively) characteristic of Neepawa and peak 2 ($r^2 = 0.983$) characteristic of Len (Fig. 9). Plotting peak areas instead of relative peak areas versus Len concentration also yielded linear correlations with $r^2 = 0.961$, 0.992, and 0.990 for peaks 1–3, respectively. Comparable results were observed at 30° C and at a faster analysis time of 20 min. Thus, in a simple binary system, quantification of peaks characteristic of a cultivar could be used to predict cultivar proportions. On this basis, a preliminary computer program (BASIC) was devised to determine relative proportions of Neepawa and Len in the above admixtures. Peak 1, which is characteristic of Neepawa (Fig. 9) was used as the reference peak in this analysis because it was the most clearly resolved peak. A normalization routine, based upon the assumption that peak retention time differences are constant throughout a chromatogram, was included to partially compensate for slight variations in peak retention times among analyses. Experience has shown, however, that variations in peak retention times are not necessarily equal throughout a chromatogram, and therefore a more complex normalization routine will be required in the future.

The proportion of Neepawa present in the mixture was obtained by first determining the ratio of the reference peak area in the normalized mixture chromatogram to that of the standard Neepawa chromatogram. This ratio (or scaling factor) then was utilized to prepare and plot a difference chromatogram representative of the Len component of the binary mixture. The ratio of the total area of the difference chromatogram to that of the standard Len chromatogram was calculated to provide an estimate of relative proportions of Len and Neepawa in the admixture.

Computer analysis provided reasonably good predictions as indicated by a standard error of the estimate of 2.33% determined from regression analysis of predicted versus known proportions of Len present in admixtures ($r^2 = 0.99$). However, considerable scatter was observed for predictions below about 5%. This poses some problems in Canada because of export grade specifications that limit the proportion of nonregistered cultivars in the top three CWRS wheat grades to 1.5, 3.0, and 5.0%, respectively. To ascertain if variability in protein extraction might contribute to the error, aliquots of Neepawa and Len extracts were mixed to give solutions equivalent to admixtures of 50, 25, and 10% Len. Predicted proportions for these solutions were 50.4, 25.3, and 10.4%, respectively. The accuracy of these predictions suggests that extraction variability contributes in part to the standard error.

A comparison of Len (Fig. 9) and difference Len chromatograms derived from admixtures of 15–75% (for example, Figs. 10A [25% Len]) revealed a high degree of similarity. However, as illustrated by Figure 10B (10% Len), difference chromatograms derived from admixtures containing 10% or less Len showed significant deviations from the known Len chromatogram. This could seriously hamper the identification of cultivars present at low levels (< 10%).

Computer analysis of chromatograms obtained from 20-min analysis also was carried out. However, because of rapid changes in signal voltages and because of greater overlap between adjoining peaks, it became very difficult to assign the beginning and end of the reference peak. It was found that a change of only a few seconds could alter predicted proportions drastically, and thus this type of computer analysis was not suitable for chromatograms derived by rapid analysis.

