The Effect of Injection Volume on the Quantitative Analysis of Wheat Storage Proteins by Reversed-Phase High-Performance Liquid Chromatography[1]

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ABSTRACT

The relationship between injection volume and the binding of gliadins and glutenins to reversed-phase high-performance liquid chromatography (RP-HPLC) columns was studied. Significant quantitative changes were observed, concomitant with increasing injection volume (5–80 μl), in the amount of protein and in relative proportions of protein peaks eluted in chromatograms. Rapid decreases in the recovery of proteins, in particular the least hydrophobic gliadins, were observed as injection volume increased. Analysis of solvent peaks by RP-HPLC and sodium dodecyl sulfate gradient polyacrylamide gel electrophoresis indicated that as injection volume increased, progressively more gliadin and glutenin proteins did not bind to the column upon injection and were eluted in the solvent peak (i.e., void volume). The effects of column temperature, extraction solvent, and type of column on this phenomenon were examined. A remedy to the problem was devised entailing the use of multiple 5-μl injections of protein extract prior to initiation of the gradient program. Virtually 100% of proteins were bound to the RP-HPLC column using this technique.

The quantitative capability of reversed-phase high-performance liquid chromatography (RP-HPLC) for analysis of cereal proteins is possibly one of the most useful attributes of this technique. Quantitation of cereal storage proteins by RP-HPLC has been used in studies designed to relate wheat gliadins and glutenins (Burnouf and Bietz 1984; Huebner and Bietz 1985, 1986, 1987) and barley hordeins (Marchylo et al 1986) to quality characteristics, as well as to assist in the identification of barley (Marchylo and Kruger 1984) and wheat (Marchylo et al 1988) cultivars.

In a previous study (Kruger and Marchylo 1985), it was observed that significant levels of gliadin and glutenin proteins were eluted in the solvent peak when large volumes of 1-propanol extracts of wheat storage proteins were injected onto a reversed-phase column. This result was ascribed to overloading of the column. Recently, Huebner and Bietz (1987) also reported the elution of gliadin proteins, extracted with 70% ethanol, in the void volume in larger scale RP-HPLC preparative work. These studies suggest that elution of proteins in the solvent peak might affect the quantitative analysis of RP-HPLC chromatograms by changing the relative proportions of protein components. In turn, this could influence quality determinations, since the relative amount of high molecular weight glutenin subunits has been shown to be related to certain wheat quality characteristics (Huebner and Bietz 1985). Therefore, the purpose of this study was to characterize more fully the relationship between injection volume and binding of wheat storage proteins during RP-HPLC analysis.

MATERIALS AND METHODS

Materials

Sequanal-grade trifluoroacetic acid and 1-propanol were obtained from Pierce Chemical Co. (Rockford, IL); dithiothreitol (DTT), 4-vinylpyridine, and Tris were from Sigma (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 used in all solutions. Neepawa, a hard red spring wheat cultivar, was used throughout the study.

Sample Preparation and Extraction Procedures

Wheat was ground in a Udy cyclone sample mill equipped with a 1-mm sieve. One-gram samples of ground grain were extracted with 6.0 ml of 50% or 40% 1-propanol containing 1% (w/v) DTT, or alternately 70% or 50% ethanol, as described previously (Marchylo et al 1988). Aggregated proteins in ethanol extracts (Bright and Shewry 1983) were reduced to subunits prior to RP-HPLC by addition of an equal volume of the appropriate ethanol solution containing 2% (w/v) DTT. Reduction was carried out over 30 min at 60° C with mixing every 10 min.

Preparation of Serial Dilutions of Protein

Serial dilutions of protein were made using storage proteins precipitated from wheat extracts prepared as noted above. To precipitate protein, aliquots of extracts (5 ml of 50% 1-propanol + 1% DTT extracts or 10 ml of 70% ethanol + 1% DTT extracts) initially were cooled to below 0° C, after which 1.5 volumes of cold water (4° C) was added with mixing. The resultant mixture then was centrifuged at 27,500 × *g* at 4° C for 10 min. Supernatant was discarded and the protein pellet was redissolved in 1.0 ml of the appropriate solvent at 60° C for 6–24 hr with mixing. This yielded a solution with an approximate fivefold increase in protein concentration. The concentrated extract was centrifuged in an Eppendorf microcentrifuge model 5414 (Brinkman Instruments Inc., Westbury, NY) at 15,600 × *g* for 10 min. Supernatant was filtered through millex HV4 0.45 μfilters (Millipore Ltd., Mississauga, ON). The protein extract then was diluted serially 2, 4, 8, and 16 times with appropriate solvents.

