

Separation of Wheat Proteins by Two-Dimensional Reversed-Phase High-Performance Liquid Chromatography Plus Free Zone Capillary Electrophoresis¹

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ABSTRACT

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Gliadins and glutenins from four hard red winter wheat cultivars were separated by a novel two-dimensional (2D) technique. Protein extracts were separated by reversed-phase high performance liquid chromatography as the first dimension with each 30-sec interval collected separately. Those fractions were then separated by free-zone capillary electrophoresis (FZCE) for the second dimension. Data was combined into 2D surface

contour plots similar to traditional gel electrophoresis 2D maps. For HPLC, C₈ and C₁₈ columns were used in the first dimension to separate gliadins and glutenins, respectively. Uncoated fused silica capillaries (27 cm × 25 μm, i.d.) were used for the 2D FZCE separations. Differences in the 2D maps of both gliadin and glutenin fractions were found between pairs of both closely related and sister lines that varied in quality.

Wheat proteins are considered to be the major determinant of end-use quality in bread (Finney 1943). Because of this, wheat proteins have been extensively studied for many years. Traditionally, polyacrylamide gel electrophoresis (PAGE) and high-performance liquid chromatography (HPLC) have been the dominant analytical methods for separating wheat proteins. Recently, high-performance capillary electrophoresis (HPCE) was introduced for the separation of cereal proteins. Although PAGE, HPCE, and reversed-phase HPLC (RP-HPLC) are capable of extremely high resolution, the highest resolution separations of wheat proteins are typically obtained using a two-dimensional (2D) technique (Bietz and Simpson 1992). More than 1,000 components of wheat proteins have been resolved using a combination of isoelectric focusing (IEF) and sodium dodecyl sulfate PAGE (SDS-PAGE) (Tkachuk and Mellish 1987). Although proteins may have similar properties in any single dimension (e.g., size, charge, pI, hydrophobicity), it is unlikely that many proteins will have identical properties across two dimensions. Thus, 2D techniques are often able to achieve resolution not possible with any single separation technique.

Traditionally, 2D separations were performed by combining two types of gel electrophoresis, IEF and SDS-PAGE (O'Farrell 1975), separating first by pI then by size. Many other types of 2D electrophoresis have been used to separate wheat proteins.

Recently, HPCE has been combined directly (on-line) with size-exclusion chromatography (Lemmo and Jorgenson 1993), gel-permeation chromatography (Yamamoto et al 1990), and RP-HPLC (Bushey and Jorgenson 1990), providing extremely high-resolution 2D separations. With these techniques, the HPLC columns are directly connected to the HPCE capillaries and separations are performed continuously from the first dimension to the second dimension.

Although HPLC peaks can be sampled several times, entire HPLC fractions are not analyzed because of the small amounts of sample used in HPCE (typically <1 nL).

In the current study, we present the combination of RP-HPLC and free-zone capillary electrophoresis (FZCE) for separating wheat proteins. Although the two techniques were not directly coupled, because all HPLC fractions were analyzed the results are nearly the same as directly coupled HPLC-FZCE. This technique does not require specially adapted equipment and can be done with currently available commercial instruments and software. With the use of an automated fraction collector, the technique can be almost entirely automated.

MATERIALS AND METHODS

Wheat Samples

Four hard red winter (HRW) wheats, TAM 105 and TAM 107 (crop year 1995) and Shawnee and Ottawa Selection (crop year 1981), were obtained from the USDA-ARS Hard Winter Wheat Quality Laboratory in Manhattan, KS. Flours from each wheat were produced by a Brabender Senior experimental mill.

Sample Preparation

Gliadins were extracted with 70% ethanol at a 3:1 ratio of solvent to flour for 1 hr at room temperature (Lookhart et al 1982). Samples were vortexed for 30 sec, every 15 min. Gliadins (400 μL) were then precipitated with three volumes of ice cold acetone. Precipitated proteins were redissolved in 400 μL of either 24% acetonitrile (ACN) + 0.1% trifluoroacetic acid (TFA) (w/v) or 500 mM acetic acid. After solubilization, samples were centrifuged for 10 min at 14,000 rpm with an Eppendorf minicentrifuge with an 18-place rotor.

For extraction of glutenins, flours were first extracted with 60% ethanol (Wieser et al 1994) at a 4:1 ratio of solvent to flour for 30 min at room temperature. The remaining pellet was then extracted with 50% *n*-propanol + 1% dithiothreitol (DTT) (4:1 ratio of solvent to flour) for 1 hr at room temperature. Samples were vortexed for 30 sec, every 15 min during all extractions. Glutenins were precipitated with three volumes of ice cold acetone. Precipitated glutenins were redissolved in 200 μL of 500 mM acetic acid or 24% ACN + 0.1% TFA (w/v). After resolubilization, glutenin samples were centrifuged in the same manner as gliadins. Both gliadins and glutenins were analyzed by RP-HPLC immediately after being centrifuged. In all cases, patterns of the resolubilized proteins were identical to patterns of proteins in the original extracts.

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Reversed Phase-High Performance Liquid Chromatography

A Hewlett-Packard 1090A chromatograph was used for all RP-HPLC analyses. Gliadins were analyzed on a Zorbax SB C₈ column (4.6 mm × 15 cm) (Marchylo et al 1992). A linear gradient (water and ACN, each containing 0.1% TFA) was used to separate the gliadins. The gradient increased from 24 to 50% ACN over 14 min. The column was rinsed for 2 min with 75% ACN and then held at initial conditions for 10 min between each separation. Temperature was maintained at 50°C. Extracted gliadins were injected (15 µL) for collection runs on the HPLC.

