

Quantitative Analysis of Corn Zeins by Capillary Electrophoresis

NICHOLAS PARRIS,^{1,2} LELAND DICKEY, and JAMES CRAIG**ABSTRACT**

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Kjeldahl analysis is commonly used to measure zein proteins in corn maize ($N \times 5.7$) with no attempt to eliminate contribution from other nitrogen sources. In this study, dry milled corn was extracted with 70% ethanol or 0.1N NaOH and the zein content of the extract measured using capillary electrophoresis in the presence of sodium dodecyl sulfate. The amount of zein protein in alcohol extracts, using this method, was in

good agreement with that determined by Kjeldahl nitrogen analysis. However, less than half of the Kjeldahl nitrogen in the alkaline extracts could be attributed to corn zein. Reproducibility expressed as relative standard deviation for migration time and peak area was 0.10 and 1.05, respectively. The technique permits rapid analysis of a large number of samples without interference from other compounds present in the extracts.

Rapid methods have been reported for quantitation of zein in ground corn. Kjeldahl analysis has frequently been used to measure the total protein content in corn. For example, zein content has been measured from alcohol extracts of corn transferred to filter paper disks and washed to remove nonzein impurities (Dalby 1974, Jones et al 1975). The zein nitrogen of the disk was then determined by micro-Kjeldahl analysis. A rapid turbidimetric method has been developed for the analysis of zein in corn (Paulis et al 1974). Zein, from either whole grain or endosperm, was measured at 590 nm after being precipitated from 70% ethanol and 0.5% sodium acetate with 1% sodium chloride. A colorimetric solid-phase determination for zein using a bicinchoninic acid assay has been reported (Chan and Wasserman 1993). Using this method, zein protein is allowed to react with the acid, resulting in a soluble purple chromophore that is quantified by absorbance (562 nm) measurements.

Reversed-phase chromatographic profiles of zein have been used to identify maize variants (Paulis and Bietz 1988, Wilson 1991, Dombrink-Kurtzman 1994, Peplinski et al 1994). Electrophoretically, SDS-PAGE has been used for the identification and quantitation of corn proteins including zein (Paulis et al 1975; Tsai 1980; Wilson 1986, 1988; Wallace et al 1990; Hamaker et al 1995). Analysis of corn proteins by SDS-PAGE is labor intensive and has a number of drawbacks, such as speed of analysis, reproducibility, and accuracy. Recently, capillary electrophoresis (CE) has been used for the fractionation and identification of wheat proteins (Bietz and Lookhart 1993, Bietz and Schmalzried 1994, Lookhart and Bean 1996, Lookhart et al 1996). These methods should also be applicable to determination of the prolamine fraction in other cereal grains.

For most applications, gel electrophoresis does not provide sufficiently quantitative results, and HPLC does not provide adequate selectivity. Capillary electrophoresis has the advantage of using very small samples that can be separated rapidly with high resolution. In addition, SDS-CE is capable of identifying the molecular weight of unknown proteins by comparison with similar protein standards.

In this study, a SDS-CE method was used to quantify zein proteins in alcohol and alkaline extracts of dry milled corn. Alkaline extracts were examined because new, more efficient methods for extracting zeins are done under alkaline conditions (Wallace et al 1990, Hamaker et al 1995), even though it is well known that deamidation of zein glutamine residues can occur even with relatively mild alkaline treatments. The procedures, used to extract the zein from ground corn, were developed so as to be practical and inexpensive when scaled-up in pilot plant runs.

MATERIALS AND METHODS**Materials**

Yellow dent corn was obtained from Davis Feeds (Perkasie, PA); OptaZein, OZP92001 (>96% protein) from Opta Food Ingredients (Bedford, MA); lysozyme (\approx 95% protein), amyloglucosidase (50 U/mL), and corn gluten, enzymatic hydrolysate from Sigma Chemical Co. (St. Louis, MO); and a protein analysis kit (CE-SDS, Bio-Rad Laboratories, Hercules, CA).