RP-HPLC

A Waters HPLC and Waters 840 data and chromatography control station were employed in this study, as described previously (Marchylo et al 1988). Analyses were carried out with the following RP-HPLC columns: Supelcosil LC-308 (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.); Supelcosil LC-308 (C_8, 300Å, 5 μm, 25 cm × 4.6 mm i.d.) preceded by a Supelguard column as noted above; Vydac 228TP (C_8, 300Å, 5 μm, 15 cm × 4.6 mm i.d. (The Separations Group, Hesperia, CA); Synchropak RP-18 (C_{18}, 300Å, 6.5 μm, 25 cm × 4.1 mm i.d.). Proteins were eluted using gradient programs A (120 min) or B (126.0 min) as described in Table I. Eluted proteins were monitored at 210 nm and 0.5 absorbance units full scale (AUFS; 1,000 mV = 0.5 AU), and chromatograms were quantified as reported previously (Marchylo et al 1986) with the exception that, where necessary, the baseline correct mode of the Waters 490 programmable multiwavelength detector or the "subtract mode" of the LC multisystem version 3.0 software was used to correct for the positive slope observed in "blank" baselines. Analyses were carried out in duplicate unless noted otherwise.

Collection and Analysis of Solvent Peaks

The delay time between detector response and elution from the

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column (Supelcosil LC-308, 5.0 × cm 4.6 mm i.d.) first was determined (38 sec). Collection of solvent peaks began 38 sec after initial detector response of the solvent peak and continued for 2 min. The RP-HPLC analysis then was aborted, and bound protein was removed by flushing for 10 min at 1 ml/min with solvent consisting of 90% acetonitrile, 10% H_2O, and 0.1% trifluoroacetic acid. Three consecutive analyses were carried out, and solvent peaks were collected for each injection volume of 5, 15, 40, and 80 μl. Resultant solvent peak samples (6 ml) were concentrated to approximately 50 μl in Bio-Rad Unicep Ultracent 30 ultrafiltration cartridges (molecular weight cutoff 30,000) (Bio-Rad, Richmond, CA). Aliquots of solvent peak concentrates were used for SDS gradient PAGE (SDSGPAGE) analyses (20 μl) and for RP-HPLC analyses (15 μl). Aliquots were reduced and alkylated prior to SDSGPAGE analysis (1 μl sample load), essentially as reported previously (Marchylo 1987).

RESULTS AND DISCUSSION

The relationship between injection volume and the elution of wheat storage proteins from RP-HPLC columns was investigated by first preparing serial dilutions (2-, 4-, 8-, 16-fold) of a protein concentrate. Initially, 50% 1-propanol containing 1% DTT was used for preparation of the protein concentrate and subsequent serial dilutions. This solvent has been shown to be extremely efficient for extraction of gliadins and glutenins (Byers et al 1983), and for this reason it has been employed in wheat cultivar identification studies in this laboratory (Marchylo et al 1988). Protein concentrate and serial dilutions were analyzed in sequence using injection volumes of 5, 10, 20, 40, and 80 μl, respectively. Thus, equal amounts of protein were injected onto the RP-HPLC column irrespective of injection volume. Resultant chromatograms are illustrated in Figure 1 (A–E) for analyses carried out at a column temperature of 50° C. Elution profiles were qualitatively the same at all injection volumes. Significant quantitative changes were observed, however, in the amount of protein and in the relative proportions of peaks eluted concomitant with increasing injection volume. Chromatograms were subdivided into four groups of peaks (Fig. 1A), and their areas were quantified using the Waters LC multisystem version 3.0 software. Relative recoveries (group area [serial dilution]/group area [protein concentrate] × 100) were plotted as a function of increasing injection volume to illustrate observed quantitative changes in protein elution characteristics. As shown in Figure 2 (middle), all protein groups exhibited a decrease in relative recovery for an 80-μl injection. Group one proteins showed the most dramatic and rapid decrease in chromatographic peak area with increasing injection volume such that about 80% was recovered following a 10-μl injection whereas only a 10% relative recovery was observed for an 80-μl

TABLE I
Gradient Programs for Separation of Gliadins and Glutenins by Reversed-Phase High-Performance Liquid Chromatography

Time of Gradient Segments (min)	Gradient Composition (% acetonitrile)		Gradient Type[a]
	Start	End	
Program A			
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
Program B			
0.0– 3.0	10	10	isocratic
3.0– 6.0	10	24	linear
6.0– 9.0	24	24	isocratic
9.0–111.0	24	48	linear
111.0–114.0	48	48	isocratic
114.0–115.0	48	24	linear
115.0–126.0	24	24	isocratic

[a] Flow rate = 1 ml/min.

