A Fast Method for Wheat Cultivar Differentiation Using Capillary Zone Electrophoresis[1]

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ABSTRACT

Capillary zone electrophoresis (CZE) parameters, including capillary length, inside diameter, buffer, temperature, voltage, and extraction conditions, were studied to reduce analysis time and increase resolution of wheat proteins. Conditions for the shortest analysis time include: capillary inside diameter, 20 μm; shortest possible capillary length, 27 cm (20 cm to detector); temperature, 45°C; voltage, 22 kV; and pressure injection for 4 sec (0.25 nL). Three alcohol-water based solvent systems were studied to improve extraction and analysis of gliadins; 30% ethanol-water was optimum. Gliadins were extracted from cultivars representative of hard red winter, hard red spring, and soft wheat classes and separated by CZE. Three separate sets of cultivars that were not differentiable by polyacrylamide gel electrophoresis at pH 3.1 were differentiated in less than 10 min each by CZE. Cultivars that were closely related (sister lines or intercrossings) were readily differentiated, and cultivars that were not genetically close exhibited quite different CZE patterns.

Considerable interest and research has been generated to distinguish cereal cultivars that provides distinction significant to differences in quality and agronomic properties. The use of instrumental biochemical analyses to identify or characterize cereal proteins has been ongoing for many years. In research, the ability to accurately identify a sample before research begins may save years of effort. Jones et al (1982) found that 3% of samples from breeders and the USDA National Germplasm Center were mismarked. Today, it is apparent that varietal identification is one of the most important and controllable parameters that one can use for predicting end-use quality.

Polyacrylamide gel electrophoresis (PAGE) has been the most useful laboratory method of grain identification during the last two decades. Gels require at least 1 hr to analyze and stain, even in the fastest form. For some situations, faster methods would be valuable. Bietz (1983) first reported the use of high performance liquid chromatography (HPLC) to characterize wheat proteins. HPLC analyzed wheat gliadins with quantitative digital output in 45 min. Lookhart and Albers (1988) compared and contrasted HPLC, PAGE, and sodium dodecylsulfate (SDS)-PAGE for cultivar identification and protein characterization. A 15-min HPLC procedure was reported by Lookhart et al (1986) for identifying wheat cultivar genotypes by gliadin analysis. Faster procedures are still being sought and reported.

One of the newest instrumental methods is capillary zone electrophoresis (CZE). It incorporates speed, high resolution, minimal sample requirement, and quantitative digital analyses. It is the first electrophoretic method that can be fully automated. For a general description of CZE, see Novotny et al (1990). CZE is an old concept; workers conducted electrophoresis in tubes even before slab gels were utilized. However, as a commercial instrument, CZE is a relatively new methodology, only five years old. Today, more than 10 companies produce full commercial CZE equipment. CZE separates materials based on analytical principles different than those of HPLC or PAGE, and therefore provides an alternate (complementary) procedure. Of those methods, CZE is the most effective for miniaturization, resolving power, and ultratrace analysis (with laser-induced fluorescence).

Preliminary information on the use of CZE for wheat varietal identification was first presented at AACC Annual Meetings (Bietz and Schmalzried 1992, 1993) and at the Gluten Workshop in

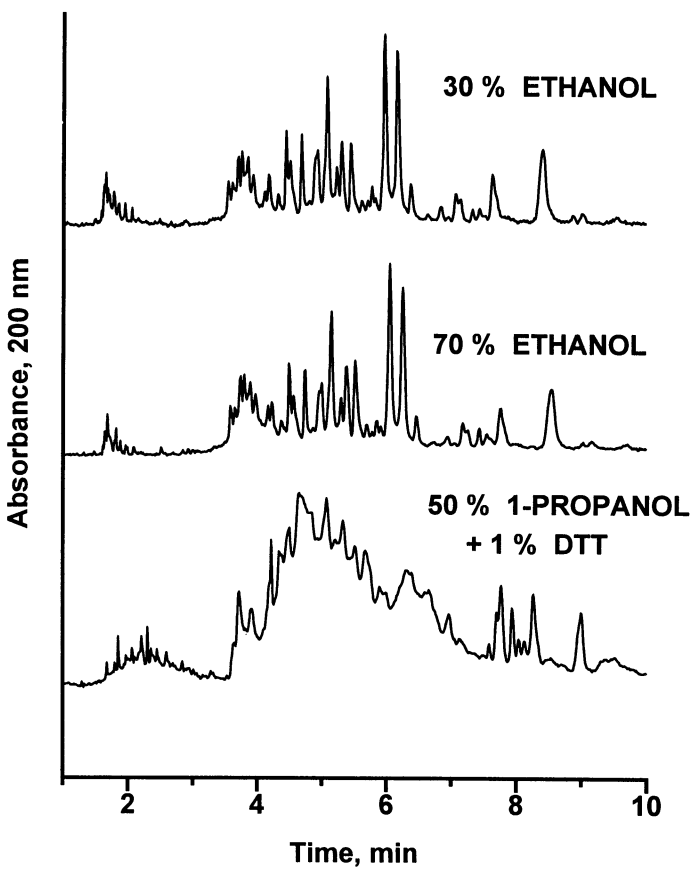

Fig. 1. Effect of extraction conditions on capillary zone electrophoresis patterns of wheat proteins of the hard red winter cultivar TAM 107. Wheat proteins extracted with 30% ethanol, 70% ethanol, and 50% 1-propanol plus 1% dithiothreitol (DTT) were pressure injected (4 sec, 0.25 nl) into a 27-cm long (20 cm to detector) × 20-μm i.d. capillary and separated at 45°C at 22 kV for 10 min using a 0.1*M* phosphate buffer pH 2.5, containing a polymeric additive. All patterns normalized to largest peak.

