Evaluation of the Precision of High-Performance Liquid Chromatography for Wheat Cultivar Identification[1]

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ABSTRACT

The reproducibility of computer-derived reversed-phase high-performance liquid chromatography (RP-HPLC) quantitation parameters following prolonged use of a single commercially available column was studied. Using a standardized experimental procedure, more than 65 chromatograms were evaluated based on gliadin extracts from bulk-ground meal and composite grinds of four kernels of the bread wheat cultivar Neepawa. Statistical results are reported for a set of more than 30 chromatogram peaks with respect to the precision of retention times, computed peak areas and heights, and percentage peak areas and heights. Whereas chromatographic resolution was relatively constant over time, prolonged column use significantly retarded peak retention times especially for early eluting components. Results show that without appropriate normalization, peak retention times lack sufficient long-term precision in order to obtain reliable results for cultivar identification and other RP-HPLC comparative analysis applications.

The separation of wheat protein extracts by electrophoretic or chromatographic procedures for computerized wheat cultivar identification and other comparative analysis applications requires precise quantitative parameters to achieve reliable results (Sapirstein and Bushuk 1985a). Previous studies on the use of reversed-phase high-performance liquid chromatography (RP-HPLC) for these applications have dealt extensively with optimization of experimental procedures (Bietz et al 1984, Bietz and Cobb 1985, Kruger and Marchylo 1985). These studies sought to improve wheat protein separation. Little attention has been focused on aspects of chromatographic reproducibility, although some reports have addressed short-term effects (Bietz and Cobb 1985, Marchylo et al 1988). This study attempts to evaluate quantitative precision of chromatograms using a standardized experimental procedure, carried out over a two-month period, in order to determine if sufficient long-term analytical precision exists for automated wheat cultivar identification.

MATERIALS AND METHODS

Materials

HPLC grade acetonitrile and ethanol were obtained from Fisher Scientific (Fair Lawn, NJ). Sequanal grade trifluoroacetic acid (TFA) was purchased from Pierce Chemical Co. (Rockford, IL). Water was distilled and then purified with a Millipore Milli-Q system (Mississauga, ON). A certified seed sample of Canadian hard red spring wheat cultivar Neepawa was used for this study. The authenticity and homogeneity of the grain was verified by polyacrylamide gel electrophoresis (Sapirstein and Bushuk 1985a).

Apparatus

HPLC analyses were performed on a Hewlett-Packard 1090M liquid chromatograph incorporating a DR5 two-solvent reservoir delivery system, auto-injector, auto-sampler, heated column compartment, and diode array detector. Analytical control and quantitation were provided by an HP-310 computer running HP 79994A software for the analytical workstation and HP 79995A operating software. A 55 Mbyte hard-disk was used for system backup and data storage. A Supelcosil LC-308 reversed-phase column (5 μm silica particle size, 300 Å pore size, C8 bonded phase, 25 cm × 4.6 mm i.d.) was used with a Supelguard LC-308 5 cm × 4.6 mm i.d. guard column of the same packing material (Supelco Canada Ltd., Oakville, ON). Peak retention times and other quantitation parameter data were transferred to the University of Manitoba Amdahl 6280 computer for statistical analysis.

Protein Extraction

Samples comprising 100 mg of whole wheat meal or four pulverized kernels were extracted with 400 μl or a 4:1 ratio of 70% ethanol in 1.5-ml microcentrifuge tubes. Four kernels were chosen, instead of single kernels, in order to decrease variation in peak height and area. The varieties chromatographed were certifiably pure and did not consist of biotypes. However, should there be doubts about purity, single kernel extractions should be carried out. The mixture was initially vortexed and left to stand at room temperature for 15 min, with vortexing at 5-min intervals prior to centrifugation for 15 min at 8,800 × g. The clear supernatant was filtered through a 0.45-μm nylon filter (Micron Separations Inc., Honeoye Falls, NY) into a disposable microvial in preparation for chromatography.