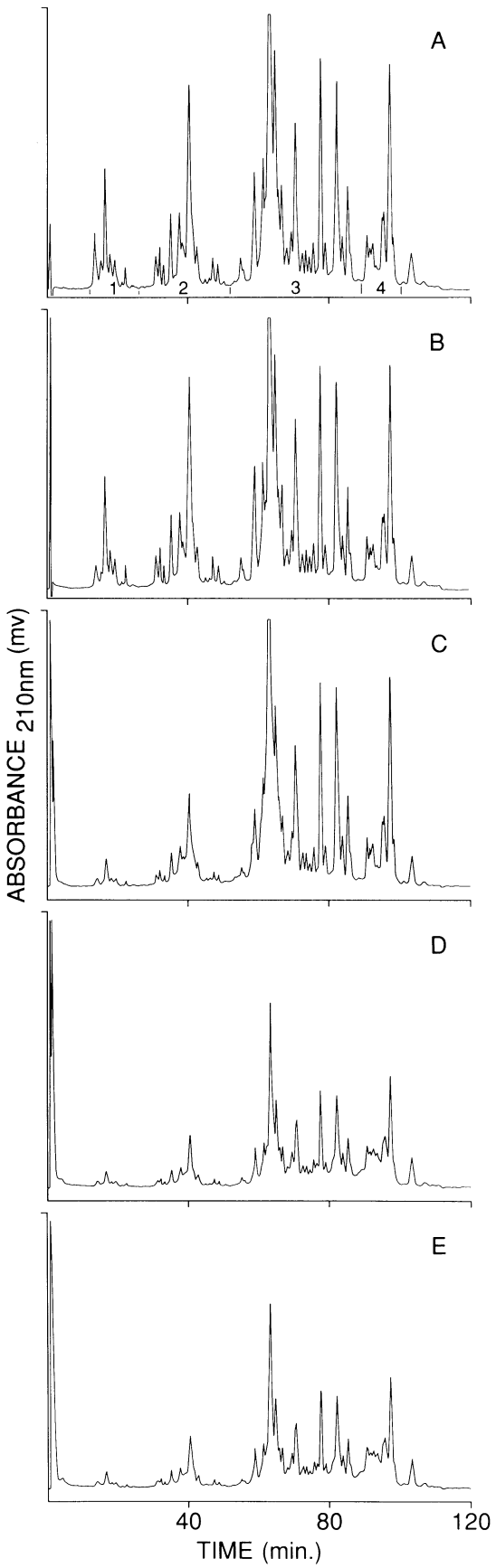

Fig. 1. Reversed-phase high-performance liquid chromatography separation of gliadin and glutenin proteins (cultivar Neepawa) using a Supelcosil column (C_8, 5 cm × 4.6 mm i.d.) maintained at 50° C. Equal amounts of protein solubilized in 50% 1-propanol containing 1% DTT were injected in volumes of 5 (**A**), 10 (**B**), 20 (**C**), 40 (**D**), and 80 (**E**) μl. Chromatograms were scaled equally to 1,000 mV full scale and were subdivided into four groups of peaks (**A**) for quantitative analysis.

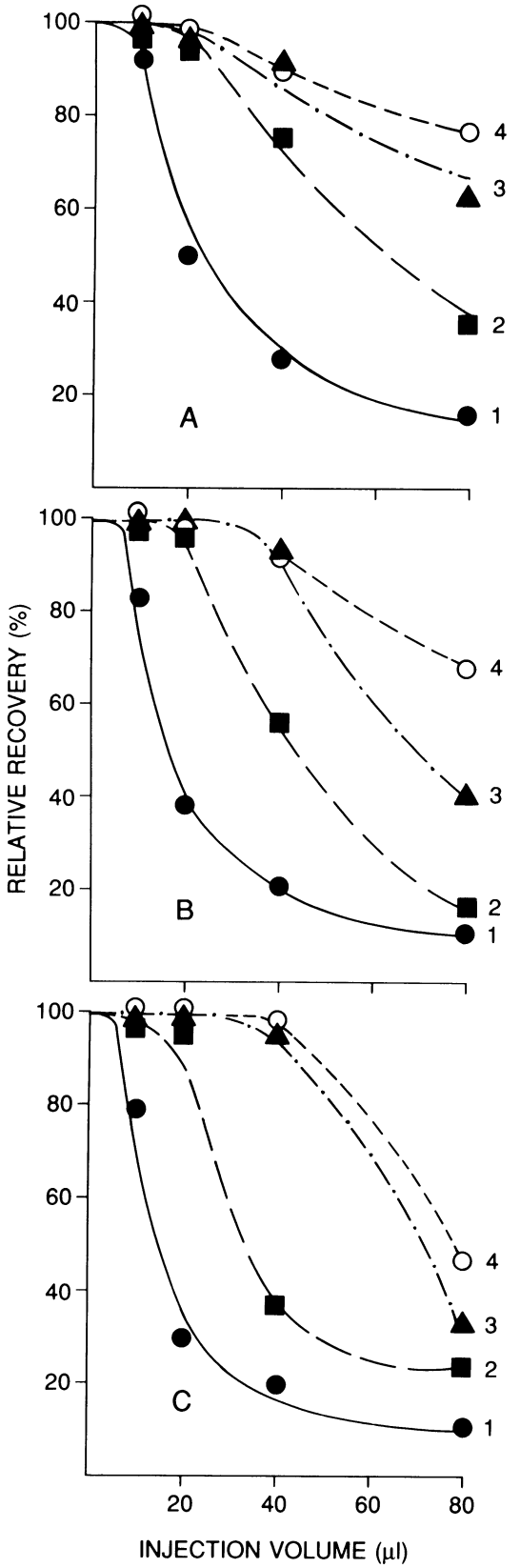

Fig. 2. Relative recovery of storage proteins (cultivar Neepawa) [group area (serial dilution)/group area (protein concentrate) × 100], solubilized in 50% 1-propanol containing 1% DTT, as a function of injection volume and column temperature. The numbers 1–4 represent the four groups of protein peaks illustrated in Fig. 1A. **A,** 30° C; **B,** 50° C; **C,** 70° C.

