Rapid and Sensitive Wheat Protein Fractionation and Varietal Identification by Narrow-Bore Reversed-Phase High-Performance Liquid Chromatography[1]

F. R. HUEBNER[2,3] and J. A. BIETZ[2]

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ABSTRACT

For wheat varietal identification by reversed-phase high-performance liquid chromatography (RP-HPLC), gliadins are typically extracted from ≈50 mg of flour with 70% ethanol. Aliquots 10–15 µl are analyzed on 15- or 25-cm × 4.6-mm (i.d.) columns at 1.0 ml/min for ≈1 hr. A gradient of increasing acetonitrile concentration, containing trifluoroacetic acid, is commonly used to elute proteins. However, such solvents are expensive, and present a disposal problem. Smaller RP-HPLC columns offer shorter analysis times, reduced flow rates, decreased disposal problems, and lower cost per analysis. The ability of such columns to fractionate gliadins and to differentiate cultivars was investigated. Using a 3.2-mm × 15-cm C_4 column, flow rate, sample size, analysis time, and solvent consumption were reduced by half, while preserving excellent resolution of extracted proteins. Gliadins can also be extracted from as little as 1–2 mg of flour or ground endosperm. With a 2.1-mm × 15-cm C_4 column (Vydac), further reductions of flow rates and analysis time (about one fourth that of a standard column) are possible, while maintaining excellent resolution. Under these conditions, solvent use is about one eighth that of standard RP-HPLC analyses. To further reduce analysis time and sample consumption, gradient times can be reduced to as little as one tenth that of standard columns, and separations can be done in only 9–10 min. Some resolution is sacrificed for such rapid analyses; while this is often acceptable, it may sometimes prevent differentiation of genetically similar cultivars that are otherwise identifiable. Similar RP-HPLC procedures can be used to analyze reduced subunits of glutenins. The ability to perform such rapid, high-resolution analyses of small samples, with low solvent consumption, suggests that narrow-bore RP-HPLC may become especially useful for selection and identification during wheat breeding.

Research during the past few years has used reversed-phase high-performance liquid chromatography (RP-HPLC) to separate wheat proteins, especially gliadins (Bietz 1983, 1990; Huebner and Bietz 1994). Most of these separations have used columns ≈25 cm long and 4.1 or 4.6 mm in diameter. Flow rates have generally been ≈1 ml/min (Huebner and Bietz 1987). In some studies, investigators have tried to decrease the analysis time to more rapidly identify cultivars. Such studies have generally used higher flow rates however, and resolution was reduced, making it difficult to identify wheats with similar gliadin compositions (Bietz and Cobb 1985, Lookhart and Bietz 1990). Reduced analysis time has been possible when only few cultivars, with very different gliadin patterns, had to be identified (Cressey 1987).

Another consideration in RP-HPLC is the amount of acetonitrile (ACN) and trifluoroacetic acid (TFA) used. These reagents are expensive, as is their disposal. Several narrow-bore RP-HPLC columns are now commercially available. They require less solvent, and offer the possibility of rapid separations with resolution comparable to that of normal analytical columns. Such columns also permit use of smaller samples. Development of methods employing such columns has been somewhat slow, however. In part, this is because specialized HPLC pumps and detector may be necessary, and the large dead volumes of many automatic injectors necessitate manual sample injection. Using an HPLC system with low dead volume pump and detector, we have now tested such columns, and here report procedures for high-resolution RP-HPLC of wheat proteins for varietal identification.

MATERIALS AND METHODS

Samples

Samples of hard red winter (HRW) wheats (cultivars Arkan, Buckskin, Brule, Collin, Karl, Katepwa, Newton, Norstar, Rough Rider, Sentinel, and TAM-107) and hard red spring (HRS) wheats or flours (cultivars Butte 86, Fortuna, Guard, Len, Marshall, McKay and Sawtell) were obtained from breeders at state universities. Five or six kernels of each cultivar were pulverized (Wig-L-Bug, Crescent Dental, Lyons, IL) to yield representative samples (Huebner et al 1990, Kubiczek et al 1993).

