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Journal of Chromatography A, 814 (1998) 25–41

JOURNAL OF CHROMATOGRAPHY A

Review

High-performance capillary electrophoresis of cereal proteins[1]

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Received 21 April 1998; accepted 18 May 1998

Abstract

Cereal grains are widely used for human foods and animal feeds throughout the world. Cereals provide dietary protein, which also often has a functional role, as wheat gluten does in bread. Cereal proteins are unique in many ways: they are highly complex and heterogeneous, are often difficult to extract, and aggregate readily, making them difficult to characterize. Because of the economic importance and widespread use of cereal proteins, however, many techniques have been used for their analysis. High-performance capillary electrophoresis (HPCE) is one of the newest techniques to be so used. This review describes the development of charge- and size-based HPCE methods for analysis of cereal grain proteins, and the use of these methods for cultivar identification, classification, and prediction of quality. HPCE is versatile, rapid, easily automated, readily quantified, and provides high-resolution separations. Clearly, HPCE is a valuable addition to other methods of cereal protein analysis and should, in time, be applicable to all protein classes from all cereals. Published by Elsevier Science B.V.

Keywords: Reviews; Food analysis; Proteins; Prolamines; Glutelins; Gliadins

Contents

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[1]Cooperative investigations, U.S. Department of Agriculture, Agricultural Research Service, and the Department of Grain Science and Industry, Kansas State University. Contribution 98-419-J, Department of Grain Science and Industry, Kansas State Agricultural Experiment Station, Manhattan, KS 66506. Mention of firm names or trade products does not constitute endorsement by the U.S. Department of Agriculture over others not mentioned.

0021-9673/98/$19.00 Published by Elsevier Science B.V.
PII: S0021-9673(98)00437-3

1. Introduction

1.1. Importance of cereals and their proteins

The critical importance of cereal grains to humanity has long been recognized. Indeed, these grains produce the natural resources for 'two of man's oldest technologies — the baking of bread and the fermentation of alcoholic beverages' [1] and, as such, are an integral part of human history. Cereal grains are major foods in every country, either directly or indirectly as animal feeds [2]. Of the world's major 22 crops, cereal grains are by far the most widely grown [3]. As an example, wheat is grown on approximately 220 million hectares, with a worldwide total yield of 550–600 million tons per year [4]. Similarly, depending on environmental and agronomic practices and on cultural preferences, rice, maize, and other cereal grains are also widely grown.

In addition to being a major source of energy, cereals are often a primary provider of nutritious protein, out-producing all other major crops in terms of annual protein yield (on a dry mass basis) by a wide margin [3]. Cereal proteins also contribute important functional properties to many foods. This is especially true in wheat, whose gluten proteins have unique viscoelastic properties that permit bread to be produced [5]. In fact, for more than 250 years chemists have attempted to relate protein amount, composition, or structure to cereal quality and other functional characteristics [6,7].

Knowledge of cereal proteins is valuable in many ways. For example, because of their heterogeneity and near-invariant expression, storage proteins provide fingerprints that differentiate cereal genotypes and cultivars [8–10]. This ability to differentiate cultivars is important during breeding, marketing, utilization, and in research. Through protein analysis, breeders can identify and select optimal parental genotypes and progeny during cultivar development, and can ascertain varietal purity. In production agriculture, varietal identification can ensure use of lines with optimal economic return, quality, yield, genetic resistance, or adaptability. Identity preservation also makes varietal identification mandatory. Millers, bakers, brewers, maltsters, and other processors must guarantee that grains and flours are suitable for high quality food and nonfood products. Thus, knowledge of cereal proteins is important both for quality prediction and for identification.

Protein amount alone is not sufficient to provide such information, however, because of the extreme heterogeneity and complexity of cereal proteins [5,11,12]. In most cereals, storage proteins termed prolamins and glutelins are most abundant. Both protein classes are insoluble in water and in salt or buffer solutions. Prolamins are soluble in aqueous alcohol blends, while glutelins are soluble only in

acids, alkali, detergents, denaturants, or after disulfide bond cleavage [7]. During cereal evolution, duplication of one or a few ancestral genes, combined with nonlethal mutations, led to the presence in any cereal of many highly homologous proteins having highly unusual solubilities and compositions. Polyploidy further increased the number of proteins in wheat [13]. As a result, any wheat variety has 100 or more unique prolamins (gliadins in wheat) and glutelin polypeptides ('glutenin,' in wheat). Gliadin and glutenin can each be further subdivided into subclasses based on differences in structure, size, solubility, or reactivity. Many of these proteins also tenaciously associate with each other through non covalent (primarily hydrogen and hydrophobic) or covalent (disulfide) bonds. As a result, native wheat glutenin occurs as polymeric complexes of molecular masses up to $50 \cdot 10^6$ — indeed, glutenin appears to be nature's largest and most complex protein [14].

1.2. Previous methods and problems of cereal protein analysis

Because of the importance and complexity of cereal proteins, many analytical methods have been developed for or applied to their analysis [8,9,15]. Gel electrophoresis in starch [16] or polyacrylamide [17], providing charge-based separations, has been widely used. Polyacrylamide gel electrophoresis (PAGE) in the presence of the detergent sodium dodecyl sulfate (SDS) [18] frequently provides size-based separations of cereal proteins. Isoelectric focusing [19] and two-dimensional methods [20] provide further separation possibilities.

Similarly, many methods of chromatography permit fractionation of cereal storage proteins. Size-based separations by gel filtration chromatography [21] and ion-exchange chromatography [22] are especially useful. The introduction of high-performance liquid chromatography (HPLC) methods [23,24], however, made chromatography especially powerful and useful [25].

Nevertheless, many of these methods have serious deficiencies. Gel electrophoresis procedures are relatively slow and labor-intensive. Attaining satisfactory resolution and reproducibility in electrophoresis is also troublesome, and achieving good quantitation is especially difficult. HPLC overcame many of these difficulties, but still could not perform some separations as well as electrophoresis. The extreme complexity of cereal proteins also demands the availability and use of numerous complementary methods, including those based upon electrophoresis.

1.3. Possibility of applying high-performance capillary electrophoresis to cereal proteins

A need was still perceived, therefore, for improved electrophoretic methods of cereal protein analysis, with better speed, automation, and quantitation. Two types of studies led to realization of this goal. First, in the early 1980s, methods in which solutes were separated in an electrical field in free solution in capillary tubes began to develop. Rapid, reproducible, high-resolution separations resulted from the use of high voltage and efficient cooling was possible because of the narrow capillaries used. Samples could be automatically analyzed, and accurate quantitation could be provided by on-column detection. Proteins, as well as small molecules, could be fractionated by capillary electrophoresis. Commercial instruments with these capabilities soon became available. These developments are discussed in many excellent recent reviews (e.g., [26–31]).

Early studies also suggested that this separation technique might prove valuable for cereal proteins. The first electrophoretic separations of wheat proteins were in fact done nearly 40 years ago by a technique analogous to HPCE, moving boundary electrophoresis [32], which might be considered the forerunner to all modern electrophoretic techniques [33]. In this method, proteins migrate in an open tube under the influence of an applied electric field. The successful use of this method to fractionate wheat gluten strongly suggested that HPCE should also be a valuable technique for separating these proteins.