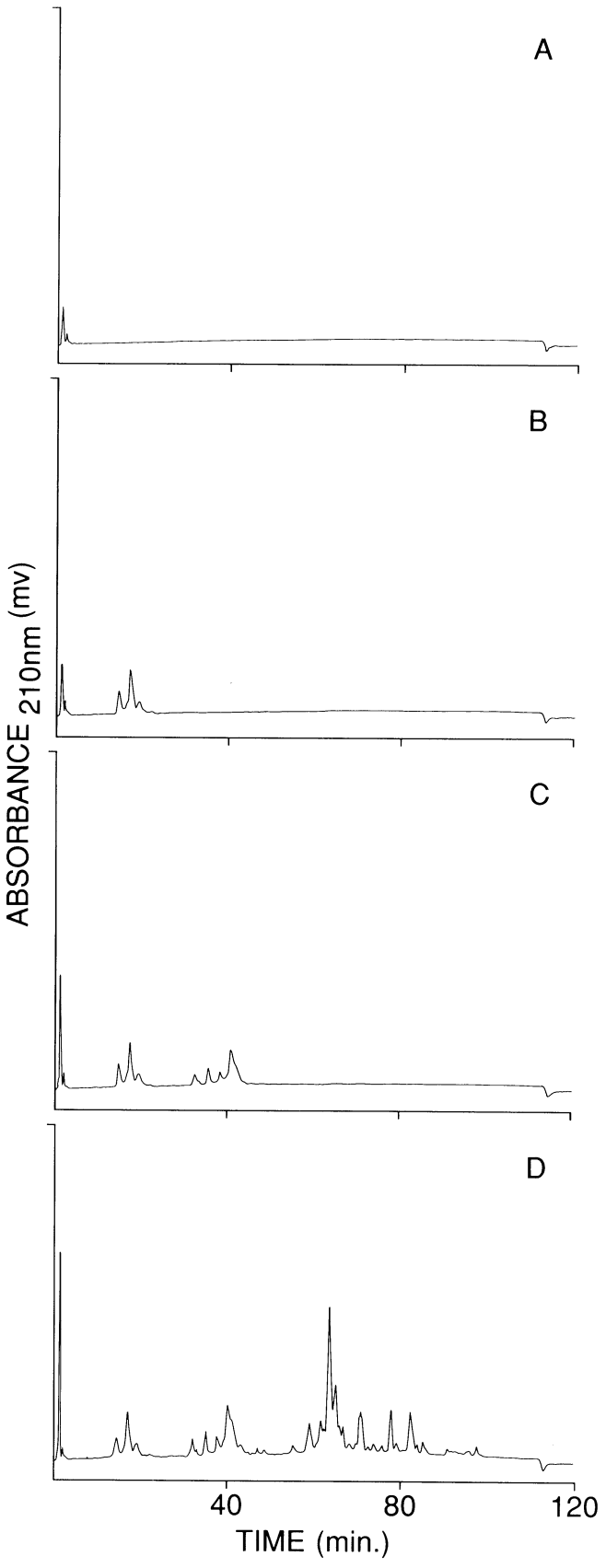

Fig. 3. Reversed-phase high-performance liquid chromatography separations of storage proteins (cultivar Neepawa) eluted in solvent peaks obtained following injections of 5 (**A**), 15 (**B**), 40 (**C**), and 80 (**D**) µl. Analyses were carried out with a Supelcosil column (C_8, 5 cm × 4.6 mm i.d.) maintained at 50° C, and chromatograms were scaled equally to 650 mV full scale for comparison.

injection. Decreases in relative recoveries at each injection volume were less drastic for successive protein groups two, three, and four, but were quite noticeable at 20-μl or greater.

Thus, the amount of a particular protein group relative to total chromatographic area (individual group area/total area) changed with increasing injection volume. Although total areas of all groups decreased, earlier eluting groups decreased faster than later eluting groups. For example, comparison of quantitative data for 5- and 80-μl injections revealed that the relative proportion contributed by group one proteins decreased from about 8 to 2%, whereas group four proteins increased from about 14 to 25%, respectively.

Column temperature also was varied to determine if temperature affected relative protein recovery. A comparison of results at temperatures of 30, 50, and 70° C (Fig. 2A–C, respectively) suggested that this was the case because somewhat higher relative recoveries were observed at 30° C as compared to 70° C.