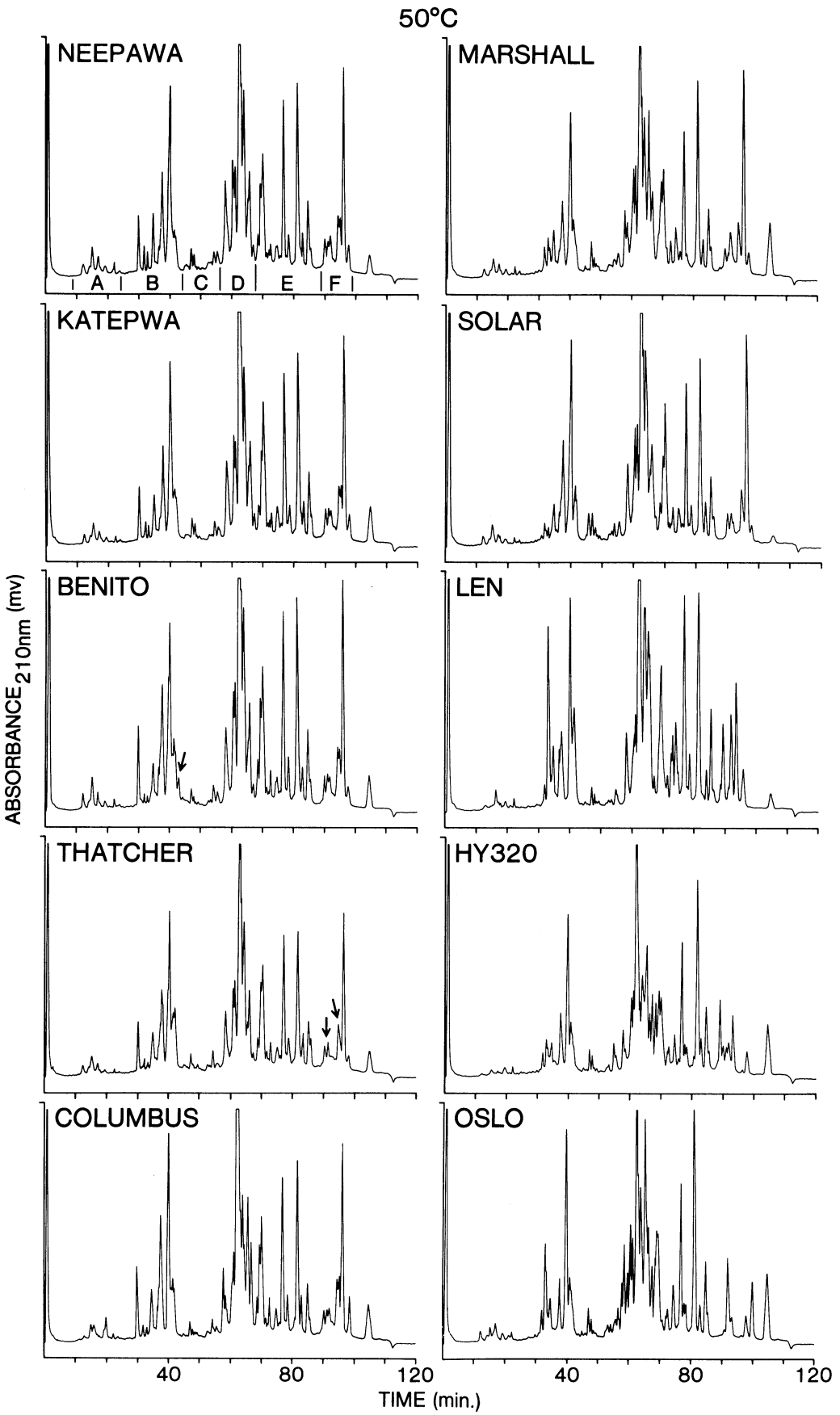

Fig. 7. Comparison of reversed-phase high-performance liquid chromatograms of storage proteins for Canadian and American wheat cultivars.

