THE APPLICATION OF CAPILLARY ELECTROPHORESIS TO EXAMINE PROTEIN MODIFICATIONS IN BAKED VERSUS FRIED TORTILLA CHIPS

by

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A thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Master of Science

Graduate Program in Food Science

written under the direction of

Professor Karen M. Schaich

and approved by

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New Brunswick, New Jersey

October, 2012

ABSTRACT OF THE THESIS

The Application of Capillary Electrophoresis to Examine

Protein Modifications in Baked versus Fried Tortilla Chips

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Although lipid oxidation is recognized as a major chemical reaction limiting shelf life of foods, its role in degrading food quality and the mechanisms involved remain incompletely elucidated. Interactions of oxidizing lipids with other food molecules have been largely ignored, even though these reactions can have dramatic impact on food properties. Lipid co-oxidation of proteins occurs extensively in nearly all processed foods and degrades textures, flavors, color, and nutritional value. It is important to measure both lipid and protein co-oxidation products to understand the full extent of oxidative deterioration during food storage.

This thesis is part of a larger project examining baked and fried tortilla chips to differentiate thermal damage to proteins from lipid co-oxidation during processing and storage. In a previous study, gel electrophoresis revealed modification of protein surfaces that affected dye binding, as well as formation of sizeable protein aggregates too large to enter normal gels involving disulfide, free radical, and other crosslinks. As an alternative

to polyacrylamide gels, capillary electrophoresis can separate peptides without molecular weight limits, by modes that may be more sensitive to side chain modifications, and requires only a few nanoliters of sample. Thus, this study investigated the use of capillary electrophoresis for tracking fragmentation and crosslinking in co-oxidized proteins.

Results corroborated observations that fried tortilla chip samples had greater changes than baked tortilla chip samples and higher incubation temperature resulted in more protein damage, most notably in fried reducing fractions. In addition, surface modifications altered protein charge, which interfered with migration in capillary electrophoresis. Peptide detection was limited to zeins of about 50 kDa because the sample filtration step intended to prevent capillary blockage also removed higher molecular weight fractions, including glutelins. However, fragmentation products not distinguishable in gel electrophoresis were detected.

Overall, results of this study suggest that capillary electrophoresis has intriguing possibilities for supplementing SDS-PAGE and other protein analyses, particularly in verifying the presence of surface modifications. However, significant hurdles—such as reasons for lack of high molecular weight peptide loading and migration—remain to be overcome before capillary electrophoresis can become a primary method for analysis of modified proteins.

ACKNOWLEDGEMENTS

First, I would like to thank my advisor, Dr. Karen M. Schaich. Not only did she guide me through the research presented in this thesis but she provided counsel through the other aspects of my graduate studies as well. It has been a privilege to work with her.

I thank my thesis committee members, Dr. Chaim Frenkel and Dr. Michael Rogers, for their support of my thesis and defense.

The help of my lab mates was invaluable. Elah Steltzer, Spurti Ravi, Brandon Bogusz, Wan Zunair Wan Ibadullah, and Yuan Dong were all instrumental in my completion of this research project.

Finally, I would like to thank my husband, Brian, for his love and patience throughout my many late nights and weekends in class and the lab and my daughter, Eleanor, for joining me in the home stretch.

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I. INTRODUCTION

While lipid oxidation is recognized as one of the primary reasons for diminished shelf life in foods, neither its role in the degradation of food quality nor the mechanisms involved are well-elucidated. It is acknowledged that lipid oxidation has an impact on other aspects of food systems such as proteins but little work has been conducted to trace co-oxidation pathways or effects. Not only are oxidative modifications to non-lipids molecules important in overall loss of food integrity and sensory quality, but reactions of lipid radicals and products with proteins can have an apparent antioxidant effect: transfer of oxidation potential to proteins removes lipid oxidation intermediates and products from the analytical stream, resulting in deceptively low readings and a false conclusion of very low lipid oxidation. Therefore, it is important to examine both lipid oxidation and protein co-oxidation products to determine the true extent of the impact on food quality and to generate an accurate picture of the role of each in degradation during storage.

The work contained in this study is part of a long-term project to examine the reactions and associated damage involved in protein co-oxidation. The objective is not a trivial task since protein co-oxidation occurs primarily in processed foods which have undergone various thermal treatments that also degrade proteins. It is thus important to distinguish damage induced by processing (thermal, shear) from damage arising from oxidation of endogenous lipids.

The process of separating sources of damage to proteins can be daunting unless system variations are limited and as many characteristics as possible are held constant. One interesting food system in which comparisons are simplified is baked and fried tortilla chips. The formulation, cornmeal treatments, and manufacture are identical up to

the point of cooking. Then one stream is directed to baking and the other to frying. This generates two products with parallel processing steps but with one critical difference: baked chips contain only the lipids that are naturally introduced with the corn while fried chips have lipids adsorbed during frying. Studying these products thus makes it feasible to distinguish co-oxidation damage to proteins from changes induced by heat processing.

Previously in this project, protein changes and underlying chemistry were investigated by gel electrophoresis (Dong 2011). Results showed that the dominant heat effects were disulfide crosslinking. The presence of adsorbed oxidizing lipid complicated matters tremendously, rapidly modifying side chains to reduce Coomassie blue dye binding and dramatically increasing disulfide, free radical, and other forms of crosslinking without significantly reducing protein solubility. Formation of very large crosslinked but loosely aggregated masses too large to enter even the stacking gel matrix were proposed to explain the extensive loss of peptides on gels yet minimal loss of protein solubility. Clearly, alternate analytical methods were needed to detect and characterize both modified and polymerized maize proteins.

Capillary electrophoresis is a powerful tool for fast and quantitative analyses that has been widely applied to proteins in general but is still in relative infancy in food industry applications. Capillary electrophoresis offers numerous advantages over gel electrophoresis, most notably requiring only very small quantities of sample and chemical reagents (Righetti et al. 1998). Mapping of purified zein protein as well as a wide range of isolated native proteins has been published (Bean and Lookhart 2000, Bean et al. 2000, Erny et al. 2007, Parris et al. 1997, Righetti et al. 1998). However, capillary electrophoresis has not been applied to characterizing complex samples of proteins

extracted from food systems. The research reported here studies the potential of capillary electrophoresis to analyze protein modifications induced by heat and lipid oxidation in foods, specifically maize proteins in baked versus fried tortilla chips.

II. LITERATURE REVIEW

A. Maize Proteins

Corn, or maize (*Zea mays L.*), can trace its roots as a crop to the Americas more than 8000 years ago. It was a component of the Native American diet and continues to be consumed throughout Central America where the grain is cooked in water containing lime, washed, and then ground. The resulting masa is used to make products such as tamales and tortillas. Maize has become a particularly integral ingredient in the Western diet in the form of snack foods and represents the second most important cereal crop worldwide, with approximately 40% being produced in the United States. Yellow Dent is the variety most commonly grown for animal feed as well as human consumption.