Experimental Procedure

Chromatography of cultivar Neepawa samples described in this report was part of a larger study involving numerous wheat cultivars registered in Canada and elsewhere for the purpose of establishing a computerized data base of genotypes for RP-HPLC comparative analysis. For chromatography, solutions of water (+0.1% TFA) and acetonitrile (+0.1% TFA) were used as solvents A and B, respectively. Solvent degassing was by helium sparging (Brown et al 1981) with a helium flow rate of 30 ml/min for 3 hr prior to analyses and then at 8 ml/min during chromatographic runs to prevent back diffusion of oxygen (Lloyd 1983) and baseline drift effects (Brown et al 1981). Column temperature was 50.0° C; solvent flow rate was maintained at 1.0 ml/min and 10 μl of the protein extracts were chromatographed. Proteins were eluted in 65 min using the conditions of Table I. The initial isocratic period preceding the linear gradient (Table I) eliminated spurious baseline noise integrations that sometimes accompany solvent peak elution. No blank runs were carried out during a set of runs for subsequent chromatographic subtraction, since the change in acetonitrile concentration from 25 to 55%, as used in this study, does not lead to major changes in the spectral properties of the TFA/acetonitrile/water system (Winkler et al 1985).

Column effluent was monitored at 210 nm. A data acquisition sampling rate of one point per 960 msec was used. This gave a total of approximately 4,000 data points per chromatogram. On average each peak was represented by over 50 data points, which is sufficient to satisfy accurate peak integration requirements (Hippe et al 1980). In a preliminary study, peak integration parameters were optimized to yield a consistent maximum number of integrated components. These optimum parameters were 0.15 min, 0 and −1 for peak width, area rejection, and threshold, respectively.

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Chromatography of replicate gliadin extracts from Neepawa and other cultivars (crushed kernels) typically followed the sequence: Neepawa meal extract, three test cultivars, Neepawa kernel extract, three test cultivars, Neepawa meal extract. To facilitate removal of adsorbed protein, the sequence was followed by three short cleanup elution sequences (three 2-min gradients [15–85% B], and a 4-min hold at 85% B), and one long clean ("flush") using a 50-min gradient (15–85% B). Depending on HPLC utilization, a more extensive cleanup protocol was employed using modifications of procedures described elsewhere (Bietz 1986, Wehr 1987). More than 240 chromatograms in total were obtained during the two-month period associated with this study.

RESULTS AND DISCUSSION

Based on direct inspection of chromatograms and integration results, a set of 30 peaks was chosen in the range 18–50 min (Fig. 1) that were judged to be identifiable as matching components among different replicate chromatograms of Neepawa samples run during the two-month experimental period. In order to compare results in relation to short- and long-term column performance, two periods of two and eight weeks were arbitrarily designated. A random sample of 15 chromatograms acquired in the two-week period was compared with 25 counterpart chromatograms sampled over the eight-week period. Matching chromatogram components were identified, and statistical analysis of computer-generated peak integration data evaluated the precision of the following quantitation parameters: peak retention time, peak area, percent peak area, peak height, and percent peak height. The values for percent peak area and height are normalized values, since the total peak area or height for the chromatogram is summed and individual peak areas or heights are expressed as a percentage of their respective totals.

Precision of Peak Retention Time

Highly significant differences were observed in the reproducibility of peak retention times during short- and long-term periods of column use (Fig. 2). The mean coefficient of variation (CV) for short-term acquired peak retention times was 0.24%. This concurs with published CVs for 60–120 min analyses of wheat storage proteins, which range from 0.33% (Bietz 1983) to 0.20% (Marchylo et al 1988) for repeated injections of one extract. Lower variability may be achieved by using shorter analysis periods; for example CVs of 0.09% and 0.13% were reported for 12-min (25–50% B) analyses by Bietz and Cobb (1985) and Marchylo et al (1988), respectively. Nevertheless, the level of mean retention time variability obtained here over a two-week interval probably represents a practical minimum in experimental error for different protein extracts analyzed using the superior resolving powers of 60-min analyses.

In contrast to the low level of peak retention time variability for short-term periods of column use, results over the long-term are substantially different (Fig. 2). The mean retention time CV for chromatograms obtained during eight weeks of analyses was 1.78%, more than seven times higher than for data acquired over two weeks. In addition, the earlier the elution, the greater the variability. For the earliest eluting peaks uncertainties over the long term approached 3% relative error. It appears that proteins of low hydrophobicity, which elute earlier, bind to (and/or elute from) the column in a way that is more affected by column aging processes (refer to following discussion). Consequently, considerable difficulty was encountered in matching common protein peaks among chromatograms obtained early in the column's life with those obtained later, being especially problematic for components with low retention times.