One possible explanation for all of the above results is incomplete binding of protein to the column upon injection, resulting in elution of protein in the solvent peak, i.e., void volume. Fifty percent 1-propanol, which has a high solvent strength, probably competed with the stationary phase for protein. Group one proteins, for example, which are the least hydrophobic, would not be tightly bound to the column under an initial eluting solvent condition of 24% acetonitrile (Table I, program A). Thus, as the volume of 50% 1-propanol injected onto the column increased, the amount of protein remaining soluble in the 50% 1-propanol also could be expected to increase. This protein then would be eluted in the solvent peak. As the hydrophobicity of the protein increased, the 50% 1-propanol would be less able to compete with the stationary phase, and less protein would be eluted in the solvent peak. Similarly, the increased solubility of storage proteins at higher temperatures (Byers et al 1983) might explain the slight effect of temperature on protein recovery. This behavior would be consistent both with the concept that RP-HPLC columns operate on the basis of a selective desorption process (Regnier 1983) or the hypothesis that protein solubility is related to elution of proteins (Cohen et al 1986).

To test this theory, solvent peaks collected following injections of 5, 15, 40, and 80 μl were concentrated and analyzed by RP-HPLC and SDSGPAGE for the presence of protein. A 15-μl injection was included because this is a standard injection volume used in past studies (Marchylo et al 1988, Marchylo and Kruger 1985). As shown in Figure 3, analysis of solvent peaks showed progressive increases in amounts of the more hydrophobic protein groups eluted with increasing injection volume. Thus, for 5-μl injections, protein was not detected in the solvent peak, which indicated complete binding of protein upon injection. In contrast, for 80-μl injections, all four protein groups were detected in the solvent peak, indicative of incomplete binding. SDSGPAGE analysis confirmed these results. As illustrated in Figure 4 electrophoregrams, protein bands were not detected in 5-μl solvent peaks, whereas an increasing number of protein bands is apparent in the solvent peak as injection volume increased to 80 μl. Solvent peaks from 15-μl injections contained primarily the least hydrophobic ω-gliadins, whereas α-, β-, and γ-gliadins and low molecular weight and high molecular weight glutenin subunits were detected in 80-μl injection solvent peaks.

The relationship between injection volume and elution of proteins also was studied using three additional columns differing in packing material, length, and manufacturer. Differences in relative recoveries were observed among columns (Fig. 5) but in all cases, relative recoveries decreased significantly at higher injection volumes for at least three of four protein groups. Increased column length did appear to have a slight beneficial effect as indicated by somewhat higher relative recoveries, particularly for the more hydrophobic group three and four proteins when comparing the 5- and 25-cm Supelcosil C_8 columns (Fig. 2 middle, and Fig. 5A, respectively). Differences among packing materials and manufacturer also were observed. For example, the 15-cm long Vydac C_8 column (Fig. 5B) exhibited complete recovery of group four proteins, whereas the 25-cm long Synchropak C_{18} column showed the poorest recoveries.

The effect of protein extraction solvent on this phenomenon was studied by using 70% ethanol, a common extracting solvent for gliadins, in conjunction with the Synchropak C_{18} column (Bietz 1985, Huebner and Bietz 1985). As illustrated in Figure 6A, relative recoveries for each protein group again were observed to decrease concomitant with increasing injection volume. A comparison of Figures 5C (50% 1-propanol) and 6A (70% ethanol) illustrates, however, that the effect of injection volume is less drastic for 70% ethanol. A slight improvement in relative recoveries also was observed for 70% ethanol extracts in conjunction with the 5-cm Supelcosil C_8 column (Fig. 7A). Presumably, this is because 70% ethanol is of lower solvent strength than 50% 1-propanol.

Huebner and Bietz (1987), in noting elution of protein in the void volume in larger scale preparative studies, suggested two possible remedies to this problem. These remedies were tested in the present study to determine if they would alleviate the problem on the analytical scale. The first possibility presented was to decrease the alcohol concentration. As illustrated in Figure 6B, reduction in the ethanol concentration from 70 to 50% did result in higher relative protein recoveries, particularly of the more hydrophobic protein groups two, three, and four. Relative recoveries for the least hydrophobic group one proteins (mainly ω-gliadins), however, remained poor at higher injection volumes (>20 μl). Similar results were obtained when the 1-propanol concentration was decreased to 40% from 50% (results not shown). Thus, although reduction in alcohol concentration did provide some improvement, the effect of injection volume on protein elution remained significant. It also is of note that the use of these lower alcohol concentrations in extracting solvents will result in an undesirable decrease in the amount of protein extracted. The second possible remedy entailed equilibration of columns at lower acetonitrile concentrations. To test this, a 5-cm Supelcosil column was equilibrated to 10% acetonitrile prior to analysis of 70% ethanol + 1% DTT and 50%