There are four major parts of a corn kernel: endosperm, germ, bran, and tip cap. The endosperm contains most of the starch present in the kernel while the germ contains the bulk of the protein and lipids. Table 1 lists these parts and their molecular components. There are four major classes of corn proteins, categorized by their solubility in selected solvents: albumins, globulins, prolamines (zeins), and glutelins. Table 2 shows the relative amounts of each protein present in the endosperm and corresponding solubilities.

Table 1: Average kernel composition on a moisture-free basis (Earle et al. 1946).

Fraction	Kernel %	Starch %	Protein %	Lipid %	Sugar %	Ash %
Whole grain	--	71.5	10.3	4.8	2.0	1.4
Endosperm	82.3	86.4	9.4	0.8	0.6	0.3
Germ	11.5	8.2	18.8	34.5	10.8	10.1
Bran	5.3	7.3	3.7	1.0	0.3	0.8
Tip Cap	0.8	5.3	9.1	3.8	1.6	1.6

Table 2: Relative amounts of protein fractions as a percentage of total protein in the endosperm and their solubilities.

Protein fraction	Percentage [a]	Solubility [b]
Albumins	3.2	Water soluble
Globulins	1.5	Salt soluble
Prolamine (zeins)	47.2	70-80 % ethanol soluble
Glutelin	35.1	Alkali soluble

[a] Inglett 1970, [b] Jimenez 1966

Albumins and globulins are the metabolically active proteins of the endosperm (Lasztity 1996). Ion exchange chromatography has been useful in separating these components into sub-fractions. Albumins resolve into five peaks that are unaffected by reducing agents indicating that they do not contain intermolecular disulfide bonds. When subjected to gel electrophoresis, albumins and globulins separate into approximately 20

peptides. It is possible that some of these bands are identical to bands from reduced glutelins. Glutelins are sometimes considered to be a sub-set of zeins due to their co-extraction when reducing agents are added to extraction buffers but are, in fact, a distinct fraction.

Zeins, found primarily in the endosperm in amounts varying from 6 to 12 %, are alcohol-soluble proteins assigned to the prolamine class (Shukla and Cheryan 2001). They typically contain high proportions of α-helix structures (Mossé 1966). It is believed that these proteins are the primary storage site for nitrogen in maize plants. Zeins are particularly rich in three hydrophobic amino acids and glutamine (Table 3) (Gianazza et al. 1977, Wilson 1983), but possess only trace amounts of basic amino acids.

Table 3: Relative amounts of the four major amino acids in zeins (Shukla and Cheryan 2001).

Amino Acid	Amount (%)
Glutamic Acid	21-26
Leucine	20
Proline	10
Alanine	10

Zeins have very low levels of lysine, tryptophan, and methionine (Bressani and Mertz 1958). This mostly non-polar composition is the reason that zeins are insoluble in water, except in the presence of alcohol, high concentrations of urea, high pH (11 or

greater), or anionic detergents. Zeins are also responsible for the hardness of the corn kernel. In order to improve nutrition and physical robustness of maize, there has been an interest in seeking mutations to reduce levels of prolamines (called opaque, brittle, and floury variants); these were found to have improved nutritional quality (Misra et al. 1972). Morphological differences between these mutant varieties are apparent in the photographs shown in Figure 1. Table 4 lists the amino acid contents of zein fractions. Table 5 summarizes their characteristics, including molecular weight of the subdivisions which have a range of 10 to 27 kDa.

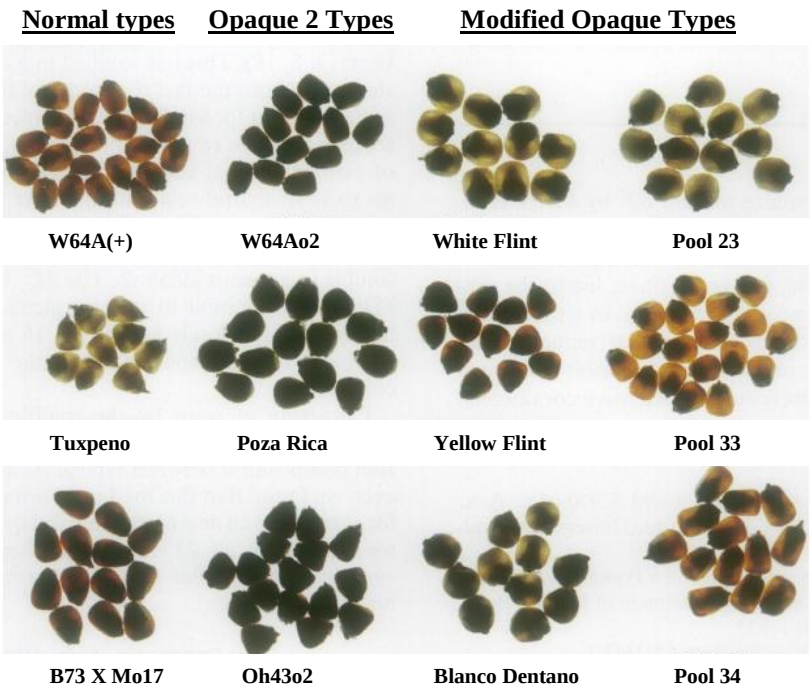

Figure 1: Photograph of backlit normal, opaque-2, and Quality Protein Maize (QPM) types of corn (Wallace et al. 1990).

Table 4: Amino acid composition zeins (mol%) from Dong (2011).

Amino acid	**A-zein (α-zein)**	**B-zein (α-zein)**	**C-zein (β-zein)**	**RSP-1 (γ-zein)**	**10 kDa zein (δ-zein)**
Lysine	0.2	0.4	0.2	0.2	0.1
Histidine	1.1	1.1	1.0	7.2	2.3
Arginine	1.1	1.1	2.6	2.1	0.0
Aspartic acid	5.7	5.7	2.4	0.5	3.4
Asparagine	---	---	---	---	---
Threonine	3.1	3.1	3.0	4.3	4.0
Serine	6.4	7.4	5.3	4.3	5.8
Glutamic acid	21.4	20.6	20.2	16.0	14.6
Glutamine	---	---	---	---	---
Proline	8.7	10.0	11.6	24.9	15.4
Glycine	2.5	2.9	8.4	7.3	3.5
Alanine	13.6	12.8	12.6	5.7	6.6
Cysteine	0.8	0.4	4.6	5.5	---
Valine	5.0	3.2	3.1	7.5	4.2
Methionine	1.3	0.4	6.7	0.8	18.4
Isoleucine	3.7	3.6	1.2	2.0	2.6
Leucine	18.4	18.7	11.0	10.0	13.1
Tyrosine	2.7	3.2	6.2	2.1	1.2
Phenylalanine	3.7	5.3	1.9	1.0	4.9
Tryptophan	0.0	0.0	0.0	---	---

Table 5: Characteristics of zein proteins.

