

# Optimizing Quantitative Reproducibility in High-Performance Capillary Electrophoresis (HPCE) Separations of Cereal Proteins<sup>1</sup>

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## ABSTRACT

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High-performance capillary electrophoresis (HPCE) is capable of producing high-resolution, rapid separations of cereal proteins. Furthermore, HPCE is highly reproducible in terms of migration time. However, little work has focused on the quantitative reproducibility of cereal protein separations. Several factors such as sample matrix, sample evaporation, voltage ramp-up time, sample injection time, and capillary end-cut were evaluated for involvement in quantitative reproducibility. These experiments showed that preventing sample evaporation, using optimum injection times, and ensuring a clean, square cut on the capillary all improved the reproducibility of peak areas. Combining these factors into an optimized procedure produced reproducibility with peak areas varying by 1.76%

relative standard deviation (RSD). Migration time was also excellent under these conditions, varying by only 0.45% RSD. Other variables such as peak area percent, peak height, and peak height percent also showed good reproducibility with RSD < 4%. Increasing the voltage ramp-up time from 0.17 to 0.68 increased peak efficiency by ≈150%. This factor had no effect on quantitative reproducibility, however. The gradual buildup of contaminants on the capillary walls occurred over time and decreased both separation efficiency and reproducibility. Rinsing capillaries periodically with appropriate solvents delayed this effect. Peak efficiency was a good marker for capillary performance and lifetime.

High-performance capillary electrophoresis (HPCE) is capable of producing high-resolution, rapid, automated separations of proteins. HPCE produces digital output that can be easily quantified and stored. HPCE separations do not require postseparation detection steps such as staining necessary with slab gel electrophoresis. In addition, HPCE uses very low amounts of buffer, which means little hazardous waste production. Given these benefits, it is not difficult to understand the interest in developing suitable methods for protein separations by HPCE. Several methods have been reported for cereal protein separations by HPCE (Bean et al 1998a; Bean and Lookhart 2000a; Lookhart and Bean, *in press*). Progress has been made in terms of resolution, separation time, capillary coating protocols, capillary rinsing procedures, and sample preparation.

One important aspect of HPCE is reproducibility. Recently, considerable research has been done on migration time reproducibility for cereal proteins (Bietz and Schmalzried 1995; Lookhart and Bean 1995, 1996; Yan and Liu 1997; Capelli et al 1998; Bean and Lookhart 1998, 1999; Day et al 1999; Lookhart et al 1999; Olivieri et al 1999; Yan et al 1999; Bean et al 2000a). In general, migration time reproducibility has very good injection-to-injection repeatability of <1% relative standard deviation (RSD) and day-to-day and capillary-to-capillary migration time repeatability of <5% (Bean and Lookhart 2000a). Several studies on factors influencing the overall reproducibility of HPCE in general have reported that HPCE is very reproducible if sufficient attention is paid to relevant details such as proper capillary storage, buffer selection and replenishment, capillary end-cut, sample matrix evaporation, sample carryover, and similar factors (Smith et al 1991; Strege and Lagu 1993; Ermakov et al 1994; Ross 1995, 1997; Shihabi and Hinsdale 1995; Yang et al 1996; Kitagishi 1997; Whatley and Chapman 1998; Altria and Campi

1999a,b; Fallor and Engelhardt 1999; Mayer and Muller 2000; Schaeper and Sepaniak 2000).

One of the strengths of HPCE is the ability to easily quantify data, which is difficult to do with slab gel electrophoresis. While considerable work has been done on investigating the migration time reproducibility, little work has focused on the reproducibility of the quantitation of cereal proteins by HPCE. The ability to easily and reproducibly quantify cereal protein fractions is an attractive feature of HPCE. It is often desirable to quantify the amounts of cereal proteins to gain better understanding of their functional role in foods. Protein expression in different environments or under different environmental stresses can also be measured by quantifying the proteins during kernel development.

While researchers have investigated the factors that can influence the overall reproducibility of HPCE separations, other than capillary rinsing protocols and sample preparation, little attention has been paid to the factors contributing to the reproducibility of cereal protein separations. In addition, virtually no work on quantitative reproducibility has been done. Investigating the factors specifically related to the quantitative reproducibility of cereal proteins is necessary and will further develop HPCE as a tool for the study of cereal proteins and their functionality in foods. This study investigated the factors that could specifically influence the separation and quantitative reproducibility of cereal protein separations by HPCE.

## MATERIALS AND METHODS

### Free-Zone Capillary Electrophoresis

All separations were made using a Beckman PACE 5510 instrument (BeckmanCoulter Inc., Fullerton, CA). Separations were performed using uncoated fused silica 27-cm capillaries (20-cm inlet-detector [Ld]) × 50 μm i.d. (Polymicro, Phoenix, AZ). The separation buffer was 50 mM iminodiacetic acid (IDA) containing 20% acetonitrile (ACN) and 0.05% hydroxypropylmethyl-cellulose (2% = 4,000 cps) (Bean and Lookhart 2000a). Separations were made at 30 kV (1,111 V/cm) and 45°C (Bean and Lookhart 2000a). Proteins were detected by UV at 200 nm. Samples were injected by pressure for 1–10 sec at 0.5 psi. Capillaries were equilibrated before use by rinsing with separation buffer for 30 min at 20 psi (Bean and Lookhart 1998) and between runs for 0.5 min at 20 psi (Bean and Lookhart 1998; Bean and Lookhart 2000a). When not in use, capillaries were flushed with 500 mM acetic acid and N<sub>2</sub> and stored empty (Bean and Lookhart 1998). Data analysis (i.e., peak efficiency) were calculated using Beckman PACE station software.

