Reversed-Phase High-Performance Liquid Chromatographic Analysis of Wheat Proteins Using a New, Highly Stable Column[1]

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ABSTRACT

A new class of sterically protected monofunctional-silane bonded phase columns (C8 and CN) was evaluated to determine resolution capabilities and long-term stability in the reversed-phase high-performance liquid chromatography (RP-HPLC) separation of wheat storage proteins. Up to 450 separations were performed using the high temperature (50°C) and low pH (< 3) conditions required to resolve gliadins and glutenins. Superior resolution of these proteins was achieved with the new wide-pore, sterically protected columns as compared to that from conventional silica-based RP-HPLC columns. Statistical analysis of retention times and quantified peak areas also indicated improved stability and reproducibility as compared to that from conventional RP-HPLC columns. Column-to-column variability also appeared minimal. Analysis of storage proteins (gliadins or gliadins and glutenins) of different wheat varieties and high molecular weight glutenin subunits exemplified the suitability of these columns for varietal identification and the study of quality-related wheat storage proteins.

Reversed-phase high-performance liquid chromatography (RP-HPLC) is now used widely for the analysis of cereal proteins. The examination of these proteins has been useful for identifying varieties (Marchylo and Kruger 1984, Bietz and Cobb 1985, Lookhart 1985, Paulis and Bietz 1986, Marchylo et al 1988, Hussain et al 1989, Scanlon et al 1989b) and for studying their relationship to quality (Burnouf and Bietz 1984, Huebner and Bietz 1986, Marchylo et al 1989, Weiser et al 1989, Kruger and Marchylo 1990). The popularity of this procedure, as pointed out by Bietz (1985), may be attributed to various features of the method, including its excellent resolution, automation, quantitation, and computerization, and its complementary nature to other separatory procedures. One area of concern, however, in the application of RP-HPLC to the separation of the cereal proteins has been poor column stability.

Glajch et al (1987) demonstrated that conventional bonded-phase columns used for RP-HPLC are not stable over relatively short periods of time when used at low pH in the presence of trifluoroacetic acid (TFA). Column instability was shown to influence significantly the long-term reproducibility of separations of wheat storage proteins (Marchylo et al 1988, Sapirstein et al 1989, Scanlon et al 1989a). This limitation has serious ramifications for varietal identification (Marchylo et al 1988; Scanlon et al 1989a,b) because changes in the protein fingerprints could result in incorrect variety identification. Similarly, variation in retention time or column characteristics could influence the identification or quantitation of quality-related storage proteins such as the high molecular weight (HMW) glutenin subunits (Marchylo et al 1992). Variation in separations also can influence quality prediction (Scanlon et al 1990). Although normalization procedures have been developed to minimize these effects (Sapirstein et al 1989, Scanlon et al 1989b), the development of more stable RP-HPLC columns undoubtedly would further enhance the usefulness of this procedure for the study of the cereal proteins.

Recently, Kirkland et al (1989) reported the development of a new class of silane-modified silica that exhibited highly reproducible and stable RP-HPLC separations of proteins at low pH. This new class of silica uses a monofunctional silane with at least two bulky groups (e.g., isopropyl) on the silane silicon atom. One reason proposed for the instability of silica packings is that the usual methyl groups on the silane silicon atom offer little steric protection to the hydrolytically sensitive Si-O-Si groups on the packing surface. The utilization of bulky side groups in place of the methyl groups has been found to sterically protect the Si-O-Si bond formed between the silane and the surface of the silica (Glajch and Kirkland 1990). This approach has resulted in a significant improvement in column stability at low pH in the presence of TFA while maintaining column plate numbers and efficiency.

The purposes of this study were to evaluate the separation of wheat storage proteins using RP-HPLC columns packed with this new class of silica and to assess the stability of these columns under the low pH conditions used to separate gliadins and glutenins.

MATERIALS AND METHODS

Wheat Samples

The Canada Western Red Spring wheat variety Neepawa was used throughout the majority of this study. This variety has been grown in substantial quantities in western Canada and is used as a standard in plant breeders' trials and as a reference variety in electrophoretic studies (Sapirstein and Bushuk 1985). Varieties used in the varietal comparison portion of the study were derived from pure seed maintained by plant breeders.

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Sample Preparation and Extraction Procedures

The Neepawa wheat used in this study was ground in a Udy cyclone sample mill (Udy Corp., Boulder, CO) equipped with a 1-mm sieve. The ground grain was thoroughly mixed before extraction. For varietal analysis, single kernels were ground into fine powders with a mortar and pestle before extraction.

Gliadins and glutenins were extracted from ground grain (1 g) with 6 ml of 50% (v/v) 1-propanol containing 1% (w/v) dithiothreitol and with constant mixing at 60°C for 30 min as previously described (Marchylo et al 1989). Single kernels were extracted in a similar fashion using 0.3 ml of the same extraction solvent, or alternately, gliadins were extracted using 70% (v/v) ethanol (EtOH) without reducing agent. Precipitation, resolubilization, and alkylation of HMW glutenin subunits were performed as noted before (Marchylo et al 1989).