Glutenin subunits were separated on a Vydac C₁₈ column (4.6 mm × 25 cm). A multistep linear gradient (water and ACN each containing 0.1% TFA) was used to separate glutenins. The gradient increased from 24 to 39% ACN over 15 min. The gradient then increased to 45% ACN over 6 min, and then further increased to 55% over the next 2 min. The column was rinsed for 2 min with 75% ACN and then held at initial conditions for 10 min between each separation. Temperature was maintained at 45°C. Glutenin samples were not alkylated for RP-HPLC analysis. Glutenin extracts (80 µL) were loaded onto the HPLC column in eight 10-µL injections. The column was held at initial conditions for 2 min between repeated injections (Marchylo and Kruger 1988). The flow rate of all HPLC separations was 0.5 mL/min to facilitate the analysis of collected fractions by FZCE. Peak widths did not increase upon decreasing the flow rate, which effectively concentrated the collected fractions. All separations were monitored at 220 nm.

Fractions were collected every 30 sec from the HPLC for separation by FZCE. Prior to collection, the column was rinsed with 0.2 mL of 6M guanidine hydrochloride (D. D. Kasarda, *personal communication*). Collected high molecular weight glutenin subunits (HMW-GS) showed evidence of contamination with lower molecular weight proteins unless this rinse was performed (data not shown). Analysis by SDS-PAGE of the HMW-GS collected after the rinse procedure revealed only HMW-GS; no other bands were detected when the gels were silver-stained (data not shown). The exact start and stop times of collections depended on the samples being analyzed. Fractions were collected continuously once started until no more peaks were evident on the HPLC. The collected fractions were loaded directly into the autosampler of the CE instrument and analyzed. Generally, two 2D separations could be completed in a 24-hr period.

HMW-GS were identified by analyzing collected fractions on SDS-PAGE with BioRad minigels as described by Tilley et al (1993). Gels were silver stained by the method of Nesterenko et al (1993).

Gliadin subclasses were identified by comparing both the HPLC and FZCE patterns to previous results of Lookhart and Albers (1988) and Lookhart and Bean (1995b).

Free-Zone Capillary Electrophoresis

A Beckman P/ACE 2100 was used for all FZCE separations. All samples analyzed by FZCE were separated on 25 µm × 27 cm uncoated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ). Capillaries were placed in cartridges with a detection aperture of 100 × 800 µm. Samples were analyzed at 12.5 kV and

45°C and were detected by UV absorbance at 200 nm. Fluorinert FC-77 (3M) was used as the coolant in the Beckman instrument. Separation buffer and capillary cleaning protocols were the same as those used by Lookhart and Bean (1996). The procedure used to initially equilibrate the capillaries was modified as described by Bean (1996). Briefly, capillaries were rinsed with 0.1M NaOH for 15 min, followed by 1.0M HCl for 10 min, and then allowed to stand for 15 min with the 1.0M HCl. Next, the capillaries were rinsed with 1.0M phosphoric acid for 60 min, followed by a 20-min rinse with separation buffer. Temperature was maintained at 45°C during the equilibration procedure. Recently purchased capillaries (from Polymicro Technologies) equilibrated without using the hydrochloric acid often exhibit no peaks, higher than normal currents, and plug frequently.

Collected HPLC fractions were injected by pressure for 16 sec. Total gliadin and glutenin extracts were injected for 5 sec. For FZCE, precipitated total gliadin and glutenin samples were dissolved in 24% ACN + 0.1% TFA to simulate the sample matrix of the collected HPLC fractions. Mixtures of 38 and 50% ACN + 0.1% TFA were also used on some samples to test the effects of various ACN concentrations in the sample matrix. TAM 107 was analyzed before every data set; the subsequent electrophoregram used as an external standard as described by Lookhart and Bean (1996).

Data Handling

Data from the FZCE along with the HPLC times of the collected fractions were combined into surface contour plots or 3D surface plots with the software package Origin (MicroCal Software, Inc. Northhampton, MA). The Origin software was used to flatten any sloping baselines in the FZCE separations, and all baselines were set to a common y-value to avoid baselines from giving false "spots" on the surface contour maps. Contour values were chosen to show as many proteins as possible without picking up baseline noise.

RESULTS AND DISCUSSION

Capillaries

Bietz and Schmalzried's (1995) initial FZCE separations of wheat proteins were done with 50-µm i.d. capillaries. Lookhart and Bean (1995a) demonstrated that 20-µm i.d. capillaries produced separations in less than half the time of the larger 50-µm-i.d. capillaries with the same resolution. However, use of smaller i.d. capillaries limits the path length of the detector cell, which in turns limits the detection sensitivity of the technique. Initially in the current studies, collected HPLC fractions were analyzed on 20-µm capillaries. However, many collected peaks could not be detected unless large or preconcentrated samples were used, resulting in poor reproducibility and long analysis times. With 50-µm i.d. capillaries, collected peaks could be detected, but analysis times and reproducibility were unsatisfactory. A compromise was the use of 25-µm i.d. capillaries, which gave nearly three times the absorbance of the 20-µm i.d. capillaries (due to increased illumination path and increased sample size for the same injection

TABLE I
Reproducibility of the Three Two-Dimensional Separations of the Gliadins from Cultivar Shawnee

Run	HPLC Elution Time (min) Peak					Free Zone Capillary Electrophoresis Migration Time Peak				
	1	2	3	4	5	1	2	3	4	5
1	9.9	9.8	9.4	11.3	13.7	21.0	18.6	16.5	12.1	12.2
2	10.1	10.0	9.6	11.5	14.0	21.0	18.6	16.6	12.2	12.2
3	10.5	10.5	10.0	12.0	14.5	20.9	18.5	16.5	12.0	12.3
Average	10.2	10.1	9.7	11.6	14.1	21.0	18.6	16.5	12.1	12.3
STD ^a	0.31	0.36	0.31	0.36	0.40	0.06	0.06	0.06	0.10	0.17
RSD(%) ^b	3.00	3.57	3.16	3.11	2.87	0.28	0.31	0.35	0.83	1.41

^a Standard deviation.