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## SDS-CE

SDS-CE separations were made using a Beckman PACE 2100 instrument. Separations were done using BioRad SDS-CE reagent modified by the addition of 15% ethylene glycol (Bean and Lookhart 2000b).

## Sample Extraction

For free-zone capillary electrophoresis (FZCE), initial samples were extracted from flour (250 mg) after removal of albumin and globulin proteins as described in Bean and Lookhart (1998). Under optimized conditions, samples were extracted from 100 mg of flour. When testing the effects of different solvents, samples were extracted with 30% ethanol after removal of albumin and globulins as described in Bean and Lookhart (1998). The supernatants from 10 samples were pooled and then divided into 400- $\mu$ L aliquots and lyophilized. These lyophilized fractions were then redissolved in various solvents at the original volume (400  $\mu$ L) by shaking on a vortexgenie2 (Scientific Industries, Bohemia, NY) for 30 min. For SDS-CE, glutenin extracts were prepared as previously described (Bean and Lookhart 2000b).

## Size-Exclusion Chromatography

Size-exclusion chromatography (SEC) separations were made using a Hewlett-Packard 1090 HPLC system. Samples were separated using a Biosep SEC4000 column (Phenomenex Torrance, CA). Mobile phase was 50% ACN + 0.1% TFA (w/v) (Batey et al 1991) with a flow rate of 1 mL/min. Column temperature was maintained at 40°C.

## Reagents

UV-grade ACN and 1-propanol were from Burdick and Jackson (Muskegon, MI). All other reagents were from Sigma-Aldrich (St. Louis, MO) and were of the highest possible purity.

## RESULTS AND DISCUSSION

### Effect of Sample Matrix

The first factor investigated for its impact on the separation was sample matrix. A wide range of solvents can be used to extract cereal proteins, including aqueous alcohol mixtures, acids and bases, chaotropes, and detergents. We would expect this diverse group of solvents to have much different conductivities and therefore have different effects on sample stacking. The viscosities of these solvents may also differ significantly, which could affect the amount of sample injected in a given timeframe (relative to the other solvents); given the small volumes injected, the exact magnitude of the viscosity is unknown.

To examine the impact of the sample matrix on the quantitation of cereal proteins, aliquots of gliadins extracted with 30% ethanol (v/v) were lyophilized and then redissolved in 50% 1-propanol (v/v), 50% ACN (v/v), 50% methanol (v/v), 50% ethanol (v/v), 4M urea, 500 mM acetic acid, 50% ethylene glycol (EG) (v/v), 1% SDS (w/v), and 50 mM sodium phosphate (pH 2.5). It should be stressed that these solvents, when used to directly extract flour, may extract a different range of proteins. For this reason, lyophilized samples were used so each solvent would contain the same set and same amount of protein. Likewise, we chose to use 30% ethanol as the solvent for initially extracting these samples because this solvent extracts less gliadin than 50% 1-propanol (Bean et al 1998b), which we typically use in preparing extracts for FZCE analysis (Bean and Lookhart 1998). This ensured that each solvent would be able to completely redissolve the lyophilized samples. To verify this, samples were analyzed by SEC and the gliadin areas were compared. Each solvent showed similar gliadin profiles, thus each solvent effectively redissolved the lyophilized proteins (data not shown).

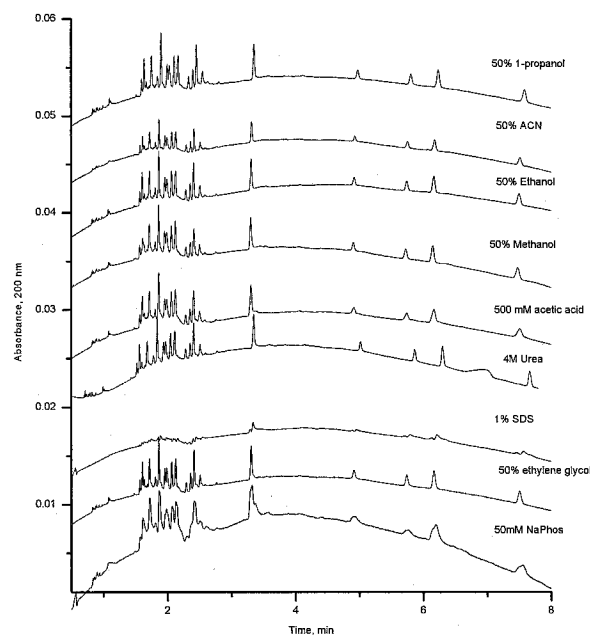
When these samples were analyzed by FZCE, there were no significant effects on the separation resolution except for two of the solvents, 1% SDS and 50 mM Na-Phosphate buffer (Fig. 1). These

two solvents would have much higher conductivities than the other solvents tested due to the sodium ions present. Therefore, these solvents would have stacking problems, especially when using an isoelectric buffer such as IDA. Capelli et al (1998) reported that low conductive sample matrices may have to be used with isoelectric buffers due to the extremely low conductive nature of these buffers.