TABLE I
Solvent Composition Changes for Chromatography

Time (min)	% Solution B	Explanation
0	25	Initial conditions
0–5	25	Isocratic
5–65	25–55	Linear gradient (0.5% solution B/min)
65	25	Return to initial conditions
65–80	25	Equilibration at initial conditions

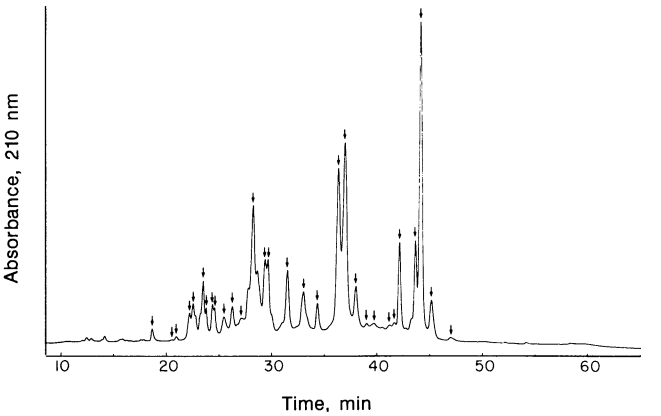

Fig. 1. Neepawa chromatogram showing (denoted by arrows) the 30 components that were consistently integrated.

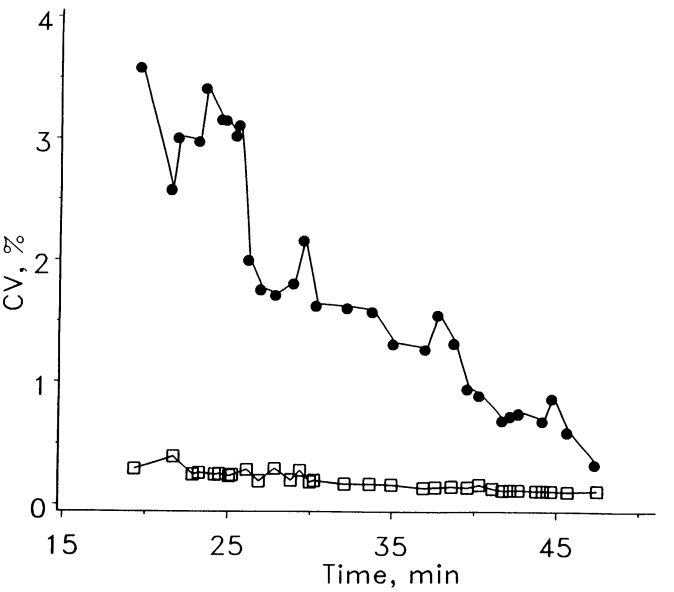

Fig. 2. Comparison of elution time precision for short- (□) and long-term (●) column use.

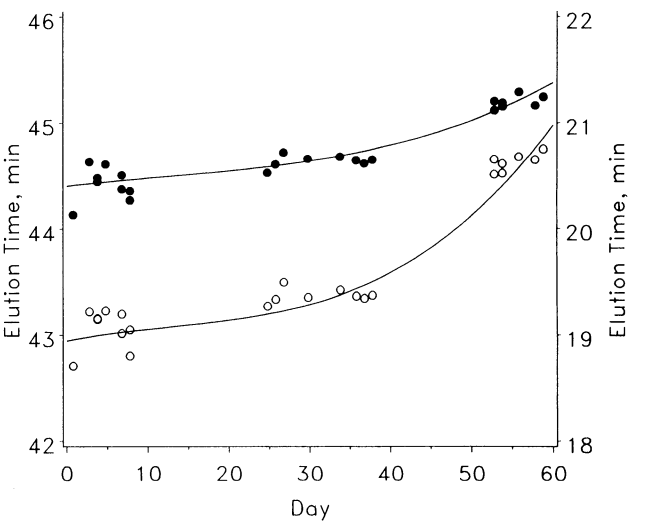

Fig. 3. Elution time variation for an early (o) and a late (●) eluting peak as the column ages. Elution times on right and left axes, respectively.

Evidence that peak retention time variability was not simply due to random errors accumulating over time can be obtained by monitoring peak retention times for individual components. Figure 3 shows typical results for an early (18.7–20.7 min) and a late (44.1–45.4 min) eluting peak during the eight week period. Loss of precision in the data can thus be attributed to a systematic increase in peak retention times. Whereas the effect is more pronounced for peaks eluting at low concentrations of acetonitrile, all proteins required greater concentrations of acetonitrile to be eluted as the column aged.