^b Relative standard deviation.

time, from 0.63 nL/sec for the 20 μm to 0.153 nL/sec for the 25 μm capillary) but are still small enough to effectively dissipate heat and prevent joule heating during high voltage separations (Lookhart and Bean 1995a, Bean 1996). The slight increase in diameter necessitated a small decrease in separation voltage as compared to the 20- μm i.d. capillaries, but total separation time was increased by only ≈ 2 min. Thus, the 25- μm i.d. capillaries could be used to directly analyze the collected HPLC fractions

without any preconcentration and without excessively large injection volumes, while still maintaining rapid separations.

Sample Matrix

Because samples collected at different times during the HPLC separation will contain different concentrations of acetonitrile, gliadin samples were analyzed in various concentrations of acetonitrile. No changes were observed in the various samples, indicating

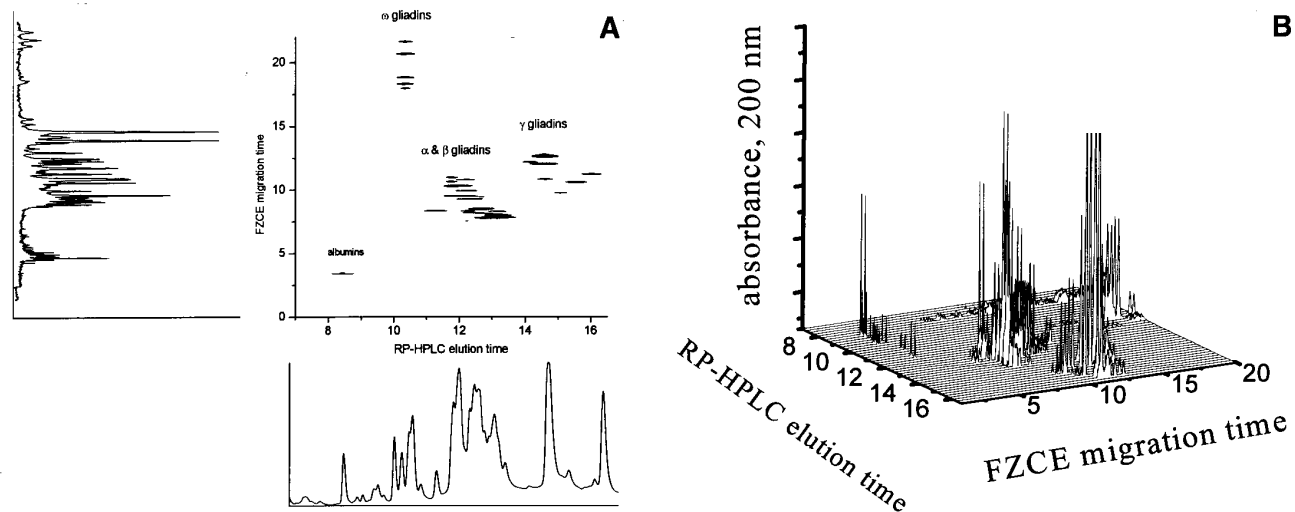


Fig. 1. A, Contour map of two-dimensional (2D) separations (reversed-phase HPLC and free zone capillary electrophoresis [RP-HPLC \times FZCE]) of gliadins from the cultivar TAM 105. **B**, Surface plot (3D) of the 2D (RP-HPLC-FZCE) separation of the gliadins from the cultivar TAM 105. Individual FZCE separations plotted vs. time of collection from the HPLC separation. RP-HPLC separations of gliadins were obtained with a multistep linear gradient of acetonitrile and water each containing 0.1% TFA at 0.5 mL/min. Collected HPLC fractions were separated on a 25 μm i.d. \times 27 cm fused silica capillary with a pH 2.5 phosphate buffer containing 20% acetonitrile at 12.5 kV and 45°C. Detection at 220 nm for HPLC and 200 nm for FZCE.