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1993 (Bietz 1994). Werner et al (1994) also reported at the 1993 Gluten Workshop on the use of CZE for wheat varietal identification by a charge-reversal coating (microcoat) to separate gliadins. Lookhart and Wrigley (1994) have also described the potential use and benefits of CZE for cereal cultivar identification.

This article reports optimized parameters for using CZE to characterize wheat gliadins and differentiate wheat cultivars. It provides a major improvement over the previous reports by reducing analysis times, using common reagents while maintaining resolution. The speed, resolution, voltage conditions, injection amounts, capillary dimensions, and reproducibility of CZE for characterizing wheat gliadins were studied.

MATERIALS AND METHODS

Wheat Flour Samples

Ten hard red winter (HRW) wheats (Eagle, Larned, Sage, Scout, Scout 66, TAM 105, TAM 107, Triumph, Triumph 64, Triumph Improved); six hard red spring (HRS) wheats (Butte, Grandin, Len, Lew, Marshall, Shield); and two soft wheats (Arthur, Nugaines) were from the Hard Winter Wheat Quality Laboratory in Manhattan, KS. Flours from each wheat were produced by a Brabender Quadromat Senior experimental mill.

Extraction of Proteins

Three alcohol-based solvent systems (70% ethanol in water [Lookhart et al 1982], 50% 1-propanol containing 1% dithiothreitol [DTT] [Marchylo et al 1988], and 30% ethanol in water [Bietz 1994]) were used to identify an optimal solvent system for CZE analysis. The first two solvent systems have been used consistently in PAGE and HPLC analyses. The third system was suggested by Bietz (1994) as appropriate for CZE. Proteins (mainly gliadins) were extracted for 30 min from flour samples (250 mg) with the alcohol solvents (1.0 ml). All extracts were filtered through 0.8-μm filters (Millex-PF, Millipore Corporation, Bedford, MA). Extracts were analyzed within 24 hr.

Capillary Zone Electrophoresis

A Beckman P/ACE system 2100 was used to separate each gliadin extract. Capillaries were uncoated fused glass 27 cm long (20 cm to detector) and 10-, 20-, or 50-μm i.d. The 10-μm i.d. capillary gave results similar to those of the 20-μm i.d. capillary, but the 10-μm i.d. capillary was easily plugged. The 75-μm i.d. capillary gave results similar to those of the 50-μm i.d. capillary, but the analysis times were considerably longer. Therefore, only

TABLE I
Capillary Electrophoresis: Sizes and Volumes[a]

	Injection Volume[b] (nL/sec)			Total Volume (nL)		
Length, cm	20 μm[c]	50 μm[d]	75 μm[e]	20 μm	50 μm	75 μm
27	0.063	2.440	12.360	85	530	1,192
37	0.046	1.780	9.020	116	726	1,634
47	0.036	1.400	7.100	148	924	2,075
57	0.030	1.160	5.850	179	1,119	2,517

[a]Calculated from Beckman Technical Information Bulletin, TIBC-103.
[b]Assuming a buffer viscosity equal to water at 30°C.
[c]0.02 mm, i.d.
[d]0.050 mm, i.d.
[e]0.075 mm, i.d.

