Acetonitrile as a Buffer Additive for Free Zone Capillary Electrophoresis Separation and Characterization of Maize (*Zea mays* L.) and Sorghum (*Sorghum bicolor* L. Moench) Storage Proteins

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An improved method for separating and characterizing maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L. Moench) storage proteins by free zone capillary electrophoresis (FZCE) was developed. Previous electrophoretic methods for analyzing these proteins required high concentrations of urea to maintain protein solubility during separation. To overcome disadvantages of urea, we developed a FZCE method that mimicked reversed-phase high-performance liquid chromatography (RP-HPLC) in that it used high levels of acetonitrile (ACN) at low pH. The optimized FZCE buffer system consisted of 80 mM phosphate−glycine buffer, nominal pH 2.5, containing 60% ACN and a cellulose derivative to dynamically coat capillary walls. Resolution was similar to or higher than that previously achieved by FZCE buffers utilizing 8 M urea as a buffer additive. ACN concentrations of at least 50% were necessary to achieve acceptable separations; this ACN concentration is approximately that necessary to extract these storage proteins. ACN was equally effective as traditional ethanol solvents and 8 M urea for solubilizing maize and sorghum proteins. The ACN-based FZCE buffer system gave high repeatability (<0.3% relative standard deviation, measured over 15 consecutive injections) for migration time. Subclasses of maize and sorghum storage proteins were identified, and genotypes of each cereal were successfully differentiated using ACN-containing buffers. This FZCE method may be applicable for the analysis of other hydrophobic proteins without the use of urea.

Keywords: *Maize; sorghum; proteins; acetonitrile; capillary electrophoresis; hydrophobic*

INTRODUCTION

Maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L. Moench) are cereal grains that are used mainly as animal feed in North America but are widely used as human food in other parts of the world. This is especially true of sorghum, a human food staple in parts of Africa. Maize and sorghum vary in protein content from 6−18% (Lasztity, 1984), and storage proteins generally comprise 70−90% of the total protein (Lookhart, 1991; Laszity, 1984). Zeins are the major storage proteins of maize (Wilson, 1987; Esen, 1987), while those of sorghum are kafirins (Laszity, 1984).

Like storage proteins of other cereals, zeins and kafirins may be divided into subclasses. Many systems of nomenclature have been proposed for these proteins (Esen, 1987; Wilson, 1991), leading to confusion as to their identities. For this work, the nomenclature of Esen (1987) is followed, dividing zeins into α, β, and γ groups based on solubility, molecular weight, immunology, and structure. This same nomenclature has been applied to sorghum proteins (Shull et al., 1991), using the criteria developed by Esen (1987). For maize, α-zein comprises 75−85% of total zein, β-zein 10−15%, and γ-zein 5−10% (Esen, 1987; reviewed in Hamaker et al., 1995). For sorghum, α-kafirin represents 66−84% of total kafirin, β-kafirin 7−8%, and γ-kafirin 9−12% (Watterson et al., 1993; reviewed in Hamaker et al., 1995).

While all cereal proteins provide interesting separation challenges, zeins and kafirins are probably the most difficult to successfully separate. They generally have a relatively small molecular weight distribution (Cooke, 1984), so sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) provides limited information (Lookhart, 1991). These proteins are also more hydrophobic than prolamins of other cereals and often aggregate and precipitate under separation conditions used successfully for prolamins of cereals such as wheat and barley (Taylor and Schussler, 1984). Because of this, polyacrylamide gel electrophoresis (Taylor and Schussler, 1984) or isoelectric focusing of maize and sorghum prolamins often necessitates the presence of high concentrations (5−8 M) of urea (reviewed by Lookhart, 1991).

In addition to these slab gel methods, two procedures for separating zeins by capillary electrophoresis (CE) have been reported. The first uses a commercially

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available SDS−CE reagent (Parris et al., 1997) and gives separations analogous to those of SDS−PAGE. This method reliably quantifies zeins, but resolution is not high (two to four major peaks), consistent with zein's narrow molecular weight distribution. The second CE method is free zone CE (FZCE), using a novel aspartic acid isoelectric buffer in conjunction with 8 M urea to maintain protein solubility (Righetti et al., 1998). Unlike SDS−CE, in FZCE proteins separate based on differences in charge density. This FZCE buffer system gave high resolution and could differentiate maize genotypes. This later methodology was used in combination with multivariate statistics to differentiate 21 maize inbred and hybrid genotypes (Olivieri et al., 1999).

While urea is an effective protein solubilizer, high concentrations of urea can cause problems. Urea solutions are prone to crystallization, necessitating frequent cleaning of instruments (Wehr et al., 1998). The presence of high levels of urea may also increase background absorbance of buffers in the low UV range (Wehr et al., 1999), and under certain conditions, urea can modify proteins (O'Farrel, 1975). The formation of charged species in the urea also shortens the shelf life of urea-based buffers (Olivieri et al., 1999).

Like electrophoresis, reversed-phase high-performance liquid chromatography (RP-HPLC) has been successfully used to separate maize (reviewed in Paulis and Bietz, 1994) and sorghum (Sastry et al., 1985; reviewed in Smith, 1994) storage proteins. In these separations, proteins typically separate at low pH in the presence of high levels of an organic solvent, usually acetonitrile (ACN). Maize and sorghum prolamins are considerably more hydrophobic than storage proteins of other cereals; for maize and sorghum, most elute upon RP-HPLC at 50−70% ACN (Paulis and Bietz, 1994; Smith, 1994). Under these conditions, no problems with protein solubility occurred and good separations resulted. We hypothesized that similar "RP-HPLC"-like conditions − i.e., low pH combined with high levels of ACN − should be possible in FZCE. Such a method would avoid the inconveniences and problems associated with use of concentrated urea solutions. Zhu et al. (1989) also hypothesized that high levels of ACN may prove useful in FZCE separations of hydrophobic proteins.

As described here, ACN was found to be a highly suitable buffer additive for FZCE of maize and sorghum storage proteins, as it is for FZCE separations of other cereals (Bietz and Schmalzried, 1995; Lookhart and Bean, 1996; Bean et al., 1999; Lookhart et al., 1999). No problems with solubility occurred; in fact, ACN was equally effective as a solvent for maize and sorghum prolamins as were traditional 8 M urea and ethanol solvents. ACN is also an attractive buffer modifier since it avoids potential problems of crystallization and protein modification often associated with urea and has a low UV cutoff, making it a suitable buffer modifier even at high concentrations.