	Solubility[a]	Percent of total zein[b]	Molecular weight
α-zein	70 % ethanol or isopropanol	80 %	22 and 19 kDa[a]
β-zein	50-70 % aqueous alcohol		14 kDa[a]
δ-zein	Aqueous alcohol w/ reducing agent		27 and 16 kDa[c]
γ-zein	SDS, aqueous alcohol		10 kDa[c]

[a] Sundaram 2001 [b] Lending and Larkins 1989 [c] Rodriguez-Nogales et al. 2006

There are four main methods for processing corn: dry milling, wet milling, alkaline processing, and a dry grind process. The products of dry milling or alkaline processing are used for human consumption while dry grind products are used in ethanol production. Briefly, dry milling is when dry corn is cleaned with steam, the pericarp (hull), germ, and tip cap are removed in a process called degerming, and the remains are ground into the appropriate size for the desired application (Rodriguez-Nogales et al. 2006). Food manufacturers use the endosperm products in snack foods and breakfast cereals. Many studies have shown that disulfide linking occurs naturally in zeins (Turner et al. 1965, Lending and Larkins 1989) and also that during cooking there is extensive polymerization of zeins as a result of disulfide linking (Batterman-Azcona and Hamaker 1998).

B. Lipid Oxidation

Lipids provide energy to cells and, therefore, are present in most foods. Unsaturated lipids are naturally susceptible to oxidation, which causes the formation of hydroperoxides and, in turn, aldehydes, ketones, acids, and alcohols (Vercellotti et al. 1992). The formation of these compounds can then result in the generation of off-flavors and off-notes as well as loss of nutritional value. It is widely accepted that lipid oxidation occurs very rapidly in the presence of oxygen and is triggered and catalyzed by free radicals, light, metals, and heat (Schaich 2005). Lipid oxidation can also be catalyzed by lipoxygenase enzymes (Egmond et al. 1972, deGroot et al. 1975a, deGroot et al. 1975b). Given the ubiquitous nature of these catalysts, it is clear why lipid oxidation is so prevalent in food systems and why there is such an interest in better understanding these reactions.

Autoxidation of lipids has long been recognized as a free radical chain reaction (Farmer 1946, Lundberg and Chepault 1947, Bolland 1949, Swern 1961). The first stage is initiation which is the formation of a lipid acyl radical by exposure to a radical initiator, or thermal or photochemical cleavage of hydrogen from the lipid (Porter et al. 1995). The second stage is propagation during which the lipid acyl radical combines with oxygen to form a lipid peroxyl radical which then abstracts a hydrogen atom from a neighboring lipid to form a stable hydroperoxide and generate a new acyl radical in the target molecule. The new radical, in turn, re-enters the cycle, reacts with more oxygen, generates another lipid peroxyl radical, and abstracts another hydrogen atom to continue the chain reaction (Uri 1961, Scott 1965, Schaich 2005). The final stage, termination, occurs when free radicals recombine to form stable products, or when alkoxyl radicals

generated from lipid hydroperoxide decomposition undergo scission to form various secondary products such as alkanes and aldehydes.

C. Protein Oxidation

1. Overview

Oxidative modification of proteins occurs on the peptide backbone and susceptible amino acid side chains, notably histidine, tryptophan, tyrosine, methionine, cysteine, cystine, glutamic acid, aspartic acid, and lysine (Means and Feeney 1998). A variety of factors trigger protein modification, including pH and the microenvironment in which it is surrounded. Proteins may react with other proteins (or with themselves) by cross-linking which is the formation of a covalent bond between two or more side chains or a side chain with another substance. Amino groups of proteins (e.g. lysine, arginine, and glutamine) can undergo reductive alkylation or condensation with a carbonyl compound from sugars or oxidizing lipids (Maillard reaction), resulting in an imine such as a Schiff base aldehyde which then undergoes an irreversible Amadori rearrangement (Means and Feeney 1998). These derivatives show an increased propensity for binding to hydrophobic surfaces, interacting with emulsions, and solubilizing in organic solvents (Means and Feeney 1998). Sometimes, the Amadori products proceed to an oxidation step involving an additional amino acid side chain to yield any number of end products called advanced glycosylation end-products (AGEs) as well as flavor compounds including pyrroles, furans, pyrazines, etc. (Means and Feeney 1998).

It is rare that food manufacturers intentionally induce protein modifications due to concerns about consumer acceptance. However, protein modifications do occur naturally

in food products and often contribute to the sensory attributes of that product such as color, flavor, and aroma.

It has been accepted for some time that protein co-oxidation does occur in parallel with lipid oxidation (Schaich 1975, 1976, 1980, 2008). This is important for several reasons. First, lipid co-oxidation of proteins is responsible for some of the off-flavors attributed solely to lipid rancidity, particularly in warmed-over flavor (Schaich 2008). Second, co-oxidations can obfuscate the occurrence of lipid oxidation since the proteins take up the lipid oxidation reaction intermediates and products, making them unavailable for the traditional detection techniques and giving the impression that little or no lipid oxidation is taking place. Co-oxidation effects such as those listed in Figure 2 may or may not correlate with measured lipid oxidation, but they certainly provide indicators that lipids did indeed oxidize in a given system.

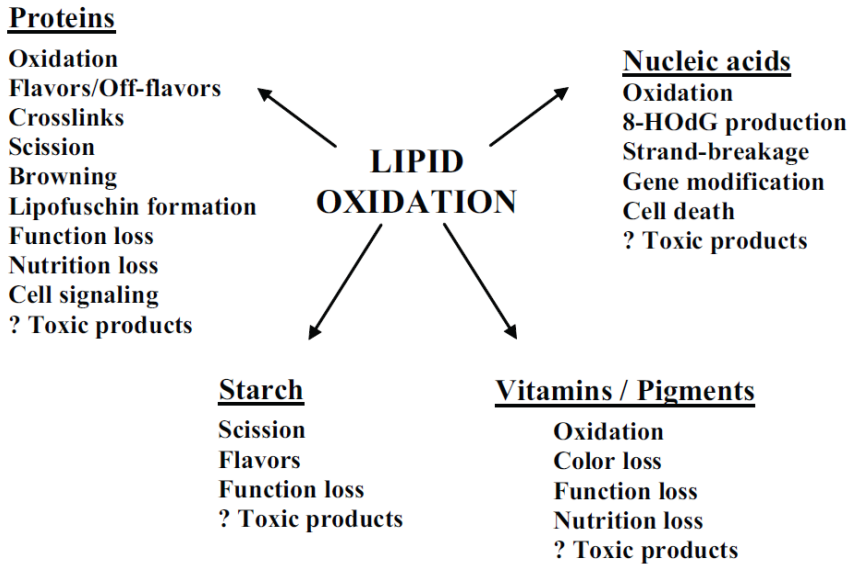

Figure 2: Types of damage that occur when lipids co-oxidize (Schaich 2008).