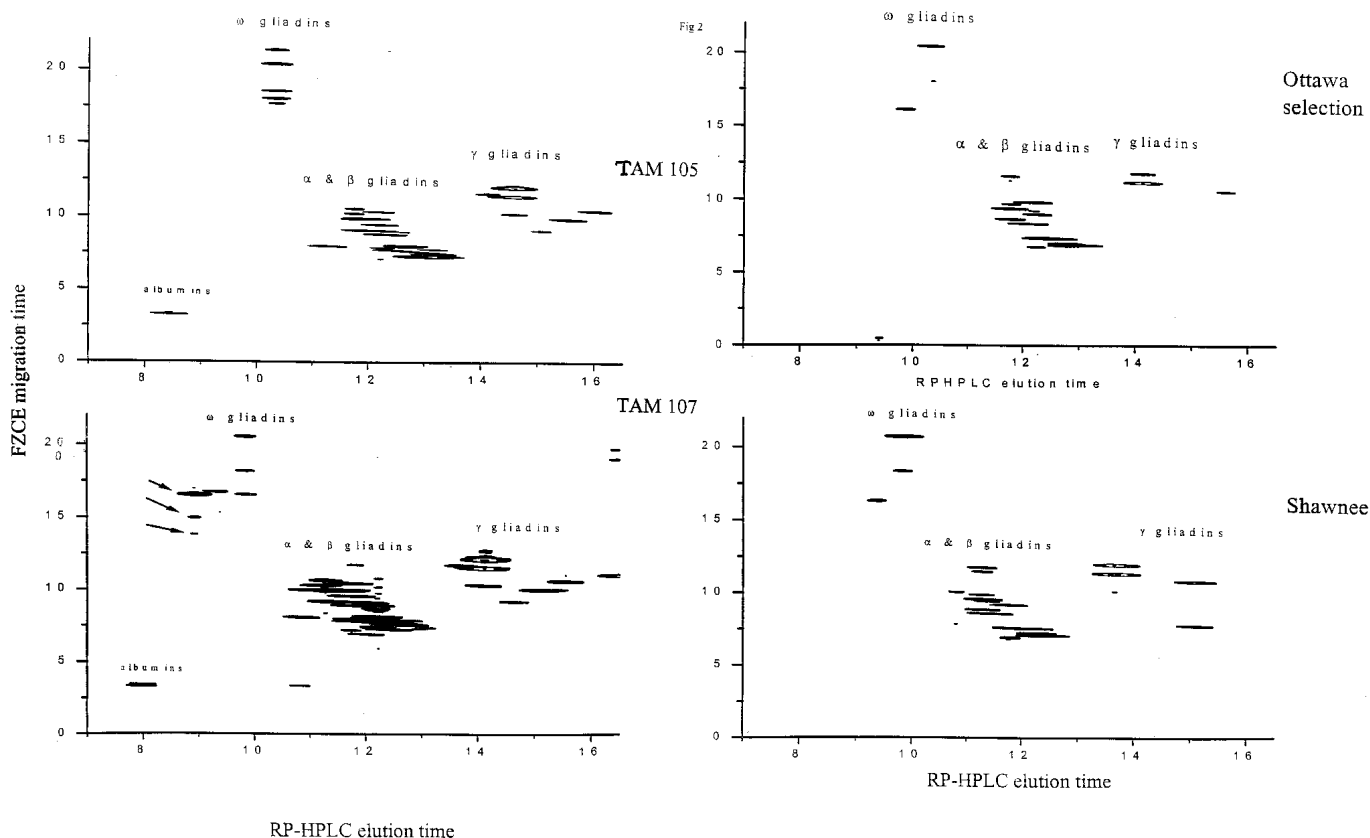


Fig. 2. Gliadin contour maps of cultivars TAM 105 and TAM 107 (**A**) and from sister lines Shawnee and Ottawa selection (**B**). The rye secalins in the cultivar TAM 107 are identified by arrows (**A**). Reversed-phase HPLC separations of gliadins were obtained with a multistep linear gradient of acetonitrile and water each containing 0.1% TFA at 0.5 mL/min. Collected HPLC fractions were separated on a 25 μm i.d. by 27 cm fused silica capillary with a pH 2.5 phosphate buffer containing 20% acetonitrile at 12.5 kV and 45°C. Detection was at 220 nm for HPLC and 200 nm for FZCE.

that the acetonitrile in the sample matrix did not interfere with the FZCE separation. Similar results were found by Zhu et al (1989) for peptides. Earlier studies by Lookhart and Bean (1995a) and Bietz and Schmalzried (1995) also showed that various concentrations of organic solvents in the sample matrix do not affect the FZCE separations of wheat proteins.

Gliadins

The 2D separation of gliadins from the cultivar TAM 105 is shown in Fig. 1A. The RP-HPLC pattern of the gliadin extract is shown along the *x*-axis with the FZCE separation shown along the *y*-axis. It should be pointed out that due to baseline noise, very small peaks cannot be detected by the contour plots. Careful study of the contour plots and the individual FZCE electrophoregrams should be made when evaluating data from this procedure. Data can also be plotted as 3D surface plots, as shown in Fig. 1B, where the individual FZCE electrophoregrams are plotted along the *y*-axis with the time of their HPLC collection along the *x*-axis. Both types of plots provide important information about the 2D separations and are easy to construct with commercially available software. Figure 1B also reveals quantitative information that is not available with the 2D surface contour maps.

Because gliadins have been well characterized by HPLC, performing 2D separations of this type provides valuable information about the FZCE separation itself. Lookhart and Bean (1995b) separated the classical Osborne (1907) fractions and identified the migration order of the gliadin subclasses on FZCE. These early separations were not performed as described in this article, and while they revealed the migration order of the various gliadin subclasses, they did not provide the resolution of the 2D technique. The 2D maps, therefore, provide more complete information about the FZCE separation. Gliadins formed three well-defined clusters, following the migration order found by Lookhart and Bean (1995b), with the ω -gliadins well separated from the rest of the gliadins. These gliadins, which are the sulfur-poor gliadins, are perhaps more structurally different from the other gliadin subclasses (Shewry et al 1986).

Faint traces of albumins were also seen, apparently coming from some early eluting peaks of the gliadin HPLC separations. Several studies have shown that extraction of wheat with aqueous alcohols solubilizes considerable amounts of albumins and globulins with the gliadins (Lookhart and Chung 1994, Wieser et al 1994, Lookhart and Bean 1995b, Fu and Sapirstein 1996).

The 2D maps also revealed some proteins that overlapped in any single dimension. Thus, some peaks on the HPLC are resolved into multiple peaks by FZCE and some proteins that overlap in the FZCE separation can be resolved by first separating them by RP-HPLC. This observation agrees with the complementary nature of RP-HPLC and FZCE.