MATERIALS AND METHODS

Sample Preparation and Extraction. Whole maize and sorghum kernels (~1 g) were ground in a commercial coffee grinder for 30 s using two 15-s pulses. Sorghum samples (genotypes NK KS710, DK-40y, Pioneer 8500, NC+6B50, Mycogen 1482, DK-35, Mycogen 1506, and Pioneer 8699) were obtained from R. L. Vanderlip, Department of Agronomy, Kansas State University, Manhattan, KS 66506. Maize genotypes (B73, Mo17, A632, Mo17×B73, H99, A632×H60, H99×A632, and H60) were obtained from L. F. Bauman, Department of Agronomy, Purdue University, W. Lafayette, IN 47907. For all extractions, 250 mg of ground material was used. Albumins and globulins were first extracted as described previously (Bean and Lookhart, 1998). Storage proteins were then extracted with various solvents, as described in the text. All samples were extracted with 1 mL of solvent for 45 min with continuous vortexing, using a Vortexgenie2 equipped with a 30-place vial holder (Scientific Instruments, Bohemia, NY).

Table 1. RP-HPLC Gradient Used for Zein Separations[a]

time (min)	% B[b]
0	28
20	60
35	75
40	85

[a] Flow rate was 0.5 mL/min, and temperature was 55 °C. [b] Solvent B was ACN/0.1% TFA (w/v), and solvent A was water/0.1% TFA (w/v).

Table 2. RP-HPLC Gradient Used for Kafirin Separations[a]

time (min)	% B[b]
0	25
40	72
46	80

[a] Flow rate was 0.5 mL/min, and temperature was 50 °C. [b] Solvent B was ACN/0.1% TFA (w/v), and solvent A was water/0.1% TFA (w/v).

FZCE. All separations were done on a Beckman PACE 5510 or a PACE 2100 instrument, using 25- or 50-μm i.d. × 27-cm (20 cm to detector) uncoated fused silica capillaries (Polymicro Technologies, AZ). Capillaries were equilibrated with separation buffer for 30 min before each series of analyses (Bean and Lookhart, 1998). Capillaries of 25-μm i.d. were rinsed between separations for 2 min with 500 mM acetic acid and then with separation buffer for 4 min (Bean and Lookhart, 1998). When using 50-μm i.d. capillaries, rinse times were reduced to 0.5 min with 500 mM acetic acid between runs and 1 min with separation buffer. When not in use, capillaries were rinsed with 500 mM acetic acid for 10 min, followed by water for 10 min and finally by nitrogen gas for 10 min. Separation voltages varied from 12.5 to 20 kV, and separation temperatures varied from 25 to 50 °C. Buffers were composed of phosphate−glycine, having a nominal pH of 2.5; buffer concentration varied from 20 to 100 mM. Acetonitrile was tested as a buffer additive at concentrations of 0−70%. Isoelectric aspartic acid buffer composition and separation conditions were as by Righetti et al. (1998), except that separation temperature was 45 °C; all separations were monitored at 200 nm.

Samples were injected with pressure (0.5 psi) for 1 s when using 50-μm i.d. capillaries and for 4 s when using 25-μm i.d. capillaries with glycine−phosphate buffer. When using the isoelectric buffer in the 50-μm i.d. capillaries, samples were injected for 5 s.

RP-HPLC. RP-HPLC separations were done using a Hewlett-Packard 1090D system equipped with a 4.6-mm × 250-mm Jupiter 300 Å C_{18} column (Phenomonex, Torrance, CA) with security guard columns (Phenomonex, Torrance, CA). Maize proteins were separated using a gradient modified from that of Paulis and Bietz (1986) (Table 1). Sorghum proteins were separated using the gradient shown in Table 2.

Subclass Identification. Subclasses of maize and sorghum proteins were located in FZCE by collecting 1-min aliquots of RP-HPLC separations (Bean and Lookhart, 1997) and analyzing them by SDS−PAGE and FZCE. Aliquots were lyophilized immediately after collection and redissolved in either 50 μL of 70% ethanol + 0.5% Na acetate + 5% β-mercaptoethanol (β-ME) for FZCE or 50 μL of 123 mM Tris-HCl buffer, pH 8.5, containing 1% SDS, 2.5% β-ME, 5% sucrose, tracking dye, and 4 M urea for SDS−PAGE. Subclasses were identified by comparing SDS−PAGE separations of the collected fractions to separations shown in Esen (1987) and Shull et al. (1991).

This allowed identification of subclasses in both the RP-HPLC and FZCE separations. The selective extraction procedure of Shull et al. (1991) was also used to identify protein subclasses. Samples were extracted according to Shull et al. (1991) and aliqouts analyzed by FZCE and RP-HPLC and also by SDS−PAGE. By comparing the results of the RP-HPLC and FZCE separations to the SDS−PAGE results, the locations of subclasses could be determined.

SDS−PAGE. SDS−PAGE was done using 15% T acrylamide gels in a minigel system (Novex, San Diego, CA). All gels contained 5 M urea (Hamaker et al., 1995). Gels were electrophoresed at 100 V until the dye front was approximately 1 cm from the bottom. Gels were then stained with gelcode blue (Pierce, Rockford, IL) according to manufacturer's directions and destained with water. For fractions from RP-HPLC separations, gels were silver-stained by the method of Blum et al. (1987).

RESULTS AND DISCUSSION

Optimization of Separations. Both maize and sorghum storage proteins have been successfully separated by RP-HPLC (Bietz and Paulis, 1994; Sastry et al., 1985; Smith, 1994). Typically, RP-HPLC is conducted at low pH in the presence of high levels of an organic modifier, usually ACN. Thus, we first attempted to determine if 'RP-HPLC conditions' could be successfully used in FZCE to separate maize and sorghum proteins without using urea or a charged detergent to maintain solubility. We first tested the effect of adding ACN to a low-pH FZCE phosphate−glycine separation buffer. This buffer has been used successfully with wheat, oats, rice (Bean and Lookhart, 1998), and barley (Lookhart et al., 1999) and has good solubility in ACN. Low-concentration buffers (20 mM) were used to mimic typical RP-HPLC conditions. Results of increasing ACN concentration on separation of maize and sorghum storage proteins are shown in Figure 1. Addition of ACN has a profound effect on FZCE separations of maize and sorghum storage proteins. When up to 40% ACN was used to separate maize proteins, poor, irreproducible separations resulted, and capillaries had to be rinsed extensively with separation buffer before reuse. At ACN concentrations above 50%, however, resolution improved and was best at 60% and 70% ACN. Similarly, use of 50−70% ACN gave good separations of kafirins. The optimum ACN concentration was judged to be 60% for both maize and sorghum.

The effect of increasing buffer concentration was next studied (Figure 2). For both maize and sorghum proteins, resolution increased as buffer concentration increased from 20 to 80 mM. At 100 mM, resolution of sorghum proteins decreased slightly, particularly at 10−12 min (Figure 2B); thus, a buffer concentration of 80 mM was chosen as optimal for both cereals.

When the separation temperature was decreased from 50 to 25 °C, little change in resolution was seen (Figure 3). These results were somewhat surprising, since elevated temperature is often beneficial in RP-HPLC separations of wheat and maize proteins (Bietz and Cobb, 1985), presumably due to disruption of hydrogen bonds. Other cereals, when separated by FZCE in low-pH phosphate buffers, also show improved protein separations at high temperatures (Lookhart and Bean, 1995; Bietz and Schmalzried, 1995). Decreasing the temperature in FZCE to 25 °C (Figure 3) slightly modified selectivity and improved resolution of some closely migrating peaks but also lengthened separation time. Thus, 45 °C was adopted as the separation temperature for routine analysis.