RP-HPLC

A Waters HPLC and a Waters 860 networking computer system (Waters Associates Inc., Milford, MA) were used. Analyses were performed using the following columns: a) a preproduction Zorbax Rx-300-C8 column (C8, 300-Å pore size, 5-μm particle size, sterically protected with isopropyl groups, 15 cm × 4.6 mm i.d.); b) an experimental Zorbax Rx-300-CN column (Cyanopropyl, as in a); and c) a conventional Supelcosil LC-308 RP-HPLC column (C8, 300-Å pore size, 5-μm particle size, 5 cm × 4.6 mm i.d. [Supelco Inc., Bellefont, PA]). Commercial columns of the Zorbax Rx-300 type are now available as Zorbax 300SB-C8 (and CN) from Chromatographic Specialties Inc. (Brockville, ON) or from Dionex (Mississauga, ON) as Dionex RP300-C8. The Supelcosil column was preceded by a guard column of the same packing material (Supelguard LC-308, 2 cm × 4.6 mm i.d.). Guard columns for the Zorbax-300-Rx columns were not available at the time of this study but can now be purchased from the above noted companies. Column temperature was maintained at 50°C unless otherwise noted; the column effluent, which contained 0.1% TFA, was monitored at 210 nm.

Storage proteins were resolved using gradients optimized for the respective columns as follows: a) Zorbax Rx-300-C8—a linear gradient extending 23–48% acetonitrile, 1-ml/min flow rate (column pressure ~ 1,000 psi), using a 60-min gradient program comparable to that reported previously (Marchylo et al 1988) (alternatively, for rapid analyses, a linear gradient extending from 24 to 50% acetonitrile using a 10-min gradient program at a 2-ml/min flow rate, column pressure ~1,500 psi); b) Zorbax Rx-300-CN—a linear gradient extending from 25 to 43% acetonitrile, 1-ml/min flow rate, using a 60-min gradient program comparable to that in a); c) Supelcosil LC-308—a 60-min gradient as in a). Alkylated HMW glutenin subunits were resolved with a gradient described previously (Marchylo et al 1989). Injection volumes were 5 μL for rapid analyses and 15 μL for all other analyses.