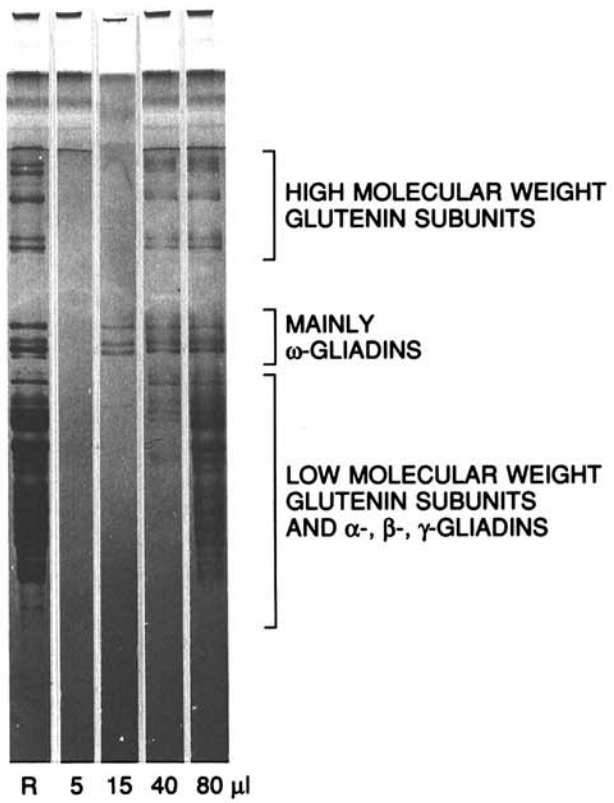

Fig. 4. Sodium dodecyl sulfate gradient polyacrylamide gel electrophoregrams of storage proteins (cultivar Neepawa) in a reference protein concentrate (in 50% 1-propanol containing 1% DTT) (**R**) and in solvent peaks obtained following injections of 5, 15, 40, and 80 μl onto a Supelcosil column (C_8, 5 cm × 4.6 mm i.d.).

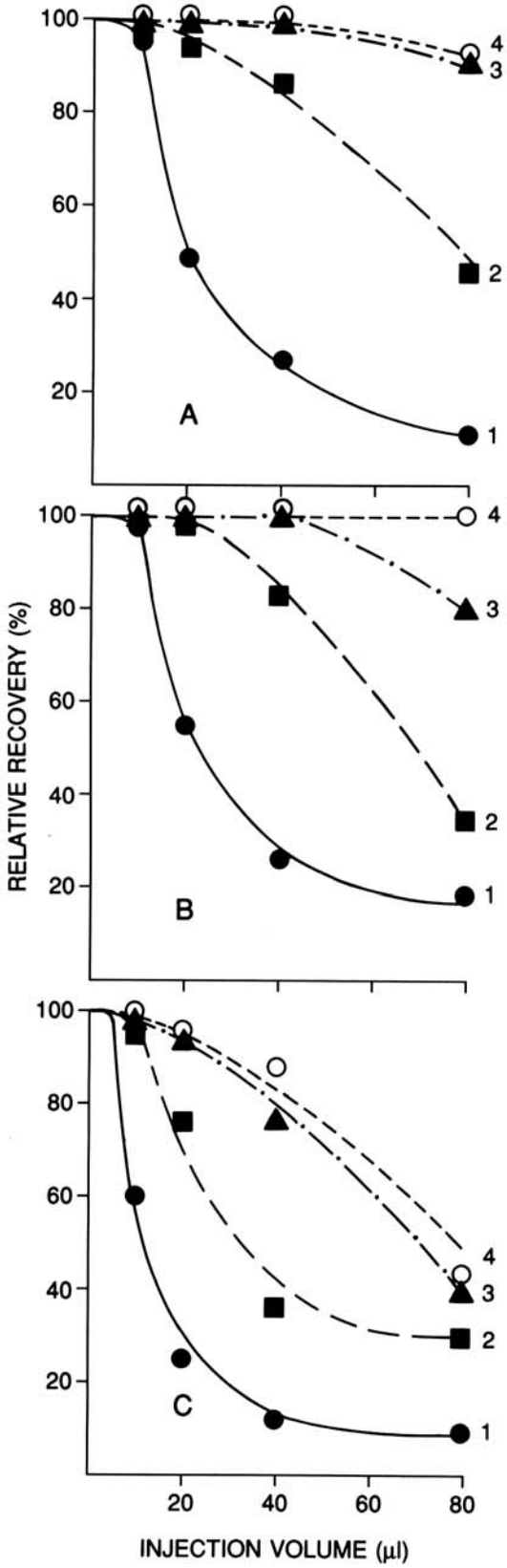

Fig. 5. Effect of reversed-phase high-performance liquid chromatography columns on relative recovery of storage proteins (cultivar Neepawa), solubilized in 50% 1-propanol containing 1% DTT, as a function of injection volume. Chromatographic columns used included: **A,** Supelcosil (C_8, 25 cm × 4.6 mm i.d.); **B,** Vydac (C_8, 15 cm × 4.6 mm i.d.); **C,** Synchropak (C_{18}, 25 cm × 4.1 mm i.d.). The numbers 1–4 represent four groups of protein peaks comprising the protein chromatogram (comparable to Fig. 1A).