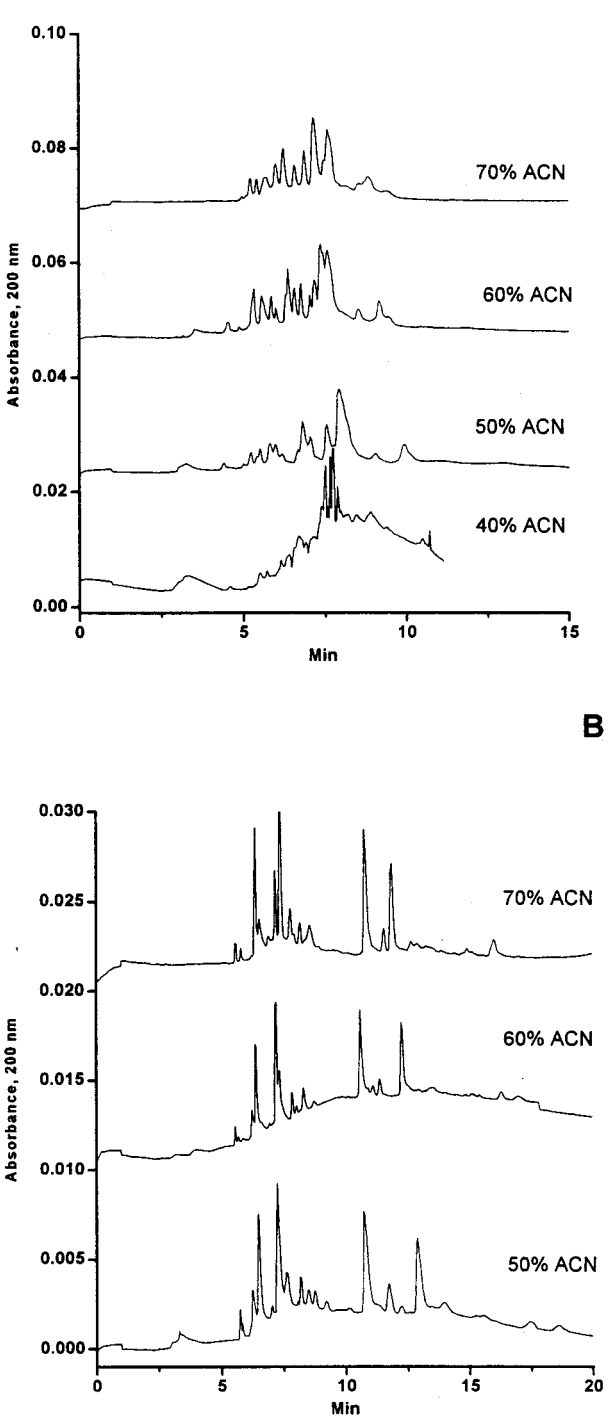

Figure 1. Effects of ACN addition on FZCE separations of zeins and kafirins. Samples were separated in 20 mM phosphate−glycine buffer, pH 2.5, containing 0.05% HPMC and levels of ACN indicated. Samples were analyzed in 25-μm i.d. × 27-cm (20-cm L_d) uncoated capillaries at 12.5 kV and 45 °C. Proteins were extracted with 70% ethanol + 5% β-ME as described in the text. Samples were injected by pressure at 0.5 psi: (A) zeins; (B) kafirins.

Due to the high levels of ACN and low conductivity of the phosphate−glycine separation buffers, low currents were generated (Bean and Lookhart, 1998). In an attempt to reduce separation time, voltage was increased to 20 kV. This did not disturb baselines and resulted in more rapid separations but decreased resolution (data not shown). Thus for routine analysis, 12.5 kV was adopted as the separation voltage. Higher voltages may be useful for rapid genotype discrimina-

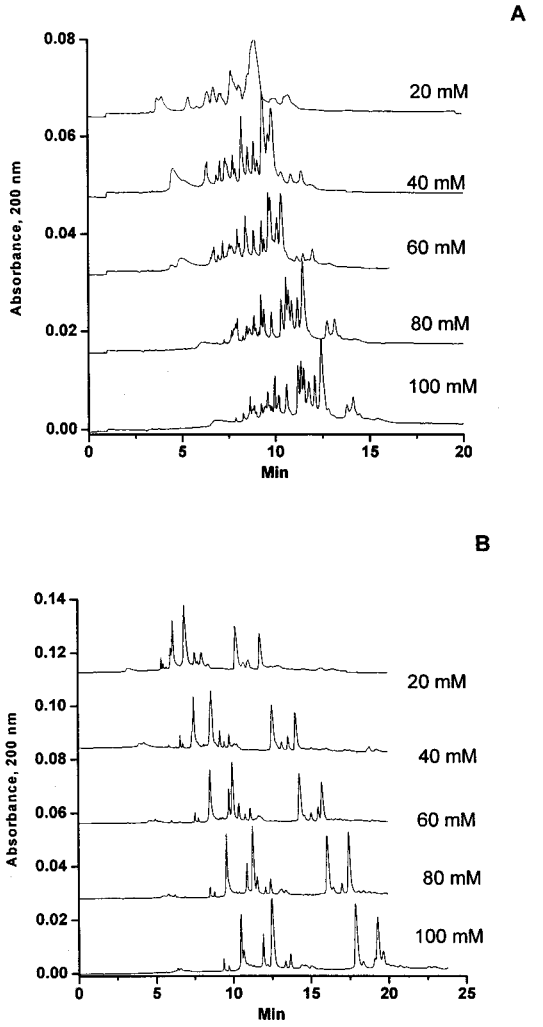

Figure 2. Effect of increasing buffer concentration on FZCE separations of zeins and kafirins. Extraction and separation conditions were as in Figure 1: (A) zeins; (B) kafirins. Separation buffer concentrations are indicated. Each buffer contained 60% ACN and 0.05% HPMC.