An increase in elution time with column use has been reported by Bietz and Cobb (1985), Simpson et al (1985), and Marchylo et al (1988). For the eight-week period of analyses in the present study, an increase in peak elution times ranging from 120 to 66 sec for the earliest and latest eluting protein components, respectively, was observed. These results, consistent with observations by Marchylo et al (1988), indicate that considerable challenges exist in using chromatography results for comparative analysis especially for data acquired over the long term. Thus chromatogram library search methods based on retention times (Lea et al 1983) would probably generate erroneous results without prior correction of the data using suitable standards (Bietz and Cobb 1985) or making periodic adjustments in the gradient program to compensate for retention time drifts (Noyes 1983).

Precision of Peak Quantitation Parameters

As with electrophoresis studies (Sapirstein and Bushuk 1985b), RP-HPLC chromatogram characterization for comparative analysis requires information concerning protein peak (band) quantitation in association with the presence or absence of components. Area is frequently used for peak quantitation because of its analytical significance (Novak 1975). Height can also be used but is more prone to imprecision due to peak dispersion as components move through the column, although the continually increasing solvent strength in gradient elution decreases this dispersion (Snyder et al 1979). Because of incomplete separation of components, drop points or tangent-skimming methods of area determination will have to be employed creating uncertainty in true peak area quantitation. Accordingly, the precision of peak quantitation parameters (peak areas, peak heights, and their respective normalized values) was assessed in order to select a suitable reproducible secondary variable in addition to peak retention time.

Figure 4 compares the reproducibility of integrated peak area and peak height data for a set of protein components detected in at least 20 replicate Neepawa chromatograms (from ground meal extracts) analyzed over an eight-week period. Mean CVs were 25.1 and 21.0% for peak areas and heights, respectively, with differences attributable to the early eluting peaks (<35 min). Significantly better precision in peak quantitation was obtained from normalized peak areas or heights. Normalizing peak areas to percent peak areas reduced the mean CV from 25.1 to 16.1%. The counterpart mean CV for normalized peak heights was likewise reduced: in this case, from 21.0 to 11.6%. Figure 5A shows the effect for peak height and percent peak height. The normalization effect was generally uniform, and significantly lower CVs were obtained for the majority of detected components across the chromatogram. These data suggest that normalized peak height represents the most satisfactory quantitation parameter in addition to peak retention time. Although changes in selectivity of components with prolonged column use may account for changes in peak quantitation (refer to following discussion), no clear trends in peak quantitation parameters were observed with prolonged column use similar to those seen for peak retention times.

Because a common ground meal sample was the basis for generating these peak quantitation results, grain protein content

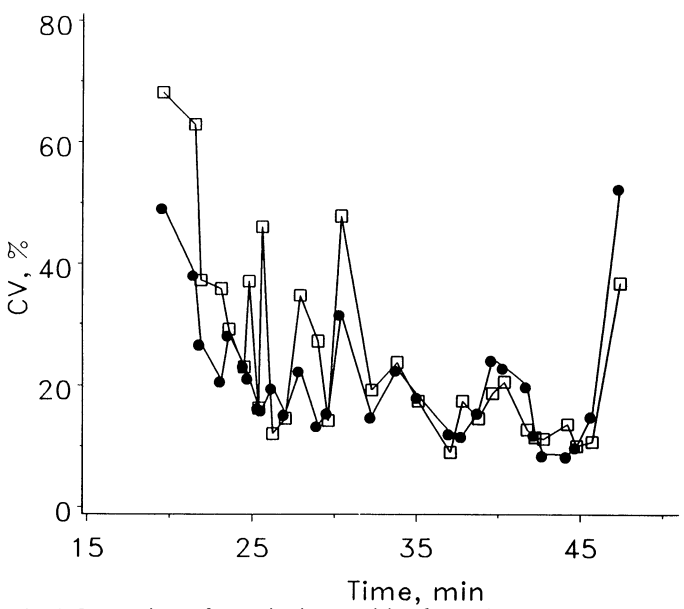

Fig. 4. Comparison of quantitation precision for peak areas (□) and peak heights (●).