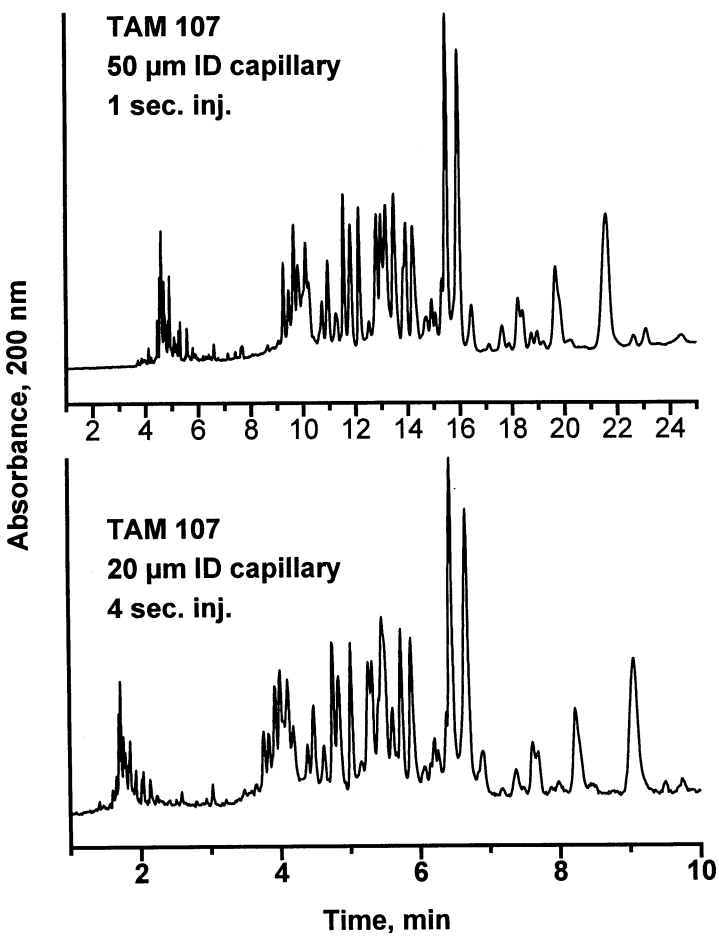

Fig. 2. Effect of capillary i.d. on capillary zone electrophoresis resolution. Gliadins were extracted from TAM 107 with 30% ethanol and analyzed on a 27-cm long (20 cm to detector) 20- and 50-μm i.d. capillaries. All patterns normalized to largest peak.

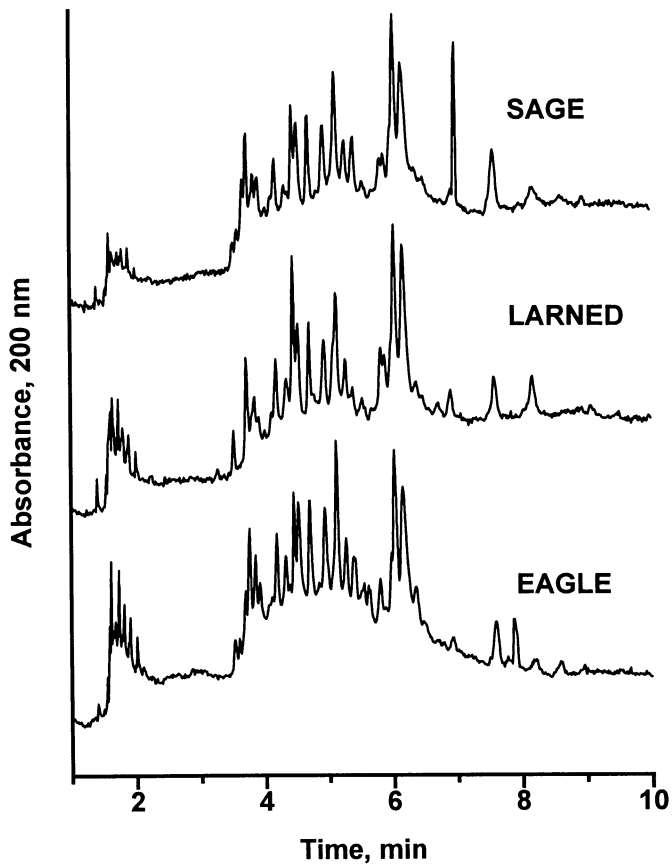

Fig. 3. Capillary zone electrophoresis patterns of gliadins of the hard red winter cultivars Eagle, Larned, and Sage. Proteins were characterized by the recommended method with 30% ethanol, pressure injected (4 sec, 0.25 nl) into a 27-cm long (20 cm to detector) 20-μm i.d. capillary, and separated at 45°C and 22 kV for 10 min in 0.1*M* pH 2.5 phosphate buffer containing a polymer additive. All patterns normalized to largest peak.

the 20-μm i.d. and 50-μm i.d. capillaries are discussed in detail. The temperature, voltage gradient, and amount injected varied with the capillary i.d. The buffer used in the inlet and outlet vials was a 0.1M pH 2.5 phosphate buffer containing a polymer additive (BioRad 148-5011, Hercules, CA).

Capillary Cleanup

Capillaries were rigorously washed in both forward and reverse directions in a manner similar to that suggested by Bietz (1994). After each analysis, the capillary was rinsed for 4 min (reverse pressure) with 1M nitric acid, 4 min (reverse pressure) with 0.1N sodium hydroxide, and 4 min (reverse pressure) with distilled water. Before each analysis, the capillary was rinsed (high pressure) for 4 min in a forward direction (+ to −) with the 0.1M pH 2.5 phosphate buffer containing the polymer additive.

50-μm i.d. Capillary

The total calculated volume of the 27-cm long, 50-μm i.d. capillary was 530 nL (Table I). Column volumes and 0.5 psi pressure injection volumes per sec (Table I) were calculated for 20-, 50-, and 75-μm i.d. capillaries (27, 37, 47, or 57 cm long, respectively) because these are the most common size combinations. Since CZE sample injections should actually be no more than 2% of total column volume (Hathaway 1992), injection time should be less than 4 sec. Reproducible peak migration times were found from 1 sec (2.44 nL) pressure injections. The Ohm's law plot of current versus voltage was linear up to 10 kV. Analyses were accomplished at 40°C by a voltage gradient from 7 kV to 10 kV over 5 min then held constant at 10 kV for another 20 min. The gradient was used to reduce analysis time while maintaining the resolution of a lower separation voltage. Total analysis time was 25 min.