The 1% SDS sample solvent would also be expected to impart a negative charge on the proteins, which could influence migration in the low pH buffer used here. In some cases, it is possible to disrupt the bound SDS from the proteins by mixing the sample with an organic solvent and still obtain good separations of cereal proteins extracted with SDS (Bean et al 2000b). When 1% SDS samples were mixed 1:1 with 100% 1-propanol, resolution was partially restored (Fig. 2), although the resolution was not as good as the other solvents tested (compare Figs. 1 and 2). Mixing the sample with 1-propanol should also lower the conductivity of the sample plug and thereby improve sample stacking. When this was attempted with the 50 mM Na-Phosphate sample, however, little improvement was seen (data not shown). Therefore, when extracting cereal proteins for FZCE analysis, a low conductive sample matrix should be used.

To test the quantitative effects of the different solvents, gliadin subclasses were measured and compared. All solvents produced roughly the same subclass composition (Fig. 3). However, significant differences were seen in the total amount of sample injected, especially for ACN. This may have been related to sample stacking or possibly due to the viscosity of the sample matrices. All samples were injected for a constant time, thus less material would have been injected from the more viscous samples. This would explain variation in the total amount of material injected. However, this does not adequately explain the low total areas seen with the samples redissolved in ACN. The reason for the large difference with these samples is currently unknown.

The solvent viscosity should be considered when trying to compare samples prepared in different solvents if a constant injection time is used. Sample stacking differences between different solvents should also be considered if comparing samples prepared in different solvents. Sample matrices with relatively low conductivities



**Fig. 1.** Effect of sample matrix on cereal protein separations. Lyophilized extracts were redissolved in various solvents and analyzed on uncoated capillaries (50  $\mu$ m  $\times$  27 cm [20-cm inlet-detector]) at 45°C and 30 kV. Run buffer was 50 mM iminodiacetic acid + 20% acetonitrile and 0.05% hydroxypropylmethyl-cellulose. Samples were injected for 1 sec and voltage ramp-up time was 0.17 min.

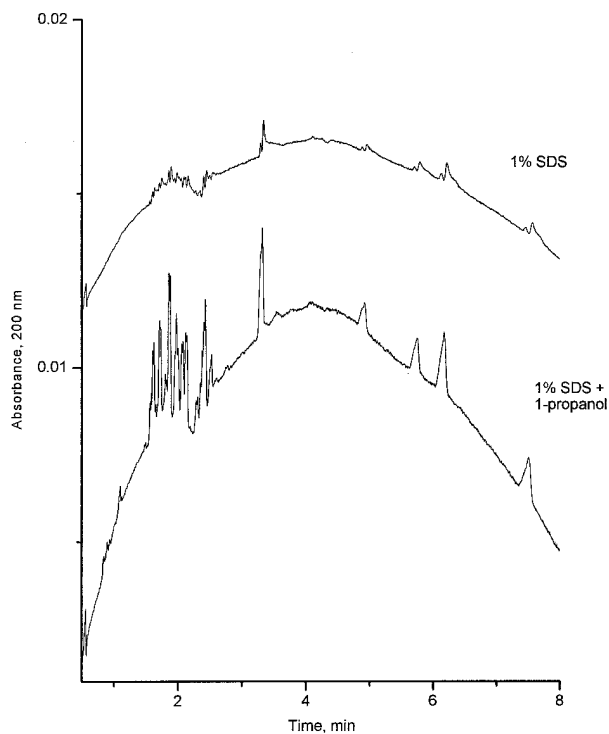
should be selected to enhance sample stacking, especially when using isoelectric buffers (Capelli et al 1998).