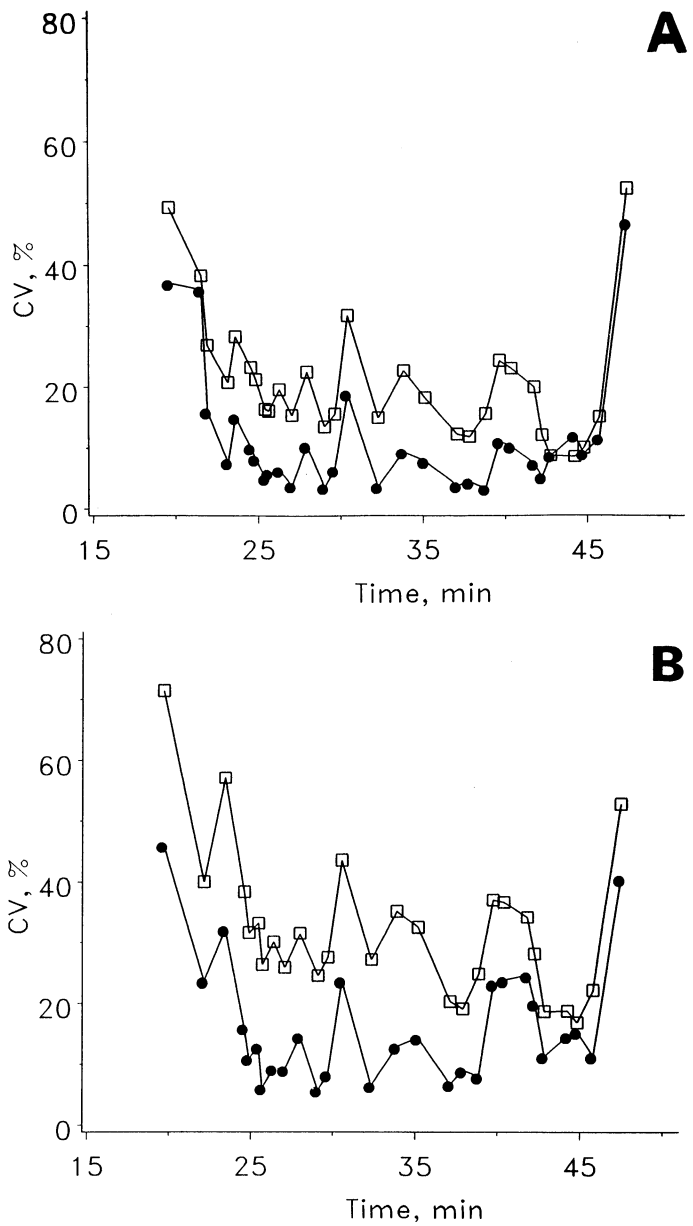

Fig. 5. Comparison of quantitation precision for peak heights (□) and percentage peak heights (●) for **A**, ground meal extracts, and **B**, four-kernel grind extracts.

per se had no bearing on the improvement in reproducibility of peak heights following normalization. Better precision in the data arises from minimizing experimental error in protein extraction and in quantitation errors associated with baseline specifications by integrator software, which can have marked effects on peak quantitation. This base level of uncertainty in peak quantitation can be inflated by differences in protein extraction arising from errors in variable materials such as wheat grains (Huebner and Bietz 1988). In this study, replicated protein extracts from ground four-kernel samples increased the relative error of all quantitation parameters by approximately 50% (Fig. 5B); mean CVs of 32.4 and 16.5% were obtained for peak height and percentage peak height, respectively.

In comparison to electrophoretic results (Sapirstein and Bushuk 1985b), it can be concluded that RP-HPLC of wheat storage proteins appears to be quantitatively less stable. Experience has shown that even for electrophoresis of single kernels, differences among common bands in terms of staining intensity are usually small. The superior sensitivity of RP-HPLC logically accounts for its relatively high level of peak quantitation variability. What effect this may have on the performance of an automated wheat cultivar identification methodology remains to be investigated. However, for comparative analysis of RP-HPLC results, peak retention time, by specifying relative protein hydrophobicity, naturally represents the primary quantitative feature. In electrophoretically based analyses, considerable tolerance can be given to differences in relative band densities, but they are still useful secondary discrimination parameters complementing band mobilities (Sapirstein and Bushuk 1985b). Likewise, the inherent variabilities in relative RP-HPLC peak heights obtained in this study should not present major difficulties for automated RP-HPLC wheat cultivar identification. However, this will only be true if adequate correction of results is performed in order to compensate for retention time changes as the column ages. A set of standards that elute at various concentrations of acetonitrile will be required in order for retention time corrections to be specific for given regions of the chromatogram. This point is dealt with in greater detail in a companion paper (Sapirstein et al 1989).

Column Factors Affecting Reproducibility

Retention of proteins on reversed-phase columns involves multisite binding by a number of phenomena including ionic interactions with residual silanol groupings, hydrogen bonding, hydrophobic interactions, and exclusion effects (Wehr 1987). Any changes in column properties with time will alter the influence of the various protein retention mechanisms and hence retention times.