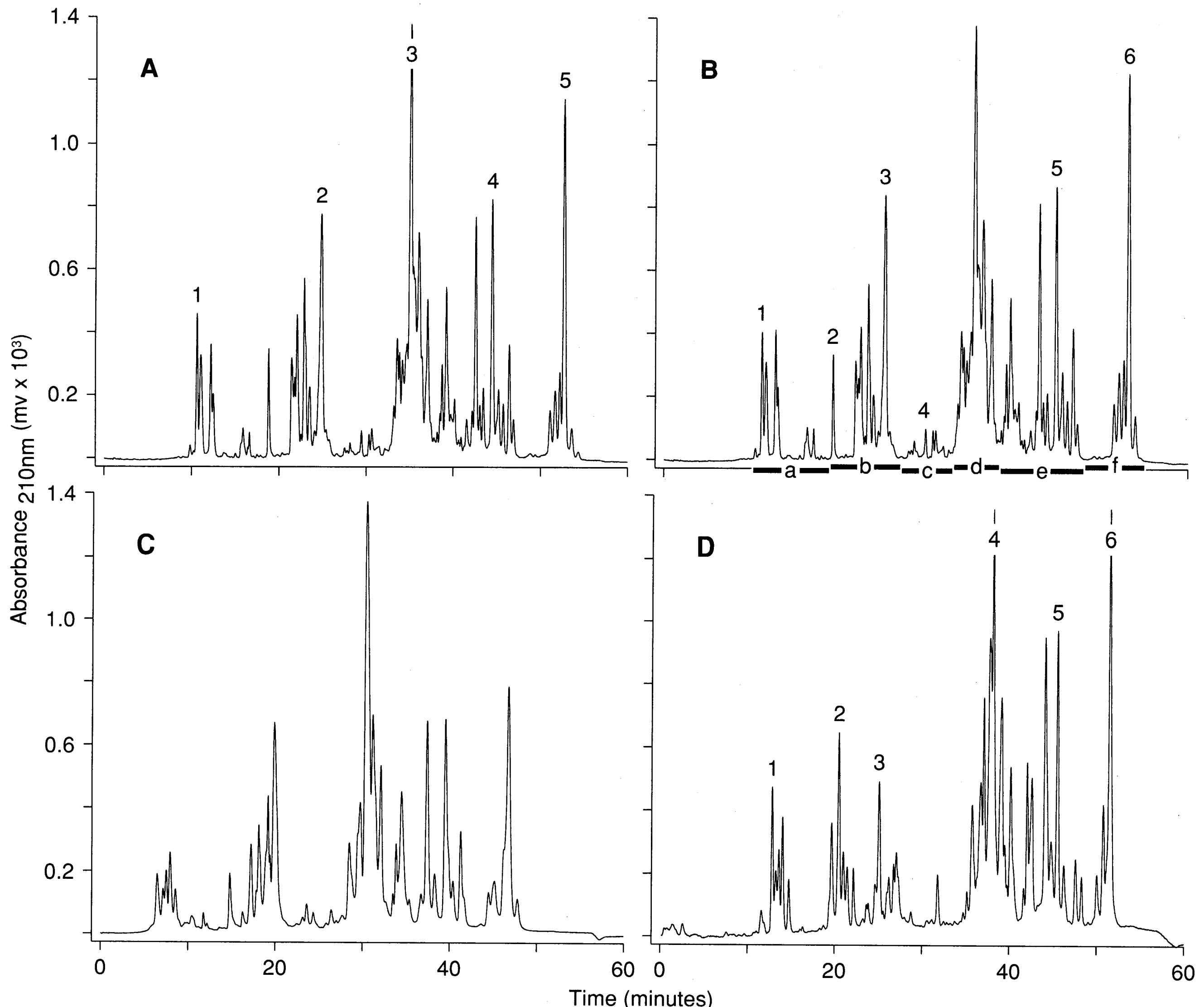

Fig. 1. Comparison of reversed-phase high-performance liquid chromatograms (obtained using 60-min gradients) of gliadins and glutenins extracted under reducing conditions from the wheat variety Neepawa using the following columns: **A,** Zorbax Rx-300-C8 (first analysis); **B,** Zorbax Rx-300-C8 (450th analysis); **C,** Supelcosil LC-308 (first analysis); **D,** Zorbax Rx-300-CN (first analysis). 1,000 mv = 1 AU.

The exception was for the HMW glutenin subunits, for which 30 μL injection volumes in conjunction with a multiple 5-μL injection technique (Marchylo and Kruger 1988) were used. Quantitative analysis was accomplished with Waters Expert Ease software using baseline-corrected chromatograms.

RESULTS AND DISCUSSION

The storage proteins (gliadins and glutenins extracted under reducing conditions) of Neepawa wheat were separated by RP-HPLC to evaluate the resolution capabilities of the new sterically protected Zorbax Rx-300 columns. Chromatograms obtained for first analyses with Zorbax Rx-300-C8 and Rx-300-CN columns are shown in Figure 1A and D, respectively. For comparative purposes, the separation of these proteins achieved with a Supelcosil LC-308-C8, wide-pore 5-cm column is also shown (Fig. 1C). As reported in a previous column evaluation (Marchylo et al 1988), the Supelcosil column provides good resolution, reasonable column stability, and relatively good column-to-column reproducibility. For this reason we have been using the Supelcosil column routinely in our laboratory.

Resolution obtained with the Zorbax Rx-300 columns was found to be superior to that of the Supelcosil column. The Zorbax Rx-300-C8 resolved 70 major and minor peaks, the Zorbax RX-300-CN 64 peaks, and the Supelcosil 53 peaks. The chromatograms from each column were similar in general appearance, but differences in selectivity were apparent. Separations at higher temperatures of 60 and 70° C were also performed with the Zorbax Rx-300-C8 column (results not shown) because higher temperatures, which have been used for the separation of wheat proteins (Bietz and Cobb 1985), can significantly influence peak shape (Cohen et al 1984) and recovery (Wehr et al 1989). Increasing temperature did not result in large changes in resolution or shifts in relative retention times as noted previously for a Supelcosil LC-308 column with conventional C8 packing (Marchylo et al 1988), and there was no increase in recovery. Contrary to results previously reported for the Supelcosil LC-308 column (Marchylo et al 1988), with the Zorbax Rx-300-C8 column, analysis at a higher column temperature of 70°C did not influence chromatograms subsequently obtained at 50°C. This is probably indicative of the steric protection afforded the hydrolytically sensitive Si-O-Si groups by the diisopropyl-bonded phase of the Zorbax RX column (Glajch and Kirkland 1990). Thus, under the stringent conditions of high temperature and low pH, hydrolysis of the bonded phase was minimized or prevented, and there was little or no change in the retention characteristics of the column.

Reproducibility of selected peak retention times and areas throughout the chromatogram were then assessed to ascertain the comparative stability of the Zorbax Rx-C8 column. Reproducibility of peak retention times for five peaks (Fig. 1A) over 200 separate analyses was excellent. As shown in Table I, the standard deviations (SD) for each peak were essentially the same, averaging 0.04 min., i.e., 2.4 sec. The mean coefficient of variation (CV = 0.17%) was significantly lower than the long-term reproducibility (CV = 1.78%, Scanlon et al 1989a) and slightly lower than short-term reproducibilities (CV = 0.33%, Bietz 1983; CV = 0.24%, Scanlon et al 1989a; CV = 0.20%, Marchylo et al 1990) reported previously for a number of conventional wide-pore C8 columns. On a short-term basis, for 10 replicate injections of the same extract, performed after 200 previous injections, a mean SD of 0.02 min (1.2 sec) and a mean CV of 0.07% were obtained (Table I). In considering this data, it is notable that there is an inherent error in peak retention time determinations because data acquisition was taking place at one point per second. In addition, other factors such as instrument performance can influence reproducibility in retention times (Sapirstein et al 1989). Taking this into account, the retention times for the peaks in question were essentially identical for the first 200 injections.