1-propanol + 1% DTT protein solutions using gradient program B in Table I. Improvements in relative recoveries again were observed (Fig. 7B,C); however, protein relative recoveries were still affected significantly at injection volumes >20 µl.

An alternate solution to the problem then was devised that entailed the use of multiple 5-µl injections of protein extract prior to initiation of the gradient program (Table I, program A). Multiple injections were carried out using isocratic conditions at initial acetonitrile concentrations (24%, Table I, program A) and a separation time of 2 min between injections. A zero volume injection sequence was included following the final 5-µl injection to initiate the gradient program. The use of multiple injections virtually eliminated the effect of injection volume (up to 80 µl) on protein elution in the solvent peak. For example, the use of three 5-µl injections as compared to a single injection of 15-µl resulted in elution of a significantly higher proportion of group 1 proteins (Fig. 8A and B) relative to other groups. Thus, quantitative analysis indicated that the relative proportion of group one proteins in a multiple 5-µl injection was about 8% as contrasted to 3% for a single 15-µl injection. Reproducibility of retention times and peak areas for multiple injections (3 × 5 µl) was comparable to

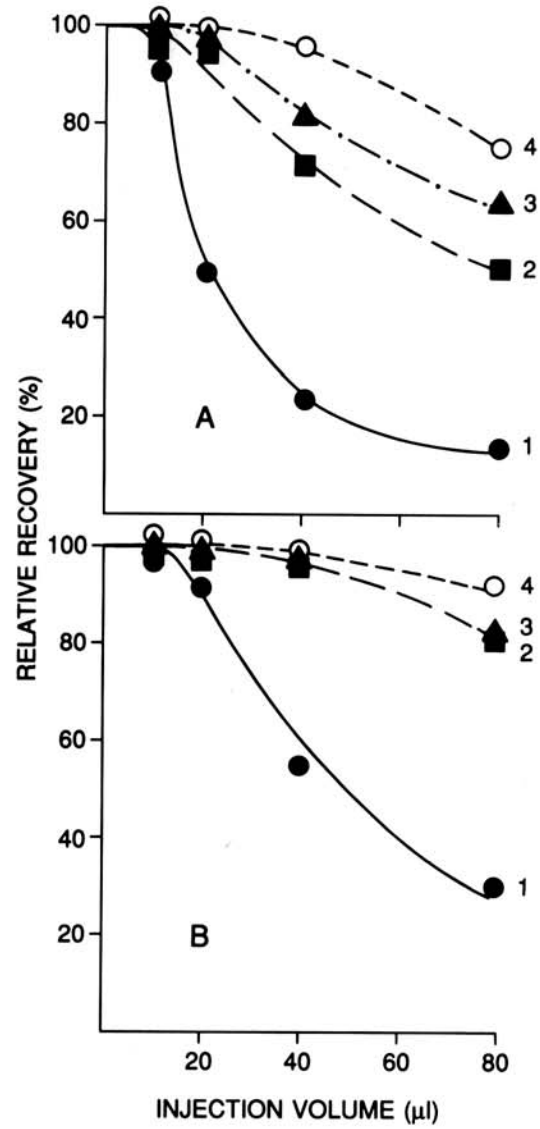

Fig. 6. Relative recovery of storage proteins (cultivar Neepawa), solubilized in 70% (**A**) or 50% (**B**) ethanol containing 1% DTT, as a function of injection volume. Reversed-phase high-performance liquid chromatography analyses were carried out on a Synchropak column (C_{18}, 25 cm × 4.1 mm i.d.) at 50° C. Quantitation was carried out on four groups of peaks comparable to those shown in Fig. 1A.

