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ARTICLE

Finding the "Bio" in Biobased Products: Electrophoretic Identification of Wheat Proteins in Processed Products

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Verification of the biocontent in biobased or "green" products identifies genuine products, exposes counterfeit copies, supports or refutes content claims, and ensures consumer confidence. When the biocontent includes protein, elemental nitrogen analysis is insufficient for verification since non-protein, but nitrogen-rich, content also may be present. However, the proteins can be extracted, separated by electrophoretic methods, and detected by UV absorption, protein stain, or immunoblotting. We utilized capillary zone electrophoresis (CZE) to separate proteins in a gliadin fraction that had been dissolved in aqueous ethanol (70%) and polyacrylamide gel electrophoresis (PAGE) to separate proteins in a gliadin-plus-glutenin fraction that had been dissolved in water containing both sodium dodecyl sulfate (SDS) and a reducing agent, dithiothreitol (DTT). We sought to verify the presence of these wheat grain proteins in wheat bread, a wheat flake cereal, wheat beer, and an enclosure for an antique automobile ignition coil reputed to contain wheat gluten. Proteins extracted from commercial wheat, corn, and soy flours served as standards, and proteins from heat-altered wheat served as process condition references. This approach successfully identified wheat proteins in these products especially if the process temperature did not exceed 120 °C. Above this temperature attenuation was nearly complete for proteins analyzed by CZE, but wheat-like patterns could still be recognized by one- and two-dimensional PAGE. Immunoblots reacted with grain-specific antibodies confirmed the identities of the cereal component especially when the protein pattern was greatly altered by thermal modification, specific protein adsorption, or protein digestion. In addition to verifying that wheat proteins are present, the complementary use of these methods can reveal whether whole wheat gluten or merely an alcohol-soluble fraction had been used in the specific product and indicate the level of thermal damage.

KEYWORDS: Biobased products; wheat; protein; gliadin; glutenin; denaturation; capillary zone electrophoresis

INTRODUCTION

Biobased products are manufactured in whole or part from minimally refined agricultural crop components such as harvest and processing residues, moderately refined plant organs, and/or highly refined starches, proteins, or other plant chemicals. Unfortunately, the identity of a biocomponent may not be apparent in the bioproduct. Unscrupulous processors may take advantage of this ambiguity by making false and fraudulent claims or substituting a petro- or old-carbon component for the biocomponent. Thus, it is in the interest of the consumer and ethical manufacturer of biobased products to have postmanufacture methods by which the composition may be verified to distinguish between the genuine and the counterfeit, uphold intellectual property claims, support or dispute content claims in product labeling and advertising, and ensure that the product poses no consumer risk. Verification methods also provide definitive support of proposed biobased content labeling guidelines and existing biobased affirmative procurement programs where more information than merely the biological or "new" carbon content is desirable (*1−3*).

The biocomponent in some biobased products may include cereal protein concentrates such as corn zein, wheat gluten, and soy protein or proteins contained in milled whole grains and/or distillers' grains. Wheat gluten protein is used in adhesives, films, fibers, coatings, polymers/resins, inks, detergents, cosmetics, hair-care products, adhesives, rubber products, milk replacers, and functional food products (*4−8*). Protein content is important in foods and food ingredients and is determined routinely by analysis of elemental nitrogen in sample combustion gases. For protein determination the analysis generally assumes that only protein nitrogen is present. The protein is estimated by multiplying nitrogen mass by 5.7 to 6.2, depending on the source matrix. The method does not identify the protein origin, and when non-protein, but nitrogen-rich compounds, such as melamine or melamine resin, are present, combustion-based protein determinations will be falsely high.

Less ambiguous analyses of protein are achieved by chromatographic and electrophoretic methods coupled with solvent-based

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leaching that may irreversibly deconstruct the protein matrix. These methods have been applied to and optimized for native cereal proteins to characterize and identify them. Cultivars have been distinguished and identified (*9*), and protein composition has been correlated to flour functional properties (*10–14*) as well as to preharvest environmental provenance (*15–17*). However, physical and thermal alteration of the protein polymer and irreversible interactions between protein and product components can present problems for analysis of processed products.

In the present study, we sought to (a) determine if a grain-sourced protein was present in biobased products, (b) estimate its botanical source and solubility characteristics, and (c) assess the level of thermal alteration during manufacture and/or use. We did this by applying capillary zone electrophoresis (CZE) and/or polyacrylamide gel electrophoresis (PAGE) to soluble protein. For CZE, which resolves on the bases of charge and size, we used a low power solvent system of 70% ethanol in water that dissolves gliadins and metabolic proteins. For PAGE, which resolves on the basis of molecular weight, we used a robust, reactive solvent system containing an aqueous detergent and a chemical reducing agent that dissolves gliadins and most glutenins. Immunoblotting of the resolved peaks was applied to specifically identify wheat (*18*, *19*).

Substrates examined were grains or berries, wheat flour, and wheat flour dough that were exposed to convective drying conditions and then analyzed to produce reference patterns for subsequent interpretation and comparison. We then examined processed products that included two types of baked bread, a cooked and dried, flake-type cereal, and a wheat beer. We also examined an antique enclosure for the ignition coil of the 1916–1918 Model-T Ford automobile. The enclosure was molded from asbestos and "reputedly" a wheat gluten binder. However, because of Henry Ford's interest in soy-derived plastics, the coils have been referred to, occasionally, as "soy" coils (*20*).

MATERIALS AND METHODS

Materials. Flour from Butte 86, a hard red spring wheat, was milled from grain produced under controlled greenhouse conditions (*21*). "Peak performer" unbleached flour was obtained from a commercial bakery supplier (Giusto, San Francisco, CA). The "Peak performer" flour is a blend of dark northern spring wheat and hard red winter wheat from Montana. A proximate analysis for this flour was 13.4% protein (N × 5.7), 5.6% lipids, 69.1% carbohydrates, and 0.6% ash (Anresco, San Francisco, CA). Intact hard red winter wheat berries of unknown cultivar(s) were obtained from a process line leading to starch and gluten separation (MGP Ingredients, Atchison, KS). Intact hard red winter wheat berries of the cultivar "Turkey" were obtained from the National Small Grains Germplasm Research Facility (National Small Grains Collection, 1691 S 2700 W, Aberdeen, ID 83210). Flour samples were used as received. Wheat berries were milled prior to analyses in a laboratory mill (Model 310; Perten Instruments, Inc., Springfield, IL).

Wheat beer, whole wheat bread, white sourdough baguette, a wheat-based flaked breakfast cereal, soy flour, and corn flour were obtained at local markets. A Model-T ignition coil was provided by Keith Townsend (Model-T Ford Club of America, Gresham, OR).