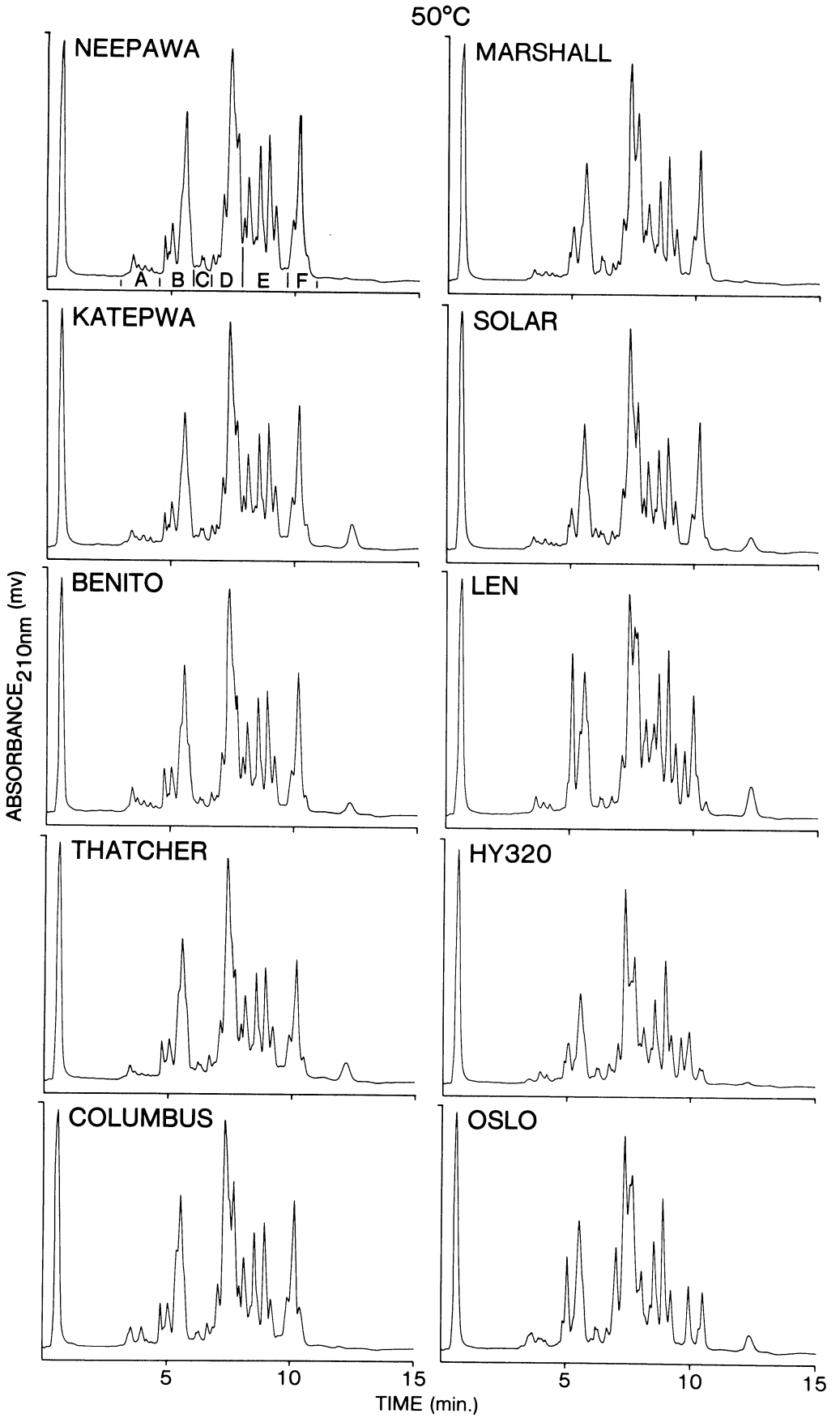

Fig. 8. Storage protein chromatograms for Canadian and American wheat cultivars obtained by rapid reversed-phase high-performance liquid chromatographic analysis.

Although this approach provided reasonably accurate predictions for computer analysis of 120-min chromatograms, considerable manual operator input was required. Because a fully automated prediction system would be desirable, a computer program (BASIC) based upon the automatic system of Bietz and Cobb (1985) was developed. A normalization routine was included to partially compensate for retention time variations. Although in theory this approach should work, problems were encountered when chromatograms extending over the whole range of mixtures were analyzed. Because negative baseline deflections were observed at and around the correct admixture concentration (i.e., the constant K, as per Bietz and Cobb 1985), it was necessary to determine a threshold value for the negative deflections. Unfortunately, a threshold value suitable for the complete mixture range could not be obtained. For example, when the threshold value suitable for a 50% Neepawa-Len mixture was used with the 90/10% Neepawa-Len mixture, a substantial prediction error was encountered (> 15%). Similar deviations of predicted from known proportions were obtained if parameters such as the absolute negative deflection, the ratio of the negative to positive deflection or change in negative deflection were used to set the threshold required for computer analysis. Insertion of the known K values yielded difference chromatograms whose relative areas correlated well with the known Len composition ($r^2 = 0.995$, standard error of estimate = 1.69%). Thus, the automatic quantitative analysis of mixtures should be possible if a general parameter is found to use in the selection of the constant K. Possibly more complete normalization of chromatograms (i.e., using more than one reference peak) might lead to a threshold value that is applicable over the entire range of admixtures.

CONCLUSIONS

RP-HPLC can be used to identify and distinguish between registered Canadian and nonregistered Canadian and American spring wheat cultivars. Although RP-HPLC can be used to determine the cultivar composition of binary mixtures by analysis of a single ground grain extract, further work is required to fully automate the procedure.

Several practical aspects of RP-HPLC technique should be given consideration for successful application to the separation of the storage proteins and subsequent identification of wheat cultivars. Care should be exercised in column selection. Although selectivity is similar among columns from different manufacturers, differences in resolution, running capabilities, and column-to-column variability are evident. Shorter columns (approximately 5–15 cm) are preferable because they are more cost effective and they provide resolution almost equal to that of longer columns. Such column-to-column variations in resolution and selectivity must be minimized, therefore, to prevent significant variations in catalogued cultivar "fingerprints." To some extent, this may be accomplished by purchasing columns from the same lot. The use of guard columns is recommended to extend column lifetime; however, they are not individually tested by manufacturers. Therefore, insertion of new guard columns should always be accompanied by analysis of a standard protein extract to ensure that resolution and selectivity are not impaired or altered. Although elevated column temperatures (70° C) improve resolution, as indicated by the number of peaks resolved, the complexity and overlap of chromatographic peaks makes analysis at somewhat lower temperatures (i.e., 50° C) more suitable for