Following the assessment of this column over the first 200 injections, analyses of extracts of other types of wheat proteins were performed as part of another research study. Included in this work were analyses of salt-soluble protein extracts that were relatively viscous and difficult to filter before injection. Concomitant with analyses of the salt solubles, column pressure and retention times increased gradually with additional injections. Precolumn inlet filters were changed on a regular basis but, as noted, a guard column was not used due to lack of availability at the time of this study. Thus, after 400 injections (Table I), retention times had increased on average 27 sec for the five peaks used as markers. However, the Zorbax Rx-300-C8 column exhibited a more consistent drift, ranging between 29 and 25 sec for peaks 1 and 5, respectively, unlike previous studies with columns using conventional C8 packing (Marchylo et al 1988, Scanlon et al 1989a), in which peak retention time drift was variable among peaks. In addition, despite the slight retention time drift, chromatograms obtained for the first analysis (Fig. 1A) as compared to the 450th analysis (Fig. 1B) were basically identical with respect to both major and minor peaks.

Thus, contrary to previous work using Supelcosil columns with conventional C8 packing (Marchylo et al 1988, Scanlon et al 1989a), the Zorbax Rx-300-C8 column exhibited the same resolution and selectivity over the long term. Although some retention time drift was observed, this drift was essentially equal for all peaks. This effect leads us to speculate that the drift was due primarily to factors other than loss of the bonded phase. In particular, as discussed by Scanlon et al (1989a), precipitation of protein or other contaminants in the column could have resulted in the similar retention time changes observed for all the peaks. Such an effect may have resulted from the viscous salt-soluble samples that were injected after the 200th analysis. Use of a guard column, which is now commercially available, may have prevented this slight change in column performance.

Reproducibility of retention times for six selected peaks (Fig. 1D) was also determined for the Zorbax Rx-300-CN column over 150 analyses. A mean SD of 0.07 min (4.2 sec), which was only slightly larger than that of the Rx-300-C8 column, was obtained. Contrasted to the C8 column, an in-depth evaluation of the Zorbax Rx-300-CN was not carried out because it was an experimental column that may not become commercially available.

Rapid analyses of 10 min, using the 10-min gradient program

TABLE I
Reproducibility of Retention Times for Zorbax Rx-300-C8 Column

Peak No.[a]	Mean Retention Time for 200 Analyses[b] (min)	SD (min)	CV (%)	Mean Retention Time for Replicate Injections[c]	SD (min)	CV (%)	Retention Time of Analysis No. 400 (min)
1	10.65	0.04	0.38	10.66	0.02	0.19	11.15
2	24.81	0.05	0.20	24.82	0.02	0.08	25.26
3	35.08	0.04	0.11	35.08	0.02	0.06	35.54
4	44.45	0.05	0.10	44.43	0.01	0.02	44.88
5	52.67	0.04	0.07	52.68	0.01	0.02	53.10
Means		0.04	0.17		0.02	0.07	

[a] Peaks as denoted in Figure 1A for the variety Neepawa.
[b] Determined using randomly selected analyses ($n = 20$) of separate extracts.
[c] Replicate injections of one extract after 200 prior analyses. $n = 10$.