Thermal Pretreatment. Grain, flour, and dough samples were exposed to convective heat transfer in up-flowing heated air at 3.1 m/s using a fluid bed dryer (Sherwood Scientific, Cambridge, U.K.). The time of exposure for wheat berries and flour was 1 h. Dough samples mixed for 7 min with a KitchenAid mixer at 65% water absorption were placed in the grooves of a Kiefer extensibility molding frame (Texture Technologies Corp., Menlo Park, CA), each lane of which had a trapezoidal cross section 4 mm deep, 4 mm at top and 3 mm at bottom, and 55 mm long to yield 1–2 g strips that were exposed to the air flow while still in the molding frame until they became milling-friable (30 min at 200 °C, 60 min at 150 °C, 150 min at 100 °C, and 3600 min at 60 and 27 °C).

Total protein content was determined using a combustion-based nitrogen analyzer (Leco Corp., St. Joseph, MI).

Capillary Zone Electrophoresis. Samples for this analysis were leached at 25 °C in 1.4 mL of aqueous ethanol (70 vol %) for 17 h with an Eppendorf thermomixer R (Brinkman Instruments Iinc., Westbury, NY). Sample amounts for leaching were 250 mg for dried dough, commercial flour blend, and flour from laboratory-milled whole berries; 170 mg of unaltered interior and exterior for baked bread (moisture content: 23% for baguette crust, 39% for baguette interior, 31% for bread loaf crust, 34% for bread loaf interior); 100 mg for flour milled from wheat cv. Turkey; 370 mg for soy flour; and 190 mg for commercial flake cereal. For the ignition coil, samples were abraded from three locations of the outer casing. The removed portion was milled in a mortar and pestel to a fibrous powder and a 5 g portion was leached in 30 mL of 70% aqueous ethanol for 24 h. The mixture was subjected to homogenization for 0.5 min (Omni Mixer Homogenizer; Omni International, Waterbury, CT) followed by 2 h leaching at 50 °C. After the leaching period, samples were centrifuged at 14000*g* (Eppendorf 5415C; Brinkman Instruments, Inc., Westbury, NY) for 10 min at ambient temperature to settle the insoluble material. The recovered supernatant was filtered with a 0.45 μm syringe prior to capillary electrophoresis analysis. For wheat beer, an aliquot was centrifuged at 14000*g*, and the supernatant was filtered with a 0.45 μm syringe prior to capillary electrophoresis analysis.

An Agilent capillary electrophoresis system was used in all separations (model G1602A; Agilent Technologies, Wilmington, DE). Capillaries were uncoated fused silica (G1600; Agilent Technologies, Wilmington, DE) with 50 μm diameter and 24.5 cm effective length. Buffer used was a 0.1 M pH 2.5 phosphate buffer containing a hydroxypropyl, methylcellulose, linear polymer additive (Bio-Rad 148-5011, Hercules, CA). All samples were pressured injected at 50 mbar undiluted. Unless otherwise indicated, injection time was 2 s for dough, flour blend, and flour milled from whole berries; 5 s for bread and wheat beer; 20 s for flake cereal; and 20 s for ignition coil. Samples were separated at 40 °C and 15 kV. Proteins were detected by UV absorbance at 200 nm (*22*, *23*).

Polyacrylamide Gel Electrophoresis. Samples were leached with an SDS buffer (2% SDS, 10% glycerol, 50 mM DTT, 40 mM Tris-HCl, pH 6.8) described previously (*24*) but with modifications depending on sample. "Butte 86" flour (50 mg) was suspended in 800 μL of SDS buffer and incubated for 1 h at room temperature with intermittent vortexing. The wheat-based flake breakfast cereal was first ground to a powder using a mortar and pestle at room temperature and leached similarly. Soy or corn flour (75 mg) was suspended in 1.5 mL of SDS buffer and boiled for 15 min (*25*). Wheat beer (600 μL) was centrifuged at 14000*g* for 15 min at 4 °C (Tomy MRX-151; Peninsula Laboratories, Inc., Belmont, CA). An equal volume of 2× SDS buffer was added to the supernate, and the mixture was incubated for 15 min at room temperature with intermittent vortexing. The pellet was suspended in 30 μL of SDS buffer and incubated for 45 min at room temperature with intermittent vortexing. A razor blade was used to cut or scrape crumb and crust from wheat bread or baguette into a mortar, and samples were ground to a powder in liquid nitrogen using a pestle. The powder (50 mg) was suspended in 800 μL of SDS buffer and incubated at room temperature for 30 min with intermittent vortexing. Material from the ignition coil (100 mg) was suspended in 150 μL of SDS buffer and incubated at room temperature for 2 h with intermittent vortexing.

Following incubation, insoluble material was removed from leachates by centrifugation at 16000*g* for 12 min at room temperature (Eppendorf 5415C; Brinkman Instruments, Inc., Westbury, NY). Aliquots were removed from the SDS extracts for Lowry colorometric protein assay (*26*) with adaptation (*21*). Prior to assay, protein was precipitated from the samples with 4 volumes of acetone. However, the wheat crust and beer samples were precipitated with 5 volumes of 0.1 M ammonium acetate in methanol to remove a brown component that interfered with the Lowry assay. Protein amounts could not be determined for the ignition coil due to a dark coloration in the sample that could not be removed with acetone or ammonium acetate in methanol precipitation methods.

Following centrifugation, proteins in the SDS supernate were separated by one-dimensional PAGE or 1-DE (*19*). Protein was applied (6 μg of protein per lane) to the SDS gel for all but the ignition coil sample, for which 15 μL of extract was loaded per lane. Following electrophoresis, proteins were visualized with Coomassie Brilliant Blue G-250. Western blots were produced according to a previously described method (*19*). Protein was applied (2 μg of protein per lane) to the SDS gel for all samples
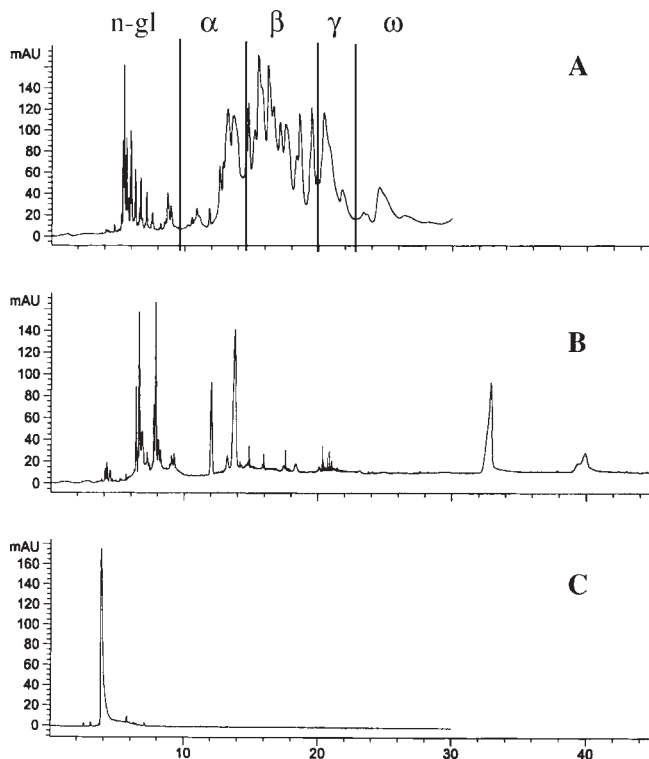
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Figure 1. CZE patterns of milled wheat (**A**), soy (**B**), and corn (**C**). Proteins extracted by 70% ethanol in water prior to electrophoresis. Wheat sample: pressure injected at 50 mbar for 5 s.