Protein Extraction

Flour or pulverized kernel samples (45–60 mg) were extracted at room temperature for 30-min with continuous vortex mixing with 0.7–1.0 ml of 70% ethanol in 10-ml polypropylene tubes. Samples were then centrifuged at 15,000 × g for 10 min. Clear supernatants were stored in small vials before automatic (Huebner and Bietz 1986) or manual injection (for the smallest column). Small samples (2–3 mg of flour) were extracted for 2 hr by a modification of the procedure of Clements (1987) using 0.1 ml of 65% ethylene glycol, 15% ethanol, and 20% water. This extraction solvent gives results very similar to those with 70% ethanol and prevents problems with small samples due to ethanol evaporation before sample analysis.

Reduced and alkylated glutenin subunit samples were prepared by first reextracting the residues remaining after gliadin extraction with 1 ml of 70% ethanol for 30 min and discarding the extract. The residue was then extracted with 1 ml of 5M urea, 0.2% dithiothreitol (DTT), and 0.05M sodium phosphate (pH 7.7) for 2 hr, and centrifuged for 10 min at 20,000 × g. To the clear solution was added 20 µl of 33% 4-vinylpyridine in 60% propanol. After 1 hr, 12 µl 33% TFA in glacial acetic acid was added to lower the pH to 3.1 ± 0.2. Samples of 2–12 µl (depending on size and type

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[2]National Center for Agricultural Utilization Research, USDA, ARS, Peoria, IL.

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

[3]Corresponding author: Food Physical Chemistry National Center for Agricultural Utilization Research Agricultural Research Service, USDA, 1815 N. University St., Peoria, IL 61604-3902. Fax: 309/681-6686.

of column used) was then injected. These sample sizes do not appear excessive for these small-capacity columns, especially for varietal identification. Obviously, however, for such small HPLC columns, sample size must be carefully optimized to prevent sample loss (Marchylo and Kruger 1988).

HPLC

Standard (4.6 mm, i.d.) and intermediate-sized (3.2 mm, i.d.) columns used a SP8700 solvent delivery system and an autosampler (SP8780XR, Spectra-Physics, San Jose, CA) (Huebner and Bietz 1987). The standard column was 150- × 4.6-mm with pore size of 300Å (Vydac C_4, Separations Group, Hesperia, CA). The intermediate size column was 150- × 3.2-mm with pore size of 300Å (W-Porex C_4, Phenomenex, Torrance, CA). Each column was preceded by a 20- × 2-mm guard column (C-130B, Upchurch, Oak Harbor, WA) containing 0.5 µm end filters and filled with Vydac reversed-phase packing. The column was maintained at a constant temperature with a column heater (CH-460, FIAtron Laboratory Systems, Oconomowoc, WI). For the smallest bore column, samples were analyzed using a SP8800 solvent delivery system (Spectra-Physics). C_4, C_8, and C_{18} columns (150- × 2.1-mm, 5-µm particle size, 300Å pore size) (Vydac) were preceded by a precolumn filter with a 2-µm frit (A-318, Upchurch). Constant column temperatures were maintained with a column heater (CH-20-C, Scientific Systems, State College, PA) modified to accept shorter columns. Flow rates and other analytical conditions are noted in the figure captions.

ACN and TFA were HPLC grade. Distilled water was further purified (NANOpure, Barnstead). RP-HPLC solvents were 10% aqueous ACN (A) and 90% aqueous ACN (B), both containing either 0.05, 0.1, or 0.15% TFA, depending on the column used. Previous studies showed that optimum results were obtained for different columns by using different percentages of TFA in the solvent (Lapvetelainen et al 1995).

Data Analysis

Proteins were detected at 210 nm (0.1 full-scale absorbance units per 10 mV) (Spectroflow 757 monitor, Kratos, Ramsey, NJ) for the 4.6- or 3.2-mm columns, or with a Spectra 100 monitor (Spectra-Physics) for the 2.1-mm columns. Data were recorded on an Omniscribe recorder (Houston Instruments, Austin, TX) or a BD 41 recorder (Kipp & Zonen, Holland) and simultaneously stored in a computer system (ModComp, Fort Lauderdale, FL). Stored data could be plotted to any convenient scale and automatically integrated between specified times.

RESULTS AND DISCUSSION

3.2-mm Column

Figure 1 shows typical analyses of HRW and HRS wheat cultivars using a 3.2-mm C_4 column. The volume of this column is only about half that of a 4.6-mm column as generally used. Thus, half as much sample and half the usual flow rate give peaks comparable in size to those from a typical analytical column. In addition, gradient time could be reduced from 50 to 35 min while maintaining satisfactory resolution. This decreases solvent use per sample to <45% of that needed for a 4.6-mm column, and permits four and five more analyses per day. Flow rate and gradient can be modified to further reduce analysis time, but resolution deteriorates, making unequivocal differentiation of genetically similar wheat cultivars very difficult.