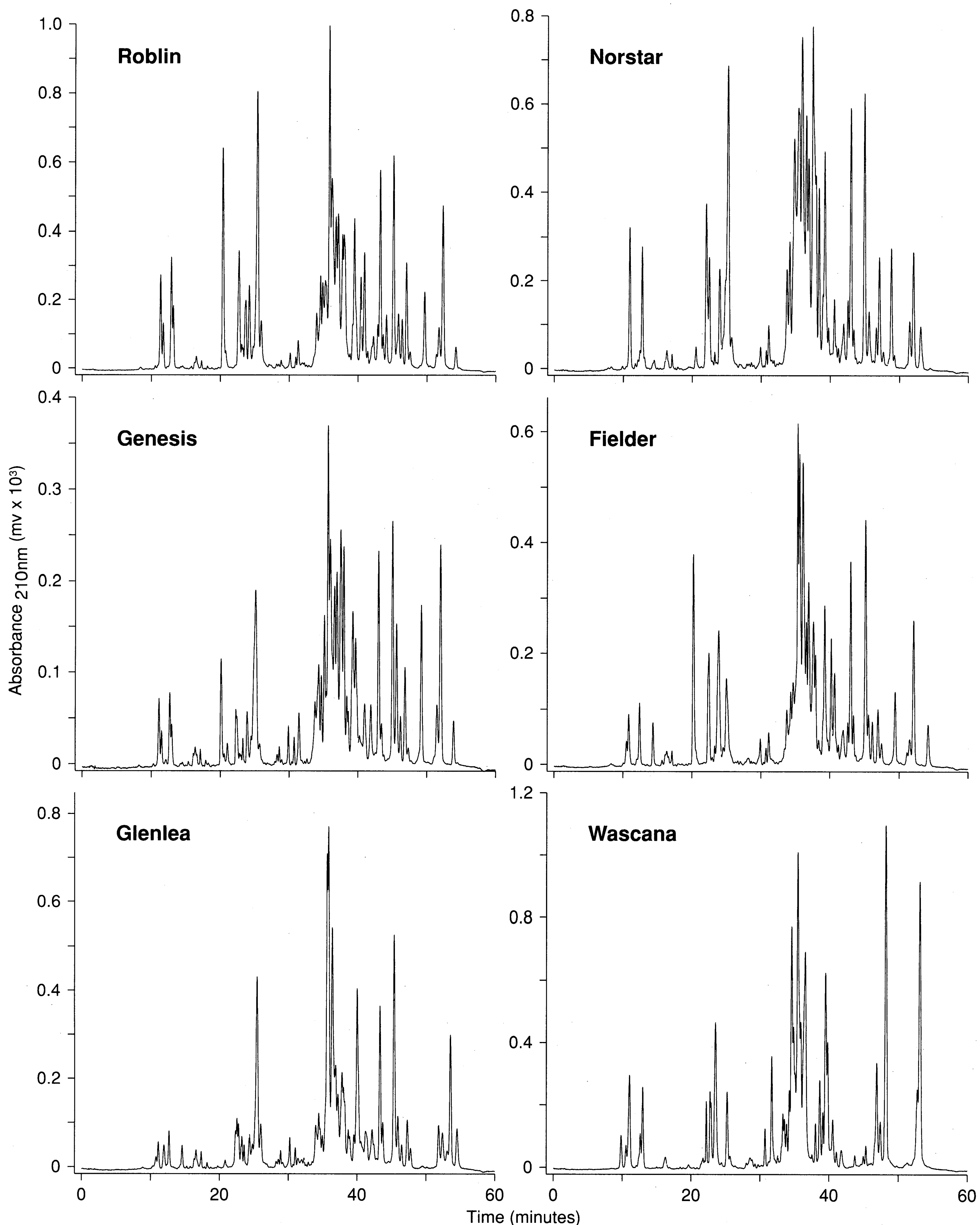

Fig. 2. Comparison of reversed-phase high-performance liquid chromatograms (obtained using a 60-min gradient) of gliadins and glutenins extracted under reducing conditions for the Canadian wheat varieties Roblin (Canada Western Red Spring); Genesis (Canada Prairie Spring wheat [white]); Glenlea (Canada Western Utility); Norstar (Canada Western Red Winter); Fielder (Canada Western Soft White Spring); and Wascana (Canada Western Amber Durum). Proteins were separated with a Zorbax Rx-300-C8 column. 1,000 mv = 1 AU.

described in Materials and Methods, were also performed using the Zorbax Rx-300-C8 column. As expected, there was a decrease in resolution with 32 peaks being resolved, although reproducibility of retention times was similar to longer analyses, with a mean SD of 0.006 min (0.4 sec) and a mean CV of 0.11% for 10 replicate injections of the same extract.

Reproducibility of six selected individual peak areas (Fig. 1B) was then determined for the Zorbax Rx-300-C8 column over the long term. Chromatogram segments and individual peaks, as denoted in Figure 1B, were quantified using 10 chromatograms randomly selected out of the first 200 analyses. The mean CVs for the six selected individual peak areas were 1.34% or 1.09% when areas were determined as a proportion of the total area. Similarly, CVs for six chromatogram segments (Fig. 1B) were 1.51 or 1.17% when areas were determined on a proportional basis. These CVs did increase, however, over 450 analyses ($N = 15$) for the individual peaks to 2.96% as is and 3.23% on a proportional basis similar to CVs of 3.12 and 2.60% for the chromatogram segments. Again, this increase in CVs may have been due to the analysis of the salt-soluble extracts concomitant with some column contamination. Despite this, reproducibility of quantitation for this column was significantly better than that previously reported for the long term (CV = 16.1%, Scanlon et al 1989a) using a conventional (Supelcosil LC-308) column.

Column-to-column variability has been shown to be a concern in previous studies (Marchylo et al 1988). Analyses using two different Zorbax Rx-300-C8 columns provided virtually identical chromatograms (as seen in Fig. 1A) with respect to retention times, resolution, and selectivity (comparative results not shown). These were preproduction columns, and we would expect that commercial columns will provide even better reproducibility.

The excellent reproducibility and stability exhibited by the Zorbax Rx-300 columns suggested that they would be suitable for wheat varietal identification and quality studies. To illustrate this, a number of varieties of different classes of wheat were analyzed using the Zorbax Rx-300-C8 column. As shown in Figure