Figure 2. Coomassie Blue stained (shown as red-filtered monochrome digital enhancement) 1D gels (**A**) of wheat (1), soy (2), and corn flour (3) and a Western blot (**B**) using a wheat-specific antibody probe to wheat prolamines. Lane "S" contains molecular weight standards. Proteins extracted by SDS/DTT prior to electrophoresis.

except the ignition coil, for which 15 μL of extract was loaded per lane. Proteins were transferred to Immobilon-P (Millipore Corp., Bedford, MA) using a Mini Protean transfer cell (Bio-Rad Laboratories, Hercules, CA); electrotransfer was performed at 100 V for 45 min. Blots were incubated with polyclonal antibodies to wheat prolamines (1:3000; Aldrich Chemical Co., Milwaukee, WI) and then with secondary antibodies conjugated to alkaline phosphatase. Proteins were visualized by reaction with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Two-dimensional PAGE or 2-DE was performed by previously described methods (*21*). Proteins were precipitated from the SDS extracts by addition of 4 volumes of acetone and incubation overnight at −20 °C. Protein was recovered by centrifugation at 16000*g* for 10 min and dissolved in urea buffer, and 18 μg was applied to the first dimension IEF gel. Proteins were separated in the second dimension by SDS gel electrophoresis and visualized with Coomassie Brilliant Blue G-250.

RESULTS

Grain Baselines. Baseline CZE data obtained for proteins from milled wheat, soy, and corn flour are shown in **Figure 1**. Each electrophoresis pattern had a characteristic pattern of proteins that was consistent with published data. The peaks in the more complex wheat electrophoresis pattern (**Figure 1A**) were clustered into five groups of structurally similar proteins. Following convention these were labeled as "n-gl" for the nongliadin protein and α, β, γ, ω representing the principal electrophoretically defined gliadins. By contrast, the soy electrophoresis pattern (**Figure 1B**) had fewer, more isolated peaks, suggesting protein groups with a smaller number of family members. The single corn protein peak (**Figure 1C**) essentially matched that of zein (not shown), the principal storage protein of corn kernels.

1-DE analyses of proteins present in wheat, corn, and soy flours are shown in **Figure 2**. Since the leaching solvent contained SDS and DTT, the protein patterns for these flours were more complex than the CZE patterns (**Figure 2A**). Protein patterns were dominated by storage proteins, which are unique for each
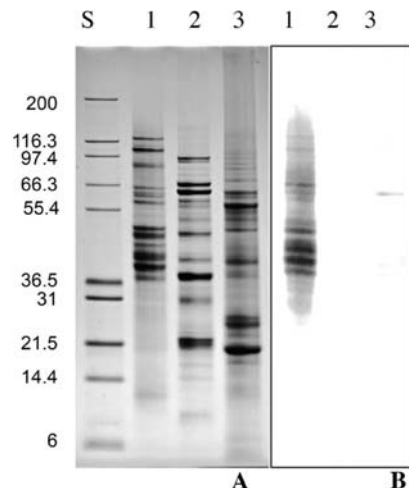
flour type. Commercial wheat polyclonal anti-prolamin antibodies were used to probe Western blots to determine their specificity to wheat, soy, and corn proteins. Wheat prolamins consist of gliadins and glutenins. The gliadins are a polymorphic collection of monomeric proteins, and the glutenins consist of high molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS) linked by interdisulfide bonds to form large insoluble polymers. As expected, the antibodies reacted with proteins in each of the prolamin groups in wheat flour (**Figure 2B**). The antibodies also reacted faintly with proteins in soy and corn flours. The weak response could be due to a wheat contaminant in the tested corn flour introduced by its manufacturer, wheat contamination of the meals used to induce the antibodies, or to nonspecific antibody reactions.