Thus, HPCE appeared to have considerable potential for cereal protein analysis. HPCE combines the high resolution of electrophoresis with the automation and ease of use of HPLC, and can give high resolution, rapid separations of proteins. Researchers have therefore begun to develop and successfully apply HPCE methods for the separation of cereal proteins. Progress to date is reviewed and summarized in this article.

2. Free zone capillary electrophoresis

2.1. Wheat

Several modes of HPCE are possible. In the simplest, free zone capillary electrophoresis (FZCE), proteins are injected into a capillary filled with buffer and separate primarily on the basis of differences in charge density. FZCE is thus analogous to acid (A)-PAGE, widely used to separate cereal proteins [8,9]. Because A-PAGE has provided good results when separating wheat proteins in slab gels, it is logical to apply similar types of separations in the capillary format. Different buffer systems have been used to separate cereal proteins by FZCE. The major methods used for wheat protein FZCE are reviewed below.

2.1.1. Borate buffer system

The first FZCE type to be applied to cereal proteins used an alkaline borate buffer containing acetonitrile (ACN) plus the detergent SDS [34]. This type of buffer was first used in HPCE by Terabe et al. [35]. The method has since become known as micellar electrokinetic chromatography (MEKC). In it, separations are based on an equilibrium between detergent micelles and solutes, which partition between micelles and electrolyte. Micelles migrate according to their mass/charge ratios, so differences of distribution in the micelle of various components form the basis of separation [36]. There is some question as to whether proteins, due to their large size, actually fully partition into the micelles or simply interact with them [37] providing mixed mode separations.

Thus, 50% methanol extracts of three wheat cultivars were separated using a 60 m*M* borate buffer (pH 9.0) containing 20% ACN and 1% SDS [34]. A 57 cm×50 μm I.D. uncoated fused-silica capillary was used at 30°C, with 10–15 s pressure injection and detection at 200 nm. Sixteen to twenty peaks resolved in ~15 min. Separation voltage was not listed, and no mention was made of separation-to-separation repeatability.

Bietz [38] and Bietz and Schmalzried [39,40] confirmed that these conditions do resolve wheat gliadins. They also systematically varied buffer concentration (30 to 60 m*M*), ACN content (0 to 40%), SDS content (0.1 to 1%), pH (8 to 9), separation temperature (20 to 50°C), capillary length (37 to 57 cm), capillary diameter (20 to 75 μm), and other experimental conditions to optimize the separation. The buffer composition used by Schwartz et al. [34] i.e., 60 m*M* borate, pH 9.0, containing 20% ACN and 1% SDS, appeared optimal. Separations were best on a 40 cm (inlet to detector)×50 μm I.D. uncoated silica capillary at 40°C and 10 kV. Proteins were detected by absorbance at 200 nm. Optimal resolution and baseline were achieved when gliadins were extracted with 30% ethanol, rather than with 70% ethanol as most commonly done.

A typical separation using these conditions is shown in Fig. 1. More than 30 components were typically resolved. Separations were rapid, automatic, and easily quantified. Using this procedure, most wheat cultivars could be readily differentiated and thereby identified on the basis of major unique qualitative and quantitative differences between varieties. Resolution compared favorably with that for the same samples analyzed by reversed-phase (RP)-HPLC. HPCE and RP-HPLC also complemented each other because of their differing separation

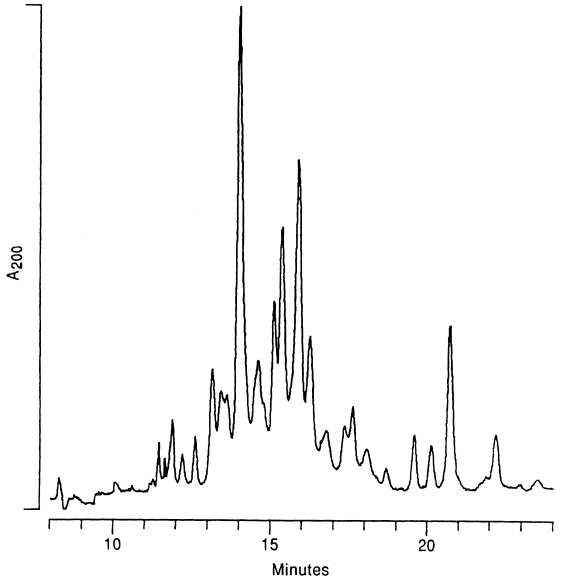


Fig. 1. CE in 0.06 *M* sodium borate buffer, pH 9.0, containing 20% ACN and 1% SDS, of Centurk wheat proteins extracted with 30% ethanol; from Bietz [38].

modes; each method could separate some proteins not resolved by the other.

Reproducibility with this HPCE procedure was difficult to achieve and maintain. Peaks typically increased in elution time upon repeated analyses, possibly reflecting binding of protein or SDS to silica walls, precipitation of protein within capillaries (changing their actual physical dimensions), or changes in electroosmotic flow. These problems can be especially severe for gluten proteins because of their interactive and aggregative tendencies and low solubilities in aqueous buffers. Extensive tests established procedures for conditioning and washing capillaries that significantly improved run-to-run repeatability. With these procedures, plus appropriate standards, acceptable results were achieved.

2.1.2. Charge reversal system

Werner et al. [41] reported a different system for gliadin fractionation. This method used a pH 2.3 aluminum lactate buffer, similar to the buffer first used in starch gel electrophoresis of gliadins [16], along with a capillary coated with a positively charged commercial polymer (MicroCoat, Perkin Elmer). This reagent dynamically coated the inside capillary walls, giving the surface a positive charge that induced reversed electroendosmotic flow (EOF) and transported the proteins through the capillary. This coating also helps prevent adsorption of positively charged proteins to capillary walls [42]. Samples were separated at 10 kV in 72 cm×50 μm I.D. capillaries. This capillary length may have been necessary for adequate separations since high EOF arises in capillaries with inner walls carrying positive charges. Separations were complete in less than 20 min, and 15 to 20 major peaks were resolved (Fig. 2), analogous to separations by acid gel electrophoresis [9]. Resolution was sufficient to easily differentiate all the wheat cultivars tested (Fig. 2), demonstrating the potential of HPCE for varietal fingerprinting and identification.

2.1.3. Phosphate buffer system

Because of reproducibility concerns found when using the borate–ACN–SDS buffer, Bietz and Schmalzried [38,40] also investigated the use of a commercially available (Bio-Rad) 100 m*M* phosphate buffer (pH 2.5) containing a polymeric additive (later reported as 0.05% hydroxypropylmethylcellulose, HPMC). Better resolution (Fig. 3) and reproducibility were reported when using the low pH phosphate buffer relative to that of the borate–SDS–ACN buffer. For these separations the separation voltage was 7 kV and temperature was maintained at 40°C. A 27 cm (effective length 20 cm)×50 μm I.D. capillary was used for these separations. Most wheat cultivars from several classes of wheat could be readily differentiated by this method with good reproducibility.