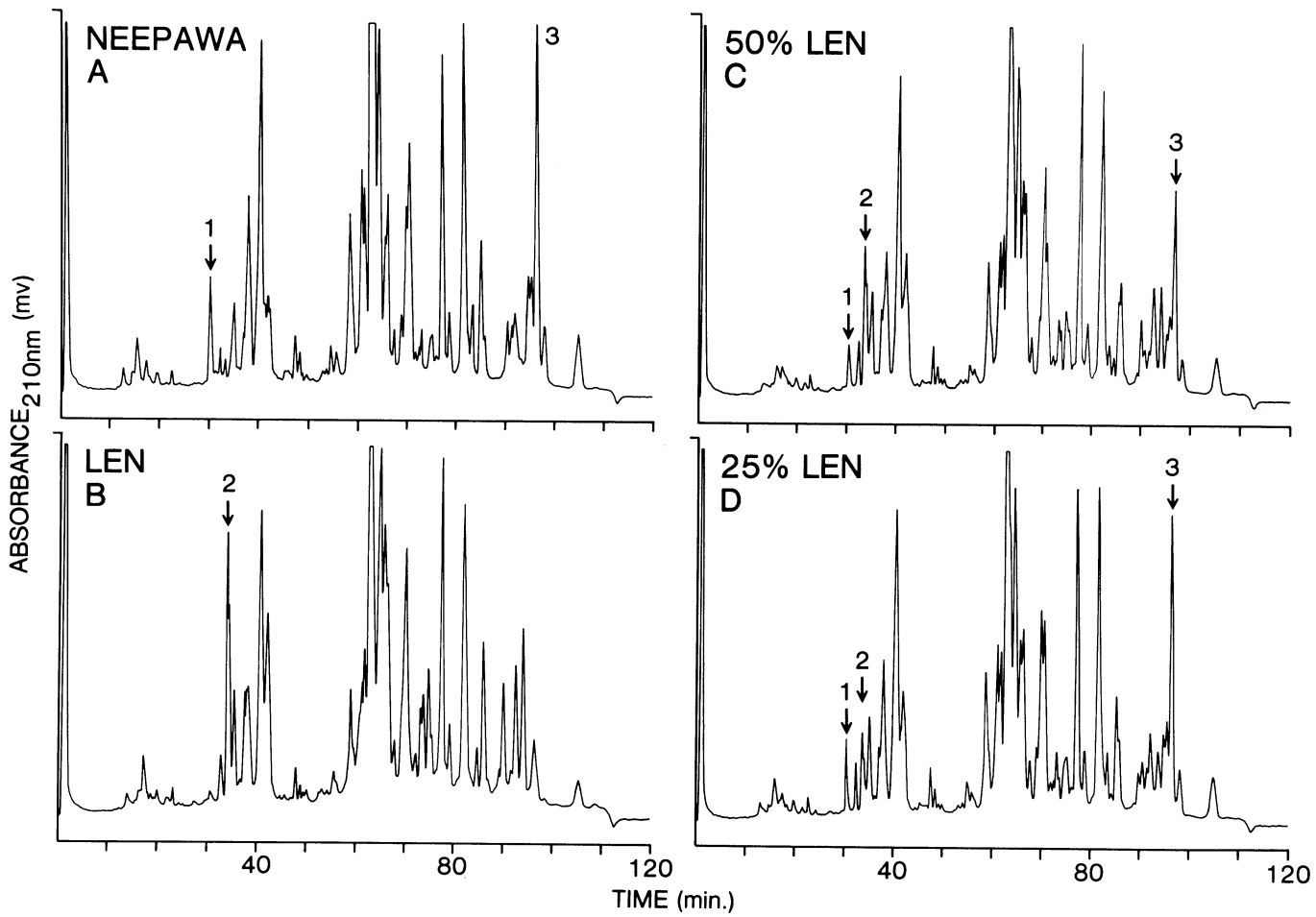

Fig. 9. Reversed-phase high-performance liquid chromatograms of storage proteins for the Canadian wheat cultivar, Neepawa (**A**), and the American cultivar, Len (**B**), and for two admixtures containing 50% Len–50% Neepawa (**C**), and 25% Len–75% Neepawa (**D**). Peaks 1–3 were used for quantitative analysis.