In cells and tissues (both plant and animal), hydroxyl radicals (HO•) or other oxidants are often blamed as the cause of protein damage because they react with the same amino acids as do oxidizing lipids and result in similar protein oxidations (Davies 1987a, Davies 1987b). However, lipid radicals and oxidation products are much longer lived and thus more likely to induce irreparable damage at sites distant from initiation. Radicals can be transferred from lipids to proteins; these protein radicals can then transfer the radicals to other constituents, oxidize other proteins, or polymerize which leads to changes in texture and membrane structure (Schaich 1980). Proteins can also react with lipid hydroperoxides, lipid epoxides, and secondary products of lipid oxidation (such as aldehydes). Studies have shown that the following amino acids are particularly prone to damage from peroxidizing lipids: cysteine, tryptophan, histidine, lysine, arginine, tyrosine, methionine, serine, threonine, glycine, alanine, valine, proline, leucine, and isoleucine (Schaich 2008). Loss of nutrition in foods is the most obvious consequence of modification or destruction of these amino acids but overall protein function is also affected and may result in increased hydrophobicity, crosslinking, and/or denaturation. Protein oxidation also has ramifications for certain diseases, including those involving protein aggregation as is the case with Alzheimer's disease (Smith et al. 1994), enzyme inactivation, cellular transport of waste, and oxidative stresses.

2. Challenges

Although it is known that oxidizing lipids degrade proteins, it is not known which lipid oxidation products have the greatest impact. The process is dynamic with different products mediating different pathways over time. Multiple pathways can also involve

several sites within a single protein at once. Models have been created to predict the effects but a food item is a complicated system and can yield unpredictable outcomes. Global co-oxidation behaviors of proteins have been broadly documented; much less is known about the detailed interaction chemistry that leads to complexes and other protein changes.

D. Conventional Method of Protein Detection

1. SDS-PAGE of Zeins

Before the development of electrophoresis, column fractionation was used to show that zeins consist of multiple components with different molecular weights, but poor reproducibility, resolution, and sensitivity were significant problems. Now, characterization of zein components is commonly performed by electrophoresis, predominately Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE is a useful tool because it provides a visual representation of samples compared to standards on the same gel and components can be separated by molecular weight.

The first conclusive data documenting zein multiplicity was generated by Turner et al. (1965) who used starch and agar gel electrophoresis. The observation that zein required reduction into components that were approximately 20 kDa before the highest molecular weight proteins could successfully enter the gel established that zeins possessed intermolecular disulfide bonds.

Despite the benefits that electrophoretic methods have offered over the years, there are several notable drawbacks to their use. Although commercially-prepared gels

are available, there are still times when a specific gradient or gel composition is desired; then, gels must be prepared by hand, which is time-consuming and requires skill. Reproducibility can also be poor because of quality of the gel preparation or differences in samples (dye-binding properties can vary, sample fronts can tend to "smile" depending on the method or voltage used, and so on). Figure 3 shows an example of SDS-PAGE analysis of zein samples.

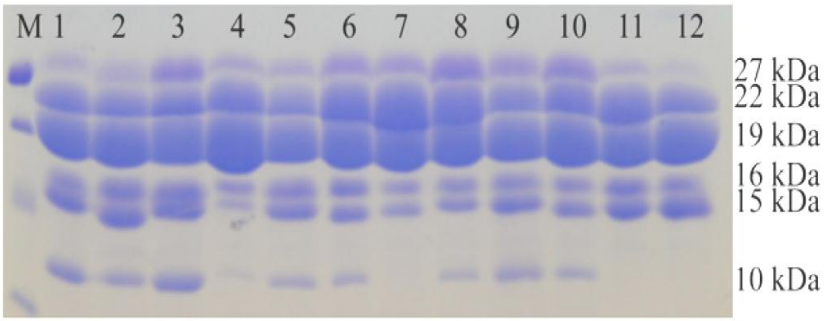

Figure 3: SDS-PAGE of 12 different maize inbred lines and genetic varieties (Wu and Messing 2010). Smiling, or slower migration in the outer lanes, is caused by high voltage and heat driving the center regions to migrate more quickly.

This thesis is part of a long-term research project focused on lipid oxidation and co-oxidation. In a previous study, zeins in tortilla chips were analyzed using SDS-PAGE to evaluate changes in protein patterns (Dong 2011). In addition to discernible normal bands, there was also extensive smearing between bands that resulted from zein modification (random crosslinking), not overloading of sample wells. Thermal degradation in baked chips and early oxidation in fried chips primarily involved disulfide crosslinking. However, additional forms of crosslinking (presumably a free radical plus a

non-reducible, non-fluorescent Schiff base) developed rapidly in proteins from fried chips with adsorbed oil. Most notably, protein solubility (reaction with Coomassie blue in solution) did not decrease, yet entire zein bands disappeared from gels even after reduction, indicating substantial modification of dye binding sites on the protein surface. New high molecular weight bands appearing at the top of sample lanes were insufficient to explain the shifts. Even when samples were analyzed in 7.5% gels, the lowest acrylamide percentages technically feasible to allow passage of very large proteins, the missing bands could not be retained. Thus, formation of very large protein aggregates too large to enter the gel but open enough to maintain solubility and Coomassie blue access were hypothesized. Aggregates remaining after reduction most likely involved free radical and perhaps other crosslinks. A major current goal, therefore, is to apply capillary electrophoresis to determine the actual size of the aggregates and elucidate mechanisms of modification.