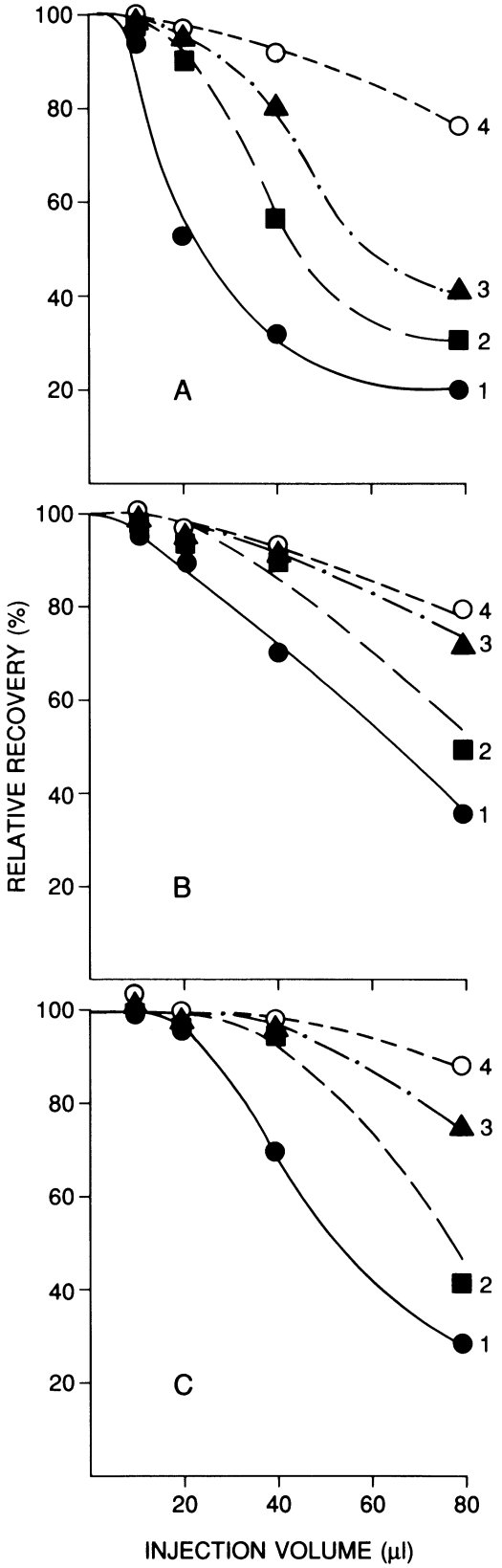

Fig. 7. Effect of initial gradient acetonitrile concentration on relative recovery of storage proteins (cultivar Neepawa), solubilized in 70% ethanol + 1% DTT (**A** and **B**) or 50% 1-propanol + 1% DTT (**C**), as a function of injection volume. Reversed-phase high-performance liquid chromatography analyses were carried out on a Supelcosil column (C_8, 5 cm × 4.6 mm i.d.) at 50° C using initial acetonitrile concentrations of 24% (**A**) or 10% (**B** and **C**). Quantitation was carried out on four groups of peaks comparable to those illustrated in Fig. 1A.

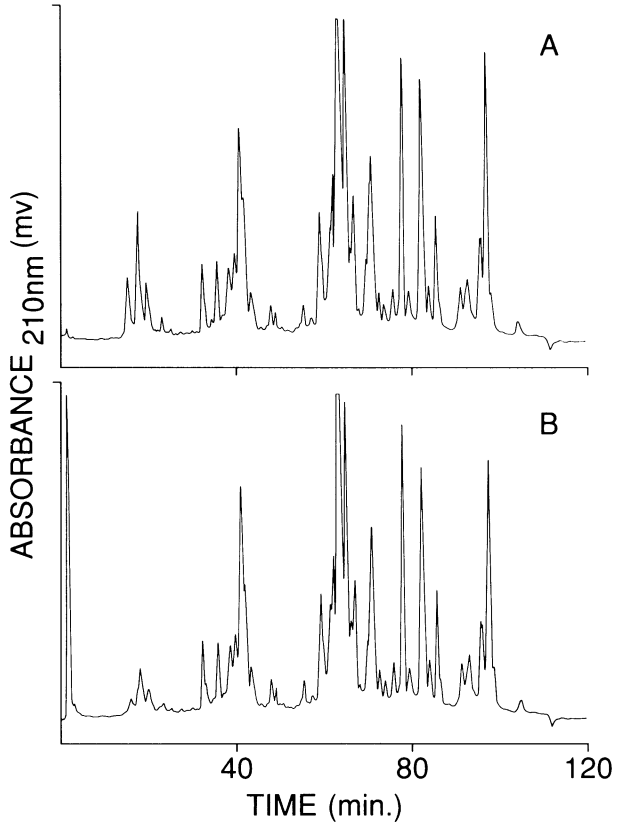

Fig. 8. Reversed-phase high-performance liquid chromatography separation of storage proteins (cultivar Neepawa) solubilized in 50% 1-propanol containing 1% DTT, using a Supelcosil column (C_8, 5 cm × 4.6 mm i.d.) maintained at 50° C. Chromatogram **A** was obtained using the multiple injection technique (3 × 5 µl), whereas **B** was obtained by a single 15 µl injection. Both chromatograms were scaled equally to 1,000 mV full scale.

previous results reported for single injection samples (1 × 15 µl) (Marchylo et al 1988). In addition, resolution remained essentially the same up to 80 µl equivalent injection volume. Analysis of serial dilutions (in duplicate) with increasing injection volumes (multiple injections up to 16 × 5 µl) showed that the relative recoveries essentially were 100% for the four protein groups (i.e., group one, 98% ± 3%; group two, 98% ± 3%; group three, 99% ± 3%; group four, 97% ± 3%; $\bar{x} \pm s_x$). Relative proportions contributed by each of the four protein groups also remained constant up to the 80-µl injection volume tested (group one, 7.8% ± 0.1%; group two, 13.7% ± 0.1%; group three, 66.4% ± 0.1%; group four, 12.1% ± 0.2%; $\bar{x} \pm s_x$). Thus, the use of multiple injections does solve the problem of incomplete binding of gliadin and glutenin protein to RP-HPLC columns as a function of injection volume. The drawback to the procedure is the added time requirements incurred for each analysis; however, in order to obtain accurate quantitation of gliadin and glutenins, it would appear at present to be the only alternative.

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