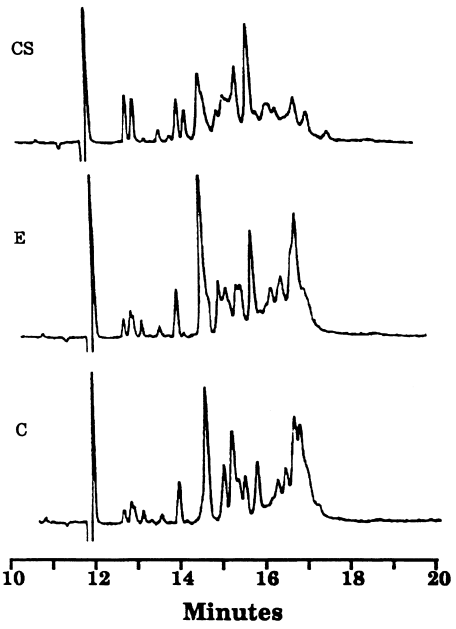


Fig. 2. HPCE separations of gliadins from single seeds of the wheat varieties Chinese Spring (CS), Eagle (E), and Cheyenne (C). Separations were performed on a 72 cm (50 cm)×50 μm I.D. capillary coated with Micro-Coat reagent and equilibrated with pH 2.3 aluminum lactate buffer; from Werner et al. [41] with permission.

Lookhart and Bean [43–45] expanded on the use of low pH phosphate buffers for the separation of cereal proteins by FZCE. Initial studies investigated the effect of reducing the capillary inner diameter from 50 to 20 μm [43]. Capillaries of 20 μm I.D. provided roughly the same resolution as 50 μm I.D. capillaries, but higher voltages could be used with the 20 μm I.D. capillaries and consequently separation time was reduced by almost 50% (Fig. 4).

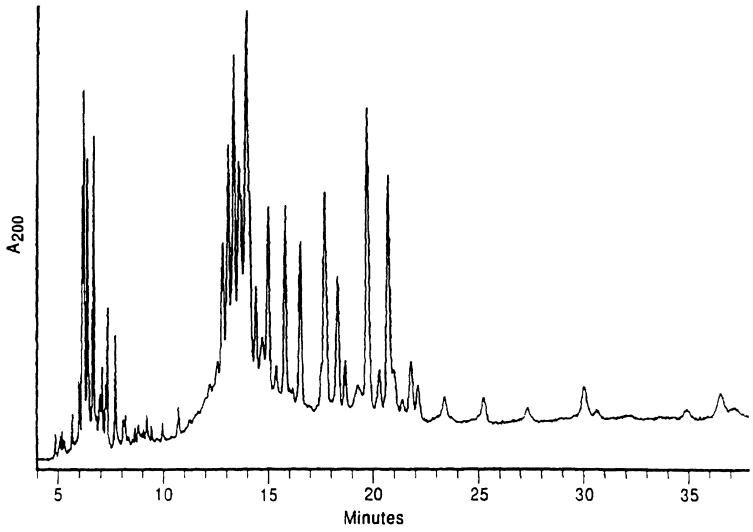


Fig. 3. CE in phosphate buffer, pH 2.5, containing polymeric additive, of 30% ethanol-soluble Centurk wheat proteins; from Bietz [38].

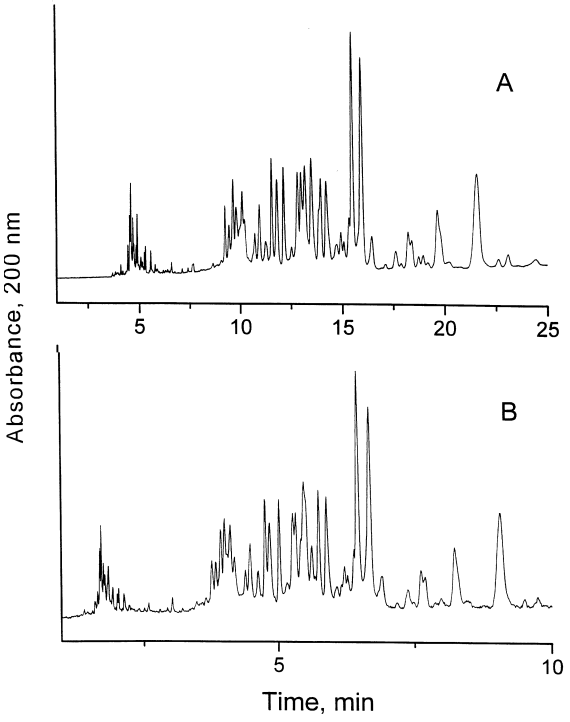


Fig. 4. Effect of capillary I.D. on separation of gliadins from the cultivar TAM 107. (A) Gliadins separated using a 27 cm (20 cm)×50 μm I.D. capillary (10 kV with an initial gradient from 7 to 10 kV). (B) Gliadins separated using a 27 cm (20 cm)×20 μm I.D. capillary (22 kV). Both separations were performed using a 0.1 *M* phosphate buffer, pH 2.5, containing 0.05% HPMC; from Lookhart and Bean [43].

With 20 μm capillaries, optimal separation voltage was 22 kV (compared to 10 kV with 50 μm capillaries), and optimal temperature was reported as 45°C [43].

It was recently suggested that 25 μm capillaries be used instead of 20 μm I.D. capillaries [46,47]. Capillaries of 25 μm I.D. offered greater sensitivity due to increased detector path length, and since more sample can be effectively loaded into the capillary per unit time. However, 25 μm capillaries were still small enough to effectively dissipate heat, and permitted rapid separations [46,47].

Attempts to improve HPCE resolution of wheat proteins in low pH phosphate buffers were reported by Lookhart and Bean [45], who tested effects of several organic solvents and detergent additives. Acetonitrile, methanol, 2-methoxyethanol, 1-propanol, and ethylene glycol were tested at concentrations of 5–20% [45]. While all modifiers had some effect on selectivity and resolution, addition of 20% acetonitrile to the buffer most significantly improved resolution of gliadins (Fig. 5). The latest modification of this FZCE buffer has been to replace sodium phosphate with β-alanine, an ion of lower conductivity. Lower currents and faster separations were reported with these buffers [48]. Recent work has found that 50 μm I.D. capillaries can be success-

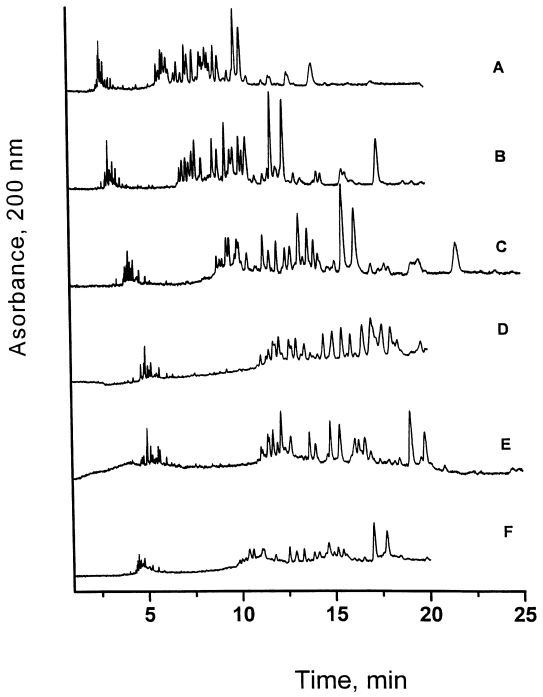


Fig. 5. HPCE patterns of gliadins from the wheat cultivar TAM 107 separated with (A) 100 m*M* phosphate buffer, pH 2.5, containing 0.05% hydroxypropylmethylcellulose; (B) buffer A+ 20% ACN; (C) buffer A+20% methanol; (D) buffer A+20% 2-methoxyethanol; (E) buffer A+20% 2-propanol and (F) buffer A+20% ethylene glycol. Separations were performed in 27 cm (20 cm)×20 μm I.D. capillaries at 15 kV (+ to −) and 45°C; from Lookhart and Bean [45].