As found earlier (Marchylo et al 1988), different RP-HPLC columns, even of the same functionality, give slightly different separations. This prevents direct comparison of results from the 3.2-mm column with those of Vydac C_4 columns used previously (Huebner and Bietz 1987, 1988). However, overall results from the two columns are quite similar, as shown in Figure 2 for the 4.6-mm column.

2.1-mm Column

Figure 2 shows typical RP-HPLC results from normal (4.6-mm) and 2.1-mm Vydac C_4 columns. Results from C_8 and C_{18} columns (not shown), while similar, show minor variations; the C_4 column appeared best for gliadins. Gradient times were 50 min for the 4.6-mm column, and 25 and 10 min for the 2.1-mm column. These results, scaled so that comparable peaks are aligned, show that the 2.1-mm column gives results very similar to those of the 4.6-mm column, except that resolution of the narrow-bore column is slightly inferior. Similarly for the 2.1-mm column, results using the 10-min gradient were only slightly inferior to those with the 25-min gradient. The balance between analysis time and resolution can also be affected by slight changes in temperature, flow rate, or gradient time.

Closely related wheat cultivars have gliadin compositions that are nearly identical, giving rise to very similar RP-HPLC patterns. To differentiate such wheats, it is necessary to use longer gradients. Figure 3 shows an example in which a 15-min gradient was necessary to differentiate the HRS cultivars Butte 86 and Len.

Narrow-bore RP-HPLC columns are also useful when sample amounts are limited (Fig. 4). A 70% ethanol extract of wheat

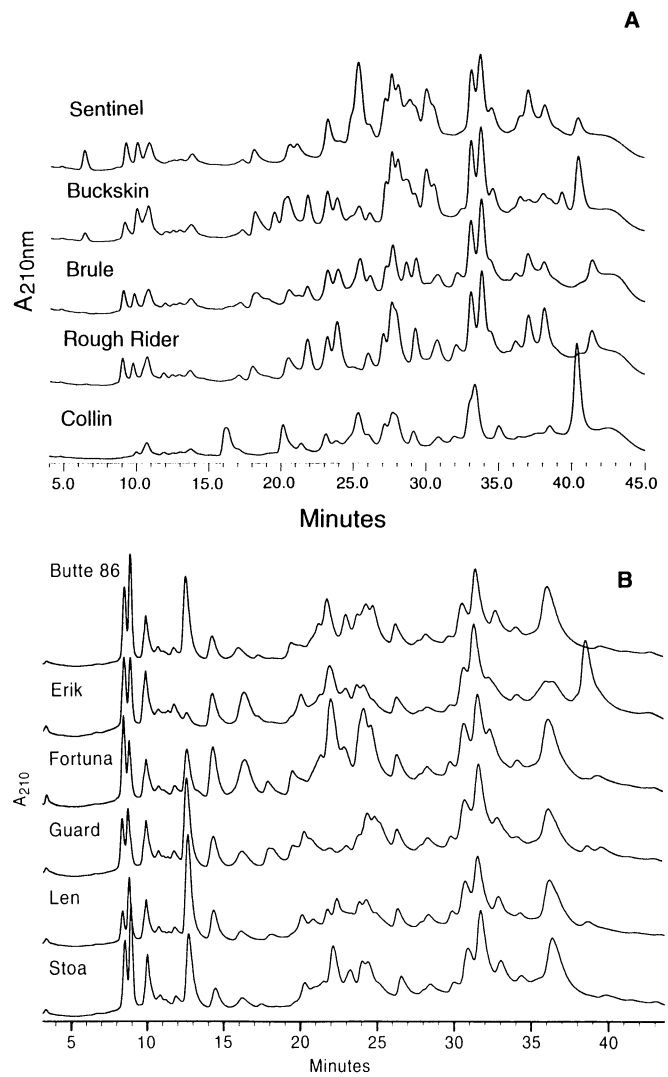

Fig. 1. Reversed-phase high-performance liquid chromatography (RP-HPLC) analyses of hard red winter **(A)** and hard red spring **(B)** wheat gliadins on a W-Porex C_4, 150- × 3.2-mm, 300Å column. Solvent A: 10% acetonitrile (ACN) + 0.05% trifluoroacetic acid (TFA). Solvent B: 90% ACN + 0.05% TFA. Gradient: 18% B initially, increasing linearly to 24% B at 2 min and then to 42% B at 35 min, held at 42% B for 2 min, and then reequilibrated at 18% B for 7 min. Flow rate 0.55 ml/min. Column temperature maintained at 60°C. Sample volumes 5–7 µl.