Heat-Damage Reference Modes. Three model modes of thermal exposure of wheat proteins were used to create reference electrophoresis patterns to alterations that might be encountered in products containing wheat or wheat protein. These modes involved simultaneous heating and drying of (1) intact wheat berries, (2) a milled wheat flour, or (3) fully developed and formed wheat dough. Resultant CZE patterns are shown in **Figure 3** with protein family groupings labeled and linked treatment to treatment to account for peak retention time variation and emphasize the pattern similarities that persist at least up to 120 or 150 °C. Unless the thermal conditions applied were severe, i.e., > 120 °C, the CZE patterns of the denatured proteins were usually readily recognizable as wheat or wheat-like with close similarity to the unheated control. Furthermore, the overall patterns (relative retention times and peak height ratios) were essentially maintained as the temperature was increased, but all peaks were greatly attenuated. In the case of drying developed dough the nongliadin peaks exhibited relatively greater attenuation as the temperature was raised when compared to the gliadin peaks. Other exceptions to the general observation were found in the 40−70 °C region where there was moderate attenuation for kernels and severe attenuation for flour of rapidly eluting proteins (α, β range of proteins) and changes to both relative peak height and ratios. We note that attenuation and peak alteration at relatively low temperature, 40−50 °C, have also been reported for CZE applied to alcohol leachates of grains stored at constant moisture and for reversed-phase HPLC applied to leachates of
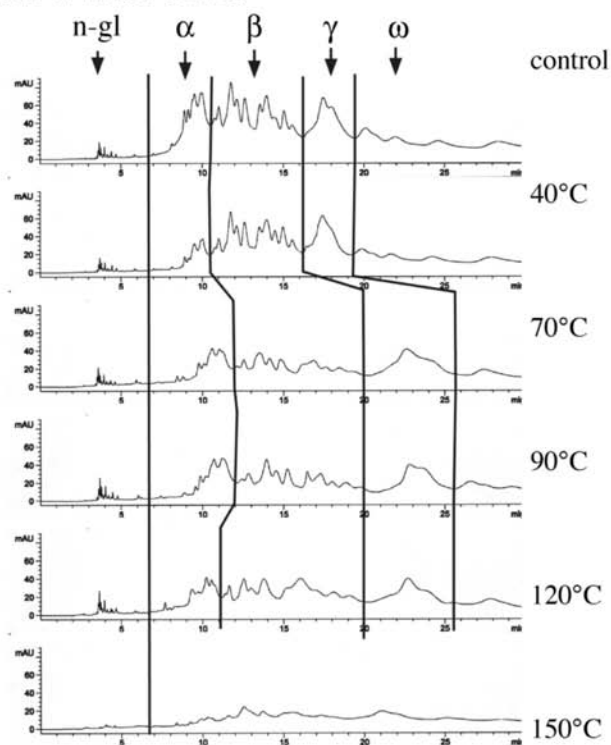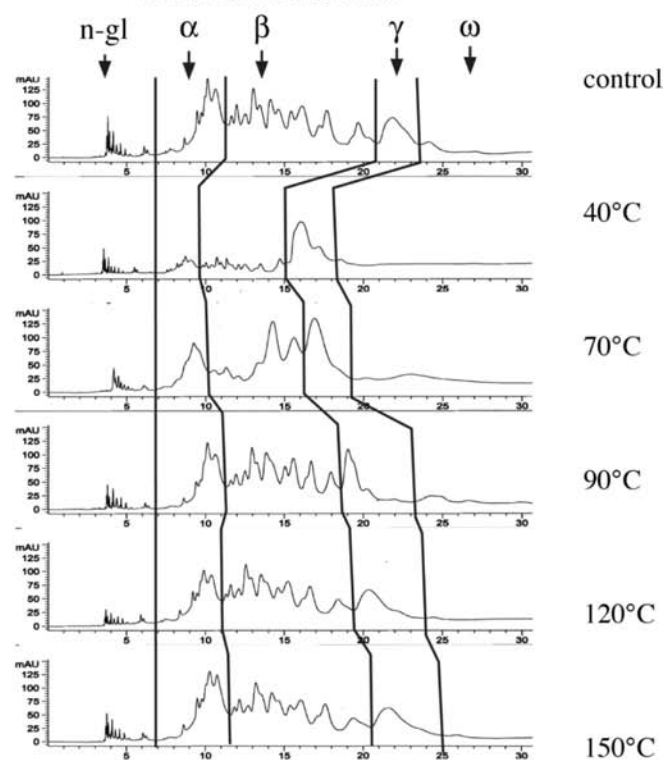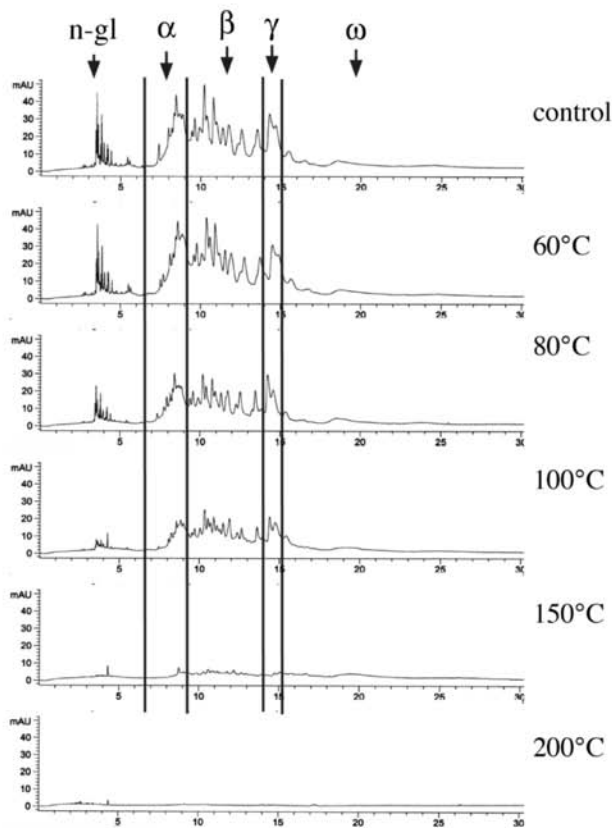
Figure 3. CZE patterns of heat-altered wheat. Mode 1: Berries in fluid bed (1 h). Mode 2: Flour in fluid bed (1 h). Mode 3 dough strips in fluid bed (0.5 h, 200 °C; 1 h, 150 °C; 2.5 h, 100 °C; 6 h, 80, 60, and 27 °C). Proteins extracted by aqueous ethanol (70%) prior to electrophoresis. Electrophoretically defined gliadin types indicated as α, β, γ, and ω. Nongliadin protein is "n-gl" and includes metabolic proteins.

wet flour meal (20% moisture) heated for 40 min at constant moisture (*27, 28*).

Proteins leached from dough produce electrophoresis patterns (**Figure 3**) with apparently improved resolution but clearly and

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consistently more rapid elution of the gliadin proteins than those leached from flour or milled berries. We suspect that these differences have their origins in differences in the microstructures and secondary molecular structures of these very complex and varied protein substrates. The addition of water to flour causes expansion, extension, and swelling that makes the substrate more accessible to leaching. Mixing of wet flour effects changes in microscopically observable macroorganization of the protein, FTIR detectable changes to secondary structures, baking performance, protein-from-starch separability, and, we expect, the manner by which the proteins enter the solvent and the surface charge/volume that they acquire (*29−31*). Also, the non-gliadin or metabolic proteins leached from dough were seen to represent a larger proportion of the total proteins in electrophoresis patterns than those leached from flour, suggesting that they are more accessible in the wet dough for structural reasons just discussed.

Analysis of Known Wheat-Based Products. Commercially available wheat bread, beer, and cereal products were analyzed by CZE. Electrophoresis patterns (**Figure 4A,C**) of interior or "crumb" samples for both a wheat bread and French bread baguette had very recognizable wheat-like patterns. However, greatly attenuated patterns, with little resemblance to either the crumb or unprocessed wheat flour, were obtained for surface or crust samples (**Figure 4B,D**). Neither the flake cereal nor the beer solubles produced wheat-like patterns by CZE (**Figure 4E,F**).

Wheat flour and wheat products were also analyzed by 2-DE (**Figure 5**). 2-D gels of wheat flour clearly resolved individual HMW-GS, LMW-GS, and α-, γ-, and ω-gliadins, illustrating an increase in protein resolution relative to 1-DE or CZE. These protein groups were also present in 2-D gels of bread, baguette, and cereal. Like the CZE patterns, the patterns for the baguette and bread crust were different from those for the crumb but, unlike the CZE data, were still recognizable as related to the wheat control. Comparison of the patterns for the baguette crust and crumb revealed that the relative levels of the HMW-GS and ω-gliadins were lower and those of the LMW-GS and α- and γ-gliadins were higher in the crust. The altered spot pattern, the lower resolution of proteins, and presence of charge trains indicated thermal alteration of the proteins during baking. Relatively few proteins were resolved in the bread crust, suggesting that heat-induced interactions during baking were more severe for the bread than the baguette. Like the baguette crust, the HMW-GS and ω-gliadins were lower and those of the LMW-GS and α- and γ-gliadins were higher in the 2-D gel pattern of the cereal proteins. The spot pattern and decreased resolution of proteins also indicated thermal alteration due to processing.