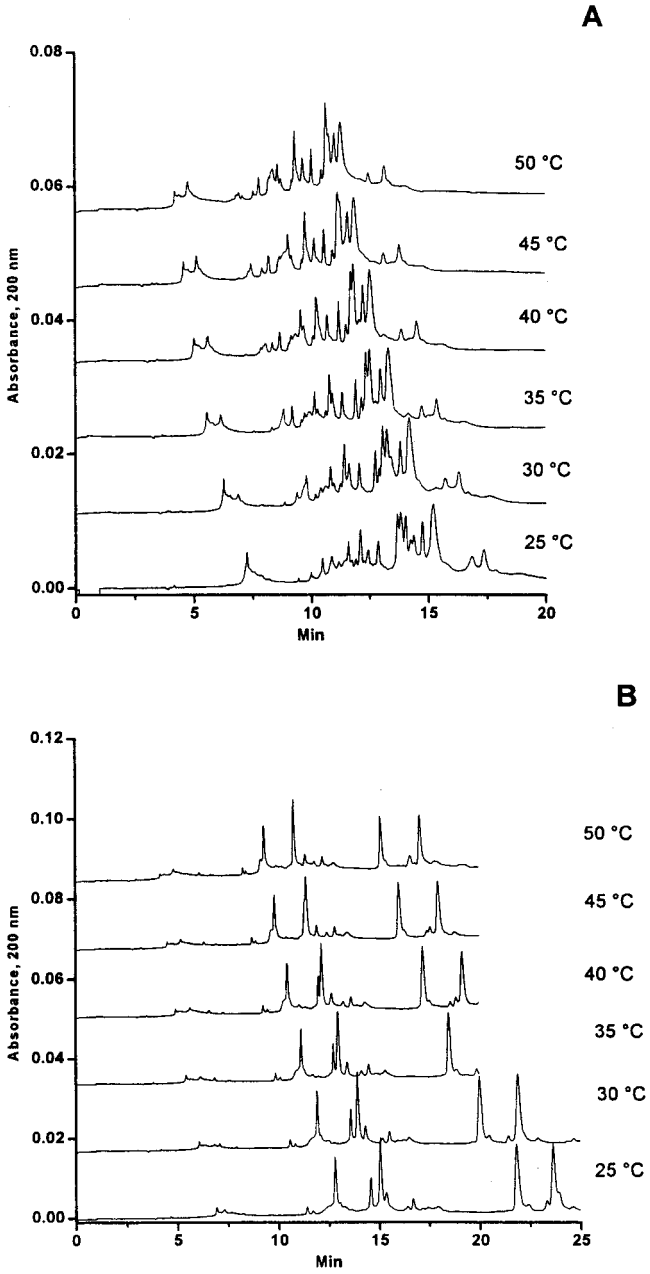

Figure 3. Effect of separation temperature on zein and kafirin FZCE separations. Zeins (A) and kafirins (B) were separated in 80 mM phosphate−glycine buffer, pH 2.5, containing 60% ACN and 0.05% HPMC. Extraction and separation conditions were as in Figure 1, except for temperature, as indicated.

tion, but further research is needed to determine whether this affects the ability of FZCE to differentiate closely related genotypes.

Using these optimal separation conditions, several hydrophilic polymeric additives were tested on separations of maize and sorghum proteins. These additives dynamically coat capillary walls, preventing protein adsorption to fused silica. Previous studies with wheat showed the importance of these polymers in cereal protein separations (Bean and Lookhart, 1998), and several types have been used successfully (Bean and Lookhart, 1998; Capelli et al., 1998). On the basis of previous studies (Bean and Lookhart, 1998; Capelli et al., 1998), we tested hydroxypropylmethyl-cellulose (HPMC), poly(ethylene oxide), hydroxypropyl-cellulose, and hydroxyethyl-cellulose (HEC) as modifiers, at concentrations of 0.5−0.05%. All gave essentially identical results with both maize and sorghum proteins (data not shown).

Buffer Comparisons. Optimized FZCE separations of zein and kafirin were next compared to previous FZCE buffer systems used to separate maize proteins (Righetti et al., 1998; Oliveiri et al., 1999) (Figure 4). Resolution and separation time for maize proteins (Figure 4A) were similar in the two buffer systems, although major differences in selectivity occurred. Sorghum proteins, however, showed quite different patterns − especially with respect to separation time − using the two buffer systems (Figure 4B), though both gave similar resolution. Thus, ACN appears to be an equally effective buffer modifier as 8 M urea, and it lacks the disadvantages typically associated with urea. For this comparison, all samples were separated in 50-μm i.d. capillaries at 45 °C; this temperature is higher than that of Righetti et al. (1998) and may explain why separations in the aspartic acid buffer were more rapid than reported by Righetti et al. (1998). Note also that separation in the phosphate−glycine buffer system had the same separation time as the isoelectric aspartic acid/urea system, despite the higher separation voltage used with the isoelectric buffer.