20-μm i.d. Capillary (Recommended Method)

The total calculated volume of the 27-cm long, 20-μm i.d. capillary was 85 nL (Table I). Since the CZE sample volume should be no more than 2% (1.7 nL) of total volume, the maximum pressure injection time should be 27 sec. However, maximum resolution and reproducible peak elution times were found with 4-sec (0.25 nL) injections. The Ohm's law plot was linear to 22 kV for the 27-cm long, 20-μm i.d. capillary. A constant voltage of 22 kV at 45°C separated the proteins in 10 min or less.

Detection

Proteins were detected by UV absorbance at 200 nm. Beckman Gold Software accumulated the absorption data at a sampling rate of 5 Hz and produced an ASCII file for analysis and presentation by ORIGIN software, (MicroCal Software, Inc., Northampton, MA). All patterns were normalized for comparisons.

RESULTS AND DISCUSSION

Extraction of Proteins

The protein patterns from CZE analyses of the extracts of TAM 107 by three solvent systems (70% ethanol, 50% 1-propanol plus 1% DTT, and 30% ethanol) were compared to optimize the extraction solvent (Fig. 1). Patterns produced from the 30% ethanol extract exhibit slightly better resolution of peaks in the 3–9 min range (20-μm i.d. capillary), but are otherwise quite similar to the pattern of the 70% ethanol extract. The patterns of the 50% 1-propanol plus 1% DTT extract exhibit many broad

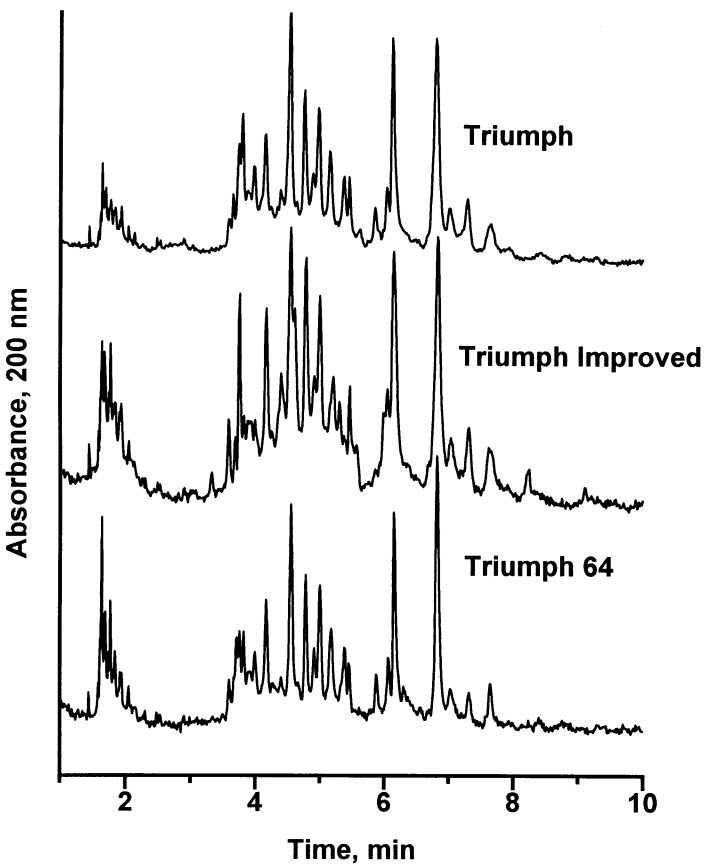

Fig. 4. Capillary zone electrophoresis patterns of gliadins of hard red winter cultivars Triumph, Triumph Improved, and Triumph 64. Proteins were separated by the recommended method with 30% ethanol, pressure injected (4 sec, 0.25 nl) into a 27-cm long (20 cm to detector) 20-μm i.d. capillary, and separated at 45°C and 22 kV for 10 min in 0.1M pH 2.5 phosphate buffer containing a polymer additive. All patterns are normalized to largest peak.