proteins was separated into four different molecular weight fractions by gel-filtration chromatography (as in Huebner and Bietz 1993). Fractions were rapidly concentrated by lyophilization and 1–4 µl aliquots were analyzed by RP-HPLC on a 2.1-mm column using a 15-min gradient. Thus, in a total analysis time of ≈80 min, we could confirm that fractions 1–4 contain primarily low molecular weight glutenins, ω-gliadins, γ-gliadins, and α- and β-gliadins, respectively (Bietz and Wall 1972, Van Lonkhuijsen et al 1992).

Use of a 2.1-mm column also permits analyses of very small samples. Typically, only 1 µl is used per analysis; analysis of 0.5 µl samples is also easily achieved. With such sensitive analyses, 2 mg of flour (or a small portion of endosperm from a kernel) is a sufficient sample; it can be extracted with 0.1 ml solvent for 10 min, and 1 or 2 µl injected for analysis. Results for such samples (not shown) are equivalent to those shown in Figures 2–4.

In an attempt to further reduce analysis time with a 2.1-mm column, a 7-min gradient was tested (Fig. 5). Each HRW wheat shown has a characteristic fingerprint identifying each cultivar. HRS wheats are differentiated in a similar manner. As noted above, however, differentiation of closely related cultivars may require slightly longer gradients.

Higher column temperature and faster flow rates also help differentiate some cultivars. For example, the analyses in Figure 5 used a temperature of 80°C, and a flow rate of 0.75 ml/min. Using such conditions, only one ninth as much solvent is used per analysis as with a typical 4.6-mm column, and six analyses are possible in the same time previously used for one run on a 4.6-mm column.

Reduced glutenins can also be analyzed using the same columns and conditions as used for gliadins. Figure 6A–C shows results of analyzing reduced and alkylated glutenin subunits on the three different size columns, using analysis conditions similar to those used for gliadins. As with gliadins, the separation is not as good for the narrower (2.1 mm) column; it may, however, be good

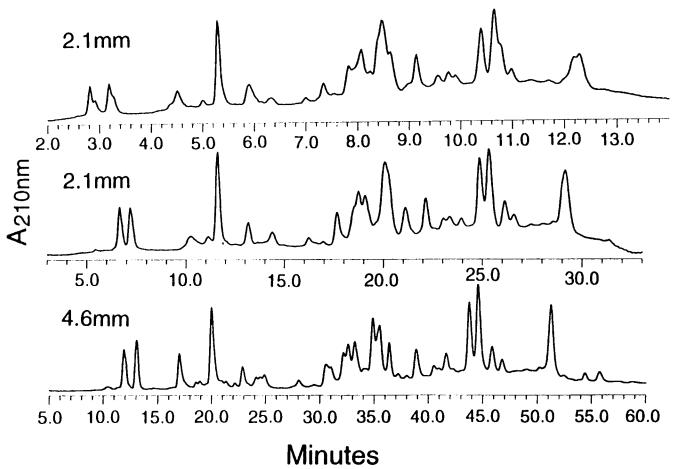

Fig. 2. Reversed-phase high-performance liquid chromatography (RP-HPLC) analyses of Butte 86 (hard red spring) gliadin on 4.6- and 2.1-mm i.d. Vydac C_4 columns. Solvent A: 10% acetonitrile (ACN) + 0.1% trifluoroacetic acid (TFA). Solvent B: 90% ACN + 0.1% TFA. Solvent for the 2.1-mm column contained 0.15% TFA. Gradient for the 4.6-mm column started at 18% solvent B, and increased to 24% B at 4 min, to 46% B at 50 min, and to 47% B at 54 min. Final gradient conditions were maintained for 4 min, and the column was then reequilibrated at initial conditions for 10 min between runs, at a flow rate of 1.0 ml/min. Temperature maintained at 60°C. Analyses on 2.1- × 150-mm column used gradient 1 starting at 20% B, increasing to 24.5% B at 1 min, to 45% B at 25 min, and to 46% B at 27. (Flow rate 0.28 ml/min at 60°C.) Gradient 2 started at 21% solvent B, increasing to 24% B at 0.4 min and to 45% B at 10 min, holding at 45% B for 1 min before returning to the starting solvent. (Flow rate 0.54 ml/min at 70°C.)