### Sample Matrix Evaporation

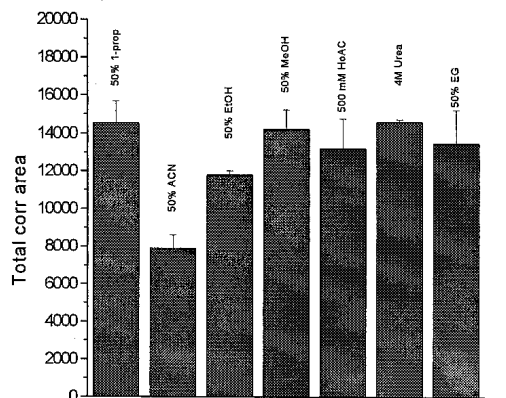
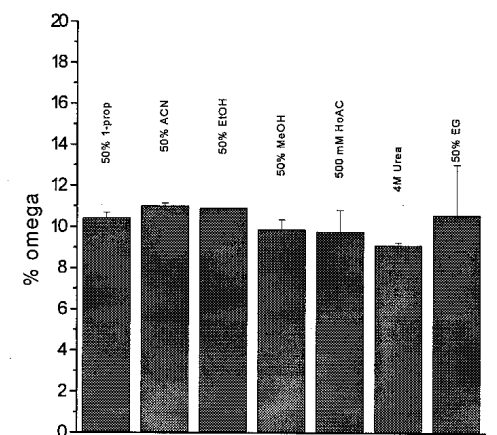
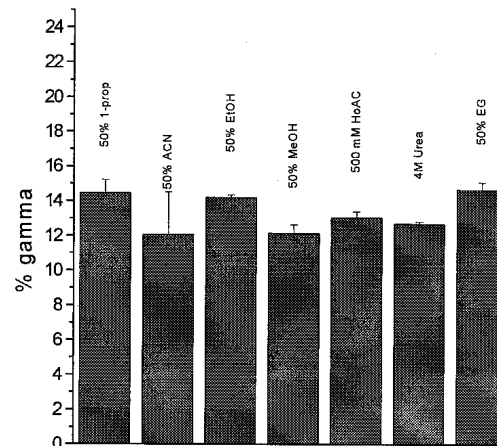
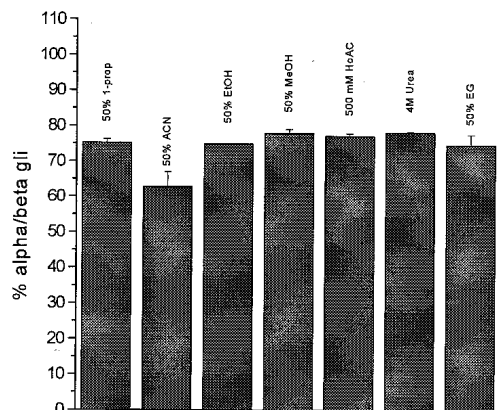
Cereal proteins are often extracted with aqueous alcohol mixtures. Due to the volatility of these solvents, evaporation of the solvent could lead to concentration of the sample and reduced protein solubility, which in turn could affect the concentration of the sample and thus the quantitative reproducibility over time. With the instrument used in this study, samples are placed in 0.5-mL centrifuge tubes set on springs in a 4-mL vial. These 4-mL vials can be partially filled with water, which humidifies the headspace above the sample tube, reducing the evaporation rate of the sample (Nelson 1993). These vials are fitted with Teflon-coated septa that allow the capillaries to enter the sample tubes, but they also close tightly to reduce evaporation (Nelson 1993). These factors could be very important in preventing evaporation of the alcoholic solvents commonly used to extract cereal proteins. Sample matrix evaporation has been indicated as a potential source of error in HPCE (Kitagishi 1997; Ross 1997; Mayer and Muller 2000). To test the magnitude of this effect on cereal protein extracts, a series of 45 consecutive injections of 50% 1-propanol extracts was made with sample vial humidified (with water in the outer vial) and nonhumidified (no water added to the outer vial). The sample volume (400  $\mu$ L) was kept constant for both tests.

The humidified samples showed little change over the 45 consecutive injections (Fig. 4). The nonhumidified samples started showing baseline disturbances by run 23. By run 45, severe shifts in the baselines were present. Resolution was also decreased, especially in the 1–2-min range. Nonhumidified samples also showed larger peaks, indicating that more sample had been loaded into the capillary, probably due to evaporation of the solvent and, thus, concentration of the sample.

To check the quantitative repeatability, the area of the largest  $\omega$ -gliadin (marked with an asterisk in Fig. 4) was integrated. This peak



**Fig. 2.** Effect of adding 1-propanol to SDS extracts. Samples were analyzed on uncoated capillaries (50  $\mu$ m  $\times$  27 cm [20-cm inlet-detector]) at 45°C and 30 kV. Run buffer was 50 mM iminodiacetic acid + 20% acetonitrile and 0.05% hydroxypropylmethyl-cellulose. Samples were injected for 1 sec and voltage ramp-up time was 0.17 min.



**Fig. 3.** Quantitative comparison of the effect of different sample matrices on gliadin subclass composition and total areas. Each sample was measured in duplicate. Error bars represent standard deviation.

was chosen as a marker to evaluate reproducibility and was used as a marker in all subsequent tests described here. For the humidified sample, the RSD for the peak area of this marker peak was 3.3%. The RSD for this same peak in the nonhumidified sample was 9.5%. Humidification of sample vials (or some other means of preventing solvent evaporation) is therefore an important factor in obtaining reproducible peak areas for cereal protein extracts, especially those samples extracted with aqueous alcohols. It was suggested that the outer 4-mL vials be filled with the same solvent as that in the sample tubes to further reduce evaporation.

The sample volume also influences the rate of evaporation (larger volumes show lower evaporation) (Mayer and Muller 2000). As much sample as possible should be placed in the vials. To keep the rate of evaporation the same and provide the highest quantitative reproducibility, all sample vials should be filled to the same level ( $\approx 450\text{--}500\ \mu\text{L}$ ).

### Voltage Ramp-up Time

The next variable investigated was the effect of voltage ramp-up during separation. Rapid voltage ramp-up times have been reported as a possible cause of capillary breakage due to the heating of the sample plug (Mayer and Muller 2000). The instrument used in this study (Beckman PACE 5510) was capable of reaching the separation voltage in 0.17 min; however, this time can be increased by the user. To determine whether the speed of the voltage ramp-up time had any effect on separation reproducibility, three different levels were tested, 0.17, 0.34, and 0.68 min. Samples (50% 1-propanol extracts) were injected 10 times at each ramp up time and the repeatability of peak areas and migration times were checked.