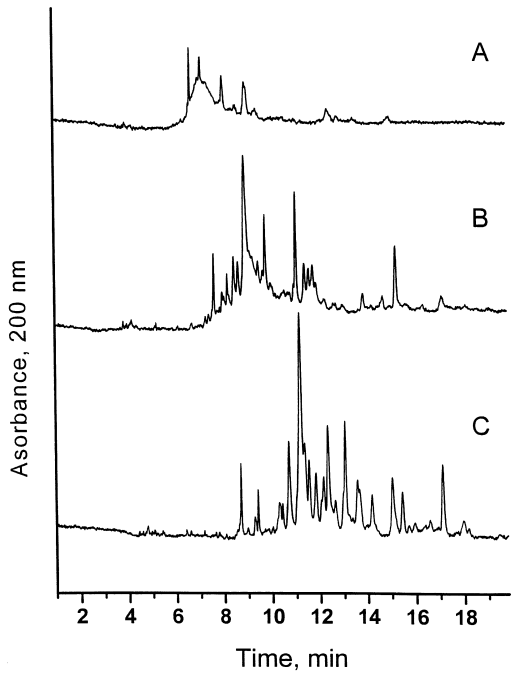


Fig. 6. HPCE separations of reduced glutenins from the wheat cultivar TAM 107. (A) 100 m*M* phosphate buffer, pH 2.5, containing 0.05% hydroxypropylmethylcellulose; (B) buffer A+ 20% ACN; and (C) buffer B+26 m*M* SB-12. Proteins were separated with a 27 cm (20 cm)×20 μm I.D. capillary at 15 kV and 45°C; from Lookhart and Bean [45].

fully used with these low conductive buffers [Bean and Lookhart, unpublished data].

In addition to organic modifiers, Lookhart and Bean [45] tested several zwitterionic and nonionic detergent additives to improve resolution. For gliadins, none of these improved resolution over that of phosphate buffer containing 20% ACN. However, resolution of glutenin subunits was further improved by adding 26 m*M* laurylsulfobetain (SB-12), a zwitterionic detergent, to the buffer (Fig. 6). Greve et al. [49] had previously used SB-12 to improve resolution of peptides that were similar in charge but differed in hydrophobicity. They speculated that the peptides may have interacted with detergent micelles, adding hydrophobic selectivity to the separation. A similar mechanism may be responsible for the improved resolution of glutenin subunits when SB-12 is used as a buffer additive [45]. Sulfobetain-based detergents were later found effective in preventing protein adsorption to the inner surface of fused-silica capillaries [50,51], which also could have played a role in the improved glutenin separations upon addition of SB-12.

The relationship between reproducibility and capillary washing procedures was also examined by Lookhart and Bean [45]. In most previous methods, capillaries were washed with base, acid, and buffer between each separation to achieve stable migration times and good reproducibility. Lookhart and Bean [45] compared several rinsing procedures and found that best reproducibility was achieved by rinsing capillaries with 1 *M* phosphoric acid between each separation followed by a short rinse with separation buffer. This protocol provided excellent repeatability

with relative standard deviations for migration times of 0.1–0.2% across twenty separations [45].

To be useful, FZCE must produce reliable results across laboratories. Thus, Bietz and Lookhart [52] initiated a study to compare reproducibility between two different laboratories. Results suggested that good inter-laboratory reproducibility can be achieved for FZCE analyses of wheat proteins. They also noted some problems, however, due to variation in composition among batches of commercial phosphate buffer. Differences in results achieved using different batches of buffer were greater when using 50 μm I.D. capillaries than for 20 μm I.D. capillaries. Narrower capillaries may therefore provide greater long term reproducibility. These data also suggest that, for optimal long-term reproducibility, buffers should be prepared with utmost care.

2.1.4. Isoelectric buffer system

A recent paper by Cappelli et al. [53] reported the use of an isoelectric buffer containing 40 m*M* aspartic acid as buffering species. Isoelectric buffers have been used in that laboratory to achieve rapid size-based separations of DNA [54] and free zone separations of peptides [55]. Righetti et al. [56] recently reviewed the theoretical and practical aspects of using isoelectric buffers.

Unlike phosphate-based buffers, the 40 m*M* aspartic acid buffer did not provide good separations when ACN was added (Fig. 7). Addition of urea to the buffer, either at 7 *M* or 4 *M* in conjunction with 20% ACN, gave the best separations of gliadins (Fig. 7). Resolution achieved using this isoelectric buffer system was sufficient to distinguish several durum and soft wheat cultivars in approximately 12 min. Interestingly, the combination of ACN and urea produced faster separations than did urea alone. Hydroxyethylcellulose (M_r=27 000) (0.5%) was used as a dynamic modifier to coat the inside of the capillary wall to reduce protein adsorption [53]. Two other additives, 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate (CHAPS) and non-detergent sulfobetaine (NDSB), did not affect resolution.

Due to the low conductivity of this isoelectric buffer, these separations were carried out in 50 μm I.D. capillaries at voltages of 30 kV (1000 V/cm). Also, due to the extremely low conductivity of the buffer, 70% ethanol was used to extract gliadins to provide more sample stacking. Interestingly, the temperature used in these separations was 25°C, which gives very poor separations with phosphate buffers [40,43]. Higher temperatures (~45–60°C) also typically produce better RP-HPLC separations of wheat proteins [57]. The reason for this temperature difference between the two different buffer systems is currently not known, but differential solubility is one possible explanation [53].

Capelli et al. [53] also addressed the issue of sensitivity and reported that samples with protein

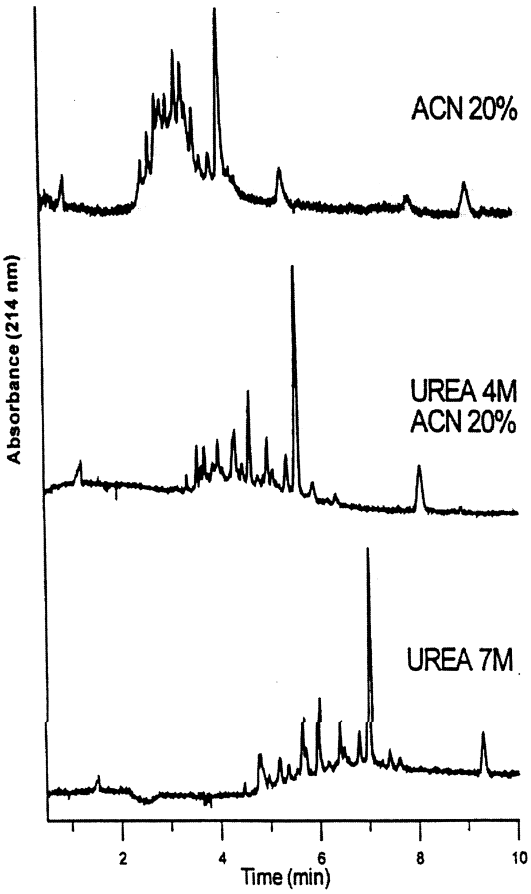


Fig. 7. Comparison of solvent effects in HPCE using a 40 m*M* aspartic acid buffer. (A) 40 m*M* Aspartic acid+20% ACN; (B) 40 m*M* aspartic acid+20% ACN+4 *M* urea; and (C) 40 m*M* aspartic acid+7 *M* urea. Proteins were separated at 25°C and 30 kV in a 30 cm×50 μm capillary; all buffers contained 0.5% hydroxyethylcellulose; from Capelli et al. [53] with permission.

concentrations of 1.25 mg/ml could be detected, although the absolute amount of protein loaded into the capillary was not reported. Good reproducibility was reported when using Asp–7 *M* urea buffer, with migration times varying by 4% over a twenty run period.