Corn Preparation

Corn was milled by a commercial feed mill. Yellow dent corn was cracked with rollers, and the pericarp was removed by aspiration. This dehulled corn was then fed to a counter-rotating, ribbed disk mill to produce a powder with a median size of 2 mm measured by shaking 50 lb of powder through a stack of sieves. Ninety-five percent of the corn mass was between 3.3 (No. 6) and 1.4 (No. 14) mm. Analysis of the extraction feed gave weight-based values of 6.9% for protein, 2.0% for lipid, and 54% for starch (measured at 12.4% moisture).

Ethanol Extraction

Corn (104.5 kg) was added to 407 L of 60% ethanol at 20°C, mixed by recirculation with a centrifugal pump for 8 hr, and left to stand in the tank overnight. The following morning, the liquid was drained from the corn and filtered through a No. 100 sieve (0.15 mm) and pumped through the centrifuge at \approx 100 kg/hr. After holding for 2.5 hr, the 300 kg of centrifugate was diluted to 40 wt% ethanol by adding 170 kg of tap water to the tank. The tank was chilled to 2°C and held for 16 hr with gentle mixing. The product (253 g) was buoyant in the liquid and removed by scooping off the top of the tank, and another 1,311 g with similar chemical composition was removed by centrifugation. The products were freeze-dried and milled with the laboratory mill (essentially to break up aggregates formed in the centrifuge). The buoyant material was subjected to particle size distribution and electrophoretic analyses.

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Alkaline Extraction

Sodium hydroxide (0.1N, 36.3 L) was added to 4.54 kg of the extraction feed at 20°C, pH 12.3. The mixture was cooled to 13°C and held with gentle mixing for 3 hr. The liquid was then separated from the mixture and pumped through a filter with 0.3-mm holes and then through a 104.8-mm tubular bowl centrifuge (Sharples Corp., Philadelphia, PA) rotating at 15,000 rpm, generating a force of $13,200 \times g$. The solids were discarded and the centrifugate was neutralized (to pH 6.7 at 22°C) with 795 mL of 2N HCl. After 30 min, the pH had risen to 7.6 and the pH was dropped to 7.1 with an additional 10 mL of 2N HCl. The liquid

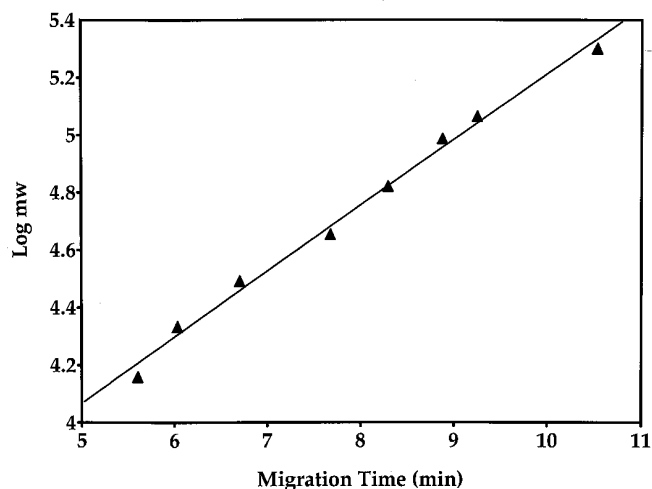


Fig. 1. Molecular weight calibration plot for standard proteins (14.4–200 kDa)

and precipitate were pumped through the centrifuge, and the precipitate was scraped from the centrifuge bowl and mixed with 19 L of 10% NaCl solution and allowed to stand for 17 hr before another 19 L of 10% NaCl were added and mixed for 30 min. The suspension was pumped through the centrifuge and the recovered solid washed in 38 L of tap water for 1 hr. Another 38 L of tap water were added and mixed for 30 min; the mixture was pumped through the centrifuge. The recovered solid was then freeze-dried using a freeze dryer (Labconco 5, Labconco Corp., Kansas City, MO), ground to a powder with a laboratory mill (Wiley No. 1, Arthur H. Thomas, Philadelphia, PA), and subjected to particle size distribution and electrophoretic analyses.