The three voltage ramp times had no effect on peak area reproducibility (data not shown). Peak separation efficiency (plates/meter [N/m]) was higher for longer voltage ramp times (Fig. 5). The  $\omega$ -gliadin peak used as a marker peak in this study showed an average N/m of 251,104 ( $n = 10$ ) for 0.17-min, 313,536 ( $n = 10$ ) for 0.34-min, and 387,207 ( $n = 10$ ) for 0.68-min voltage ramp

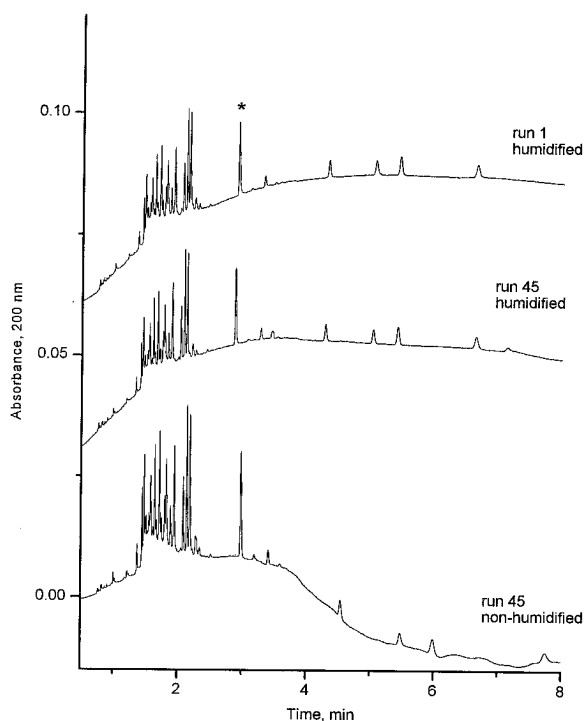
time. This change in efficiency may have occurred due to heating of the sample plug at the more rapid voltage ramp-up times, leading to decreased separation efficiency. Thus, the longer voltage ramp time may provide slight benefits in improving the separation efficiency and therefore the resolution. Using a ramp-up time of 0.68 min also improved the separation of wheat proteins in SDS-CE (data not shown).

### Injection Time

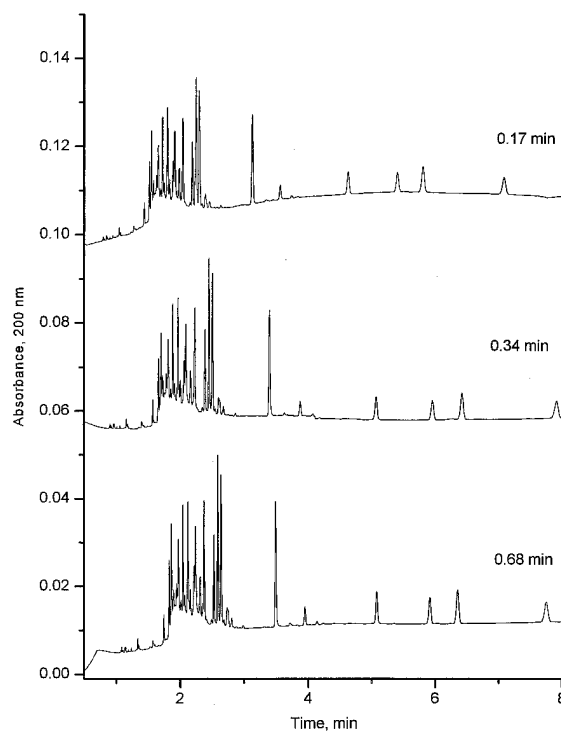
The effect of sample concentration and injection time was investigated next. Engelhardt and Cunat-Walter (1995) recommended that, for best injection repeatability, longer injection times with dilute samples should be used relative to a short injection time with a more concentrated sample. However, Schaeper and Sepaniak (2000) found that shorter injection times at higher pressure showed better peak area reproducibility than longer injection times at lower pressures. Shihabi and Hinsdale (1995) reported that injection times either too short or too long produced poor quantitative reproducibility. Relatively long injection times might overload the capillaries, leading to poor reproducibility (Shihabi and Hinsdale 1995). Longer injection times allow instruments to better compensate for slight pressure fluctuations (Whatley and Chapman 1998). These reports, although somewhat variable in their results, demonstrate that injection time can influence quantitative reproducibility.

To determine the best injection time for cereal protein separations, a sample was diluted 4 $\times$  and 10 $\times$  with solvent (50% 1-propanol). The control sample (no dilution) was injected for 1 sec, the 4 $\times$  dilution for 4 sec, and the 10 $\times$  dilution for 10 sec. This should have resulted in the same absolute amount of sample being loaded onto the capillary. No qualitative differences could be seen in the separation patterns (Fig. 6) and, quantitatively, the separations were essentially identical (data not shown).

The reproducibility of each concentration was checked over 10 runs. The 4-sec injection showed the best reproducibility, with RSD



**Fig. 4.** Effect of sample matrix evaporation on cereal protein separations. Samples were separated on uncoated capillaries ( $50\ \mu\text{m} \times 27\ \text{cm}$  [20-cm inlet-detector]) at  $45^\circ\text{C}$  and 30 kV. Run buffer was 50 mM iminodiacetic acid + 20% acetonitrile and 0.05% hydroxypropylmethyl-cellulose. Samples were injected for 1 sec and voltage ramp-up time was 0.17 min.