2.2. Oats

Oat proteins have also been separated by FZCE using an acidic phosphate buffer containing 0.05% HPMC [58]. Several pairs of closely-related oat cultivars were successfully differentiated using 20 μm I.D. capillaries and conditions similar to those used to separate wheat proteins.

Subsequent studies showed that the separation of oat prolamins was greatly improved when 20% ACN and 26 m*M* SB-12 were added to the separation buffer (Fig. 8) [45]. Using these additives, oat prolamins were separated in less than 10 min, with good resolution.

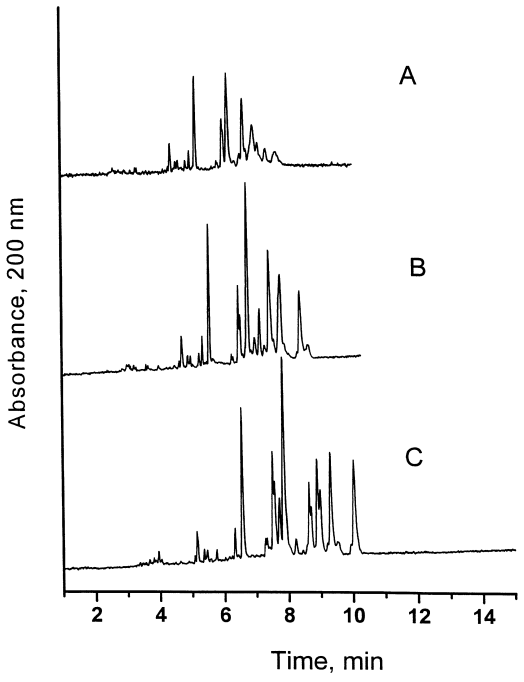


Fig. 8. Separation of prolamins from the oat cultivar Troy; (A) 100 m*M* phosphate buffer, pH 2.5, containing 0.05% HPMC, (B) buffer A+20% ACN, and (C) buffer B+26 m*M* SB-12. Separation conditions as in Fig. 6; from Lookhart and Bean [45].

2.3. Rice

The separation of rice prolamins by FZCE was also reported by Lookhart and Bean [58]. Unlike other cereal proteins, however, 20 μm I.D. capillaries could not be used, since prolamins are less abundant in rice than in wheat and oats [8] and could not be detected. Therefore, 50 μm I.D. capillaries were used. However, successful separations of rice prolamins have been carried out in 25 μm I.D. capillaries [Bean and Lookhart, unpublished data]. Using 50 μm capillaries, several closely-related cultivars could be differentiated by FZCE using 100 m*M* phosphate buffer, pH 2.5 [58]. As with oat prolamins and wheat glutenin, however, the best separations of rice prolamins were achieved when 20% ACN and 26 m*M* SB-12 were added to the buffer (Fig. 9) [45].

3. Applications of FZCE

3.1. Cultivar differentiation

Most reports of FZCE separations of cereal proteins have emphasized the potential use of the methods for cultivar identification or differentiation. Schwartz et al. [34] first demonstrated this application of FZCE by successfully differentiating three wheat cultivars. Bietz and Schmalzried [38,40] subsequently confirmed and expanded on the application of FZCE using an alkaline borate–SDS buffer system for cultivar identification. Similarly, Werner et al. [41] showed the application of a charge reversal system for wheat varietal identification (Fig. 2). Most useful, however, has been the demonstration by Bietz and Schmalzried [38,40] that a low pH phosphate buffer could successfully differentiate wheat cultivars with high resolution (Fig. 10). Subsequent studies using low pH buffers have shown that cultivars could be successfully differentiated in ~10 min or less [43,53] depending on the buffer system used and the samples being analyzed. It is also interesting to note that many cultivars can be differentiated even if buffer systems and operating

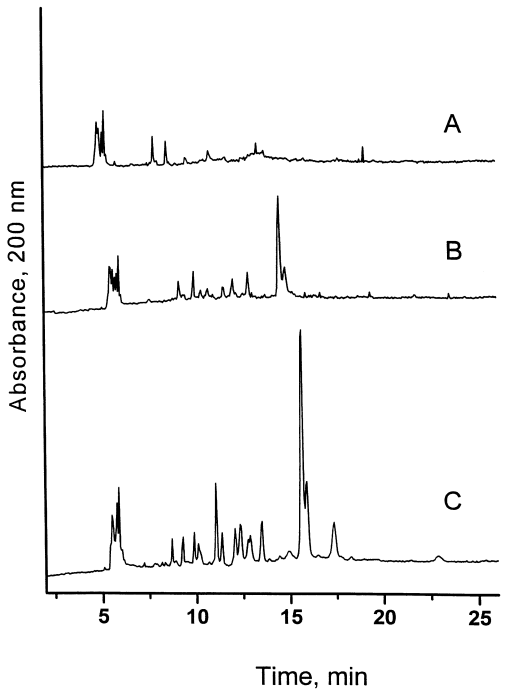


Fig. 9. Separation of rice cultivar IR-28 prolamins; (A) 100 m*M* phosphate buffer, pH 2.5, containing 0.05% HPMC, (B) buffer A+20% ACN, and (C) buffer B+26 m*M* SB-12. Separations were carried out in 27 cm (20 cm)×50 μm I.D. capillaries at 10 kV (+ to −) and 40°C; from Lookhart and Bean [45].

conditions provide less than maximum resolution. For example, Lookhart and Bean [43] reported that 100 m*M* phosphate buffer differentiated all cultivars tested, including even three very closely related lines. However, resolution of this buffer was later improved by addition of 20% ACN. This again points to the flexibility of FZCE: separation conditions can be varied to suit the needs of the researcher in terms of resolution and speed.

The ability of CE to distinguish among different cultivars is also being used in other disciplines in a somewhat analogous fashion. For example, FZCE has recently been used to differentiate fish species [59].

3.2. Characterization of proteins and protein classes

Another important use of FZCE has been to characterize cereal proteins. Werner et al. [41] purified representatives from the four gliadin subclasses and analyzed them to determine their migration order in a charge reversal buffer system. For gliadin subclasses, the migration order was ω-gliadins, followed by γ-, β-, and α-gliadins. In this buffer system, proteins migrate against the EOF that carries proteins through the capillary. Thus, α-gliadins, which are most highly-charged and mobile in the acidic buffer used, are last to cross the detector. This is the exact opposite of the order of migration of gliadins in other buffer systems, as discussed below.