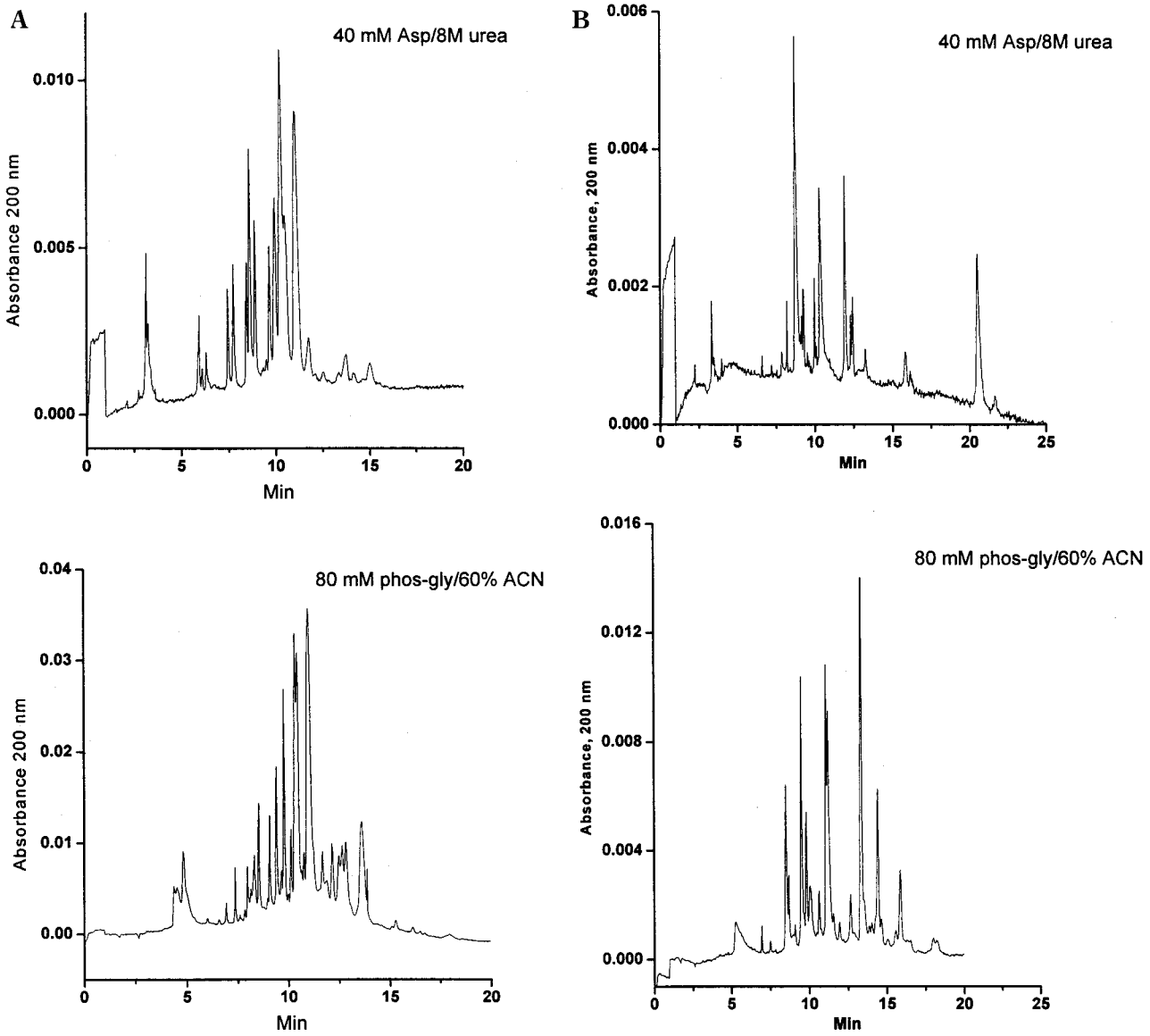

Figure 4. Comparison of FZCE separations of maize and sorghum prolamins in isoelectric/urea and phosphate−glycine/ACN buffers: (A) zeins (genotype Mo17); (B) kafirins (genotype Mycogen 1506). The 40 mM aspartic acid/8 M urea buffer contained 0.5% HEC, and the 80 mM phosphate−glycine/60% ACN buffer contained 0.05% HPMC. Samples were extracted with 70% EtOH + 5% β-ME after removal of albumin and globulin proteins. Separation conditions with phosphate buffer were as in Figure 1; separations in aspartic acid buffer were at 21.6 kV and 45 °C. For both buffers, 50-μm i.d. × 27-cm capillaries were used. Samples were injected for 1 s with the phosphate−glycine buffer and 5 s with the Asp/urea buffer.

Attempts were made to use 40 mM aspartic acid (Righetti et al., 1998), in ACN-containing buffers; however, 40 mM aspartic acid was insoluble in 60% ACN. Similarly, iminodiacetic acid (IDA) (Bossi and Righetti, 1997), also an isoelectric buffer, was tested with ACN; but at concentrations of 100 mM, IDA was also insoluble in 60% ACN. Studies are in progress to find other organic solvents or combinations of solvents that could be used with various concentrations of IDA.

Protein Solubility in Acetonitrile. The above studies show ACN to be a useful modifier for FZCE separations of maize and sorghum storage proteins. Several factors may explain its success. Organic modifiers can alter the zeta potential at the capillary wall and alter electroosmotic flow rates in FZCE (Schwer and Kenndler, 1991); this mechanism might affect maize/sorghum protein separations. High ACN concentrations may also simply maintain protein solubility during electrophoresis, as suggested by the observation that no useful separations occurred below a critical ACN concentration and that only minor changes took place at higher ACN concentrations. To test whether a minimum ACN concentration is necessary to maintain protein solubility, maize and sorghum samples were extracted with 30−70% ACN + 5% β-ME. Results were compared to solubilities in 70% ethanol + 5% β-ME and in 70% ethanol/0.5% Na acetate + 5% β-ME, which are routinely used to extract maize proteins (Paulis and Wall, 1977), and in 8 M urea + 5% β-ME. Extracted proteins were analyzed by RP-HPLC, and peak areas of extracted material were quantified by integration.

Extraction results for maize are shown in Figure 5. Relatively minor amounts of α-zein were extracted with ACN (under reducing conditions) until the concentration reached 50%, which was also the minimum necessary in FZCE to achieve useful separations. Solubilities of β- and γ-zeins were similar, except that 40% ACN was also an effective solvent, in agreement with the less hydrophobic nature of these proteins in comparison to α-zeins (Paulis and Bietz, 1994). Thus, it appears likely

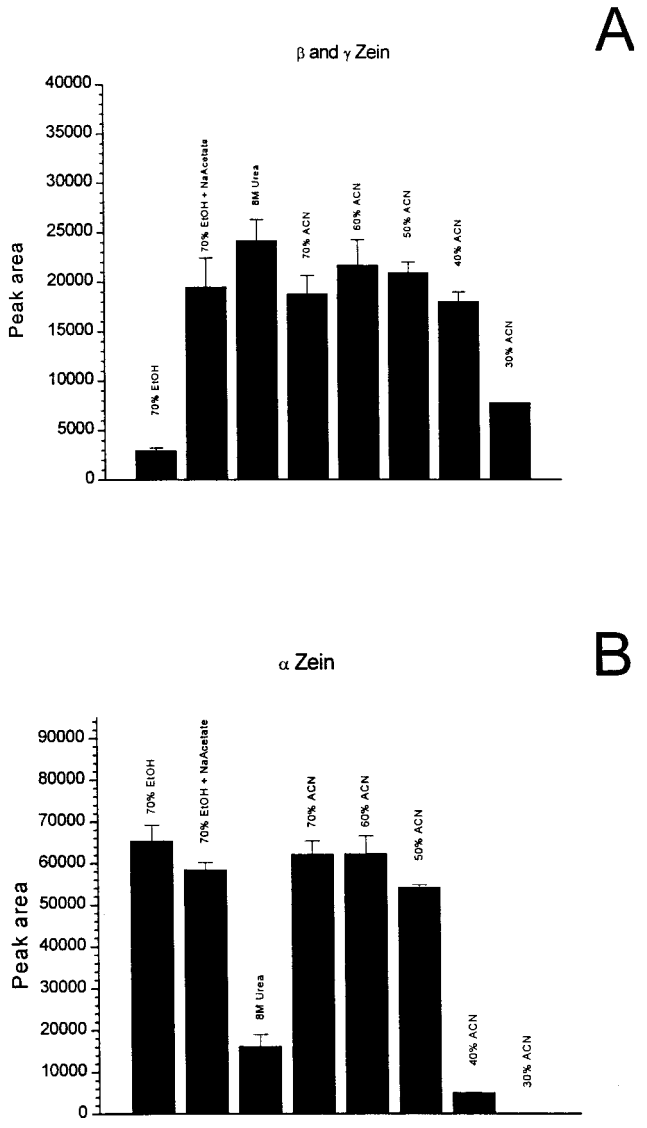

Figure 5. Extraction of zeins by various solvents. All extractions were performed under reducing conditions (i.e., in the presence of 5% β-ME): (A) β- and γ-zein subclasses; (B) α-zein subclass. Error bars indicate standard deviation ($n = 2$).