**Fig. 5.** Effect of voltage ramp-up time on cereal protein separations. Samples were separated on uncoated capillaries ( $50\ \mu\text{m} \times 27\ \text{cm}$  [20-cm inlet-detector]) at  $45^\circ\text{C}$  and 30 kV. Run buffer was 50 mM iminodiacetic acid + 20% acetonitrile and 0.05% hydroxypropylmethyl-cellulose, with voltage ramp-up times as indicated. All separations were performed on the same capillary with the same sample in consecutive analysis.

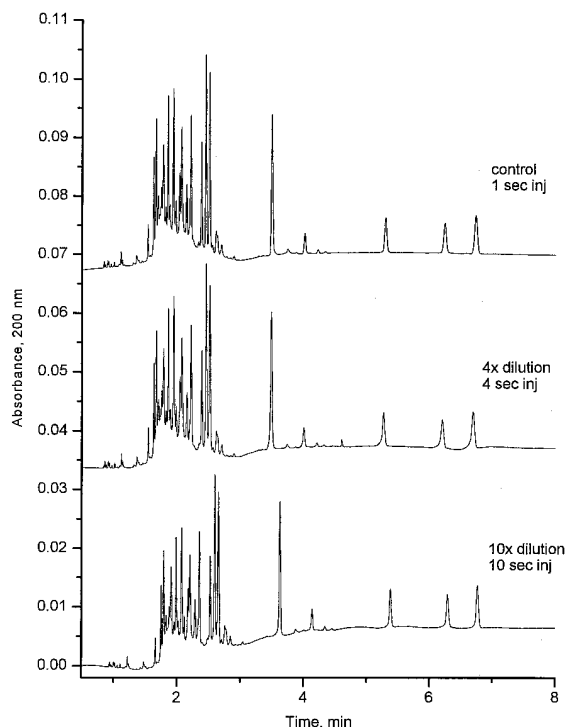
≈ 2% for corrected peak areas. The 1-sec injection showed highest variability with RSD > 5%, while the 10-sec injection had RSD ≈ 4%. These data support Shihabi and Hinsdale (1995), who reported that too short or too long an injection time leads to decreased quantitative reproducibility. The long injection time may have decreased reproducibility due to the difference between sample matrix and buffer. The longer injection time created a much larger sample plug in the capillary. This large plug of solvent (50% 1-propanol) could adversely affect the separation by creating a large zone of low conductivity that may act as a resistor and heat differently than the rest of the buffer-filled capillary. The current profile for both the 4- and 10-sec injections showed disturbances during the initial stages of the separation but the magnitude was higher for the 10-sec injection (data not shown).

### Capillary End-Cut

The final factor investigated was the capillary end-cut. A smooth, straight cut on the capillary ends may influence separation resolution and reproducibility (Roizing 1998). To examine the effects of the capillary end-cut on cereal protein separations and quantitation, a single capillary was subjected to a series of different cutting procedures. After each cutting procedure, a series of 10 separations were made; then the next cutting procedure was tested.

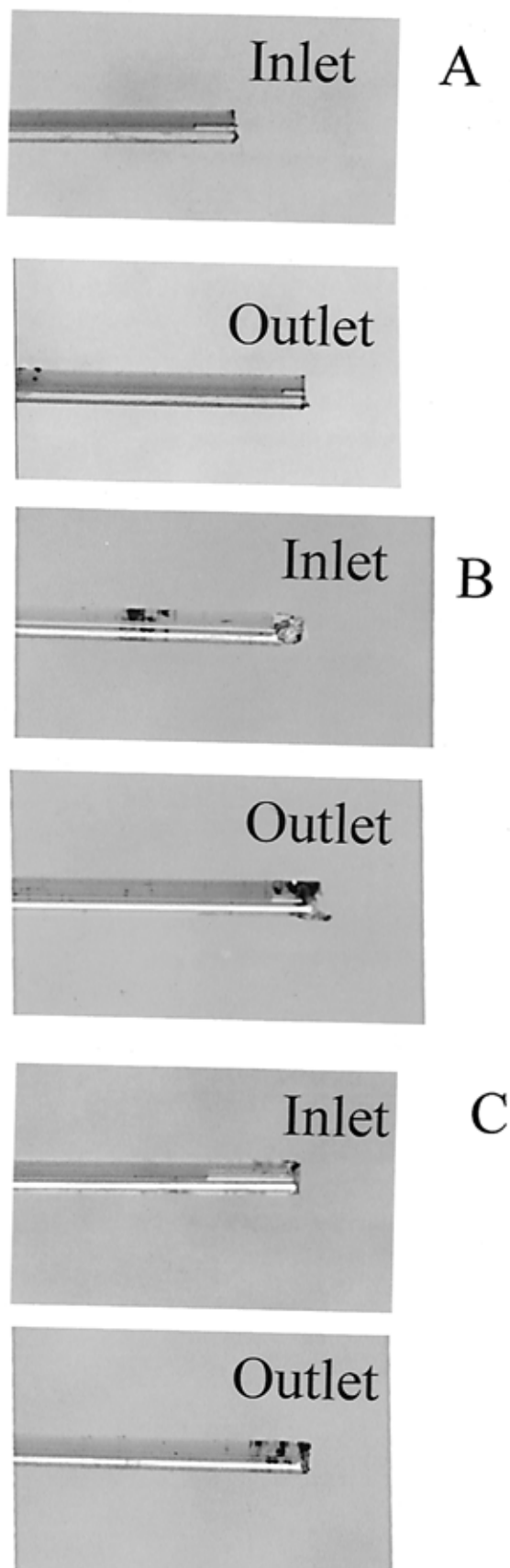
First, the capillary was cut with a diamond wheel cutter, which produced very straight capillary edges (Fig. 7A). The capillary ends were then crushed with a silica chip, producing very rough ends (Fig 7B). Finally, the capillary was cut by scoring the capillary with a silicon chip and breaking the capillary. This produced relatively smooth ends (Fig 7C) but not as smooth as the diamond wheel cut. These results are consistent with previous reports on the effect of capillary cutting procedures (Roizing 1998).