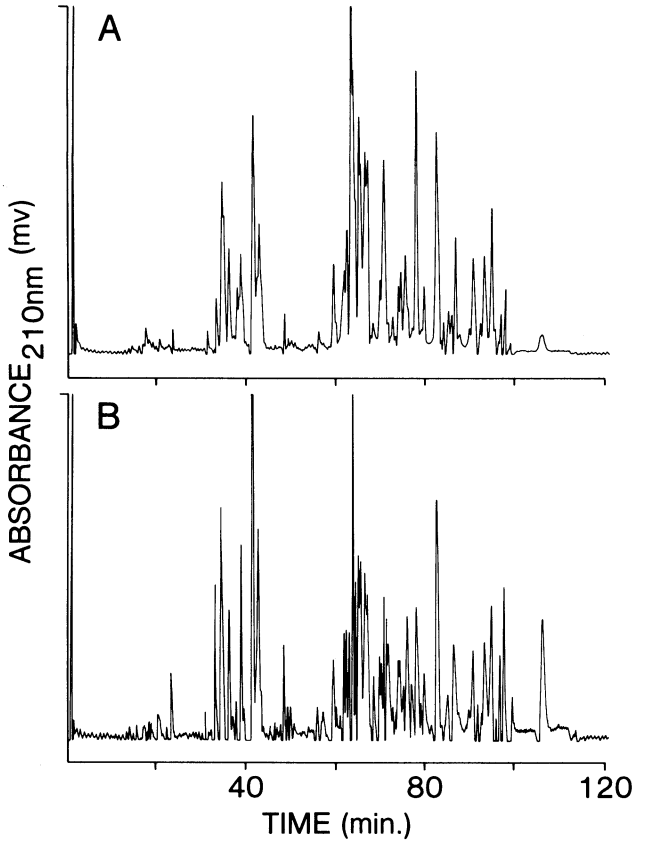

Fig. 10. Reversed-phase high-performance liquid chromatography difference chromatograms derived by computer analysis of storage protein chromatograms for admixtures of **A,** 25% Len–75% Neepawa; **B,** 10% Len–90% Neepawa. Difference chromatograms were scaled to enhance comparison of chromatograms.

cultivar analysis. Analysis at lower temperatures should be preceded by a column conditioning period at 70° C to improve column resolution. The excellent reproducibility of peak retention times and cultivar fingerprints exhibited by this technique must be routinely monitored using a "standard" chromatographic mixture to detect changes in retention times and selectivity that occur concomitant with long-term usage. Column stability is a major consideration, because changes in column performance with time can seriously influence cultivar fingerprints, which in turn can hamper the cultivar-identification process. Such deterioration of column performance was slower at elevated column temperatures. In addition, it should be remembered that the overall reproducibility of the technique is dependent upon smooth functioning of the HPLC system. In particular, care must be taken to ensure that pump performance is maintained, as small flow rate changes, for example, can result in significant changes in cultivar fingerprints.

The choice of analysis time also merits special consideration because loss in resolution with more rapid analyses can impair identification of closely related cultivars. In many situations, however, rapid analysis (< 20 min) could be used to screen out potential problem samples, which then could be analyzed using slower analysis times and consequent increased resolution. Although in this study optimum resolution was obtained with analyses times of 120 min, throughput can be increased with analyses of about 60 min that provide almost identical resolution (unpublished results).

Computerized quantitative analysis does show some promise; however, further work will be necessary to improve normalization of chromatograms, to improve prediction accuracy, particularly at low admixture concentrations (< 10%), and to obtain an automatic procedure for quantifying admixtures (binary and more complex) over a wide range of admixture concentrations. In addition, the use of pattern recognition techniques such as that described by Cohen et al (1987) should be applicable to qualitative analysis of chromatograms.

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LITERATURE CITED

ALLISON, M. J., and BAIN, H. 1986. The use of reversed-phase high-performance liquid chromatography as an aid to the identification of European barley cultivars. Euphytica 35:345-351.

BIETZ, J. A. 1983. Separation of cereal proteins by reversed-phase high-performance liquid chromatography. J. Chromatogr. 255:219-238.

BIETZ, J. A. 1985. High-performance liquid chromatography: How proteins look in cereals. Cereal Chem. 62:201-212.

BIETZ, J. A., and COBB, L. A. 1985. Improved procedures for rapid wheat varietal identification by reversed-phase high-performance liquid chromatography of gliadin. Cereal Chem. 62:332-339.