Chemical Analysis

Protein content ($N \times 5.7$) of zein extracts was obtained using Kjeldahl method AOAC 2.055 (1984). Reported results are the mean of two separate determinations.

Starch was determined as described in Sigma Technical Bulletin SAB-1. Approximately 100 mg of dried extract was weighed into a 250-mL Erlenmeyer flask to which 25 mL of deionized water was added with stirring. The pH of the mixture was adjusted to between 5 and 7, if necessary, and boiled for 3 min while stirring. The mixture was then autoclaved for 1 hr at 135°C and allowed to cool to $\approx 60^\circ\text{C}$; then 100 mL of deionized water was added. An aliquot of the starch-containing solution was mixed with 1 mL of amyloglucosidase (50 U/mL) from *Aspergillus niger* (Sigma Product No. S9144) and the solution was incubated at 60°C for 15 min. After cooling, glucose that was enzymatically liberated from starch was determined by use of a YSI glucose analyzer, model 2000 (Yellow Springs Instrument Co., Yellow Springs, OH). The amount of starch present was calculated from the glucose concentration.

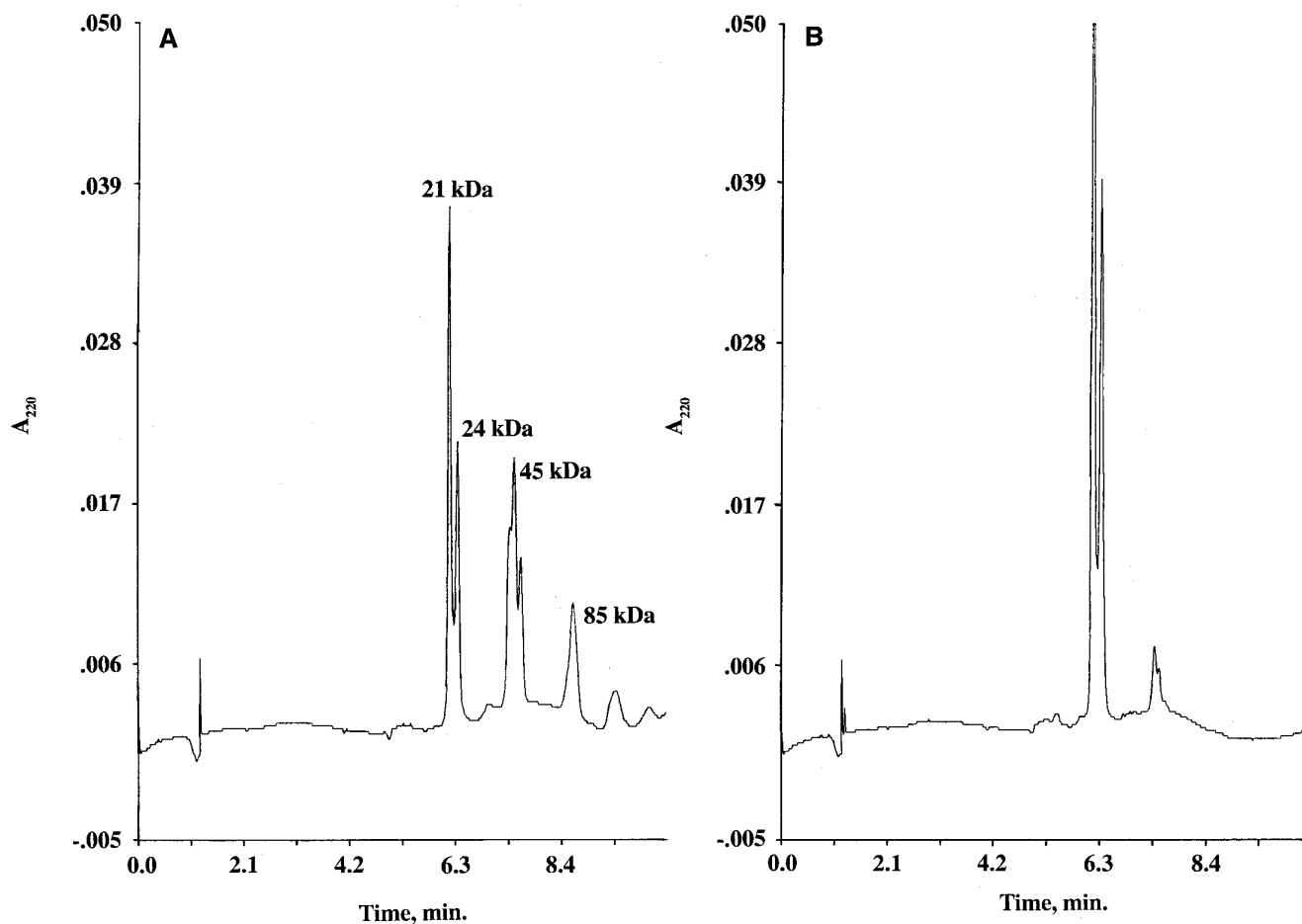


Fig. 2. Sodium dodecyl sulfate capillary electrophoresis of commercial zein: unreduced (A) and reduced (B).

Lipids were estimated by extracting 100–200 mg of dry extract with 5 mL of hexane and subsequently with 5 mL of chloroform. The hexane and chloroform extracts were collected in tared vials and evaporated to dryness (constant weight) under a stream of nitrogen. Moisture content was determined by drying samples in a 110°C oven for 24 hr.

Capillary Electrophoresis

All CE analyses were performed on a Bio-Focus capillary electrophoresis system using a protein analysis kit (CE-SDS, Bio-Rad Laboratories). Conditions were optimized for the quantitation of zein proteins. Dried zein protein extracts (2.0–6.0 mg) were solubilized in 1 mL of the sample buffer containing 10 μ L of 2-mercaptoethanol and the mixture was heated at 100°C for 20 min. Complete mixing of the solutions was accomplished by vortexing at 5-min intervals. The samples were cooled and microfuged for 5 min at 12,000 \times g. Separations were performed using an uncoated fused silica capillary (24 cm \times 75 μ m i.d.) at 15 kV and 20°C for 15 min. Samples were injected for 5 sec at 10 kV (electrophoretically) and proteins were detected at 220 nm. OptaZein (>96% protein) was used as the standard for quantitation of the extracted zein proteins.