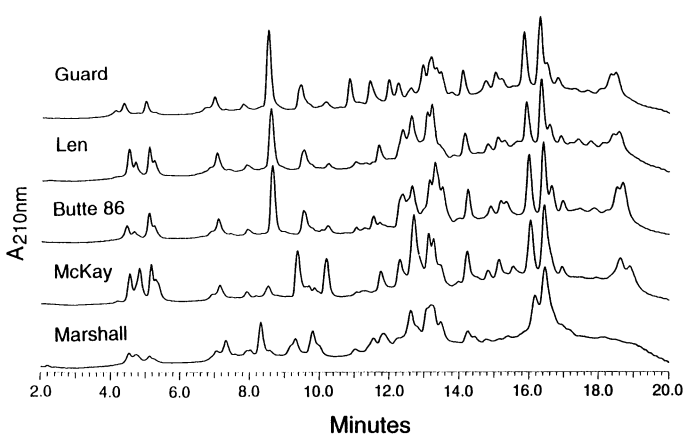

Fig. 3. Reversed-phase high-performance liquid chromatography (RP-HPLC) differentiation of hard red spring wheat gliadins on a 2.1-mm i.d. Vydac C_4 column. Solvents as given in Fig. 2. Gradient began at 21% solvent B, and increased to 24% B at 1 min and to 45% B at 15 min. Final gradient conditions maintained for 1 min. (Flow rate 0.38 ml/min at 70°C.)

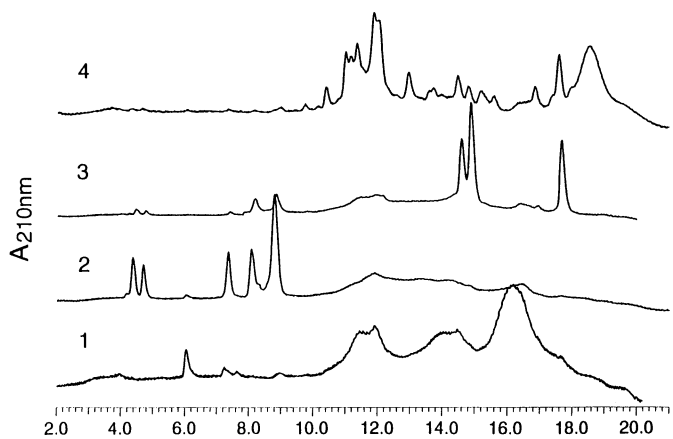

Fig. 4. Reversed-phase high-performance liquid chromatography (RP-HPLC) separations (2.1-mm i.d. Vydac C_4 column) of gliadin fractions separated by gel filtration. Samples 1–4 are fractions of decreasing molecular size. Experimental conditions as given in Fig. 3.

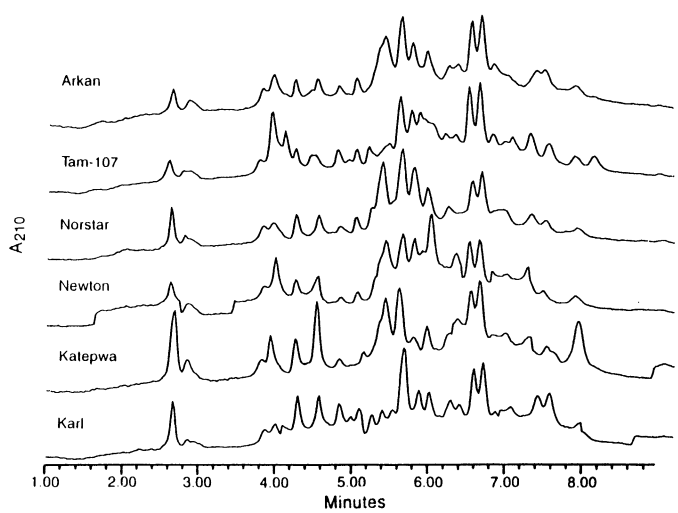

Fig. 5. Reversed-phase high-performance liquid chromatography (RP-HPLC) separations of hard red winter wheat gliadins on a 2.1-mm i.d. Vydac C_4 column. Solvent gradient (using solvents as given in Fig. 2) began at 19% B, and increased to 22% B at 0.5 min, to 44% B at 6 min, and to 45% B at 7 min. (Flow rate 0.75 ml/min at 80°C.)