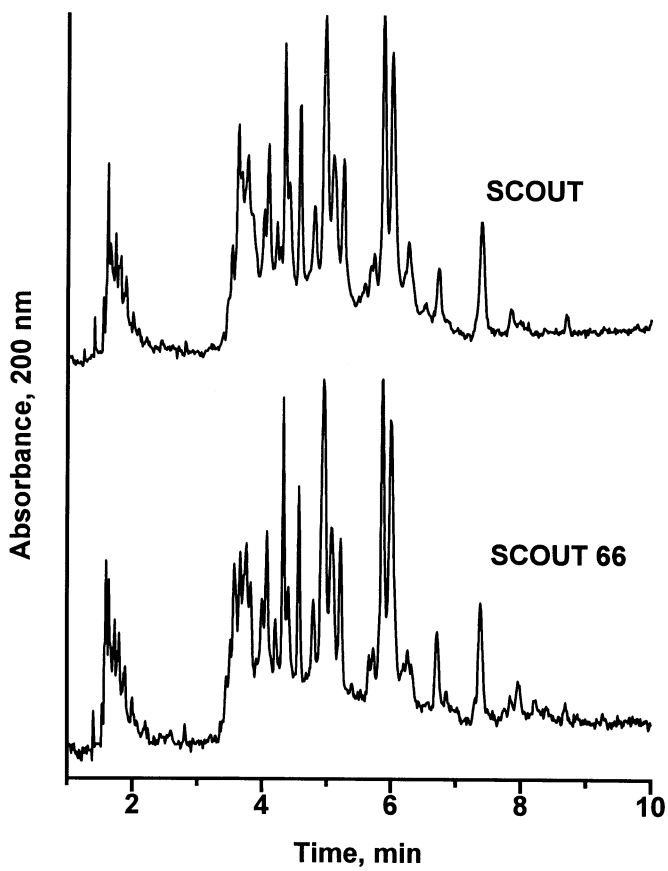

Fig. 5. Capillary zone electrophoresis patterns of gliadins of hard red winter cultivars Scout and Scout 66. Proteins were separated by the recommended method with 30% ethanol, pressure injected (4 sec, 0.25 nl) into a 27-cm long (20 cm to detector) 20-μm i.d. capillary, and separated at 45°C and 22 kV for 10 min in 0.1M pH 2.5 phosphate buffer containing a polymer additive. All patterns normalized to largest peak.

poorly resolved peaks typical of a total protein extract. Patterns of these same extracts, analyzed on a 50-μm i.d. capillary, exhibited similar tendencies. Therefore, 30% ethanol was used as the standard extraction medium.

CZE results (data not shown) for the typical Osborne protein fractions (Osborne 1907) showed that the early eluting peaks (1–3 min in Fig. 1 for the 20-μm i.d. capillary) were albumins and globulins; the later peaks (3–10 min) contained gliadins and glutenins.

Retention Time-Reproducibility

The CZE patterns for 10 consecutive injections of a single extract (30% ethanol) of TAM 107 were analyzed on 27-cm long, 20- and 50-μm i.d. capillaries. Elution times of the same five peaks selected from the gliadin patterns of consecutive injections of the same extract were statistically analyzed. The average relative standard deviation was 0.63% for the 50-μm i.d. capillary and 0.67% for the 20-μm i.d. capillary (data not shown). The relative standard deviation for the peak heights of those five peaks was 8% for the 50-μm i.d. capillary and 4% for the 20-μm i.d. capillary (data not shown).

Capillary Inside Diameter

The effect of capillary i.d. was measured by analyzing the 30% ethanol extract of TAM 107 in 27-cm long, 20- and 50-μm i.d. capillaries (Fig. 2). The peak resolution of the 50-μm i.d. capillary was slightly better than that of the 20-μm i.d. capillary. That difference in resolution is mainly due to the electrophoresis voltage conditions (10 kV vs. 22 kV). Overall, the 20-μm i.d. capillaries produced patterns nearly identical to those of the 50-μm i.d. capillaries and, because they were less affected by Joule heating during electrophoresis, provided faster analyses (Fig. 2). Temperature affects CZE resolution and stability, as might be expected when approaching the nonlinear portion of the Ohm's law plot. The 20-μm i.d. capillary separated the gliadins best at 45°C, whereas the 50-μm i.d. capillary separated the gliadins best at 40°C.

The optimum separation of gliadins with 50-μm i.d. capillary involved starting at 7 kV, increasing linearly to 10 kV at 5 min, then holding constant at 10 kV for 20 min. This system allowed the early eluting peaks to separate and then increased voltage to speed up separation of remaining proteins. The optimum separation (recommended method) for gliadins used a 20-μm i.d. capillary and 22 kV constant voltage for 10 min to provide high resolution and fast analysis. The voltage used during the major separation period in the 50-μm i.d. capillary was less than half that used in the 20-μm i.d. capillary (10 kV vs. 22 kV), and the initial voltage was less than one-third that used in the 20-μm i.d. capillary (7 kV vs. 22 kV). However, the 20-μm i.d. capillary was capable of analyzing the extracts in less than 10 min, whereas the 50-μm i.d. capillary required nearly 25 min. Since the 20-μm i.d. capillary also had excellent resolution (nearly identical to that of the 50-μm i.d. capillary) and was much faster, it was used for all further analyses.