The gliadin HPLC gradient was chosen to provide rapid separations and a manageable number of fractions (usually 20), rather than maximum resolution. Longer gradients, providing better resolution, were also tested. Such gradients gave better resolution, but showed few new spots (data not shown). Thus, HPLC resolution appears to not be critical to good 2D separations, and rapid HPLC analyses can be used.

The 2D mapping readily reveals different protein compositions of different cultivars. Figure 2A compares the two closely related backcross lines, TAM 105 and TAM 107, and Fig. 2B compares the two sister lines, Shawnee and Ottawa Selection. The gliadin patterns of these wheats differed in both the FZCE (Lookhart and Bean 1996) and HPLC separations (unpublished data) and accordingly, differences between the 2D patterns were also evident. The rye ω -secalins of TAM 107, marked by arrows, were readily evident. The 2D procedure allows for easy differentiation of the various gliadin subclasses and may provide the high resolution necessary to differentiate closely related cultivars that are not possible to distinguish with either separation technique alone.

Reproducibility

One potential drawback of using 2D gel electrophoresis is reproducibility of the separations, because separate gels must be made fresh for each 2D separation. It was hoped that by combining two instrumental techniques, where separations take place in columns and capillaries can be used multiple times, the reproducibility would be improved. Figure 3 shows three replicates of the separation of gliadins from the cultivar Shawnee analyzed on independent occasions. The patterns were nearly identical, although minor variation does exist. The migration-elution times for selected peaks are shown in Table I. Although these data do not represent a measure of the reproducibility of the overall 2D technique, they do reveal where variation in the separations came from.

Migration times for the peaks in the CE dimension differed by only $\approx 1\%$ relative standard deviation (RSD), demonstrating excellent reproducibility in the FZCE dimension. Elution times of the peaks in the RP-HPLC dimension differed by $\approx 3\%$ RSD. The elution times of the RP-HPLC peaks increased among the three replicates, which may have been caused by column aging. Migration-elution times for FZCE and HPLC, respectively, can vary with

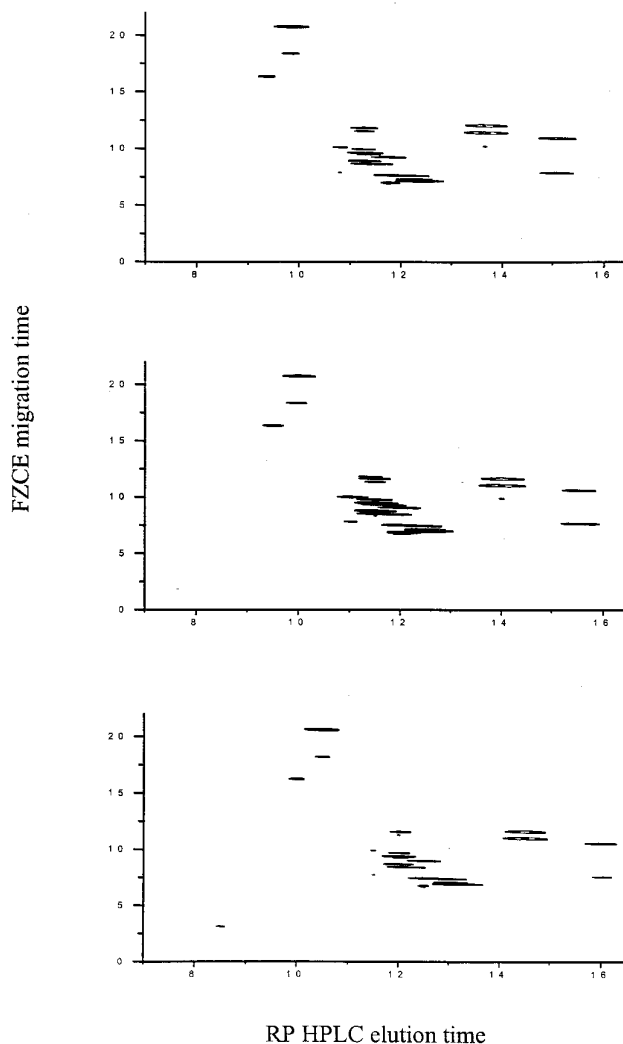


Fig. 3. Contour plots of the gliadins of the cultivar Shawnee. Separations were performed on three independent occasions. Reversed-phase HPLC separations of gliadins were obtained with a multistep linear gradient of acetonitrile and water each containing 0.1% TFA at 0.5 mL/min. Collected HPLC fractions were separated on a 25 μ m i.d. by 27 cm fused silica capillary with a pH 2.5 phosphate buffer containing 20% acetonitrile at 12.5 kV and 45°C. Detection was at 220 nm for HPLC and 200 nm for free zone capillary electrophoresis.