The separation resolution was affected by the cutting procedure. When the capillary was cut such that a smooth end was present, resolution was good (Fig. 8). However, when the capillary ends were crushed, resolution was significantly degraded. Note that when



**Fig. 6.** Effect of injection time on cereal protein separations. All samples were separated on uncoated capillaries (50  $\mu\text{m} \times 27 \text{ cm}$  [20-cm inlet-detector]) at 45°C and 30 kV. Run buffer was 50 mM iminodiacetic acid + 20% acetonitrile and 0.05% hydroxypropylmethyl-cellulose, injection times as indicated.

the capillary was cut after the ends were crushed, the resolution was restored. The results were the same when using SDS-CE (a smooth flat cut produced better resolution than a jagged cut) (data not



**Fig. 7.** Capillary ends resulting from different cutting procedures: **A**, diamond wheel cut, **B**, capillary ends crushed, and **C**, capillary scored and broken.

shown). These results are in agreement with previous results showing that the cut on the end of the capillary could affect separation resolution (Roziing 1998).

The peak area reproducibility was also affected. The capillary cut with the diamond wheel cutter showed the best reproducibility with an RSD = 5.9% ( $n = 3$ ) for the peak area. After crushing the capillary RSD = 11.4%. However, when the capillary was scored and broken, producing a more even end, RSD = 6.8%, close to that of the diamond wheel cut. Thus, it is important when separating cereal proteins by HPCE to ensure that the capillary has a smooth, straight end-cut.

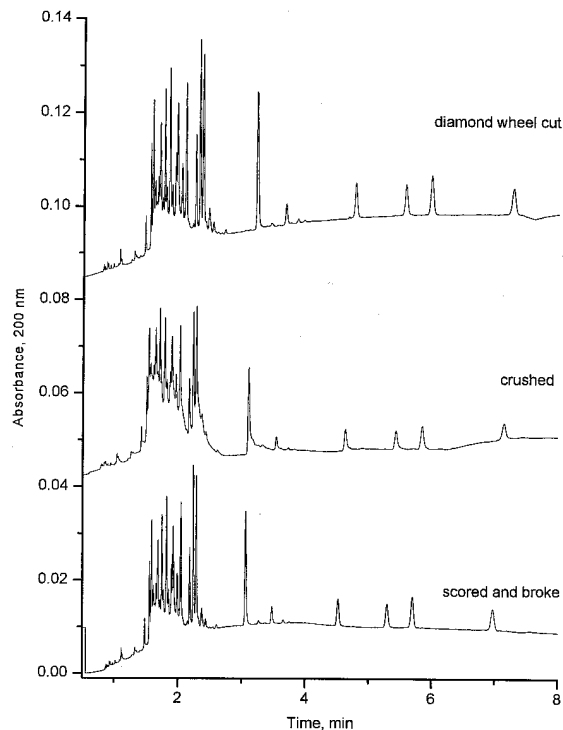
### Optimum Reproducibility

Humidification of the sample vials, sample concentration and injection time, voltage ramp-up time, and capillary end-cuts all individually affect either the separation resolution or peak area quantitation. As a final test, all these factors were combined to evaluate the overall impact on resolution, migration time reproducibility, and reproducibility of peak areas. A series of 20 consecutive injections was made with a diluted sample injected for 4 sec with a 0.68-min voltage ramp-up using a humidified vial and a capillary cut with a diamond wheel cutter. Note that, in this case, the sample was extracted with 1 mL of 50% 1-propanol from 100 mg of flour rather than the 250 mg normally used in our laboratory (Bean and Lookhart 1998). This provided a more dilute sample in one step, simplifying the procedure and reducing possibilities for reproducibility errors during dilution of the sample.

Reproducibility data under optimum conditions are shown in Table I. Corrected peak area had RSD = 1.76%. In addition to corrected peak area, several other parameters were also checked for reproducibility. Migration time, peak height, peak height percent, and corrected peak area percent also were measured. Migration time showed RSD < 1%, as reported earlier for this buffer system (Bean and Lookhart 2000a). All other variables had RSD < 4%, demonstrating good repeatability over consecutive injections.