Wehr (1987) reviewed different column changes and the conditions under which they would occur. The three main types were void formation, frit blockage, and alteration of the stationary phase. Since the operating pH was acidic, and a noncationic ion-pair reagent was used, the chromatographic conditions discount void formation. Further evidence discounting void formation is the absence of peak broadening or splitting. Because no appreciable rise in pressure was observed, frit blockage was also discounted as contributing to retention time and selectivity changes as the column aged. Therefore factors affecting peak retention times would appear to be stationary phase alterations during column use. A major stationary phase alteration is hydrolysis of siloxane groups to silanols and loss of the hydrocarbon moiety (Claessens et al 1985). The decrease in overall hydrophobic bonding ability, concomitant with this loss, would lead to a decrease in retention times (Glajch et al 1987, Noyes 1983, Wehr 1987), a result not seen in this study or by other workers dealing with RP-HPLC of wheat storage proteins.

In addition to many hydrophobic proline residues, wheat proteins contain a very high proportion of glutamic acid, most of which is present as glutamine (Schofield and Booth 1983). The amide group of glutamine has a propensity for hydrogen bonding due to its polar, albeit uncharged, nature, so silanol-protein interactions will contribute significantly to the retention mechanisms of wheat proteins, contrary to the minor role such

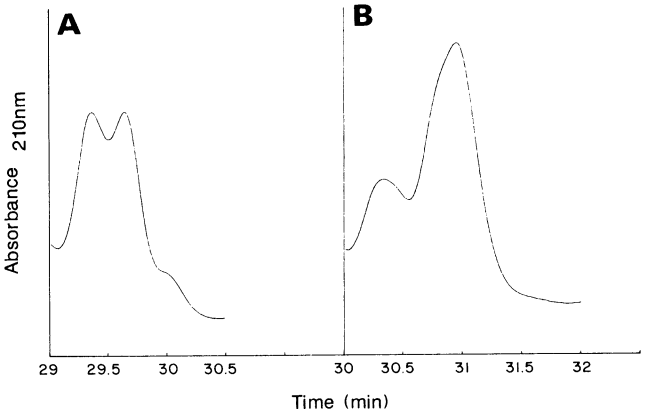

Fig. 6. Selectivity changes with time. For a Neepawa extract, **A** is a protein doublet early in column life, and **B** shows the same doublet at a later stage of column life.

interactions play in retention of other proteins (Cohen et al 1985). In this case, the loss of C8 groupings with time and the increase in the polar silanol groupings will increase the attraction between the proteins and the stationary support. More acetonitrile will be required to solvate the hydrophobic residues and induce elution, hence retention times will increase, as observed in this study and by Marchylo et al (1988). The less hydrophobic proteins eluted at the beginning of the chromatogram (ω-gliadins, according to Bietz 1983) are more affected by this increase in silanophilicity due to more hydrogen bonding between the protein's amide groups and the new silanols, which by virtue of the hydrocarbon chain loss are also more sterically accessible. Indeed, the greater glutamine content of ω-gliadins (Kasarda et al 1976) may be responsible for their eluting at lower acetonitrile concentrations (Bietz 1983).

Selectivity, and hence peak quantitation parameters, will also be affected by column aging effects. An example of such a selectivity change is shown in Figure 6, where an equal-sized doublet became a doublet of different size as the column aged. Irreversible protein precipitation can occur on the column, especially if the protein reacts with a hydrolyzed silanol grouping (Cohen et al 1984). As a result a secondary partition site can arise (Kirschbaum et al 1984), which can retain a given protein to a greater, lesser, or to the same extent (Melander and Horvath 1980), depending on the protein's amino acid composition and its conformation. A protein that is retained more by the secondary site will require more acetonitrile to elicit elution. If the numbers of the secondary sites are small then peak broadening will occur followed by front shoulder formation (Kirschbaum et al 1984). As more contaminant binds to the column, the proportion of secondary sites to unaltered column sites increases; more protein is eluted later, giving rise eventually to a secondary peak. This secondary peak will then merge with the later eluting peak creating a similar effect to that seen in Figure 6.

The high amide content of wheat proteins would be expected to contribute to increased retention times as the column deteriorates with use (Glajch et al 1987). Routine monitoring of changes in retention times of a standard would facilitate comparison of chromatograms acquired at different times. Similarly, peak selectivity changes in a standard could be used to correct quantitation parameters that may be altered by protein precipitation effects during column use (Kirschbaum et al 1984).

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