Proteins in the wheat beer insoluble and soluble fractions were analyzed by 1-DE (**Figure 6**) to determine their identities. The pattern of proteins in the wheat beer insoluble fraction of Coomassie Blue stained gels (**Figure 6A**) was similar to that of wheat flour: it contained HMW-GS, LMW-GS, and gliadins. The pattern of the soluble fractions was less complex, containing principally proteins in the gliadin/LWM-GS region of the gel. Western blots probed with the anti-prolamin antibodies (**Figure 6B**) confirmed the identities of the proteins in these fractions as being wheat-sourced. The CZE pattern for the wheat beer soluble fraction (**Figure 4**) reveals non-wheat-typical behavior.

Analysis of Suspect Wheat-Based Product. We also examined a 1916−1918 Model-T ignition coil for the presence of cereal proteins that it is reputed to contain. A photograph of an ignition coil of this type along with a dimensional assembly drawing from the period is shown in **Figure 7**. A combustion analysis determined that nitrogen was present and at a level of 7% protein if all
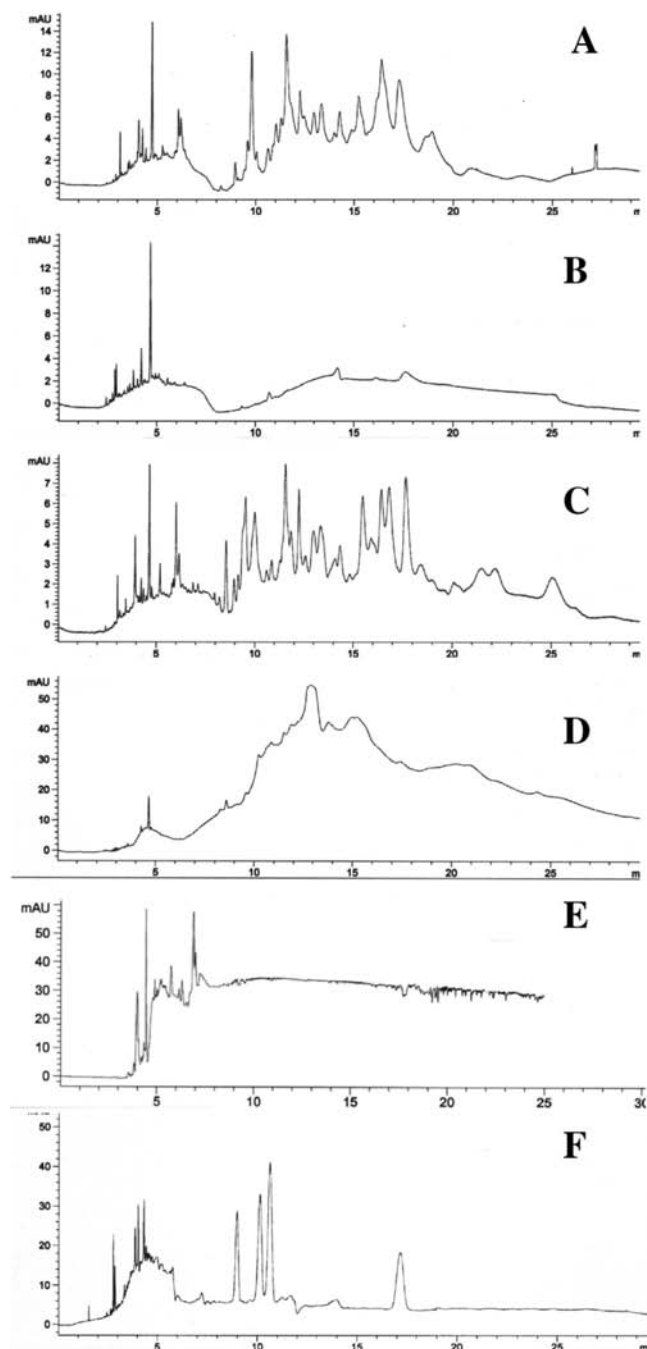

Figure 4. CZE analyses of baked bread (interior and surface). Samples are (**A**) wheat bread interior or crumb, (**B**) wheat bread surface or crust, (**C**) French baguette interior or crumb, (**D**) French baguette surface or crust, (**E**) gluten-enriched wheat flake cereal, and (**F**) wheat beer. Proteins extracted by aqueous ethanol (70%) prior to electrophoresis. Note that different vertical scales are used.

of it were protein nitrogen. This is roughly half of that normally encountered in flour or bread. A CZE pattern of ignition coil material is shown in **Figure 8B**. A comparison of the coil electrophoresis pattern to that of heated flour or heated dough (**Figure 8A,C**) suggested, although not conclusively, that wheat proteins were present in the ignition coil. Although there was a large baseline difference, matching of electrophoresis patterns for heat-damaged flour and the ignition coil revealed a nearly peak-to-peak match of 18−20 peaks with similar relative positions and magnitudes within the central regions of the traces. This alignment suggests the presence of wheat gliadins and/or LMW-GS in
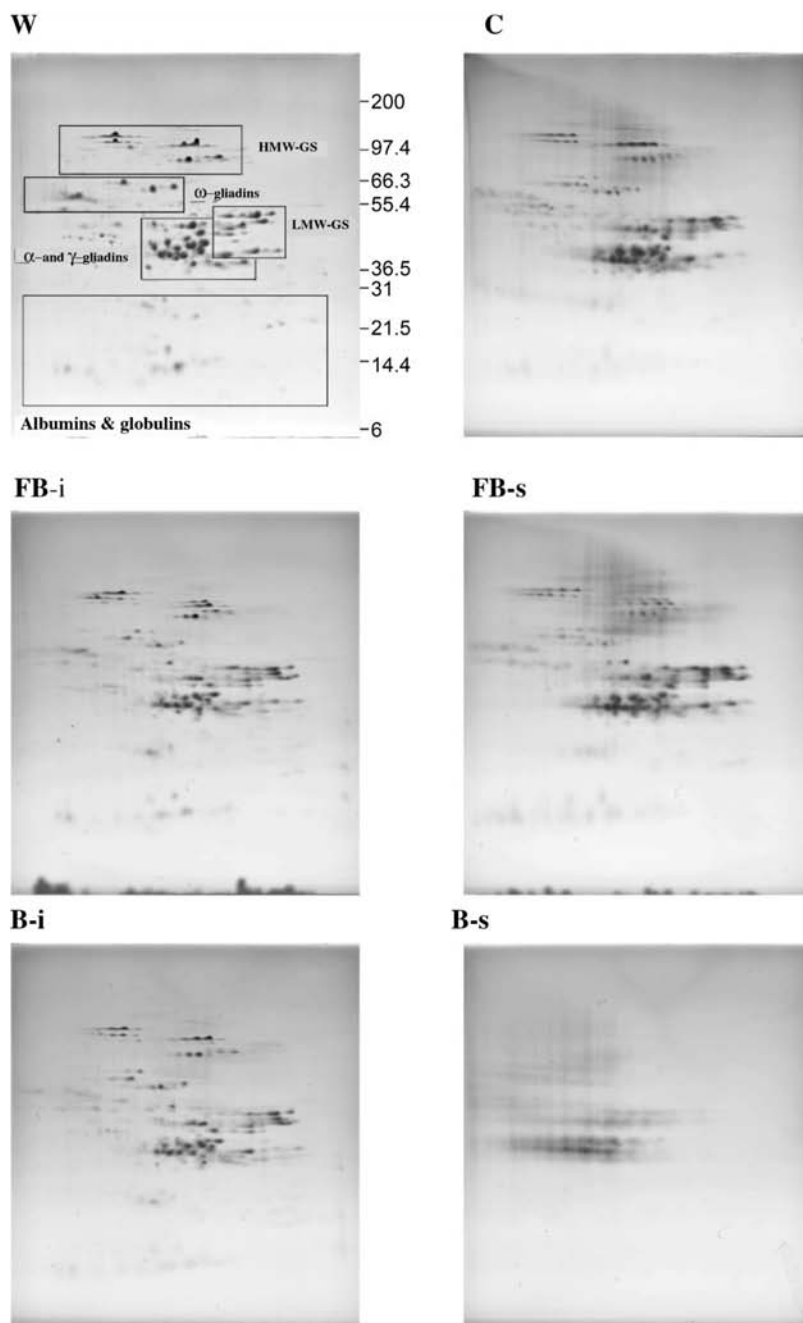
Figure 5. Analysis (2-D PAGE with Coomassie Blue staining) of protein found in wheat (W), breakfast cereal (C), French bread (FB), and bread (B) illustrating the effects of exposure to heat during processing. Surfaces or crust (s) and interior or crumb (i) exhibit more and less thermal alteration, respectively. Proteins extracted by SDS/DTT prior to electrophoresis.