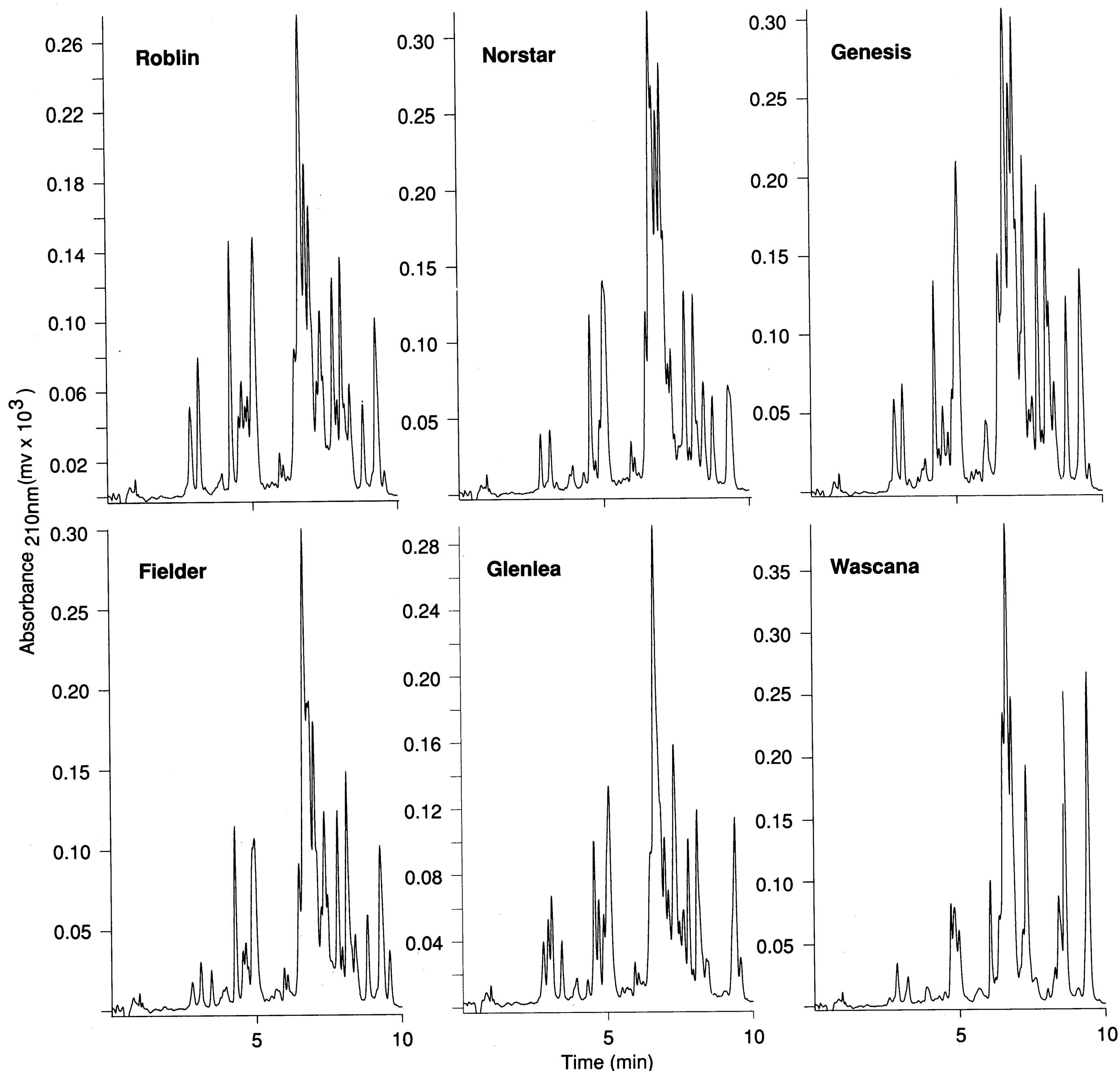

Fig. 3. Comparison of rapid reversed-phase high-performance liquid chromatograms (obtained using a 10-min gradient) of gliadins and glutenins extracted under reducing conditions, for the Canadian wheat varieties denoted in Figure 2.

2 for gliadins and glutenins extracted under reducing conditions, the excellent resolution characteristics of this column enabled all varieties to be easily discriminated. These varieties also were distinguishable by rapid 10-min analyses (Fig. 3), even though these separation conditions provided poorer resolution of gliadins and glutenins.

For illustrative purposes, unreduced 70% (v/v) EtOH extracts of these varieties, comprised primarily of gliadins, were also analyzed because many investigators use gliadins alone for wheat varietal identification. As seen in Figure 4, excellent resolution of the gliadins was also achieved and all varieties were distinguishable.

The quality-related HMW glutenin subunits in Neepawa were also separated using the Zorbax Rx-300-C8 (Fig. 5A), CN (Fig. 5B), and Supelcosil C8, 5-cm columns (Fig. 5C). These subunits were alkylated prior to analysis to facilitate their resolution (Burnouf and Bietz 1984). All three columns provided excellent resolution, but the Zorbax RX-300-C8 column had the added