2. HPLC of Zeins

In addition to a long history of separation by electrophoretic techniques (particularly SDS-PAGE), there has also been extensive documentation of cereal protein separation by chromatographic techniques. Column chromatography required large amounts of material and provided rather crude, poorly-resolved chromatograms, but the advent of High Pressure Liquid Chromatography (HPLC) moved chromatography into competition with electrophoresis for ease, resolution, and small sample requirements (Oda and Landers 1997). Three HPLC modes, in particular, have been used: ion-exchange chromatography (IEC), size-exclusion chromatography (SEC, also known as gel permeation), and

reversed-phase chromatography (RP) (Rodriguez-Nogales 2006). In IEC, all proteins in a sample are bound to the stationary phase of a column and the pH of ionic strength of the eluting buffer is shifted to release the proteins. In SEC, proteins travel through the stationary phase and are separated based on molecular size. However, proteins that are too small will elute in the void peak while proteins that are too large may not pass through the maze of beads contained in the column during a reasonable experimental time frame. In RP-HPLC, the stationary phase is nonpolar and separation occurs based on how strongly the hydrophobic amino acids react with the stationary phase which slows their path. This latter technique has shown the most long-term promise for separating zeins. Figure 4 shows an example of RP-HPLC analysis of a zein sample (Bietz 1985).

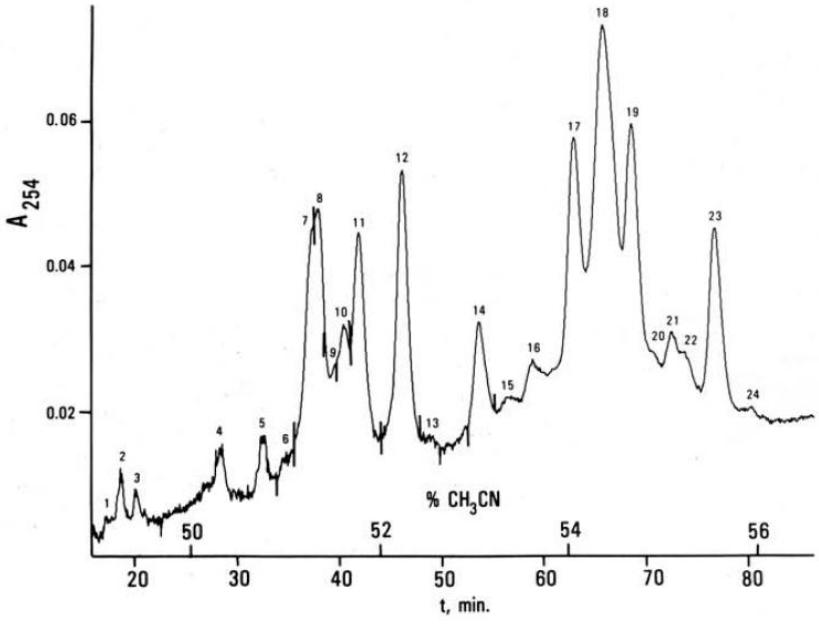

Figure 4: RP-HPLC of reduced and pyridylethylated zein (specific zein fractions not identified, peaks only numbered for counting purposes) (Bietz 1985).

HPLC provides several advantages over SDS-PAGE (Bean et al. 1998). One is that delivery of buffer by pumps provides constant flow rate and thus good reproducibility. Another is that HPLC is quantitative as well as qualitative, thanks to coupling with a detector. For proteins, the most common is an ultraviolet detector, which can be set at a desired detection wavelength such as 210 nm (peptide bond absorbance region) or 280 nm (aromatic absorbance region). Most modern HPLC systems are also equipped with autosamplers that allow many samples to be prepared at once, loaded into the instrument, and automatically injected onto the column sequentially.

The use of HPLC instrumentation is widespread in analytical laboratories (Bietz 1985). Although the requisite materials are quite expensive (large volumes of high quality reagents are required for separations and column prices begin at several hundred dollars), they are versatile instruments that can analyze a wide range of molecules and compounds with a "simple" switch of buffer and column (Snyder and Kirkland 1979). The presence of salts in buffer solutions required for proteins leads to a common error of "salting out" the instrument or fouling the column if proper rinsing is not performed; this can be avoided with diligence and know-how. Guard columns installed before the column can bear the brunt of changeover at a lower cost.

E. Detection of Proteins by Capillary Electrophoresis

1. Introduction and Theory

Capillary Electrophoresis (CE) is based on the principle of capillary induction which has only recently been mechanized to allow for fast, automated separation and quantitation of a wide range of molecules from complex matrices (Oda and Landers

1997). The underpinnings are, as the name would suggest, transport of a material by capillary action coupled with movement of moieties under the effect of an applied electric field. Capillary electrophoresis can be used as a complementary technique in tandem with other methodologies or as a stand-alone technique when traditional methods are not applicable.

In order to identify the benefits of capillary electrophoresis for elucidation of protein content, it is first necessary to give an overview of the principles and workings of the instrument. In its simplest form, the instrument consists of an inlet reservoir, an outlet reservoir, a capillary (filled with background electrolyte), a power source, and a detector (Figure 5).

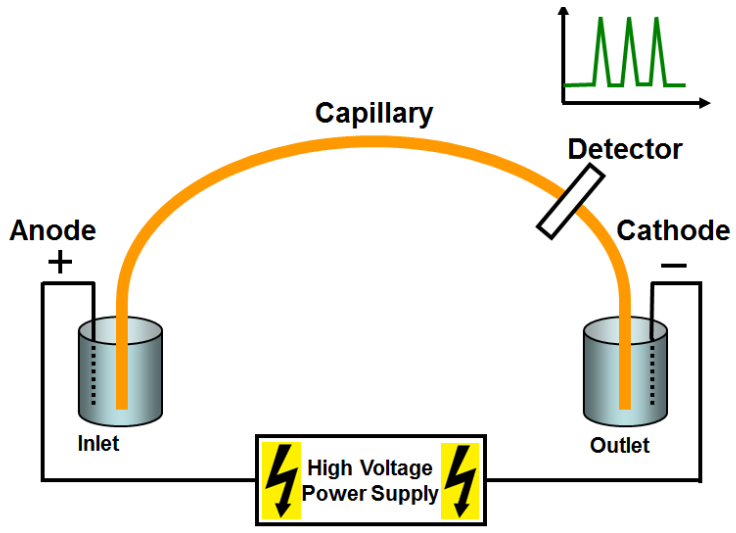

Figure 5: Diagramatic representation of a capillary electrophoresis instrument.

Capillaries are fused-silica, typically with an inner diameter of 20 to 100 µm, an outer diameter of 375 µm, and a length of 20 to 100 cm (Oda and Landers 1997). The outside of the capillary is coated with a polymer (polyimide) which allows for easier

handling of what would otherwise be a very fragile material to manipulate. A small portion of the amber-colored coating is removed via heat or acid to allow for a clear window where the detector (usually 200 or 800 μm in length) is positioned. In commercial instruments, the capillary resides in a cartridge through which circulates a coolant with high heat transfer properties. This coolant is critical for capillary electrophoresis: it efficiently conducts heat away from the capillary which reduces Joule heating within the capillary, allows a steady voltage/current to be maintained at up to 30,000 V (Scholz et al. 2002), and decreases buffer viscosity and separation time (Baker 1995). The dimensions of typical capillaries allow for analysis of very small volumes of sample, in the nanoliter to microliter range.