the ignition coil case. The similarity of electrophoresis patterns for heat-damaged flour and the ignition coil also suggests that high temperature might have been applied in manufacture of the coil and is in keeping with the close proximity of the coils to the hot Model-T engine. One notable difference between these two patterns occurs in the rapidly eluting region that is occupied normally by wheat metabolic proteins. The relatively large response detected in the coil sample can be attributed only to a possible characteristic of the source material.

Proteins were leached from the ignition coil for analysis by 1-DE for further characterization (**Figure 9**). Of the methods tried, extraction with the SDS buffer provided the best separation of proteins in 1-D gels. Unfortunately, extracts of the ignition coil case contained a darkly colored component that we suspect caused the high background staining and poor resolution of the

proteins in the gels of this sample (**Figure 9A**, 1a). However, image enhancement by two methods revealed two bands at approximately 38 and 41 kDa (**Figure 9A**, 1b and 1c). A Western blot probed with the anti-prolamin antibodies reacted with these bands, which, in agreement with the CZE patterns (**Figure 9B**, 1 and 2), are located in the region of the gel containing the gliadins and LMW-GS. A second Western blot that was probed with monoclonal anti-α-gliadin antibodies did not detect any proteins (data not shown), suggesting a very low level of α- and β-gliadins in the sample and/or that the anti-prolamin polyclonal antibodies detected only γ-gliadins and/or LMW-GS.

Unmodified source protein used in the formulation of biobased products was not available for reference and may be available only in rare situations. At the time of manufacture of the ignition coil, a large percentage of acreage in the Midwest grew a single
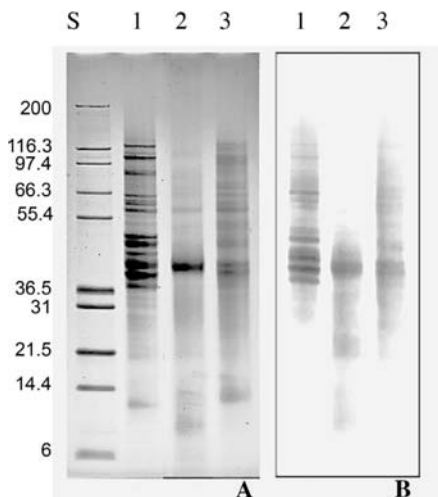
Figure 6. Detection of wheat beer proteins by PAGE amplified with Coomassie Blue (**A** shown as red-filtered monochrome digital enhancement) and amplified with antibody probe (**B**) for MW standards (S), wheat (1), beer solubles (2), and beer insolubles (3). Proteins extracted by SDS/DTT prior to electrophoresis.
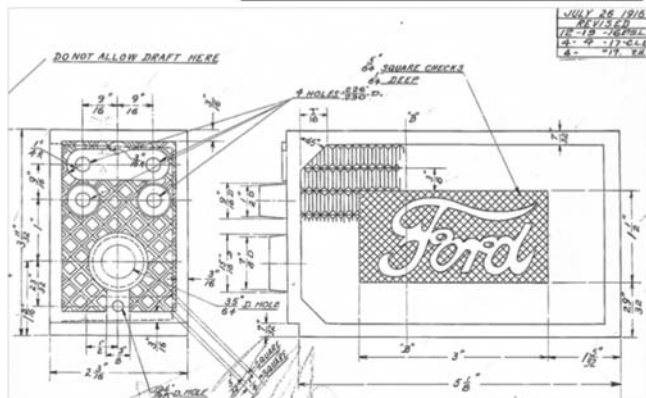

Figure 7. Photograph (top) of ignition coil or magneto that was "die cast" reputedly as an asbestos–soy or asbestos–gluten mixture. Line drawing (bottom) abstracted from larger original Ford Motor Co. assembly drawing entitled "Die casting drawing composition design": T6793.

hard red wheat cultivar known as "Turkey" or "Turkey Red", to which many current Midwestern hard red wheats are related. Chromatographic and electrophoretic signatures of proteins from wheat on the Turkey pedigree have been reported with differences in detail but with five recognizable and conserved regions: metabolic proteins, alpha, beta, gamma, and omega gliadins. Lastly, the CZE patterns of **Figure 10** show remarkable similarity between Turkey Red and the blended flour used in this study. All five primary regions of alcohol-soluble proteins are clearly represented (*12, 13, 23, 32*).

DISCUSSION

Cereal protein in products inevitably will be altered by the conditions of processing and may not be analytically accessible. Ideally, analytical approaches will extract proteins from products and detect protein subunits that can be compared to the unaltered source protein. The use of reference or model systems can provide clues to the protein modification and can be correlated to the source, unmodified protein.