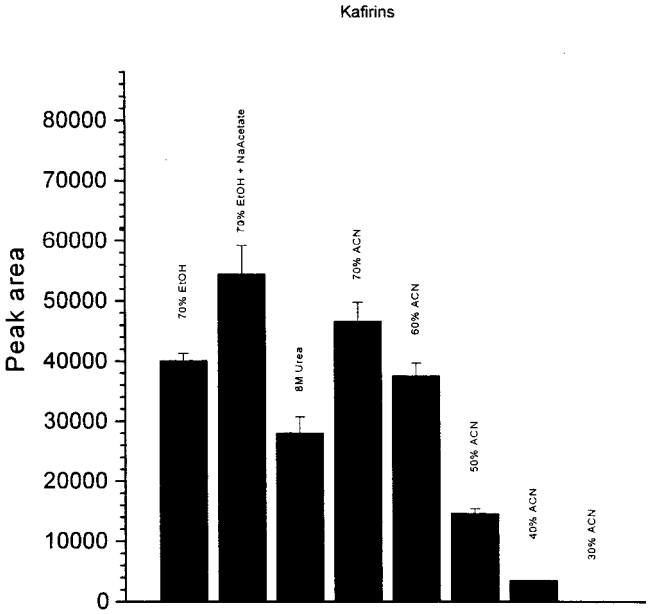

Figure 6. Comparison of solvent efficiencies for kafirin solubility. Error bars indicate standard deviation ($n = 2$).

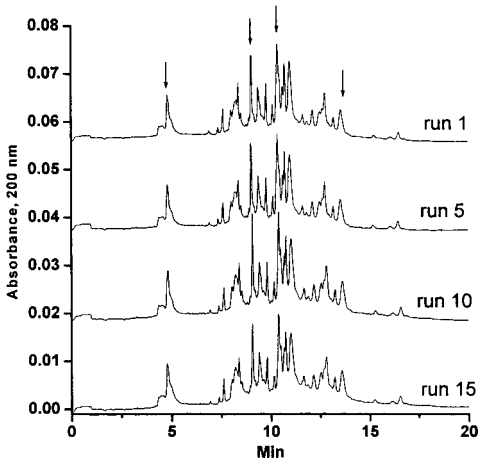

Figure 7. Repeatability of zein FZCE separations. Results for 4 of 15 consecutive injections are shown. Peaks marked with arrows were used to calculate migration time repeatability. Separation buffer was 80 mM phosphate glycine (pH 2.5) + 60% ACN and 0.05% HPMC. Separation conditions were the same as in Figure 1, except a 50-μm i.d. capillary was used.

that the effect of ACN on FZCE separations is primarily to keep proteins soluble, and comparable minimum ACN concentrations are necessary for extraction and for FZCE. This may explain the apparent lack of success previously reported (Righetti et al., 1998) in using ACN as an FZCE additive for zein separations: tested levels were simply not high enough. The data in Figure 5 also clearly show that 50−70% ACN (+5% β-ME) is as effective a solvent for maize proteins as both ethanol and urea solutions.

Extraction results were similar for sorghum proteins (Figure 6), except that a slightly higher (i.e., 60%) ACN concentration was required for maximum extraction; this agrees with the higher hydrophobicity of kafirins. Data in Figure 6 are for total kafirins, since kafirin subclasses did not resolve as completely upon RP-HPLC as did those of zein. Again, 60−70% ACN was as effective as traditional ethanol-containing solvents and urea solutions for extracting kafirins.

Capillary Diameter. Since phosphate−glycine−ACN buffers generate low currents (Bean and Lookhart, 1998), effects of increasing capillary diameter were examined. Separations in 50-μm i.d. capillaries were similar to those in 25-μm i.d. capillaries, but larger capillary inner diameters provide higher sensitivity and are less prone to plugging. Resolution was slightly higher in the 50-μm i.d. capillaries (data not shown). Capillary rinse times can also be reduced using larger inner diameter capillaries. Thus, 50-μm i.d. capillaries were used for the remainder of this project.

Repeatability. To test FZCE repeatability of the ACN buffer system, 15 consecutive injections of maize proteins were made using 50-μm i.d. capillaries (Figure 7). Run-to-run repeatability was excellent, with RSD's of <0.3% for migration time when using ACN as a buffer additive (Table 3). These values are similar to those achieved upon FZCE of other cereal proteins using similar buffers with ACN as a buffer additive (Bean et al., 1998). For maximum repeatability, samples were analyzed within 18−24 h after they were prepared.

Day-to-day and capillary-to-capillary repeatabilities were also evaluated. Day-to-day variation in peak

Table 3. FZCE Migration Time Repeatability for 15 Consecutive Injections

	migration time (min)[a]			
run	peak 1	peak 2	peak 3	peak 4
1	4.81	9.02	10.34	13.55
2	4.81	9.02	10.35	13.55
3	4.80	9.02	10.35	13.56
4	4.79	9.02	10.34	13.55
5	4.79	9.01	10.34	13.53
6	4.79	9.03	10.36	13.55
7	4.79	9.04	10.36	13.56
8	4.78	9.04	10.34	13.53
9	4.78	9.02	10.35	13.54
10	4.79	9.06	10.39	13.62
11	4.79	9.07	10.40	13.64
12	4.78	9.07	10.40	13.64
13	4.77	9.07	10.40	13.63
14	4.77	9.06	10.39	13.62
15	4.77	9.05	10.38	13.61
AVG	4.79	9.04	10.37	13.58
SD	0.01	0.02	0.02	0.04
RSD (%)	0.27	0.25	0.24	0.29

[a] Corrected peak area = peak area/migration time of peak (Altria, 1993).

migration times (measured across 3 days) was between 0.3 and 3% RSD (data not shown). Capillary-to-capillary variation (measured on four different capillaries) was between 0.3 and 5% RSD. Some differences in selectivity were noted for a few peaks between capillaries. Again, a standard sample should be analyzed with every data set to allow long-term comparisons between samples analyzed several days apart or on different capillaries (Lookhart and Bean, 1996).

Some changes in FZCE separation patterns occur in extracts much older than 24 h, especially if exposed to air; this may indicate re-formation of disulfide bonds. The importance of achieving and maintaining complete reduction of disulfide bonds is also indicated by the observation that extractability of γ-zeins and γ-kafirins is often variable and appears to change with sample age. Use of fresh samples and control extracts is recommended to ensure reproducible results.

During these studies, samples were routinely pre-extracted with salt solutions and water, thus removing albumins and globulins that may otherwise bind to capillary walls (Bean and Lookhart, 1998). If, for example, sorghum proteins are extracted directly without pre-extraction, resolution is poor and capillary surfaces become fouled. It may be possible that water- or salt-soluble compounds other than albumins and globulins interfered with separations, especially in sorghum. This phenomenon requires further investigation.

Previous FZCE methods utilizing aspartic acid/urea buffers have exhibited problems with migration time repeatability (Olivieri et al., 1999). However in the current study, excellent run-to-run migration time repeatability was achieved (Table 3). The exact reason for this discrepancy is not known but may be partly due to sample preparation differences between the two studies. In this study, albumin and globulin proteins were removed prior to analysis as these proteins, or other unidentified water/salt-soluble compounds, are known to interfere with FZCE separations, at least in wheat (Bean and Lookhart, 1998). Furthermore, a post-separation rinse with 500 mM acetic acid was used in this study to help remove contaminants from capillary walls (Bean and Lookhart, 1998). Slight differences in pH between the two buffer systems could also be responsible for differences in repeatability. The buffer in this study had a nominal pH of 2.5, while that of the aspartic acid/urea had an apparent pH of 3.9 (Olivieri et al., 1999). This higher pH would lead to a stronger negative charge on the capillary wall which may provide more interaction with the positively charged proteins, a factor discussed in greater detail in Capelli et al. (1998) and Olivieri et al. (1999).