Note that for this test, a brand new capillary was used. It was observed that as samples were analyzed over several days, N/m decreased. Reproducibility and resolution also slowly decreased (data not shown). Rinsing the capillary with 500 mM acetic acid periodically ( $\approx 40$  run intervals) decreased the rate of the decline in these parameters. The decrease in peak efficiency may be due to gradual build-up of protein (or other contaminant) on the inner capillary

walls. This build-up, in turn, may lead to the decreased reproducibility and resolution. Therefore, it is recommended that the capillaries be rinsed periodically with a solvent capable of cleaning the capillary walls. For wheat proteins, 500 mM acetic acid works very well. Other cereal proteins may require different solvents. SDS may be a good solvent to use to periodically rinse the capillaries, it has been very effective removing protein from capillary walls (Verzola et al 2000). However, caution would have to be exercised when using SDS as a rinse to make sure that all excess SDS was removed from the



**Fig. 8.** Effect of capillary end-cut on cereal protein separations. All separations were on uncoated capillaries (50  $\mu\text{m} \times 27$  cm [20-cm inlet-detector]) at 45°C and 30 kV. Run buffer was 50 mM iminodiacetic acid + 20% acetonitrile and 0.05% hydroxypropylmethyl-cellulose. Samples were injected for 1 sec and voltage ramp-up time was 0.17 min.

**TABLE I**  
Run-to-Run Repeatability Under Optimum Conditions

Run	Corrected Peak Area (mAu)	Peak Area %	Migration Time (min)	Peak Height (mAu)	Peak Height %
1	3,964	4.20	3.67	26,520	8.01
2	4,059	4.17	3.67	27,281	8.14
3	4,029	3.94	3.66	27,699	8.10
4	3,949	4.14	3.67	27,203	8.37
5	3,974	4.18	3.67	27,128	8.07
6	3,969	4.22	3.67	27,090	8.31
7	3,979	4.19	3.62	22,752	8.41
8	3,735	4.21	3.65	25,785	8.39
9	3,956	4.25	3.68	27,112	8.40
10	3,990	4.42	3.68	27,043	8.39
11	3,947	4.22	3.68	26,883	8.32
12	3,951	4.29	3.68	26,575	8.31
13	3,979	4.31	3.67	26,588	8.24
14	3,934	4.32	3.68	25,969	8.16
15	3,947	4.43	3.69	26,507	8.39
16	4,009	4.43	3.69	26,715	8.10
17	3,981	4.43	3.69	26,791	8.49
18	3,973	4.39	3.67	26,426	8.25
19	3,831	4.42	3.69	25,365	8.32
20	4,022	4.44	3.69	26,512	8.07
Average	3,958	4.28	3.67	26,497	8.26
Standard deviation	69	0.13	0.01	1,036	0.14
Relative standard deviation	1.76	3.09	0.45	3.9	1.71

system to avoid altering the charge of proteins. It is also recommended that a standard cereal protein extract be analyzed with each data set and the N/m measured and compared with previous runs. This serves as a control to ensure that the capillary is still functioning at its best and that the capillary walls have not become contaminated. Use of a standard cultivar was also recommended (Lookhart and Bean 1996) for the evaluation of separation resolution. Use of standards with every data set is very important for ensuring good reproducibility.

### Data Analysis

In HPLC, proteins are swept through the column under the flow of mobile phase. As proteins leave the column and pass through the detection cell, they all have the same velocity. This is not the case in HPCE, where each protein moves at its own velocity (Kitagishi 1997; Mayer and Muller 2000). This creates a potential problem in the integration of peak areas because the velocity of the protein affects the width of the peak and, thus, the area. This must be taken into account when integrating peak areas by correcting for the migration time. This can be done by dividing the peak area by the migration time of the peak, producing a corrected area (Mayer and Muller 2000; Kitagishi 1997; Altria 1993). All peak area data in this study were corrected in this manner. Correction of peak area must be done to properly integrate HPCE results.

The software used to integrate peak areas can also cause differences in reproducibility. Certain algorithms are more reproducible than others (Faller and Engelhardt 1999). In addition, the placement of baselines and peak start and stop markers could influence reproducibility. In this study, the software was left at default settings to integrate peaks. However, in a few instances, peaks had to be manually selected and baselines manually drawn to accurately integrate the peak of interest. Data should be carefully analyzed to ensure that the software is consistently integrating peaks the same between runs.

### CONCLUSIONS

HPCE is capable of producing high-resolution, rapid separations of cereal proteins. Previous studies have shown that HPCE is capable of very good migration time reproducibility as well as good reproducibility between laboratories for cereal protein separations (Bietz and Lookhart 1997). Although migration time reproducibility is important, the ability to reproducibly quantitate proteins separated by HPCE is also very important. Several factors can influence the quantitative reproducibility of cereal protein separations. The most relevant factors in this study were injection time, a smooth cut on the capillary, and properly humidifying the sample vials. In addition, decreasing the voltage ramp-up time led to higher efficient separations, a factor important in resolution.

With any analytical technique, attention must be paid to the details to provide reproducible results. Several factors other than those tested here can influence reproducibility: instrument coolant levels, capillary storage, buffer preparation and age, sample preparation and age, sample stability, lamp life, capillary equilibration and washing, instrument cleanliness, and operator experience. It is clear that if these factors are properly addressed, HPCE is very capable of producing excellent reproducibility. Future research on other factors that could affect quantitative reproducibility are the use of internal standards to improve capillary-to-capillary and day-to-day reproducibility and a better understanding of the interactions between cereal protein extracts and capillary inner walls and improved methods of dynamically coating the inner capillary walls.

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