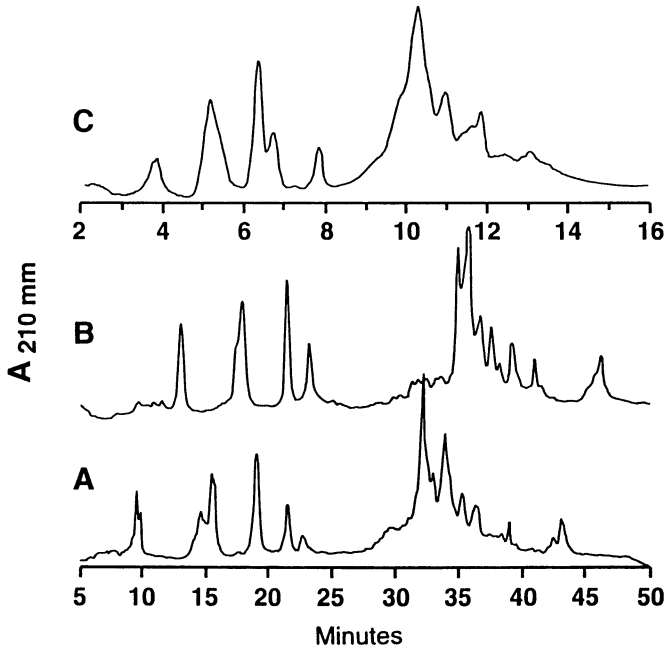

Fig. 6. Reversed-phase high-performance liquid chromatography (RP-HPLC) of Stoa (hard red spring) wheat glutenin. Solvents as in Fig. 2. **A:** Analysis on a 4.6-mm i.d. Vydac C_4 column. Gradient started at 19% solvent B and increased to 22% at 3 min, to 40% B at 40 min, and to 42% B at 42 min, and was held for 4 min before reequilibration at 19% B for 10 min. (Flow rate 1.0 ml/min at 60°C.) **B:** Analysis on a W-Porex C_4 3.2-mm i.d. column. Gradient started at 19% solvent B and increased to 23% B at 0.7 min, to 43% B at 40 min, and then to 45% B at 42 min, before returning to 19% B for 8 min of reequilibration. (Flow rate was 0.45 ml/min at 60°C.) **C:** Analysis on a 2.1-mm i.d. Vydac C_4 column. Gradient started at 19% solvent B, increased to 22% B at 0.5 min, to 39% B at 12 min, and to 40% B at 13 min, where it was held for 1 min before reequilibration. (Flow rate 0.4 ml/min at 65°C.)

enough to differentiate high molecular weight subunits of different cultivars. It is probable that this separation could also be improved by modifying analytical conditions used.

CONCLUSIONS

Some previous studies showed that when only a few wheat cultivars are involved, rapid gradients can be used to identify wheat cultivars (Cressey 1987, Lookhart et al 1986). Peak resolution was limited, however, even with improved methods (Hay and Sutton 1990). Those procedures also required increased flow rates, necessitating nearly the same amount of reagents per sample analyzed as used in standard analyses.

With narrow-bore columns, however, both flow rate and analysis time decrease, significantly reducing solvent consumption while increasing the number of samples that can be analyzed. For example, with an autosampler suitable for use with small columns, as many as 140 samples could be analyzed per day, using less solvent than previously used in a day for 22 samples. The analytical conditions we present have been optimized for each narrow-bore column tested, and appear to be optimal for our chromatographic system with these columns. However, for other systems and columns, minor modification of analytical conditions may be necessary to achieve optimal separations.

For RP-HPLC methods to be used for practical and routine applications, they must be rapid, accurate, reproducible, and cost effective. Until now, many RP-HPLC methods, while accurate and reproducible, have been too slow to be practical. Reagent acquisition and disposal costs have also been quite high. We have now shown that separations of wheat proteins on narrow-bore columns can be significantly faster than before, and that reagent-associated costs can be reduced. Resulting chromatograms can also be stored and compared to results for known cultivars using relatively low-cost computers and software, facilitating cultivar identification. These advances clearly make RP-HPLC not only a superior laboratory technique, but also show it to have the potential to be a low-cost, practical method for cultivar identification and quality prediction during breeding, marketing, and utilization of wheat.

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