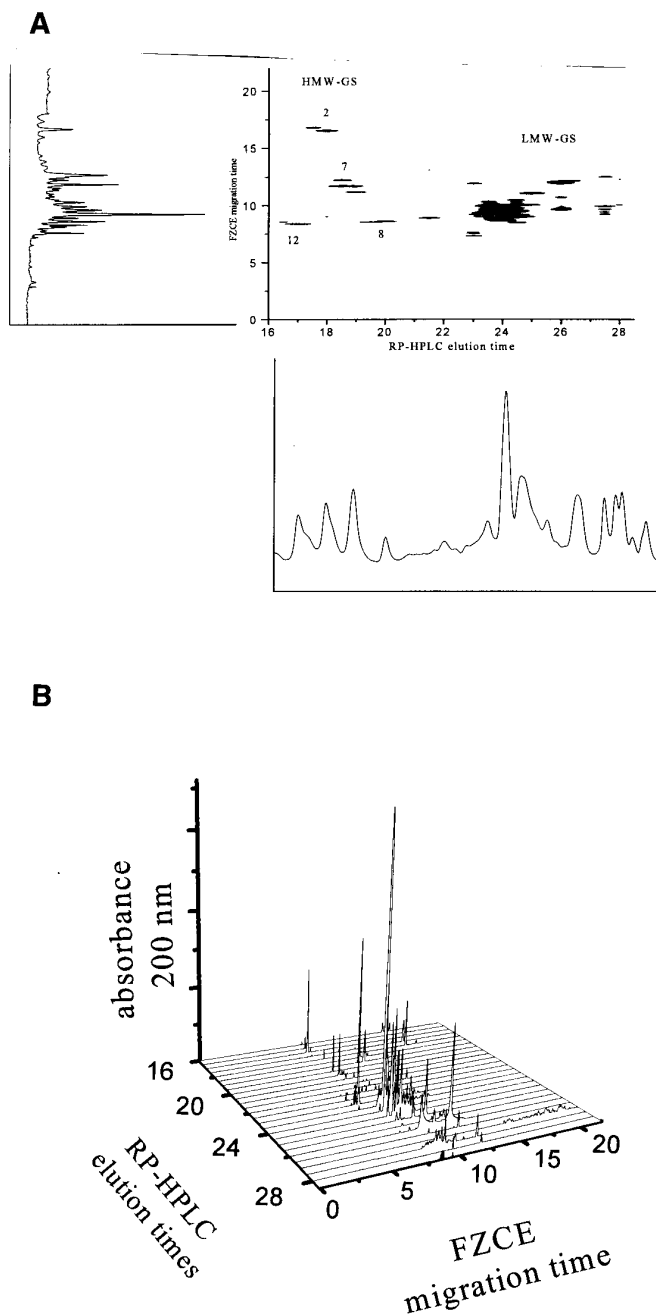


Fig. 4. Two-dimensional contour plots (A) and 3D surface plot (B) of total glutenin from the cultivar TAM 105. Reversed-phase HPLC separations of glutenins were obtained with a multistep linear gradient of acetonitrile and water each containing 0.1% TFA at 0.5 mL/min. Collected HPLC fractions were separated on a 25 μ m i.d. by 27 cm fused silica capillary with a pH 2.5 phosphate buffer containing 20% acetonitrile at 12.5 kV and 45°C. Detection was at 220 nm for HPLC and 200 nm for free zone capillary electrophoresis.

time (Marchylo et al 1988, Scanlon et al 1989, Bietz and Lookhart 1997), which in turn will affect the long-term reproducibility of this 2D technique. However, the computerized data produced by these instruments can easily be corrected for any changes that may occur. Such a method was developed by Sapirstein et al (1989) for the standardization of elution times on HPLC. That method has also been successfully used with FZCE data in our laboratory (unpublished data). Although these techniques were not used in this study, the methodology is readily available.

The type of data generated by this technique could also be easily quantified. Peak areas for the FZCE separations could be easily computed for each separation and totaled. For HPLC, corrected peak areas must be used for quantitation (Altria 1993).

Glutenins

The combination of RP-HPLC and FZCE provides high-resolution separations of reduced glutenins. Typically, glutenins, especially HMW-GS, are reduced and alkylated for separation by RP-HPLC and are not well resolved unless alkylated (Lookhart and Bietz 1994). However, alkylation of the HMW-GS leads to a loss of resolution by FZCE and produces poorly shaped peaks (data not shown). For this reason, the glutenins were not alkylated for this study.

The 2D separation of glutenins of TAM 105 is shown in Fig. 4A as a contour map and in Fig. 4B as a 3D surface map. The HMW-GS and LMW-GS, which were well separated by RP-HPLC, can be seen to overlap in the FZCE separation, with both groups forming distinct clusters on the 2D maps.

The 2D maps also revealed that FZCE separates the HMW-GS into multiple peaks. These multiple peaks may be the result of any one of numerous types of post-translational modifications, some of which have been reported in wheat (Tilley et al 1993, Buonocore et al 1994, Tilley and Schofield 1995, Lauriere et al 1996, Tilley 1996). HPLC has separated isoforms of posttranslationally modified proteins with very high resolution, even detecting changes to single amino acids on some peptides (Castagnola et al 1996, Ganzler et al 1996).

It is unlikely that these multiple spots result from contamination by additional proteins or degradation caused by the RP-HPLC separation. Only HMW-GS were seen when collected fractions were analyzed by SDS-PAGE and silver-stained (as long as the column was rinsed with guanidine hydrochloride before collection as stated earlier). In addition, ω -gliadins, which are most often found contaminating glutenin preparations, are well separated from the glutenin proteins on the 2D maps. Likewise, degradation seems unlikely because precipitated HMW-GS separated by FZCE that have not gone through the RP-HPLC separation process gave the same pattern as those collected from the RP-HPLC. Additionally, when the individual FZCE electrophoregrams from the 2D procedure were combined, the resultant electrophoregram was essentially identical to an electrophoregram of the same HMW-GS not analyzed by RP-HPLC (data not shown). Other 2D techniques have also separated the HMW-GS into multiple peaks (Payne et al 1982), as has IEF (Shewry et al 1984, Tilley et al 1993). Research is currently underway at this laboratory to fully investigate the nature of the multiple forms of the HMW-GS reported in this article.