Protein Subclass Characterization. Cereal storage proteins consist of many types, often classified on the basis of factors including solubility, size, composition, and polymeric nature. To use FZCE optimally for maize and sorghum proteins, we thus needed to identify mobility characteristics of their subclasses. To do this, procedures similar to those used to identify gliadin subclasses were followed (Lookhart and Bean, 1995; Bean and Lookhart, 1997). Proteins were first separated by RP-HPLC, and continuous aliquots were collected. Unlike with wheat, RP-HPLC fractions had to be concentrated before FZCE to permit detection of β and γ subclasses, present at low levels (Hamaker et al., 1995). We also used larger (50-μm i.d.) capillaries to enhance detection of β and γ subclasses. Due to the low conductivity of the phosphate−glycine buffer and high levels of ACN present in the buffer, poor sample stacking occurred when injecting large volumes of fractions collected from RP-HPLC separations. Using concentrated samples and larger diameter capillaries helped to overcome these limitations. In addition to collecting samples from RP-HPLC separations, the selective extraction procedure of Shull et al. (1991) was used to locate protein subclasses.

Migration and elution characteristics of maize and sorghum protein subclasses are shown in Figure 8. For maize proteins, the FZCE migration order (γ, β, α) was the same as the elution order in RP-HPLC (Paulis and Bietz, 1994) and in acidic polyacrylamide gel electrophoresis (Esen, 1987). The β-zein subclass, however, migrated within the α-zein region, but γ-zeins were well-separated (Figure 8A). Resolution of the FZCE separation was equal to or superior to that of the RP-HPLC separation for the α-zeins, but somewhat lower for γ-zeins. SDS−PAGE analysis of the collected RP-HPLC fractions used to locate the zein subclasses is also shown in Figure 8A.

The FZCE migration order of sorghum proteins was the same as in maize (i.e., γ, β, α), but again β-kafirins overlapped with the α-kafirins, as they did in RP-HPLC (Figure 8B). The resolution of the FZCE separation was slightly better than that of the RP-HPLC, and the separation time was much faster; this was also true of the maize separations. SDS−PAGE analysis of the fractions collected from the RP-HPLC used to locate the kafirin subclasses is shown in Figure 8B.

In general, FZCE resolution was equal to or better than that achieved by RP-HPLC. Thus, FZCE can provide high-resolution separations of both maize and sorghum proteins, but without the waste generated by HPLC or the expense of RP-HPLC columns.

Genotype Comparisons. Prolamins extracted from several inbreds and hybrids of maize (Figure 9) and sorghum (Figure 10) were compared by FZCE to determine its ability to differentiate genotypes. All maize genotypes could be successfully differentiated based on unique qualitative and quantitative differences. The hybrid A632×H99 was similar to both A632 and H99,

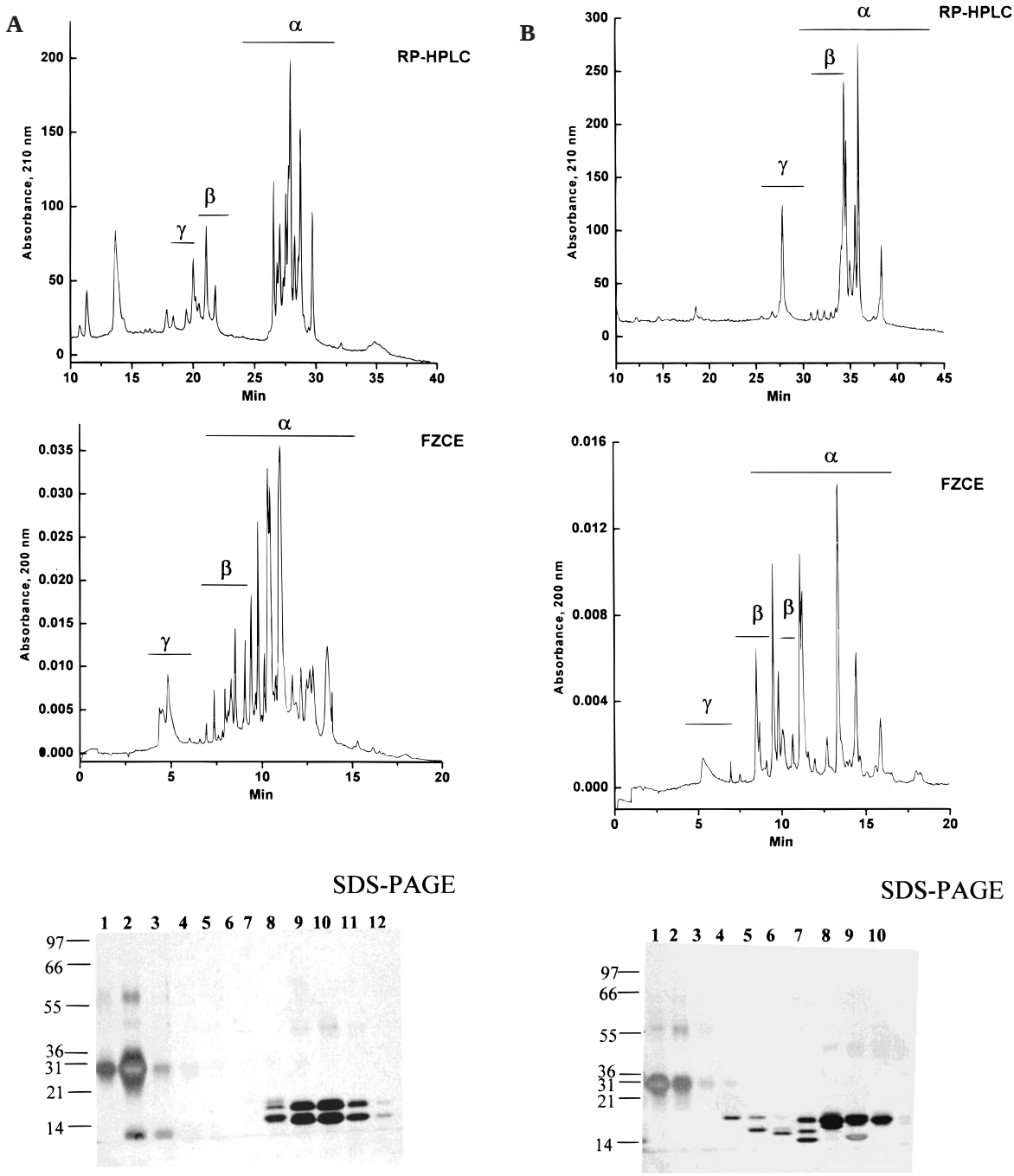

Figure 8. FZCE migration and RP-HPLC elution positions of subclasses of maize (A) and sorghum proteins (B). Proteins extracted with 70% ethanol + 0.5% Na acetate + 5% β-ME were first fractionated by RP-HPLC, and collected fractions were then analyzed by FZCE and SDS−PAGE. FZCE separation conditions were as in Figure 7. SDS−PAGE analysis of the fractions collected from RP-HPLC separations are also shown. Lane numbers on the SDS−PAGE gels correspond to RP-HPLC fraction numbers. Numbers on the sides of the gels are approximate locations of molecular weight markers.