Processing cereal-containing products exposes them to temperature and moisture regions or "states" above the glass transition temperature where physical and chemical changes are most commonly detected. Examples of properties that increase are softness, chemical reactivity such as glutenin disulfide cross-linking, water absorption, surface hydrophobicity, and darkening. Examples of properties that decrease are foaming capacity, solubility, bread loaf volume, baked gluten ball volume, and dough extensibility. Ultimately, pyrolysis occurs near 160 °C, setting an upper limit for grain-containing products. As moisture falls below 25%, the glass transition temperature is raised and wheat gluten protein becomes increasingly stable and unreactive (*6, 7, 22, 33−46*).

Most reference thermal pretreatments reported here produced patterns similar to a thermally untreated control that had good treatment-to-treatment peak tracking. When the thermal conditions exceeded about 120 °C, CZE patterns of wheat proteins were greatly attenuated. This attenuation, which we attribute to protein interactions affecting the protein solubility in aqueous alcohol, was not observed for convectively dried wheat flour. We believe that the reason for this difference may be found in the smaller size of the flour particles (~100 μm for flour particles) and the concomitant rapid rate of moisture loss. The small particles of flour would be expected to lose moisture very rapidly, thereby acquiring a higher glass transition temperature and greater thermochemical resistance. By contrast, much slower moisture removal would be expected for the larger intact grains and for the formed dough samples, both of which have a characteristic dimension of ~3 mm or more than an order of magnitude larger than the flour particles.

The flour reference data at low temperature (40−70 °C) also exhibited poor peak resolution that impeded, but did not preclude, treatment-to-treatment peak tracking. Low-temperature electrophoresis pattern behavior for flour has been noted by others who reported reduced peak resolution, peak intensity shifts, and overall peak attenuation (*27, 28*). Protein endothermic transitions have been reported as low as 60 °C (*18*). However, these may be analytic anomalies related to how the protein is deconstructed and removed from the source matrix and reassembled by the solvent and have not yet been linked to functional changes of the protein. Further, we suspect that the thermal and water activity provenance of the sample also is key to understanding this issue, but this was not pursued (*47*).

For wheat berries and flour, peaks for metabolic proteins were positionally stable, but the peak positions determined for gliadin proteins exhibited treatment-dependent variation. Peaks representing protein subunits with relatively high electronegativity (slowly migrating) were more strongly affected. Heat exposure and dehydration followed by ethanol leaching apparently produce new and irreversible protein–protein noncovalent interactions and altered net surface charge-to-diameter ratios on the soluble species. New or altered covalent disulfide bonds may also be formed during these processes, but the resolved gliadins generally do not participate in these reactions directly since most lack appropriate cysteine residues (*48*).

For wheat dough samples, in contrast to the berry and flour data, stable retention times were determined for all proteins and
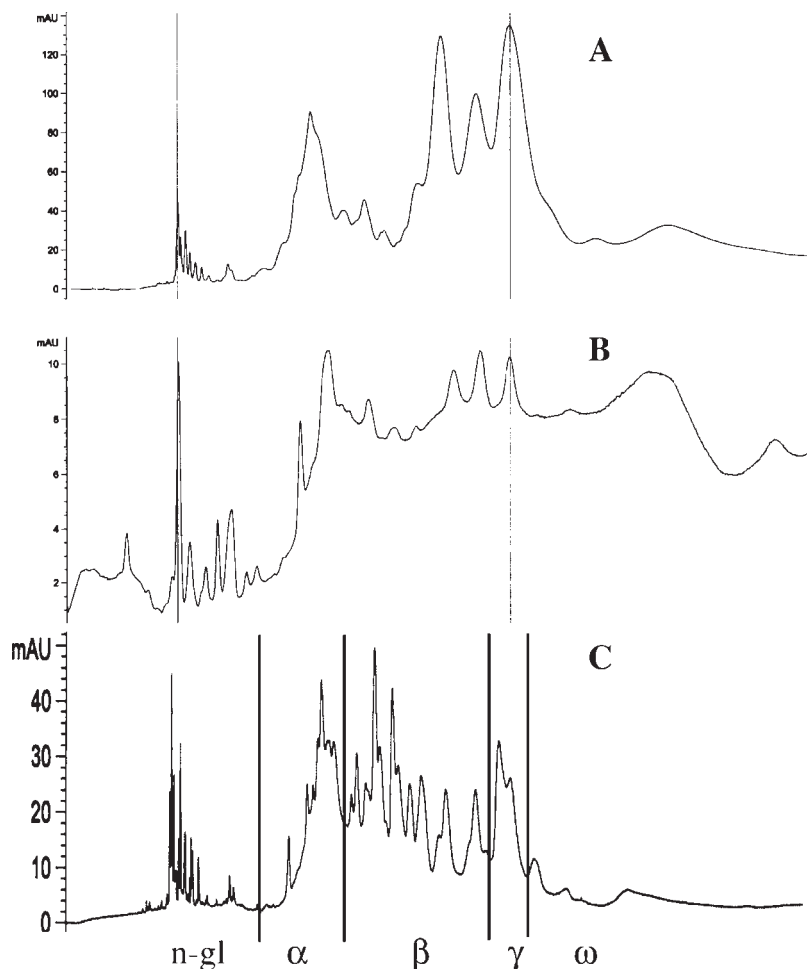
Figure 8. Comparison of CZE electrophoresis patterns for Model-T Ford casing and heat-altered flour. Electrophoresis patterns were digitally compressed or extended along the abscissa based on prominent peaks in the n-gl and ω-gliadin families. (**A**) Heated flour (70 °C), wheat flour; (**B**) coil; (**C**) heated dough from flour blend. Proteins extracted by aqueous ethanol (70%) prior to electrophoresis.
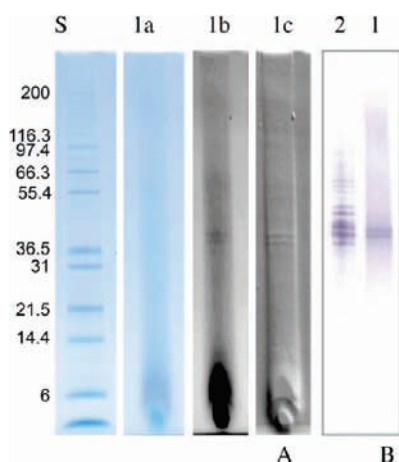

Figure 9. 1-D PAGE amplified with Coomassie Blue stain (**A**) and with antibody blot (**B**) applied to molecular weight standards (S), ignition coil (1), and wheat flour (2) and shown unenhanced (a), enhanced with red filtration (b), and enhanced with contrast embossed (c). Proteins extracted by SDS/DTT prior to electrophoresis.

treatments. This result suggests that mixing induces a state of protein aggregation and organization that is not further altered by heat, dehydration, and ethanol leaching (*31*).