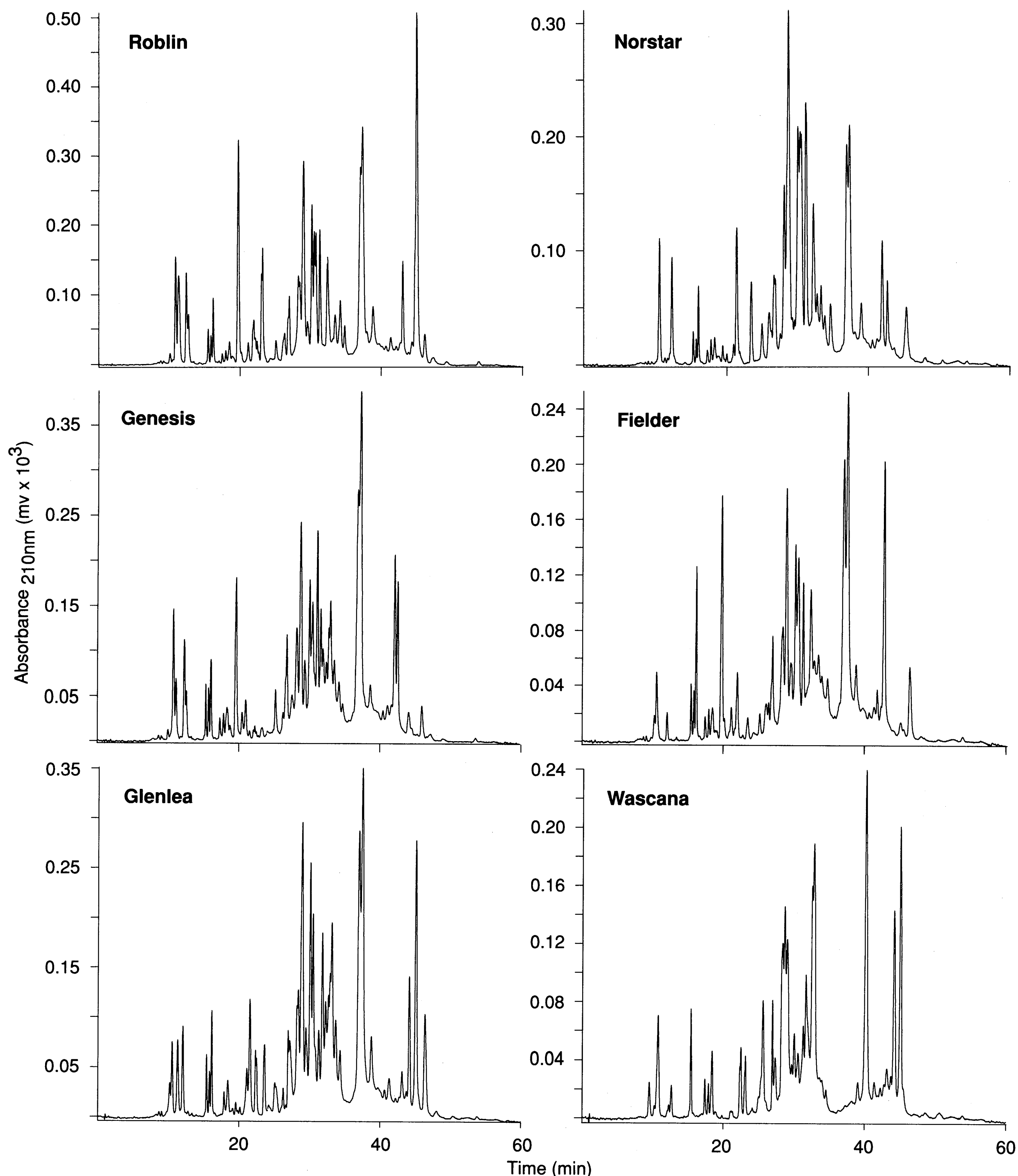

Fig. 4. Comparison of reversed-phase high-performance liquid chromatograms (obtained using a 60-min gradient) of gliadins extracted with 70% EtOH under nonreducing conditions, for the Canadian wheat varieties as denoted in Figure 2.

advantage of separating all of the HMW glutenin subunits from the small amounts of contaminating ω-gliadins present in the precipitated HMW protein fraction (Marchylo et al 1989). This was especially apparent for the Zorbax Rx-300-CN column where the ω-gliadins coeluted with subunits 9, 5, and 7* in particular. In the case of the Supelcosil column, coelution with subunit 10 was apparent as previously reported (Marchylo et al 1989). Confirmation of the presence of the coeluting ω-gliadins was determined by electrophoretic analysis of peak fractions (results not shown). Overlap of HMW glutenin subunits and ω-gliadins would result in overestimation of areas and thus influence quality analysis.

Differences in the relative retention times of the ω-gliadins and HMW glutenin subunits is indicative of differences in selectivity among these columns. The excellent resolution of the unalkylated HMW glutenin subunits achieved at a column temperature of 70°C with the Zorbax Rx-300-C8 column (Fig. 5D) is also noteworthy. In particular, subunit 10 was well separated from both the ω-gliadins and other HMW subunits. At lower column temperatures, resolution of individual peaks was not obtained with this or other columns (results not shown).

CONCLUSIONS

This preliminary evaluation of a new class of sterically protected, silica-based Zorbax Rx-300 reversed-phase columns indicated that the Zorbax columns provided superior resolution and column stability for the separation of wheat storage proteins when compared to conventional silica-based RP-HPLC columns previously studied (Bietz 1985, Marchylo et al 1988, Scanlon et al 1989a). Although the plate numbers and separating efficiencies of the Zorbax Rx-300 columns are reportedly comparable to those of conventional packings (Glajch and Kirkland 1990), we found that more gliadins and glutenins were resolved with the sterically protected columns. Column-to-column variations also appeared to be minimal with comparable chromatograms for both major and minor peaks. Reproducibility of retention times in both the short and long term was superior for the new column types. No column drift was observed over the first 200 analyses and only a small amount (27 sec, comparable for peaks throughout the chromatogram), was noted after 400 analyses. These results were achieved in the absence of a guard column that would probably have further improved the long-term stability and lifetime of this type of column. Reproducibility of quantitation was shown to be substantially improved over conventional columns, particularly in the long term. These results would suggest, as reported (Kirkland et al 1989, Glajch and Kirkland 1990), that steric protection has made these columns more resistant to degradation in column performance under the aggressive conditions of temperature and low pH required to resolve the hydrophobic wheat storage proteins (glutenins and/or gliadins).