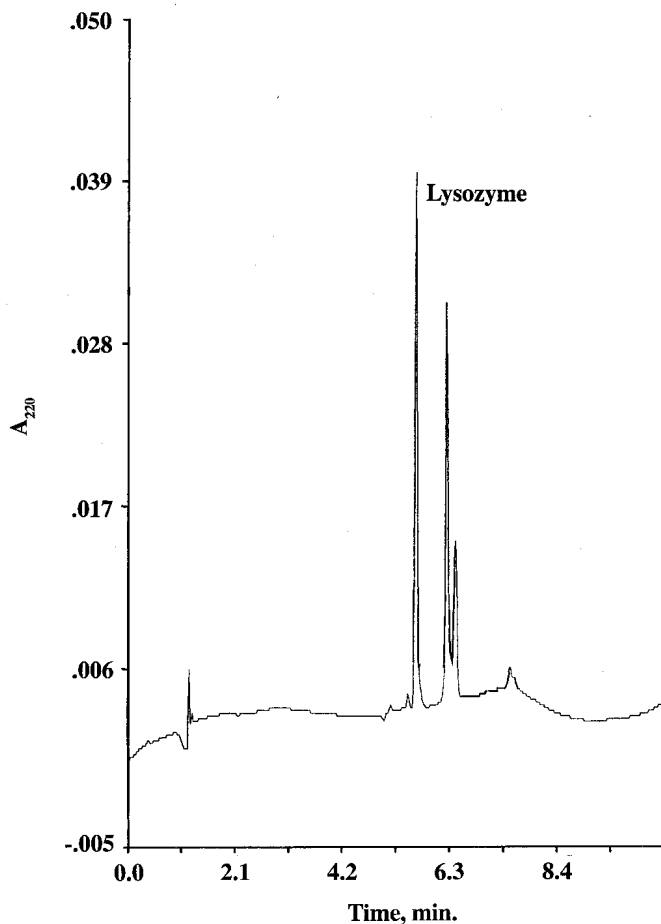


Fig. 3. Sodium dodecyl sulfate capillary electrophoresis of commercial zein spiked with lysozyme.

The standard curve was constructed by dissolving \approx 4.0 mg of OptaZein in 2 mL of sample buffer containing 20 μ L of 2-mercaptoethanol. The sample was heated and microfuged as described above and diluted with sample buffer to \approx 0.25, 0.50, 0.75, 1.0, and 2.0 mg/mL. Protein molecular weight and OptaZein standards were run before quantitation of zein extracts. Molecular weight standards (Bio-Rad Laboratories) and their corresponding molecular weights were: lysozyme, 14,400; trypsin inhibitor, 21,500; carbonic anhydrase, 31,000; ovalbumin, 45,000; serum albumin, 66,200; phosphorylase B, 97,000; β -galactosidase, 116,000; and myosin, 200,000. Zein protein molecular weights in corn extracts was estimated using the BioSize software (Bio-Rad Laboratories).

RESULTS AND DISCUSSION

Considerations in developing the CE method for the quantitation of zein proteins in different substrates were that: 1) an accurate standard curve is constructed using zein proteins with UV-absorption properties similar to those in the sample being analyzed, and 2) zein proteins are extracted reproducibly from different substrates. The proposed SDS-CE method permits the separation of proteins as SDS-protein complexes using a sieving mechanism. Base line separation of molecular weight standards (14,400–200,000 Da) was achieved in <11 min, and the calibration plot of molecular weight vs. migration time (Fig. 1) had a correlation coefficient of 0.993. The electropherogram of commercial zein consisted of two partially resolved α -zein proteins with migration times of 6.2 and 6.4 min (Fig. 2A). The α -zeins are a complex group of closely related prolamines with molecular weights of 19 and 22 kDa (Wallace et al 1990). Estimation of their molecular weight relative to the migration time of protein standards indicated that the molecular weights of the α -zeins were 21 and 24 kDa. The next two groups of peaks on the electropherogram, with migration times centered around 7.5 and 8.6 min, appear to be the dimer and tetramer of the α -zeins with molecular weights of \approx 45 and 85 kDa and are similar to previous findings by SDS-PAGE (Paulis and Bietz 1988). These polymers and other higher molecular weight polymers with migration times of 9.5 and 10.0 min appear to be associated primarily through disulfide linkages, since only

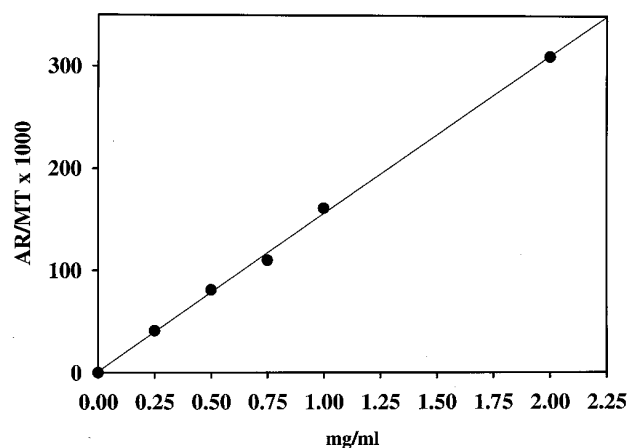


Fig. 4. Response linearity for α -zeins. AR/MT = area/migration time.