The HMW-GS were identified on the 2D maps by determining their positions in the HPLC separation by SDS-PAGE analysis of the collected HPLC fractions. Once the identity of the HPLC peak was known, it was easy to find the HMW-GS position on the 2D maps. The HMW-GS spots could then be traced to the FZCE electrophoregrams and the individual HMW-GS could be located on the FZCE electrophoregrams. Figure 5 shows a separation of total glutenins and of the HMW-GS of TAM 105 showing the location of the HMW-GS in the FZCE electrophoregrams.

The LMW-GS, which can be difficult to separate (Morel 1994), are also well resolved. Two-dimensional contour maps fail to show the true resolution achieved for these subunits because LMW-GS are much more abundant than are HMW-GS. Again, to fully interpret the 2D maps, both the contour plots and the individual FZCE electrophoregrams should be scrutinized. Resolution of the LMW-GS can be better seen in Fig. 6, which shows some representative FZCE electrophoregrams of the LMW-GS of TAM 105.

Figure 7 displays the 2D maps of glutenins from four wheat lines: Shawnee, Ottawa Selection, TAM 105, and TAM 107.

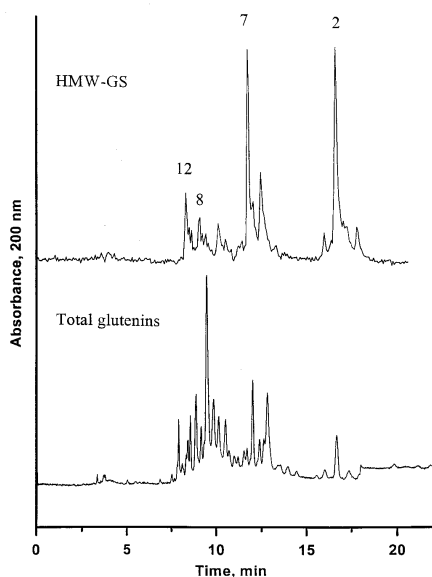


Fig. 5. Separation of total glutenins and precipitated high-molecular-weight glutenin subunits (HMW-GS) from the cultivar TAM 105 showing the locations of the individual HMW-GS in the free zone capillary electrophoregrams. Reversed-phase HPLC separations of glutenins were obtained with a multistep linear gradient of acetonitrile and water each containing 0.1% TFA at 0.5 mL/min. Collected HPLC fractions were separated on a 25 μ m i.d. by 27 cm fused silica capillary with a pH 2.5 phosphate buffer containing 20% acetonitrile at 12.5 kV and 45°C. Detection at 220 nm for HPLC and 200 nm for free zone capillary electrophoresis.

Several HMW-GS were resolved into multiple peaks with some appearing to have different patterns in different cultivars. The maps also revealed that some of the HMW-GS overlapped each other or were only slightly separated in the FZCE separations. For example, 2* and 7 in the TAM 107 pattern overlapped as did 2* and 6 in the Shawnee and Ottawa selection patterns, whereas HMW-GS 8 and 12 were only slightly separated by FZCE in the TAM 105 and TAM 107 cultivars. At this time, it is not possible to identify exactly which peaks belonged to which HMW-GS for those pairs that overlapped (e.g., 2* and 7 and 2* and 6).

CONCLUSIONS

The combination of RP-HPLC and FZCE used in a 2D system provided important information about FZCE separations of gliadins and glutenins (both HMW-GS and LMW-GS). This 2D technique is reproducible and easy to perform. This work confirmed earlier results of Lookhart and Bean (1995b) concerning the migration order of gliadins and revealed that the gliadin subclasses formed distinct clusters with the α - and β -gliadins together in one group. The ω -gliadins formed a distinct grouping well separated from the other gliadins. The glutenins were separated into clusters of HMW-GS and LMW-GS with individual HMW-GS separated into multiple forms. The identity of these different forms is unknown at present. LMW-GS were also well separated.

This technique can be used to separate proteins or groups of proteins inseparable by single dimensional analysis. Although both RP-HPLC and FZCE produce high-resolution separations by themselves, the combination provides a useful method for identifying proteins or groups of proteins. The type of data generated by this technique can easily be quantitated by computing corrected peak areas from the FZCE separations. Data could be easily standardized using external standards to provide for good long-term reproducibility.

This technique may also prove useful in fingerprinting cultivars where extremely high resolution is needed to differentiate closely related lines or in genetic analysis where proteins are related back to specific chromosomes (e.g., gliadin block analysis).

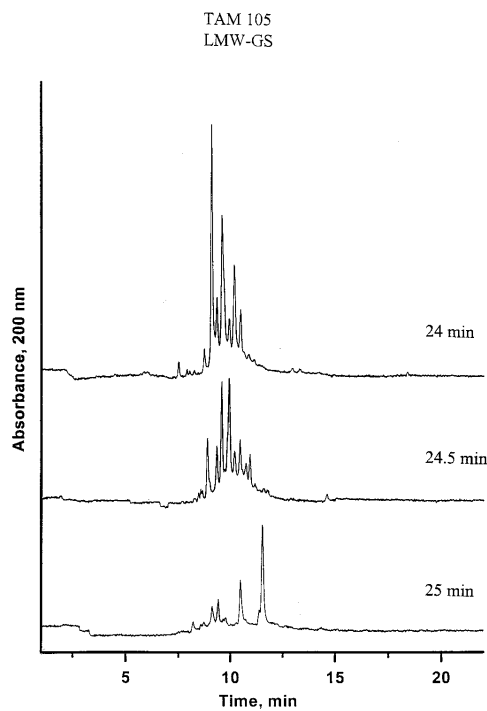


Fig. 6. Individual electrophoregrams from the free zone capillary electrophoresis (FZCE) separations of the low-molecular-weight glutenin subunits of the cultivar TAM 105. Times represent the start time of the collected fractions. Reversed-phase HPLC separations of glutenins were obtained with a multistep linear gradient of acetonitrile and water each containing 0.1% TFA at 0.5 mL/min. Collected HPLC fractions were separated on a 25 μ m i.d. by 27 cm fused silica capillary with a pH 2.5 phosphate buffer containing 20% acetonitrile at 12.5 kV and 45°C. Detection was at 220 nm for HPLC and 200 nm for FZCE.