A sample is introduced into the capillary by one of two methods (Oda and Landers 1997). The first is by applying pressure at the inlet (or vacuum at the outlet) to force a sample into the capillary. Assuming that the sample is not very viscous and the species of interest are smaller than the radius of the capillary, this is typically the preferred method because introduction of all molecules into the capillary is assured and reproducibility is greater since each sample injection is a representative portion of the bulk sample. The second method is to apply voltage to both the inlet and the outlet simultaneously to initiate ion flow in the direction of the current. In order to introduce a sample into the capillary by electrical means, the current must be in the correct orientation and the molecules of interest must have a charge; therefore this means is not appropriate if some of the molecules of interest are uncharged (a simple dipole moment is insufficient). With this method, samples should not be injected more than once from the same sample well because there is a certain bias involved with injection by voltage and

subsequent injections do not necessarily have the same composition. However, sample introduction by voltage may be preferred if only one type of molecular charge is of interest (cations or anions) or the sample is quite dilute because more of the target charged molecules may be drawn up.

Once the capillary has been filled with background electrolyte and the sample is injected, regardless of selected method, a high voltage is applied across the capillary (providing resolution equivalent to $1x10^6$ plates/m in HPLC) which causes the charged molecules to move either forward towards the detector or backwards to the inlet. Obviously, if the applied voltage were the only active effect, only a portion of the molecules would pass the detector while some would travel the opposite direction and others would not move at all. This is where capillary electrophoresis has one very important benefit over other electrophoretic techniques: electroosmotic flow (Figure 6).

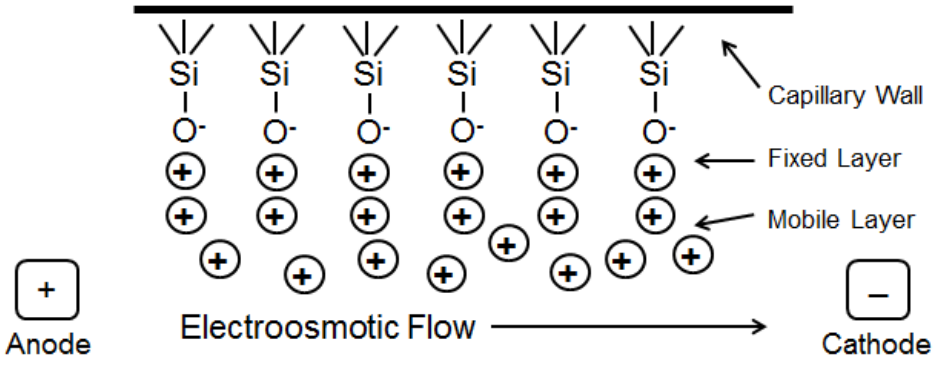

Figure 6: Diagram of the double layer that enables electroosmotic flow.

Electroosmotic flow provides the force that drives molecules that would not otherwise be drawn to the outlet (such as uncharged molecules) past the detector (Oda

and Landers 1997). It is analogous to the mechanical pump in HPLC, but the pump in capillary electrophoresis is driven by an electrical field. The capillary is made of silica glass and therefore the silicon oxide inner walls of the capillary have a pH-dependent negatively charged layer of silanol groups (SiO-). The positive ions in the background electrolyte line up along the wall with a strong attraction and form several layers of decreasing, but still present, affinity. The innermost layer of charge is called the "Inner Helmholtz" or "Stern" Layer; here, the charge is static. The next layer in is called the "Outer Helmholtz Plane" (Oda and Landers 1997). When voltage is applied, the cations in the Outer Helmholtz Plane begin to move and, due to cohesion of water, the background electrolyte is swept across the length of the capillary, carrying molecules with it. Molecules that were travelling in the direction of the detector will continue to do so. Neutral molecules will passively be swept along with the line of demarcation between positive and negative regions (called the electroosmotic front). Most importantly, assuming that the strength of the electroosmotic flow is greater than that of the pull of the charge towards the inlet, molecules that carry the same charge as the cathode (outlet) are dragged past the detector. In this way, all molecules can be detected even when voltage is applied in only one direction.

There are several conditions that affect electroosmotic flow (Oda and Landers 1997). The electrolyte concentration of the background solution is most critical. Also, the capillary walls must be clean as they can bind molecules in the sample tightly, much like with fouling of an HPLC column. This is especially true with proteins which must be stripped from the capillary surface by rinsing with acid and/or base between each sample or every few samples. Determining the electroosmotic flow is critical because it

serves as a line of delineation between anionic and cationic peaks. This can be accomplished by introducing a neutral marker into the capillary and running the desired method or protocol; some typical markers are dimethyl formamide (DMF), dimethyl sulfoxide (DMSO), and mesityl oxide.

The manner in which the background electrolyte travels through the capillary determines the shape of the flow front (Oda and Landers 1997). In HPLC, pump action propels the buffer resulting in drag against the walls of the column which has a relatively large diameter (on the order of 1 cm or larger). This generates a parabolic profile (Figure 7) and compounds elute as peaks that can be rather broad. By contrast, in capillary electrophoresis, chemical action moving the solution almost entirely eliminates drag resulting from contact with the capillary wall, and the capillary itself has a very small inner diameter. Thus, the shape of the buffer front in capillary electrophoresis is nearly flat (Figure 7), which greatly reduces peak broadening, fronting, and tailing.

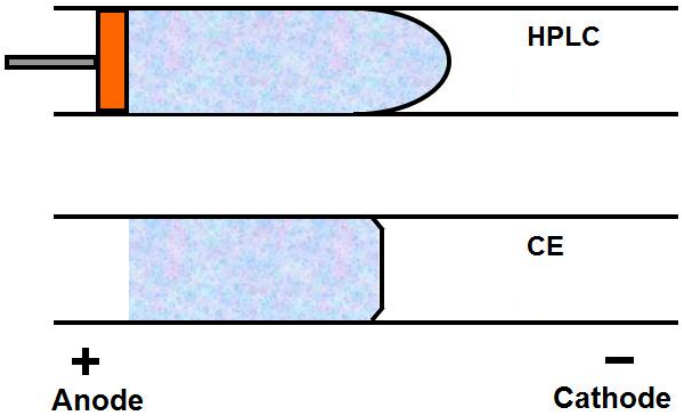

Figure 7: HPLC and capillary electrophoresis flow profiles.