TABLE I
Analysis (%) of Corn Extracts

Solvent	Chemical Analysis			α -Zeins Electrophoretic Analysis ^a	
	Protein	Starch	Oil	Moisture	SDS-CE ^b
70% Ethanol	77.68	0.15	8.11	2.31	74.4 \pm 4.1
0.1N NaOH	73.62	10.01	5.77	1.29	31.0 \pm 2.8

^a n = 6.

^b Sodium dodecyl sulfate capillary electrophoresis.

the monomers plus a small amount of dimers were present after reduction with 2-mercaptoethanol (Fig. 2B). Small peaks present in reduced zein samples before and after the α -zeins are probably albumins, globulins, (≈ 5 min) glutelins, or a small amount of unreduced dimers (7.5 min) based on SDS-PAGE assignments (Wallace et al 1990).

The reproducibility of the method was determined by spiking zein with lysozyme (14.4 kDa). The latter protein was selected because it was completely resolved from other corn proteins (Fig. 3). Normalized data were calculated from the migration time of the first α -zein peak and the total area of both α -zein peaks for six replicate samples. The percent relative standard deviation for migration time and area were 0.10 and 1.05, respectively. The concentration of α -zein in extracts was determined by measuring area/migration time relative to the standard (Fig. 4). Area/migration time was used rather than area because in CE, peaks are moving through the detection window at their electrophoretic velocity plus the rate of electro-osmotic flow. As a result, the residence time of each peak in the detection window will be different. Because the peak area depends on the UV response of the analyte and its residence time, it is necessary to compensate by normalizing peak area to migration time. The linearity of area/migration time versus the concentration of zein in the standard curve was >0.995 at concentrations ≤ 2.5 mg/mL.

Chemical analysis of both extracts indicated that they contained $\approx 70\%$ protein and $\approx 6-8\%$ oil (Table I). The alkaline extract contained $\approx 10\%$ starch and the alcohol extract contained very little (0.15%). Analysis of six replicate samples of the dried extracts by SDS-CE indicated that the alcohol and alkaline extracts contained 74.4 and 31.0% α -zeins, respectively (Table I). Comparison of

both methods indicated that almost all of the nitrogen present in the alcohol extract was protein nitrogen but less than half was present as protein nitrogen in the alkaline extract. Examination of the extract electropherograms indicated that the alcohol extract consisted almost entirely of α -zeins (Fig. 5A). The alkaline extract, however, contained α -zeins and both higher and lower molecular weight material (Fig. 5B). Since the lower molecular weight compounds (≈ 5 min) comprise $<12\%$ of the electropherogram, it appears that a significant amount of deamidation of the corn proteins occurred. Apparently the glutamine residues of the zeins were hydrolyzed to glutamic acid and did not precipitate with the α -zeins after neutralization and centrifugation.

The previous methods used for the analysis of zein proteins have unique advantages. The turbidimetric method of analysis is rapid, easy to use, and requires essentially no specialized equipment. Chromatographically, the RP-HPLC method permits the separation of proteins based on their hydrophobicity. Protein fractions can be collected from the analytical column and further characterized. SDS-PAGE is useful primarily in comparing and differentiating these fractions or other proteins samples primarily by molecular weight. The CE method is direct, requiring no preliminary delipidation or desaccharification of samples. Detection is based on light absorption by protein, eliminating problems associated with nonprotein nitrogen and nonspecific dye binding. The method is rapid and provides a means of processing a large number of samples reproducibly and economically. It will benefit food scientists and engineers by providing a more quantitative measure of zeins used in the preparation of films or encapsulating material, used to extend product shelf-life, or as a protein-based fat mimic substance.