The improved stability and resolution of the Zorbax Rx-300

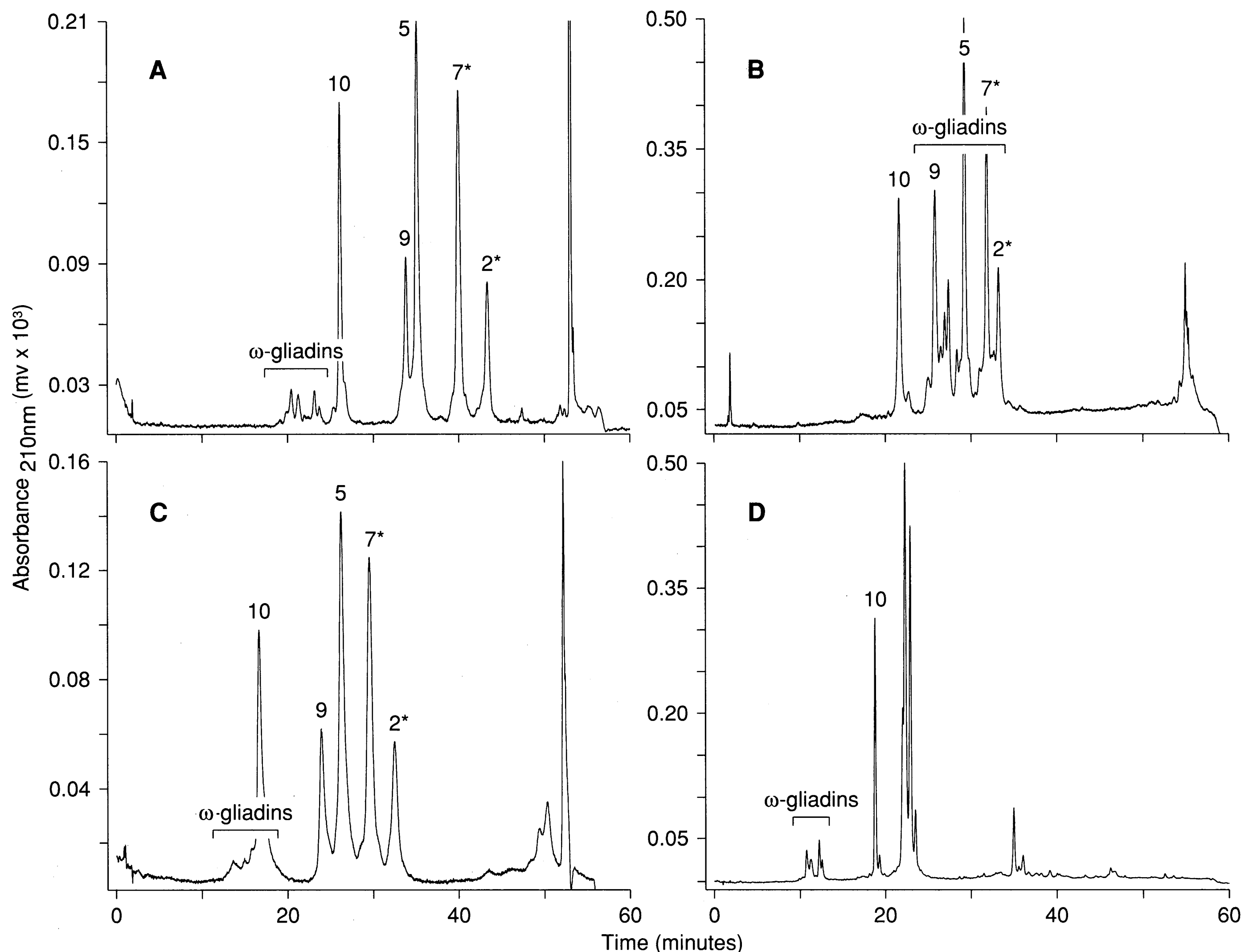

Fig. 5. Comparison of reversed-phase high-performance liquid chromatograms of the high molecular weight glutenin subunits of the wheat variety Neepawa using the following columns: **A** and **D,** Zorbax Rx-300-C8; **B,** Zorbax Rx-300-CN; **C,** Supelcosil LC-308. Subunits in **A–C** are alkylated; those in **D** are unalkylated. Analyses in **A–C** are at a column temperature of 50°C, whereas **D** is at 70°C. High molecular weight glutenin subunits are denoted per Payne and Lawrence (1983), except for subunit 7*, which is per Marchylo et al (1992). 1,000 mv = 1 AU.

columns makes them ideally suited for wheat varietal identification or quality studies. When used with normalization routines, these columns should provide highly reproducible chromatograms both qualitatively and quantitatively. Use of these columns should enhance significantly the utility of computerized wheat varietal identification methods such as reported by Scanlon et al (1989b). Improved quantitation with these new columns should also at least partially solve some of the problems encountered in using RP-HPLC for quality prediction as noted by Scanlon et al (1990). The Zorbax Rx-300-C8 column will be very useful for the qualitative and quantitative analysis and purification of the HMW glutenin subunits because of its ability to separate them from contaminating gliadins.

Although the Zorbax Rx-300-C8 column was evaluated in more depth in this study, the CN column also would appear to be useful in these applications. Differences in selectivity between these two packings could be used in a complementary fashion for the study of the wheat proteins.

The Zorbax Rx-300 columns probably will be applicable to the separation of other cereal proteins. For example, preliminary separations of the hordein proteins of barley has provided results comparable to the excellent separations achieved for the wheat storage proteins.

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