Another critical component of capillary electrophoresis is the detector (Oda and Landers 1997). Typical commercially available detectors are Ultraviolet (UV), Photo Diode Array (PDA), and Laser (single or dual beam). The least costly option is the UV detector which is similar to that of traditional HPLC instruments and carries out the same function: measuring the absorbance at a set wavelength. The standard commercial instrument (produced by Beckman Coulter) comes equipped with several filters in an array of designated wavelengths with space to add more filters, although they are difficult to obtain. Proteins are typically measured in the 200 nm wavelength range. Aqueous solutions of background electrolytes are generally low in UV absorbance. Therefore, the signal from the lamp passes through the window in the capillary and falls upon the detector which measures a baseline signal. When a molecule passes in front of the window, it absorbs the light and a negative peak is captured; the software automatically inverts the signal so that the electropherogram (analogous to an HPLC chromatogram) displays the normal peak shape which analysts are accustomed to seeing.

A more costly detector option is the PDA which scans across all wavelengths simultaneously. The benefit of this detector is that a reference wavelength can be chosen and is used to diminish the signal to noise ratio (background noise). This is especially useful when analyzing very low concentration samples.

A third option is the laser detector, which is very expensive and rarely cited in research.

The Beckman Coulter version of the capillary electrophoresis is built with modular detectors that are intended to be switched out as needed, without the use of tools or special knowledge (PA 800 plus Pharmaceutical Analysis System 2011). Use of a

PDA or laser detector instead of a UV detector will increase sensitivity but will not affect run time or resolution of peaks, which are determined by analyte interactions with the solvent, the current, and the capillary walls.

Once the sample passes the detection window, it travels through the final portion of the capillary and is dispensed into a waste vial (Oda and Landers 1997). When additional analysis of fractions is desired, it is possible to either capture each sample separately in a 96-well plate for transfer to another instrument or to couple the capillary electrophoresis to another instrument directly. The most common linkage is to a mass spectrometer (MS). Extreme care must be taken to ground the connection owing to the high voltage carrying through the capillary and there are now commercial options for doing so. In fact, much research has been conducted in the past five years with CE-MS, including some research on zeins.

2. Capillary Electrophoresis of Proteins

Capillary electrophoresis is sub-divided into many modes of separation but those most applicable to proteins are capillary zone electrophoresis (CZE), capillary iso-electric focusing (CIEF), capillary gel electrophoresis (CGE), and isotachophoresis (ITP) (Oda and Landers 1997). CZE is the most universal because it accepts a wide range of analytes (size and character). CZE is also the simplest method to prepare and perform because it consists simply of a buffer in a bare fused silica capillary, although the buffers themselves and the resulting chemistry can be quite complicated. Molecules are separated mainly based on charge density and the method can be considered analogous to Acid PAGE. Most work with cereal proteins thus far has been by CZE.

CIEF separates the molecules by their isoelectric points within the capillary and then pushes them past the window to be detected in distinct bands. This method is used more frequently now that stand-alone commercial instruments have become available, making preparation easier and standardized testing possible; however, CIEF instruments are far less versatile than traditional capillary electrophoresis instruments.

As the name implies, CGE uses a gel-filled capillary and, when the sample is treated in an SDS-containing buffer, is an analog to the slab gel method SDS-PAGE. SDS-CE separates based on molecular mass and can be used to find an approximation of molecular weight. It is more automated and straightforward than HPLC or SDS-PAGE. Much of the published research pertaining to this method uses a Bio-Rad gel made of hydrophilic polymer which acts as a molecular sieve (Bean and Lookhart 2000); unfortunately, this gel is no longer commercially available. Beckman Coulter makes a gel as well but this can only be purchased in an expensive kit. The only other option is to make the gel directly but this requires extreme diligence in selection of reagents as well as degassing to ensure that all microscopic air bubbles are removed from the system.

The most important consideration for the analysis of proteins is the buffer, since its characteristics (pH, aqueous/non-aqueous, salt concentration, etc.) will strongly affect the shape, hydrodynamic size, and charge of the protein (Oda and Landers 1997). It is also important to consider that proteins will adsorb onto the silica walls of the capillary unless precautions are taken. If adsorption is not too severe, it may be sufficient to purge the capillary with acid (such as acetic acid or 1 M hydrochloric acid) in order to rinse the proteins from the walls and re-establish the surface charge of the silica. If adsorption is a significant issue (identified by tailing of peaks—not usually seen in capillary

electrophoresis—or a rising baseline) then it may be necessary to include additives such as hydroxypropylmethylcellulose (HPMC) in the background electrolyte. Additives may have the added benefit of minimizing interactions of proteins with each other. Another option for limiting adsorption is coated capillaries. These may be purchased commercially but extreme care must be taken in selection of solvents or solutions introduced because incompatible solutions may strip the coating, leaving just a regular bare fused silica capillary. Capillaries may be coated in the lab and are generally re-coated with every sample injection. As can be imagined, stripping and recoating the capillary with every increment adds a significant amount of time to the analysis.

In theory, the velocity of a molecule (v_p) through the capillary is approximated by the following equation:

$$v_p = \mu_p E$$

where μ_p is electrophoretic mobility and E is electric field strength (Oda and Landers 1997). As such, the electrophoretic mobility of the molecule is:

$$\mu_p = \frac{z}{6\pi\eta r}$$

where z is the net charge of the molecule, η is the viscosity of the background electrolyte, and r is the Stokes radius (the radius of a hard sphere that diffuses at the same rate as the molecule). Using the Stokes radius as:

$$r = \frac{k_B T}{6\pi\eta D}$$

where k_B is the Boltzmann constant, T is temperature, and D is the diffusion coefficient, the electrophoretic mobility can also be stated as:

$$\mu_p = \left(\frac{L}{t_r}\right)\left(\frac{L_t}{V}\right)$$

where L is the distance from the inlet tip of the capillary to the detection window, t_r is the migration time to travel distance L, V is the applied voltage, and L_t is the total length of the capillary (from inlet tip to outlet tip).

For small molecules and especially ions, these equations hold true and it is possible to predict migration time (Oda and Landers 1997). However, the equations do not accurately predict migration of proteins, in which charge and size vary widely based on the nature of the protein and the solution in which it is dispersed. If a protein is folded tightly, it approximates a sphere but the surface charges can vary depending on which amino acids are present on the outside of the sphere. If a protein is unfolded to a large extent, it is not possible to know whether, once introduced into the capillary, the protein is traveling with a narrow profile (small radius) or a wide profile (large radius) relative to the electroosmotic flow.

As protein modeling and the understanding of folding behavior improves, it becomes more likely that it will be possible to predict migration behavior; for now, a protein sample must be applied to the instrument to determine its travel time. This makes method development difficult because initial analyses may have to be run for hundreds of minutes to make sure that no stray peaks migrate very late in the run. It is also possible to switch the voltage to determine if any peaks are traveling in the opposite direction from the window at a faster rate than the electroosmotic flow is pulling them towards the detector.