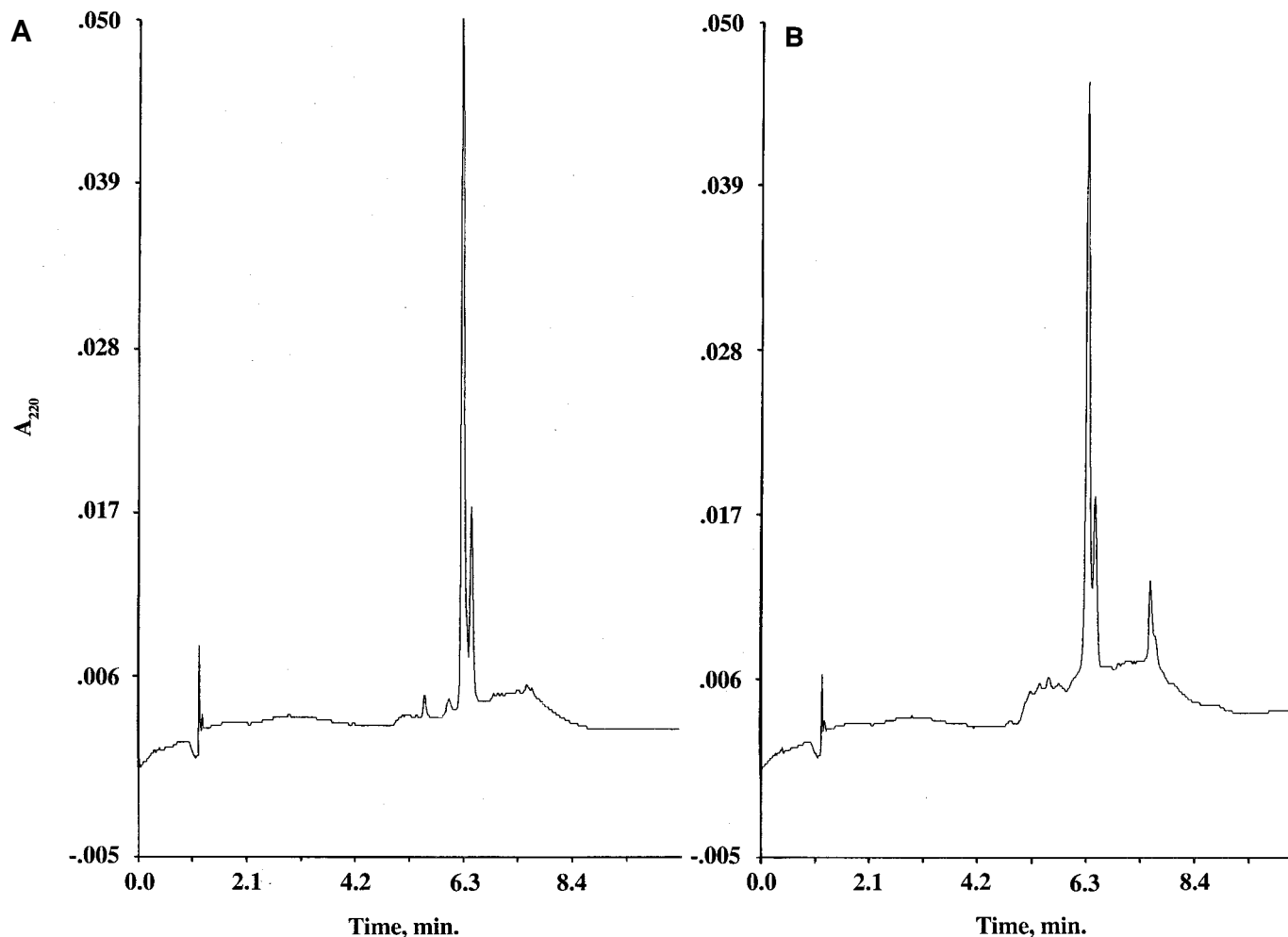


Fig. 5. Sodium dodecyl sulfate capillary electrophoresis of alcohol extract (A) and alkaline extract (B).

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