The effect of too much (large sample plug) sample injected onto a capillary is increased peak broadening (Hathaway 1992). For capillaries 27 cm long (the shortest capillary length possible in the Beckman P/ACE 2100 capillary cartridge without removing the mandril), the 20-μm i.d. capillary maximum injection volume (2% of total volume) should be 1.7 nl, whereas the maximum for the 50-μm i.d. capillary would be 10.6 nl. However, we deter-

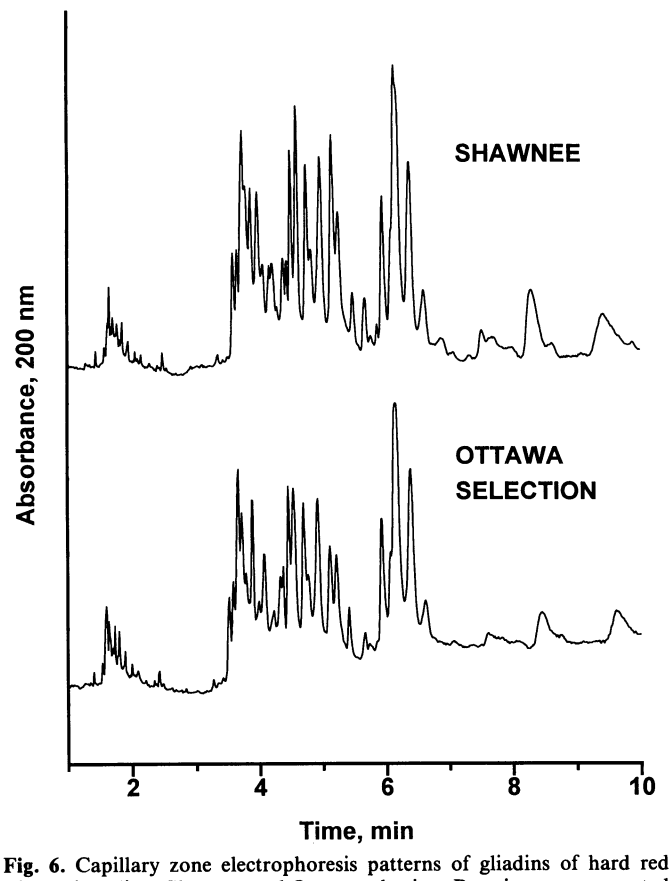

Fig. 6. Capillary zone electrophoresis patterns of gliadins of hard red winter sister lines Shawnee and Ottawa selection. Proteins were separated by the recommended method with 30% ethanol, pressure injected (4 sec, 0.25 nl) into a 27-cm long (20 cm to detector) 20-μm i.d. capillary, and separated at 45°C and 22 kV for 10 min in 0.1*M* pH 2.5 phosphate buffer containing a polymer additive. All patterns normalized to largest peak.

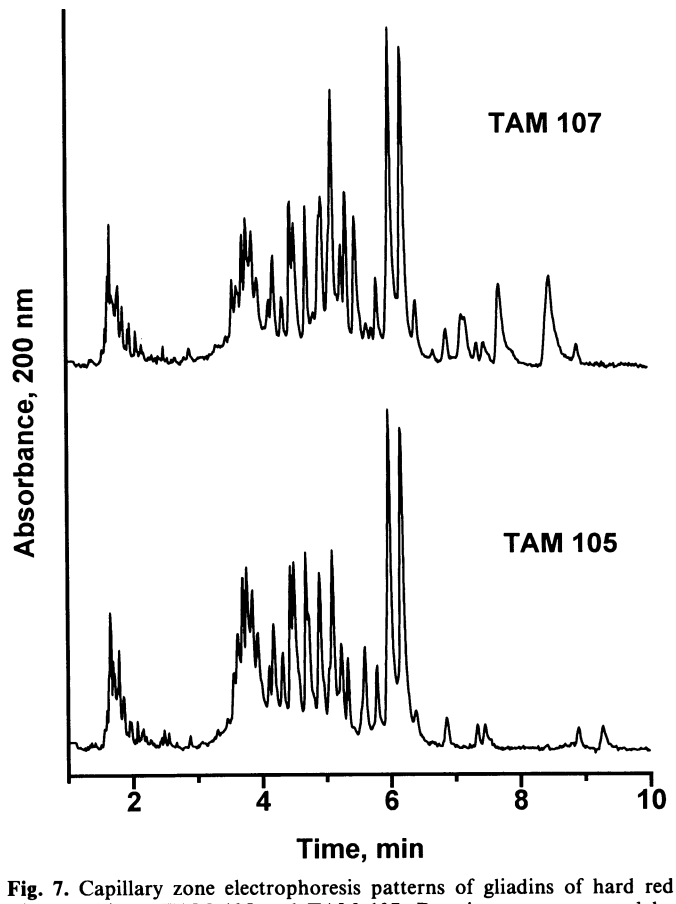

Fig. 7. Capillary zone electrophoresis patterns of gliadins of hard red winter cultivars TAM 105 and TAM 107. Proteins were separated by the recommended method with 30% ethanol, pressure injected (4 sec, 0.25 nl) into a 27-cm long (20 cm to detector) 20-μm i.d. capillary, and separated at 45°C and 22 kV for 10 min in 0.1*M* pH 2.5 phosphate buffer containing a polymer additive. All patterns normalized to largest peak.

mined empirically that optimum resolution was obtained when the injection volumes were 0.25 nl and 2.44 nl (i.e., <0.5% of total volume) for the 20- and 50-μm i.d. capillaries, respectively.