Lookhart and Bean [44] characterized the wheat protein classes resulting from Osborne fractionation [7] by FZCE. Albumins, globulins, gliadins, and glutenins were sequentially extracted from flour and analyzed on 20 μm I.D. capillaries. Albumins and globulins migrated most rapidly and appeared early (at ~2–5 min, depending on conditions used) in the electropherogram, as expected from their higher basic amino acid contents and mobilities in A-PAGE [8]. Gliadins and glutenin subunits had roughly the same mobilities and migrated more slowly than albumins and globulins.

Lookhart and Bean [44] also purified several proteins from each of the four subclasses of gliadins by RP-HPLC and then analyzed these fractions by FZCE to determine their migration order in low pH phosphate buffer. The migration order was α, β, γ, and ω, in order of decreasing effective mobility. This corresponds to the relative mobilities of these proteins upon A-PAGE, which separates proteins by the same mechanism as FZCE, that is, mainly on the basis of charge differences. This migration order is opposite to that reported by Werner et al. [41] in which α-gliadins have the lowest effective mobility.

3.3. Classification

In addition to the ability to differentiate cultivars, Bietz and Schmalzried [40] sought to distinguish hard red spring from hard red winter wheats. When the electropherograms of the wheats used in this study were averaged by class, the resultant averaged hard red winter wheat class electropherogram was found to exhibit more late-migrating gliadins (i.e. γ- and ω-gliadins) than were found in the average hard red spring wheat class electropherogram. The ability

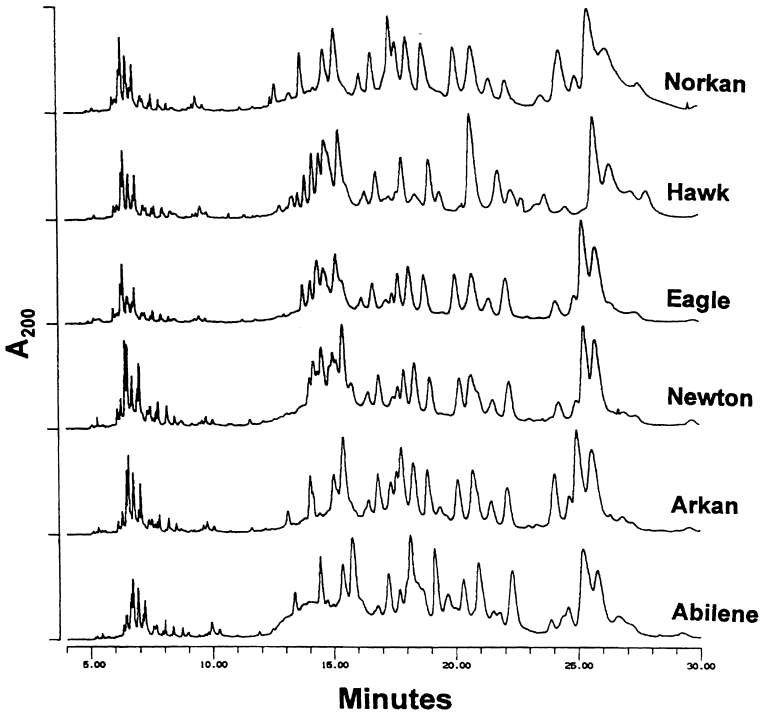


Fig. 10. Differentiation of hard red winter wheat varieties by CE, in the acidic phosphate buffer system, of 30% ethanol-soluble wheat proteins; from Bietz and Schmalzried [40].

to differentiate wheat classes is especially important in grain marketing, since wheat class is closely related to end-use.

3.4. Quality prediction and breeding

One important goal of separating cereal proteins is to relate the presence or amount of specific proteins to end-use quality parameters. With traditional slab gel electrophoresis methods, such quantitation is not easy. In fact, the ability to easily and accurately quantify proteins is one reason why automated HPLC methodology has rapidly gained favor with cereal chemists. FZCE, however, offers the similar capability of easily quantifying and identifying proteins separated by an electrophoretic process. Several reports have already shown the potential for FZCE to screen protein samples for quality-related attributes or to relate separation information to quality related end-use properties. Shomer et al. [60] used FZCE and electron microscopy to investigate the potential role of albumins and globulins in breadmaking. A later study reported similar experiments with gliadins and glutenins [61]. Lookhart and Chung [62] utilized FZCE to study the relationship between environment and protein composition in six samples of the wheat cultivar, Karl, grown in different environments. Amounts of albumins and globulins in 30% ethanol extracts were negatively correlated to bread making attributes.

Lookhart et al. [63] reported that FZCE could identify and differentiate the 1AL.1RS rye translocation from the 1BL.1RS rye translocation in flour; this could not be done by other analytical techniques such as RP-HPLC [8]. Rye translocations improve the agronomic properties of wheat, but can negatively affect bread quality and can make dough excessively sticky [8]; thus it is important to be able to screen breeding lines for the presence of these translocations.

FZCE may benefit wheat breeders by providing new methods to screen new cultivars. For example, novel high-molecular-mass glutenin subunits (HMM-GSs) introduced from wild diploid ancestors (e.g.

Triticum tauschii) or through genetic engineering can be analyzed [Bean et al., unpublished data]. Similarly, FZCE analysis of blocks of gliadins has been used in genetic studies to identify chromosome alleles that are markers of various quality traits [64].

3.5. Two-dimensional RP-HPLC×FZCE

Comparisons of FZCE and RP-HPLC results for the same protein extracts showed that both methods could differentiate most wheat cultivars, but that the resolution of HPCE was slightly higher, particularly when both methods are compared on a similar time scale [40]. FZCE often resolved multiple components from single RP-HPLC peaks [45], demonstrating the advantageous complementary nature of these two methods of protein fractionation.

One of the latest applications of FZCE in cereal protein separations has thus been the combination of FZCE with RP-HPLC, producing two-dimensional separations of gluten proteins [47]. Proteins were first separated by RP-HPLC, and fractions collected at 30-s elution time intervals using an automated fraction collector. These fractions were then directly separated by FZCE [47]. Data from each electropherogram were then combined to form two-dimensional contour plots (Fig. 11A) or three-dimensional surface plots (Fig. 11B). High-resolution separations of both gliadins and glutenin subunits were achieved.