as was expected, considering that all endosperm proteins of inbred lines are expressed in hybrids; still, differences between the hybrid and the two parents were easily seen (Figure 9A). This was also true of FZCE separations of the hybrid between H60 and A632 (Figure 9A). Likewise, the hybrid B73×Mo17 was easily differentiated from either B73 or Mo17 (Figure 9B). For the sorghum genotypes investigated, two classes of patterns were seen, differentiated by a large pair of peaks between 14 and 15 min. Five genotypes possessed this type of pattern (Figure 10A) and were readily differentiated from the remaining three genotypes (Figure 10B). Within the first grouping the patterns were similar between some of the genotypes, particularly NK KS710 and NC+5B50. The remaining three genotypes were similar to each other, although it was possible to differentiate them from one another (Figure 10B).

CONCLUSIONS

Hydrophobic storage proteins of maize and sorghum were separated by FZCE with high resolution using ACN as a buffer modifier. Conditions chosen were similar to those in RP-HPLC − i.e., separations in both systems occur at low pH and high ACN concentration.

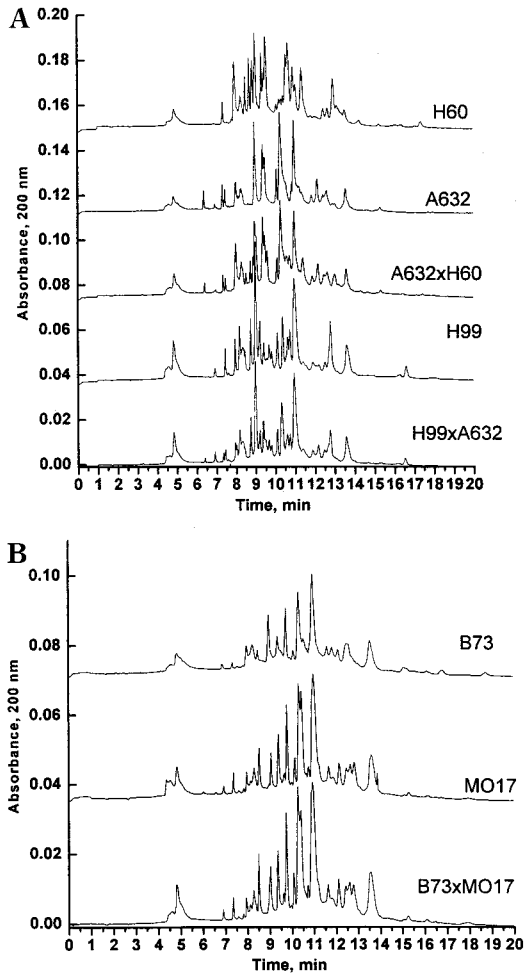

Figure 9. Differentiation of maize genotypes by FZCE analysis of extracted prolamins. Proteins were extracted with 70% ethanol + 0.5% Na acetate + 5% β-ME and separated as in Figure 7.

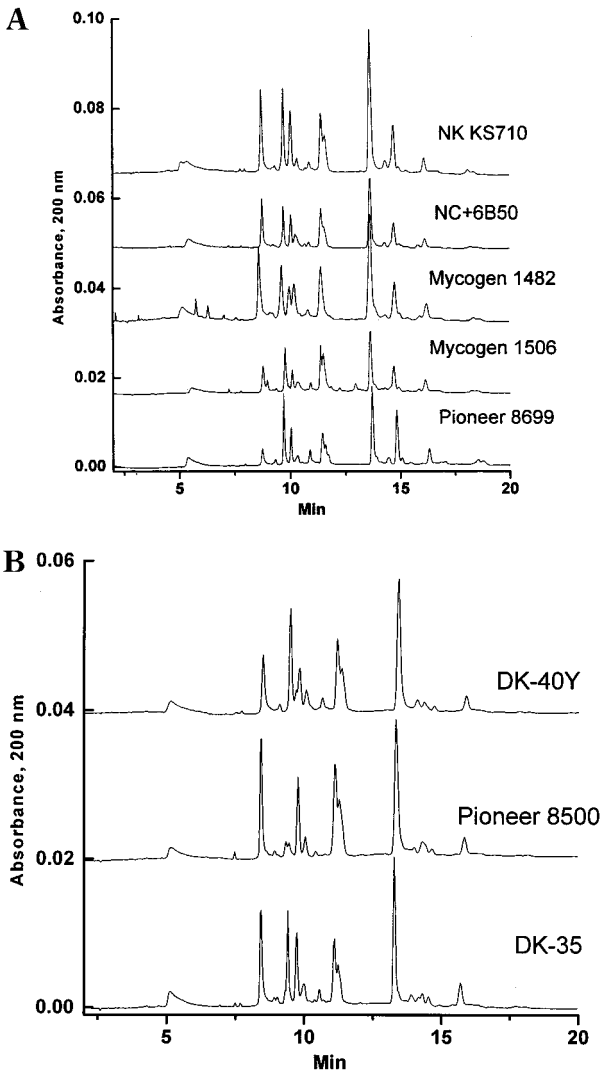

Figure 10. Differentiation of sorghum genotypes by FZCE analysis of extracted prolamins. Proteins were extracted with 70% ethanol + 0.5% Na acetate + 5% β-ME and separated as in Figure 7.

Since extraction and separation solvents are similar, protein solubility is maintained during FZCE, and adsorption and aggregation phenomena are avoided. Resulting separations display high resolution and are generally similar to those of previous methods using 8 M urea, but the ACN buffer system developed avoids the disadvantages of using concentrated urea to maintain protein solubility. Separation-to-separation repeatability is very good, with a relative standard deviation of <0.3% during 15 consecutive injections. FZCE using this ACN buffer system can differentiate most maize and sorghum genotypes on the basis of their unique prolamin fingerprints. Migration positions of subclasses of maize and sorghum prolamins were located and found to be basically the same as in RP-HPLC and PAGE. Thus, the ACN buffer FZCE procedure we developed offers an attractive alternative to other methods for separating maize and sorghum prolamins, avoiding most disadvantages of those procedures. The methodology may also be applicable to other hydrophobic proteins that have been successfully separated by RP-HPLC.

ABBREVIATIONS USED

ACN, acetonitrile; FZCE, free zone capillary electrophoresis; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SDS−CE, sodium dodecyl sulfate−capillary electrophoresis; HPMC, hydroxypropylmethyl-cellulose; HEC, hydroxyethyl-cellulose; 2D, two dimension; i.d., inner diameter; IDA, iminodiacetic acid; RSD, relative standard deviation; EtOH, ethanol.

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