Cultivar Differentiation

Most cultivars chosen for this study had been previously analyzed by A-PAGE (Jones et al 1982) and by HPLC (data not shown). Some closely related cultivars had identical patterns by A-PAGE and minor differences by HPLC. Eagle, Larned, and Sage are one such group. CZE showed both qualitative and quantitative differences among those cultivars in the 3–10 min gliadin range (Fig. 3). Differences in the 1–3 min range are also evident. However, since only albumins and globulins elute in the 1–3 min range, that range cannot be used to differentiate cultivars because albumins and globulins are not reliable genotypic indicators (Lookhart and Wrigley 1994).

The CZE patterns of three closely related cultivars (intercrossings) (Triumph, Triumph Improved, and Triumph 64) are shown in Figure 4. Triumph Improved and Triumph 64 were intercrossed and selected from the original Triumph for improved agronomic traits. Differences in peak heights and positions, especially in the 3–6 min regions, are readily apparent.

CZE patterns of two closely related cultivars, Scout and Scout 66, are shown in Figure 5. Differences in their CZE patterns are evident in the ranges of 3–4 min and near 6.25 min. It was not possible to differentiate between the cultivars in either of these two sets of cultivars by A-PAGE, and only minor differences were found by HPLC (data not shown).

Cultivars Shawnee and Ottawa selection are closely related sister lines, with opposite baking qualities. Shawnee has good baking quality and Ottawa selection has poor quality (Tilley et al 1994). Their gliadins have been extensively studied and reported by Lookhart and Albers (1988). Their glutenins were studied by Payne et al (1988). In the gliadin A-PAGE and HPLC patterns of Ottawa selection and Shawnee (Lookhart and Albers 1988), four differences were noted by each. The CZE patterns (Fig. 6) reveal many qualitative and quantitative differences, especially in the 3.5–4.6 min range and the 7–8 min range.

TAM 105 and TAM 107 are closely related cultivars, differing mainly by the presence of 1RS rye translocation segment in TAM 107. Their CZE patterns (Fig. 7) show considerable differences. The three peaks in the 7–10 min range of the TAM 107 pattern should be noted.

The CZE patterns of the 30% ethanol extracts of the six hard red spring wheats (Butte, Grandin, Len, Lew, Marshall, and Shield) are shown in Figure 8. Differences in all areas of the patterns are evident for most of these cultivars. The cultivar Shield has a unique pattern with the largest peak at 5.2 min, the pattern of peaks between 3.5 and 5 min, and the triplet of peaks eluting between 6.2 and 7 min. Marshall is differentiated by a unique set of peaks eluting between 4 and 7 min. Lew is differentiated by having its largest peak at 4.4 min and by the doublet at 5.8–6 min. Len, Grandin, and Butte have similar patterns. Len is distinguished from Grandin and Butte primarily by the combination of peaks eluting from 6 to 7 min, whereas, Butte and Grandin differ only by the peaks eluted in the 3.8–4.0 min range. The two soft wheats, Arthur (soft red winter) and Nugaines (soft white), which are very different genetically, also exhibit large differences in their CZE patterns (Fig. 9). One major difference is that the largest peak in Nugaines elutes at 4.2 min, where the largest peak in Arthur elutes at 5.1 min. Many other differences are readily seen.