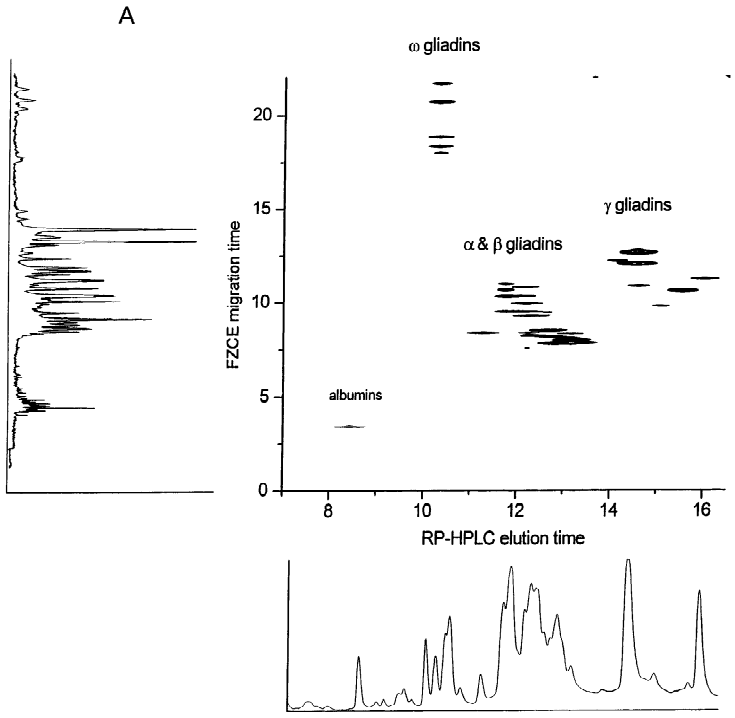
Such separations provide useful information in several ways. First, information about the proteins themselves is revealed. For example, gliadin subclasses cluster together in the two-dimensional maps, confirming their similar chemical compositions as known from sequence analyses [65–67]. Second, these two-dimensional separations provided valuable information about the FZCE separations themselves. Since it is difficult to collect material from FZCE for subsequent analyses, it can be difficult to identify all peaks separated from a complex wheat protein mixture. However, since, most cereal proteins have been well characterized by RP-HPLC (e.g. [68]), proteins can be collected from known RP-HPLC separations and then analyzed by FZCE, providing information about the FZCE separation itself. In this manner, it was determined that FZCE resolves many apparently homogeneous HMM-GSs, known to be related to bread making quality [69], into multiple peaks [47]. Locations of low-molecular-mass glutenin subunits in the FZCE separations were also determined in this manner.

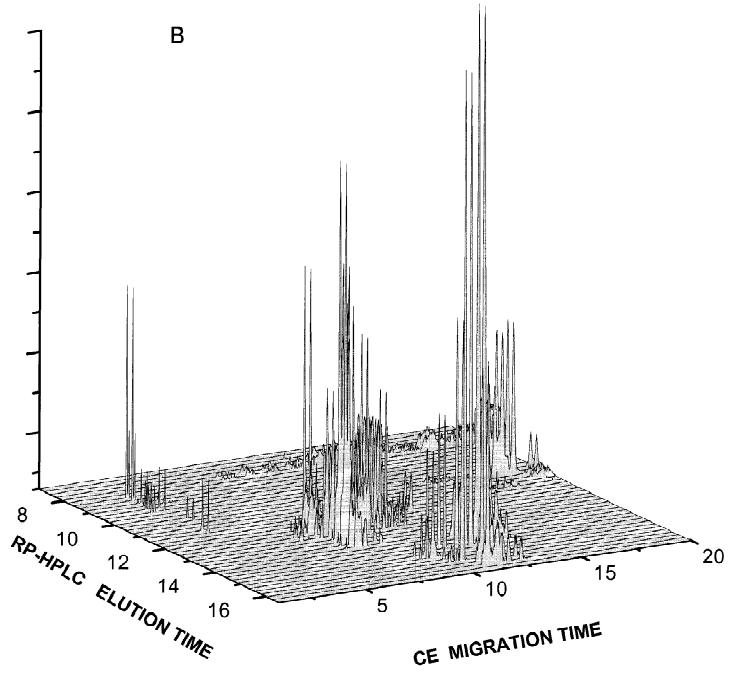
This off-line combination of HPLC and HPCE required no specialized equipment and could be almost entirely automated with use of an automatic fraction collector. Many other types of two-dimensional separations, such as size-exclusion chromatography (SEC) in the first dimension followed by FZCE or capillary isoelectric focusing (cIEF) in the second, should also be possible.

3.6. Miscellaneous

Several additional applications of FZCE have been reported. Turner et al. [70] used FZCE to determine the purity of α-gliadins isolated by combined ion-exchange chromatography and RP-HPLC. O'Keefe et al. [71] used FZCE to study the binding of metals to cereal proteins. Adams et al. [72] used FZCE to examine how friabilins and puroindolines may influence the texture of wheat breads. FZCE has also been used to separate seed proteins of other plants. Cultivars of *Phaseolus vulgaris* have been differentiated by FZCE [73]. Peanut proteins extracted from seeds, leaves, and cell cultures have been separated by FZCE [74] as have soy proteins and hydrolyzates [75]. Albumins from seeds of *Vicia* species have been separated in both coated and uncoated capillaries [76]. Electrophoresis in slab gels has been extremely successful in differentiating many plant species; FZCE may offer even more useful separations [77].

Fig. 11. (A) Contour map of a two-dimensional (RP-HPLC×FZCE) separation of gliadins from the wheat cultivar TAM 105. RP-HPLC separations were with a multistep linear gradient of acetonitrile and water, each containing 0.1% trifluoroacetic acid, at 0.5 ml/min. Collected HPLC fractions were separated on a 27 cm×25 μm I.D. by fused-silica capillary with a 100 m*M* phosphate buffer, pH 2.5 containing 20% acetonitrile and 0.05% hydroxypropylmethylcellulose at 12.5 kV and 45°C; from Bean and Lookhart [47]. (B) Surface plot (three-dimensional) of the two-dimensional (RP-HPLC×FZCE) separation of gliadins from wheat cultivar TAM 105. Individual FZCE separations are plotted vs. time of HPLC elution. Separation conditions were as in Fig. 11A; from Bean and Lookhart [47].


A
ω gliadins
γ gliadins
α & β gliadins
albumins
FZCE migration time
RP-HPLC elution time
0
5
10
15
20
8
10
12
14
16


B
RP-HPLC ELUTION TIME
CE MIGRATION TIME
8
10
12
14
16
5
10
15
20

4. Size-based separations

SDS-PAGE is widely used to separate cereal proteins, and for many years has been an extremely beneficial method. This may best be illustrated by the use of SDS-PAGE to separate wheat's HMM-GSs. These proteins were first separated by SDS-PAGE by Bietz and Wall [18] and were later shown to be related to important end-use properties in wheat [69]. Since then, cereal chemists, geneticists, and plant breeders have relied on SDS-PAGE to identify, study, and screen HMM-GSs in wheat. Because of this important role of SDS-PAGE in cereal chemistry, it was desired to be able to perform analogous separations by CE. Several recent reviews have dealt with the general theory and application of size-based CE separations to proteins [78–80]; the specific application of this technology to cereal proteins is discussed in detail below.