BIETZ, J. A., BURNOUF, T., COBB, L. A., and WALL, J. S. 1984. Wheat varietal identification and genetic analysis by reversed-phase high-performance liquid chromatography. Cereal Chem. 61:129-135.

BURNOUF, T., and BIETZ, J. A. 1984. Reversed-phase high performance liquid chromatography of reduced glutenin, a disulfide-bonded protein of wheat endosperm. J. Chromatogr. 299:185-199.

BURNOUF, T., BIETZ, J. A., COBB, L. A., and WALL, J. S. 1983. Chromatographie liquid haute performance des gliadines de variétés de blé tendre (*Triticum aestivum* L.) cultivées en France et possibilités d'identification variétale. C. R. Acad. Sci. Paris 297:377-382.

BYERS, M., MIFLIN, B. J., and SMITH, S. J. 1983. A quantitative comparison of the extraction of protein fractions from wheat grain by different solvents, and of the polypeptide and amino acid composition of the alcohol-soluble proteins. J. Sci. Food Agric. 34:447-462.

COHEN, M. E., HUDSON, D. L., MANN, L. T., VAN DEN BOGAERDE, J., and GITLIN, N. 1987. Use of pattern recognition techniques to analyze chromatographic data. J. Chromatogr. 384:145-152.

GLAJCH, J. L., KIRKLAND, J. J., and KOHLER, J. 1987. Effect of column degradation on the reversed-phase high-performance liquid chromatographic separation of peptides and proteins. J. Chromatogr. 384:81-90.

KOSMOLAK, F. G. 1979. Gliadin composition of the bread wheat cultivars BW 20 and Sinton. Can. J. Plant Sci. 59:1001-1005.

KRUGER, J. E., and MARCHYLO, B. A. 1985. Selection of column and operating conditions for reversed-phase high-performance liquid chromatography of proteins in Canadian wheat. Can. J. Plant Sci. 65:285-298.

LOOKHART, G. L. 1985. Identification of oat cultivars by combining polyacrylamide gel electrophoresis and reversed-phase high-performance liquid chromatography. Cereal Chem. 62:345-350.

MARCHYLO, B. A. 1987. Identification of Canadian and American wheat cultivars by SDS gradient PAGE analysis of gliadin and glutenin subunits. Can. J. Plant Sci. 67:945-952.

MARCHYLO, B. A., and KRUGER, J. E. 1984. Identification of Canadian barley cultivars by reversed-phase high-performance liquid chromatography. Cereal Chem. 61:295-301.

MARCHYLO, B. A., and KRUGER, J. E. 1985. Assessment of RP-HPLC columns to separate hordein proteins and identify cultivars of barley and barley malt. J. Am. Soc. Brew. Chem. 43:29-35.

MARCHYLO, B. A., and LABERGE, D. E. 1981. Barley cultivar identification by electrophoretic analysis of hordein proteins. II. Catalogue of electrophoregram formulae for Canadian-grown barley cultivars. Can. J. Plant Sci. 61:859-870.

MARCHYLO, B. A., KRUGER, J. E., and HATCHER, D. 1986. High-performance liquid chromatographic and electrophoretic analysis of hordein during malting for two barley varieties of contrasting malting quality. Cereal Chem. 63:219-231.

PAULIS, J. W., and BIETZ, J. A. 1986. Separation of alcohol-soluble maize proteins by reversed-phase high-performance liquid chromatography. J. Cereal Sci. 4:205-216.

REGNIER, F. E. 1983. High-performance liquid chromatography of biopolymers. Science 222:245-252.

SAPIRSTEIN, H. D., and BUSHUK, W. 1985. Computer-aided analysis of gliadin electrophoregrams. III. Characterization of the heterogeneity

in gliadin composition for a population of 98 common wheats. Cereal Chem. 62:392-398.
SEEDS ACT. 1986. Seeds variety order. Canada Gazette 119(20):4127.
SMITH, J. S. C., and SMITH, O. S. 1986. Environmental effects of zein chromatograms of maize inbred lines revealed by reversed-phase high-performance liquid chromatography. Theor. Appl. Genet. 71:607-612.
TKACHUK, R., and MELLISH, J. 1980. Wheat cultivar identification by high voltage gel electrophoresis. Ann. Technol. Agric. 29:209-212.
WINGAD, C. E., IQBAL, M., GRIFFIN, M., and SMITH, F. J. 1986. Separation of hordein proteins from European barley by high-performance liquid chromatography: Its application to the identification of barley cultivars. Chromatographia 21:49-54.

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