Our data for wheat-based substrates were consistent with published information for similar substrates. For instance, we obtained fully attenuated CZE gliadin protein reference patterns for > 120 °C dehydration of dough and grain samples and for bread surfaces, toasted cereal, and wheat-based beer. This is consistent with reports of bread surface or crust temperatures of ~160 °C in a 200 °C oven; French-type bread crust temperatures ~125 °C in high humidity, 250 °C ovens; flake cereal temperature exposure to 140−240 °C air in toasting ovens; and cereal beer mash cooking and liquefaction temperatures of 90−165 °C prior to enzymatic deconstruction and digestion. Also note that the attenuation of wheat bread crust was greater than that of French bread crust, reflecting an expected higher crust temperature. By contrast, we obtained normal, wheat-like gliadin protein patterns for reference exposures to less than 120 °C and for bread and French bread crumbs that would be expected to reach only about 90 °C (*49−54*).

The aggressive SDS/DTT solvent successfully produced readily recognized wheat gliadin and glutenin protein patterns for the bread samples. Hence, both bread crumb samples yielded wheat recognizable patterns. Data for higher temperature (> 120°) treatment of surface or crust were not attenuated as were the CZE patterns but were similar to those of the crumb. However, the protein spot distribution was less clearly defined for crust samples with the most severely treated samples (whole wheat crust) producing the least definition.

The "unknown" cereal protein in the Ford ignition coil enclosure was identified as a probable wheat protein by CZE with the best match to a low temperature reference (70 °C) but
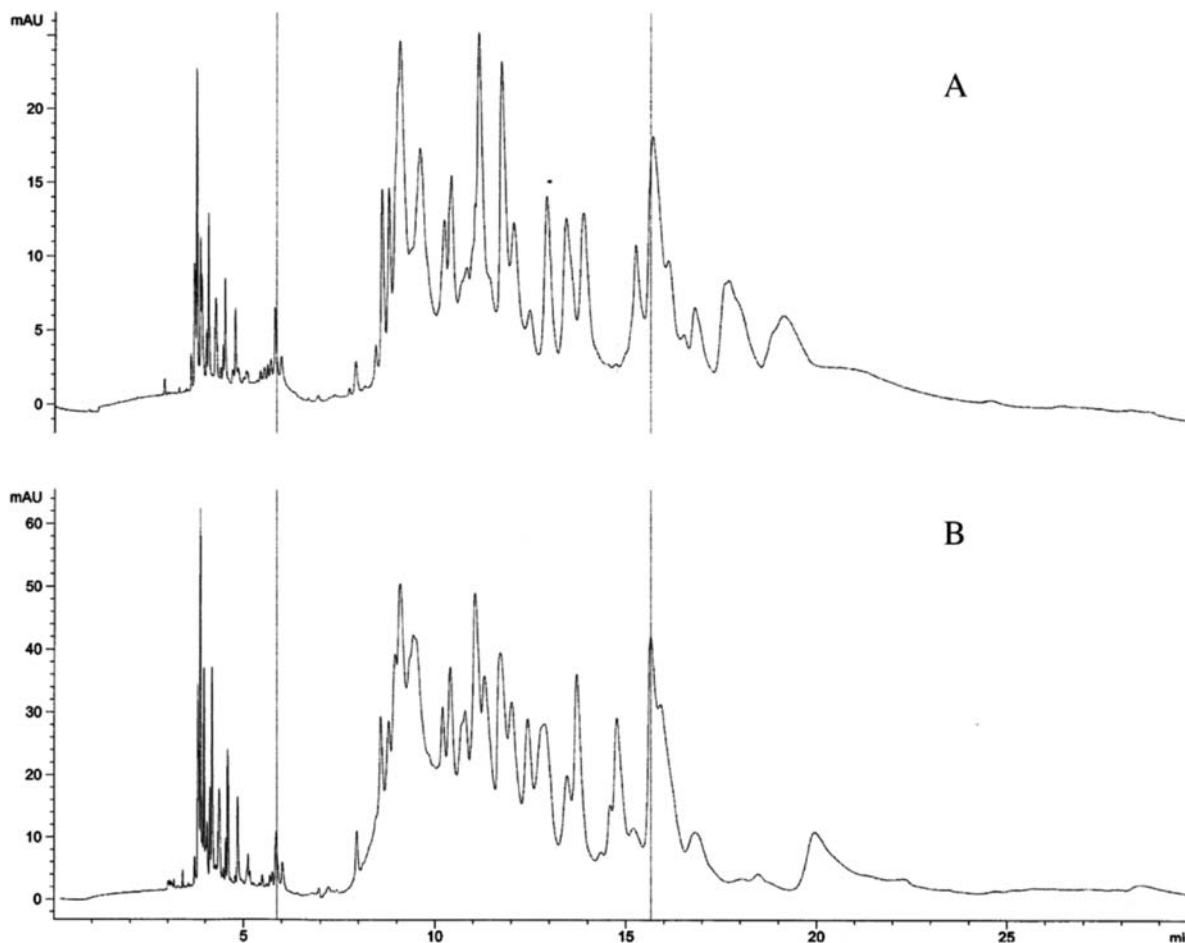
Article

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Figure 10. CZE reported for a flour from cv. Turkey or Turkey Red, a hard red winter wheat cultivar grown on 98% of the acreage in Kansas in the early 1900s (**A**), and from a modern, blended commercial flour described as a dark northern spring wheat and hard red winter wheat from Montana (**B**) used in this study. Proteins extracted by aqueous ethanol (70%) prior to electrophoresis.

reasonable matches to unmodified protein. A subset of wheat proteins were identified using 1-DE and confirmed with wheat anti-gliadin protein antibodies. The incomplete patterns revealed in 1-DE gels may be due to irreversible binding to the asbestos component and consequently poor extraction. Wheat protein binding to surfaces is proportional to both the temperature of the solid surface and molecular weight of the polymers. Starch and activated carbon have been reported to strongly bind protein especially at high temperatures, and in the case of starch, the physical state of the starch and its distribution within the gluten matrix may also influence the protein analytical response (*30*, *55*−*58*).

The long chain length and extreme susceptibility to irreversible change due to mechanical, thermal, solvent exposure, and possible proteinase exposure make it imperative to employ a variety of analytical methods for identifying the biocomponent in a processed product. In the case of wheat, the solvent employed and the analytical method utilized provide clues to the presence of a protein (gliadins in 70% ethanol, gliadins and glutenins by SDS−DTT) and the possible changes that have occurred (ethanol solubility affected strongly by temperature, SDS−DTT less strongly affected). This is further enhanced by the method of protein resolution (CZE can provide very high resolution of the gliadins; 1-DE and 2-DE provide increasingly detailed resolution of all of the storage proteins) and the specificity of detection (antibody reaction with proteins can produce a wheat identification when other methods cannot). The combination of these methods yielded convincing identification of wheat protein in

wheat beer, bread, French bread, a wheat-based flaked breakfast cereal, and the Ford ignition coil.

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