4.1. Methodology

4.1.1. Commercial sizing systems

Most size-based separations of cereal proteins to date have been performed with commercial buffer systems. The first such application was reported by Werner et al. [41], who utilized the commercial (ABI) reagent ProSort to separate total protein extracts from several wheat cultivars. ProSort is a proprietary uncrosslinked poly(acrylamide) of undisclosed molecular mass in a N-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid (TES) buffer, pH 7.0, containing 0.1% SDS [81]. Total proteins from several wheat cultivars were extracted with unbuffered 1% SDS–1% β-mercaptoethanol and separated in a 42 cm×55 μm I.D. capillary at 30°C and 12 kV. For optimum resolution of HMM-GSs, 5% methanol and 5% of a 75% glycerol solution were added to the ProSort reagent. The addition of methanol decreased reproducibility due to gradual evaporation of the alcohol; however, buffer vials were not covered in these studies. In a later study, Sutton and Bietz [82] modified the procedure of Werner et al. [41]. A precipitation step was used to enrich HMM-GSs and remove proteins that migrated close to some HMM-GSs. Addition of glycerol to the ProSort reagent was not necessary. Using this method, excellent separations of HMM-GSs were obtained (Fig. 12). This study used a Beckman CE instrument that allowed buffer and sample vials to be covered with silicon rubber caps, reported by the manufacturer to reduce evaporation during separations [83]; thus, evaporation of methanol was not a problem. Sutton and Bietz [82] also compared the use of constant voltage with constant current, and found that constant current seemed to offer better run-to-run repeatability. Like Werner et al. [41], Sutton and Bietz found that unbuffered SDS worked best as sample buffer. Slight differences in migration order of several HMM-GSs, as compared to mobilities upon SDS-PAGE, were noted by both Werner et al. [41] and Sutton and Bietz [82]. Sutton and Bietz [82] theorized that this may be due to different structures of certain HMM-GSs when moving through a fixed pore (such as those in a PAGE gel) as compared to entangled polymer solutions. HMM-GSs have been reported to possess residual structure when denatured with SDS [84,85], and are widely recognized for their anomalous molecular masses predicted from SDS-PAGE.

Another commercial sizing system has also been

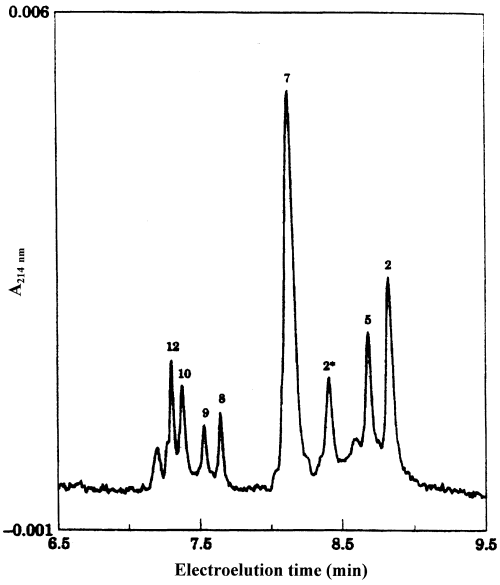


Fig. 12. Size-based CE separation, using a modified ProSort system, of high-molecular-mass glutenin subunits from a mixture of New Zealand wheat cultivars Karamu and Tiritea. Peaks are numbered according to conventional nomenclature; from Sutton and Bietz [82] with permission.

used to separate cereal proteins. Parris et al. [86] used a commercial size-based kit from Bio-Rad to separate and quantify zeins extracted from maize. The method was highly reproducible and allowed direct quantitation of zein fractions. At least one report of size-based separation of proteins from noncereal seeds has also been published. Different *Lupin* genotypes were differentiated by size-based separations using a commercial buffer from Beckman [87].

4.1.2. Other polymers

To date, most size-based separations have used commercially available kits, particularly Prosort. However, to our knowledge, this sieving matrix is no longer available. Thus efforts are underway to find better alternatives. Recently, a preliminary study comparing dextran, pullulan, and uncrosslinked poly-(acrylamide) as sieving agents for the separation of HMM-GSs was reported [48]. All three polymers worked well. Using concentrated buffers, all HMM-GSs were resolved (Fig. 13). This system may provide an alternative to the use of organic solvents such as methanol currently used to resolve HMM-GSs in size-based separation systems.

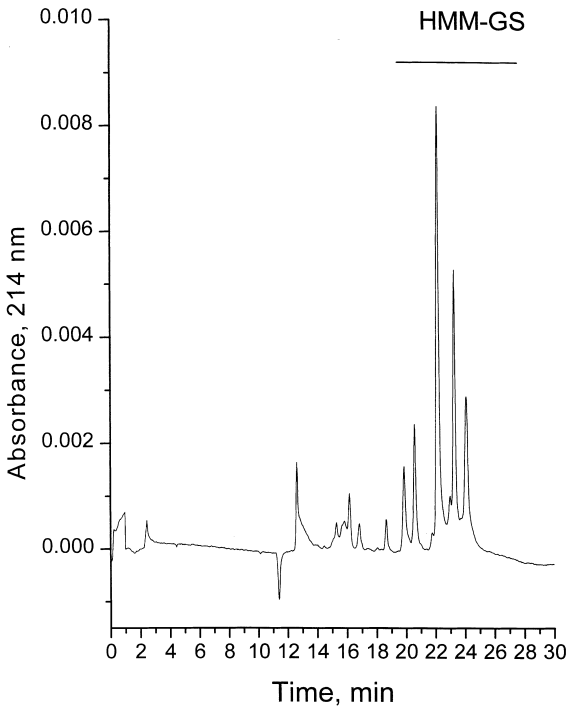


Fig. 13. Size based separation of HMM-GSs from the wheat cultivar Karl. Proteins were separated with 3% uncrosslinked poly(acrylamide) in a 400 m*M* Tris–HEPES [HEPES=4-(2-hydroxyethyl)piperazinethanesulfonic acid]+0.1% SDS buffer, pH 7.0, at 4 kV and 25°C using a 27 cm (20 cm)×50 μm I.D. uncoated capillary; from Bean and Lookhart (unpublished data).

4.2. Applications

4.2.1. Cultivar differentiation

As with FZCE, one of the first successful applications of size-based separations was differentiation of wheat cultivars. Werner et al. [41] showed that cultivars could be successfully discriminated using total protein extracts in conjunction with the ProSort reagent. Pollard et al. [87] successfully differentiated *Lupinus* genotypes utilizing size-based separations.

4.2.2. Ferguson analysis

The HMM-GSs of wheat migrate abnormally upon SDS-PAGE, giving apparent molecular masses significantly higher than those predicted by cDNA sequencing. Werner [88] used the ability of HPCE to rapidly perform Ferguson plot analysis on HMM-GSs to study this phenomenon. Molecular masses determined by Ferguson analyses closely matched those predicted from cDNA sequencing studies. HMM-GSs possessed frictional coefficients similar to those of standard proteins, but had lower charge-to-frictional coefficient ratios [88]. Werner [88] therefore concluded that anomalous SDS-PAGE migration of HMM-GSs was due to decreased SDS-binding and not unusual conformations leading to higher than normal frictional coefficients. No conclusion could be reached concerning the hypothesis that SDS-denatured HMM-GSs retain residual structure, leading to abnormal migration.

4.2.3. Quantitation and quality relationships

Weegels et al. [89] and Bekkers et al. [90] used ProSort reagent to study the composition of gluten macropolymer (GMP), an important measure of breadmaking quality. Kelfkens et al. [91] reported a rapid method for quantifying GMP. This method utilized diluted solutions of ProSort, which gave rapid separations in which all proteins essentially comigrated in one peak, permitting easy quantitation.

5. Conclusions

CE has rapidly taken its place as one of the more powerful and promising techniques available for the fractionation of complex mixtures of storage proteins from cereal grains. It is versatile, rapid, easily automated, readily quantified, and offers high resolution separations that can help identify genotypes and relate structure with functionality. Clearly, HPCE is a valuable addition to other methods of protein analysis that should, in time, be applicable to all protein classes, from all cereals.

Many variables affect precision and selectivity in HPCE, however, and some of these have not yet been optimized. But we must remember that HPCE is still in its infancy and that, as we learn to better understand and control these methods, they will become truly exceptional and useful analytical tools.

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