No specially adapted equipment is necessary for this technique, and it can be done with currently available commercial instruments and software. With the use of an automated fraction collector, the technique can be almost entirely automated. Collected fractions from the HPLC need only be placed in the autosampler of the HPCE. Currently, two 2D separations can be completed in a 24-hr period. Research is currently being conducted at this laboratory to reduce the analysis times of the FZCE separations to speed up the 2D process even more.

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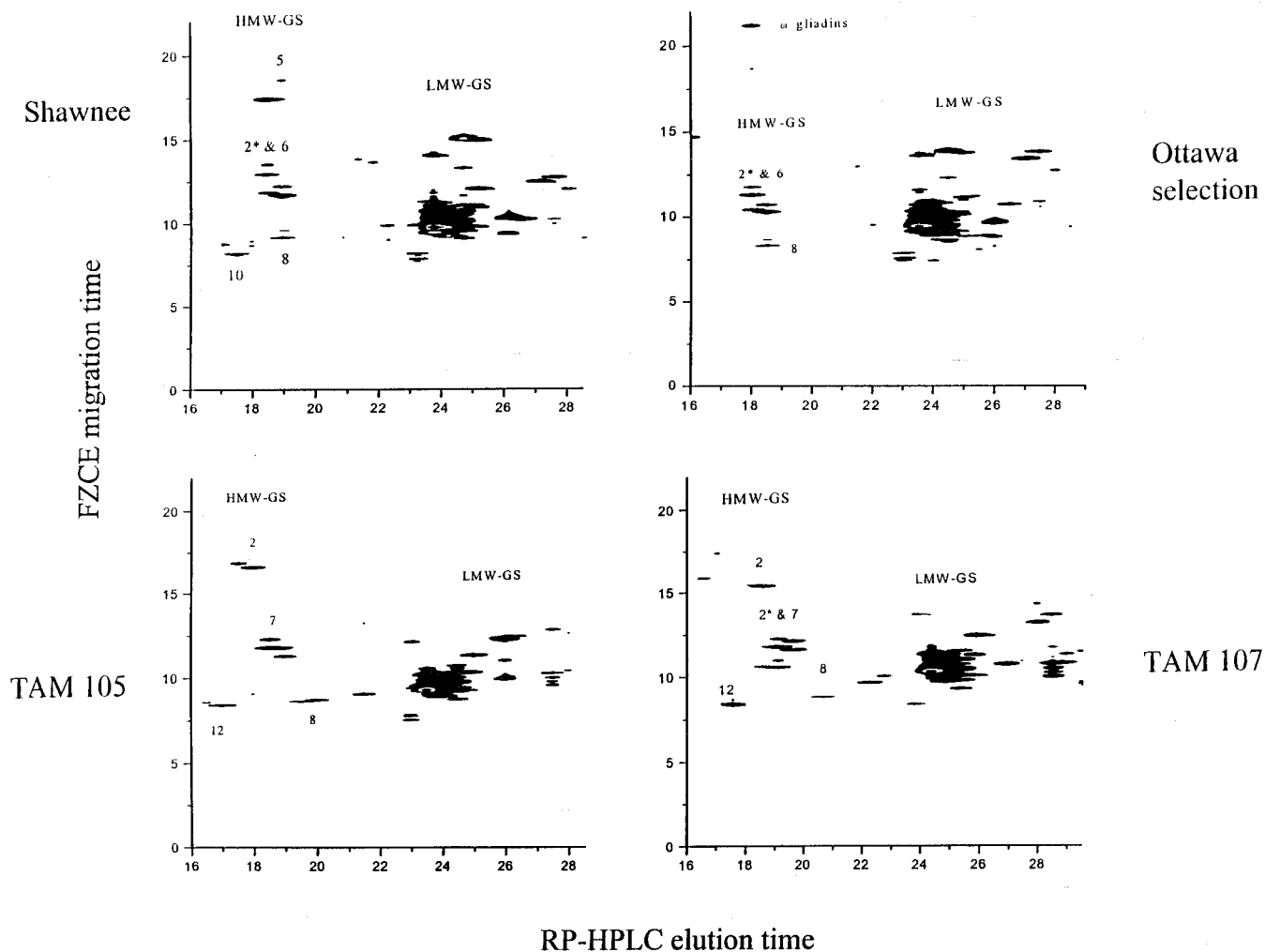


Fig. 7. Two dimensional contour plots of the glutenins from the wheat lines Shawnee, Ottawa selection, TAM 105, and TAM 107. RP-HPLC separations of glutenins were obtained with a multistep linear gradient of acetonitrile and water each containing 0.1% TFA at 0.5 mL/min. Collected HPLC fractions were separated on a 25 μ m i.d. by 27 cm fused silica capillary with a pH 2.5 phosphate buffer containing 20% acetonitrile at 12.5 kV and 45°C. Detection was at 220 nm for HPLC and 200 nm for free zone capillary electrophoresis.

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