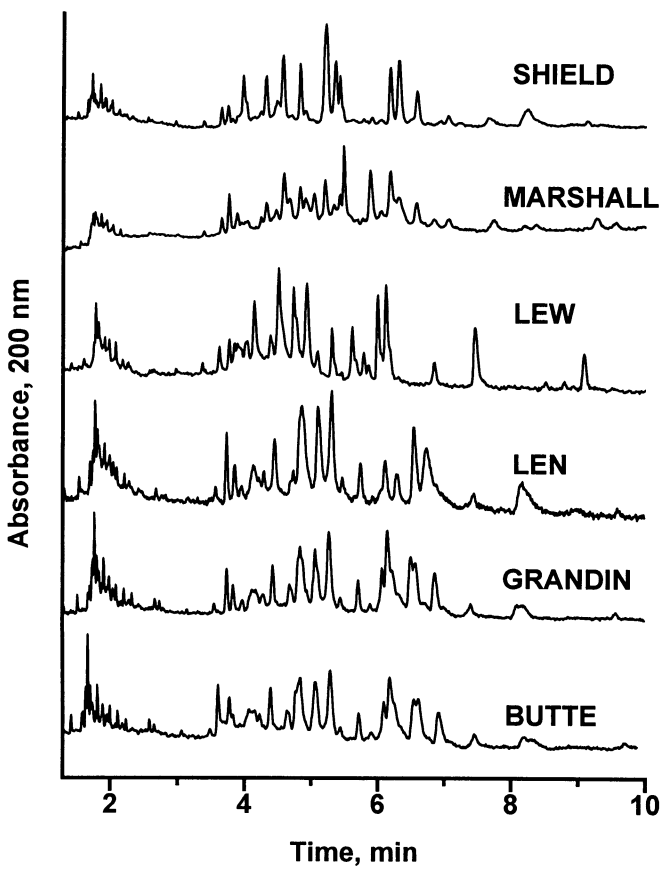

Fig. 8. Capillary zone electrophoresis patterns of gliadins of hard red spring cultivars Butte, Grandin, Len, Lew, Marshall, and Shield. Proteins were separated by the recommended method with 30% ethanol, pressure injected (4 sec, 0.25 nl) into a 27-cm long (20 cm to detector) 20-μm i.d. capillary, and separated at 45°C and 22 kV for 10 min in 0.1*M* pH 2.5 phosphate buffer containing a polymer additive. All patterns normalized to largest peak.

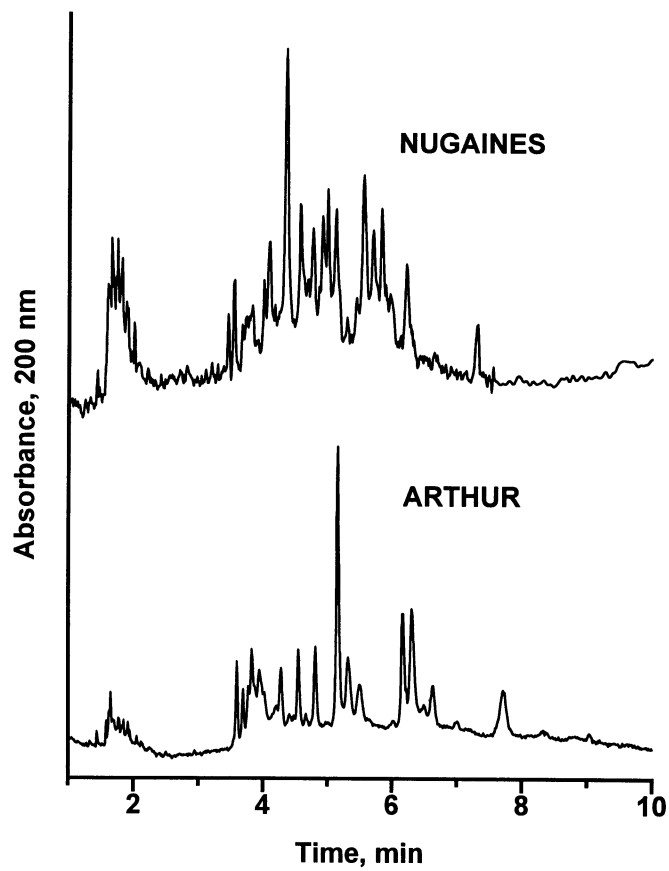

Fig. 9. Capillary zone electrophoresis patterns of gliadins of soft wheat cultivars Arthur and Nugaines. Proteins were separated by the recommended method with 30% ethanol, pressure injected (4 sec, 0.25 nl) into a 27-cm long (20 cm to detector) 20-μm i.d. capillary, and separated at 45°C and 22 kV for 10 min in 0.1*M* pH 2.5 phosphate buffer containing a polymer additive. All patterns normalized to largest peak.

CONCLUSIONS

CZE is a high-resolution, fast method for cultivar identification and purity testing. In the wheat cultivars examined, CZE gave better resolution and shorter analysis times than either A-PAGE or HPLC.

The recommended method (20-μm i.d. capillary) produced wheat gliadin separations at least as good as those reported by Bietz (1994), Bietz and Schmalzried (1992, 1993), and Werner et al (1994), and it was much faster.

Uncoated glass capillaries 27 cm long (20 cm to the detector) that are either 20- or 50-μm i.d. are useful for protein separation (fractionation) and, hence, for cultivar differentiation. The recommended method for fast CZE analyses is to use 20-μm i.d. capillaries. The 20-μm i.d. capillaries build up less heat due to lower currents than do the 50-μm i.d. capillaries. Therefore, higher voltages can be used to obtain faster analyses. The reproducibility and resolution of the 20-μm i.d. capillary was nearly identical to that of the 50-μm i.d. capillary, but separation took less than half the time.

The amount of sample injected affects the resolution of the CZE capillaries. In general, longer injection times (injection volumes >0.5% of column volume) degrade the resolution. The optimal injection time for the 20-μm i.d. capillary was 4 sec (about 0.25 nl or ~0.3% of total volume), and the optimal injection time for the 50-μm i.d. capillary was 1 sec (2.44 nl, or 0.46%).

In conclusion, CZE is a technique well-suited to separate cereal protein samples. It provides an alternative selectivity to conventional techniques of PAGE and HPLC combined with inherently high separation efficiencies. Other features that make it an attractive alternative are: 1) simplicity (i.e., no pumps, seals, or gels); 2) quantitative digital data; 3) speed (a few minutes); and 4) small sample requirements (a few microliters).

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