3. Capillary Electrophoresis of Zeins

Capillary electrophoresis is widely used in the pharmaceutical industry due to its automation, speed, and generation of low volumes of solvent waste (Sekhon 2011). It is an especially useful tool for isolating and analyzing both potential therapeutic and causative molecules. For example, studies have been conducted on proteins associated with certain diseases such as separation and identification of human squamous cell lung carcinomas and small cell lung carcinomas.

Figure 8 illustrates that, while industries such as genetic research represent the bulk of the work, food accounts for approximately 10% of capillary electrophoresis research in a recent survey. While some work has been carried out on food proteins, the study of cereal proteins is relatively in its infancy; most of the work has been conducted on wheat proteins. The initial forays into applying capillary electrophoresis to cereal proteins were conducted with wheat gliadins (Bietz and Schmalzried 1995), rice, and oats (Lookhart and Bean 1995).

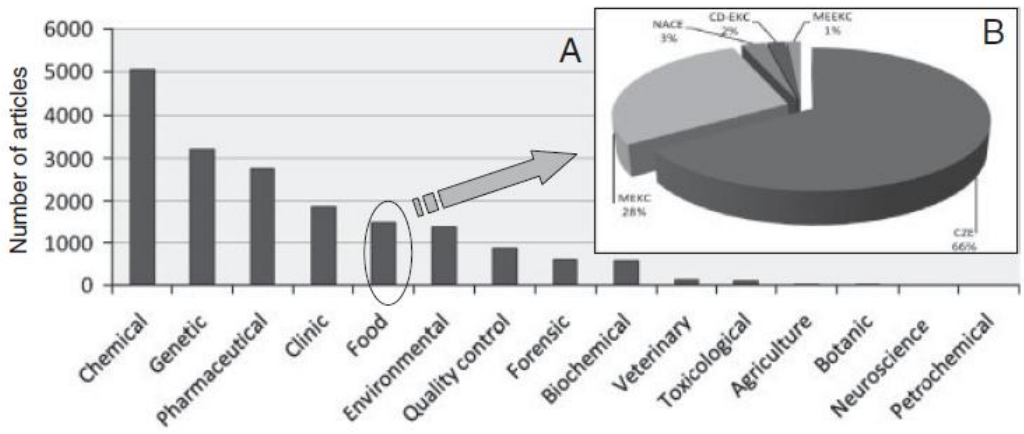

Figure 8: Methods categorized by application (A) and distribution of mode in food analysis (B) (Piñero et al. 2011).

Zeins may be the most difficult cereal protein to successfully separate due to the relatively small distribution of molecular weights among components (Cooke 1984) and poor solubility. Where zeins are concerned, the only capillary electrophoresis research reported was on extracts from pure corn samples (Parris et al. 1997, Bean et al. 2000); the vast majority of the work has been focused on differentiating cultivars rather than detecting modifications.

Typically, urea is used to solubilize maize proteins. However, high salt concentrations are incompatible with capillary electrophoresis because the salt tends to disrupt the current and lead to unstable baselines and increased heating in the capillary. Bietz and Schmalzried (1995) reported severe reproducibility problems with alkaline buffers. The use of sodium phosphate buffers as an alternative improved reproducibility somewhat but not as much as the phosphate glycine buffer identified by Lookhart and Bean (1995). The benefit of phosphate glycine buffer is a decrease in conductivity and thus an increase in detection time. Another beneficial development was the addition of acetonitrile to the background electrolyte (Lookhart and Bean 1995); research has shown that the optimal concentration range for zein solubility and capillary electrophoresis resolution is 50 to 70 % (Bean et al. 2000) (Figure 9).

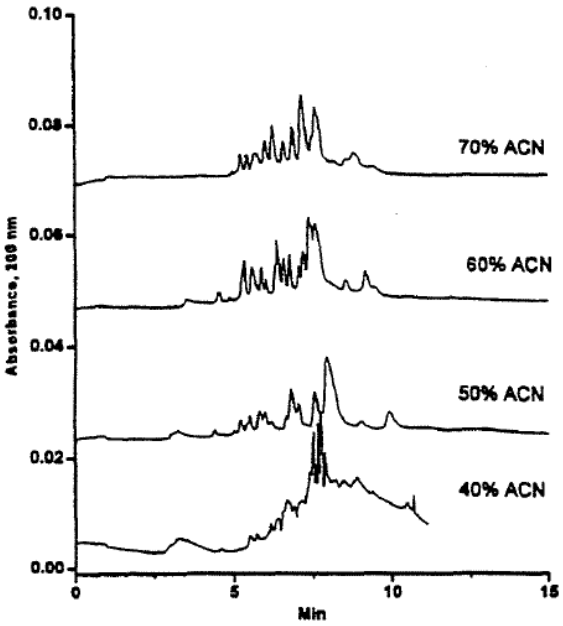

Figure 9: Effects of acetonitrile on zein separation (Bean et al. 2000).

Bean and Lookhart (2000) showed that electropherograms run by FZCE were reproducible within 18 to 24 hours after preparation; attempting to analyze samples beyond that window of time led to different separation patterns and they surmised that it was an indication of disulfide bonds being reformed. They also found that, in general, resolution was better and separation time was faster than when running comparable samples by RP-HPLC.

FZCE is the mode most frequently used to analyze zeins. Some studies did investigate the use of SDS methods in capillary electrophoresis; unfortunately, nearly all of them were conducted using the aforementioned Bio-Rad SDS sieving gel that is no longer manufactured. The only commercial kit available is manufactured by Beckman Coulter, and little information is yet available about behavior of zeins (or proteins in general) in this material.

4. Capillary Electrophoresis versus SDS-PAGE

Although they are based on similar theoretical foundations (the migration of molecules under the gradient of an applied field of voltage), capillary electrophoresis and SDS-PAGE have some important differences (Baker 1995). The most obvious benefit of capillary electrophoresis is automation; sample preparation is often quite easy and once the samples, buffers, and rinses are loaded into the instrument and the protocol programmed, the method is self-executing and can process samples accurately and reproducibly for many hours on end (several days, even). Literature cites countless instances of comparative capillary electrophoresis runs taking less than 15 minutes to fully resolve a sample compared to the several hours it can take to run, stain, and de-stain an SDS-PAGE gel. For example, Harrold et al. (1993) separated 16 anions with baseline resolution within four minutes.

Capillary electrophoresis allows for the detection of a very wide range of molecular sizes, from ions to proteins, as long as the molecules are fully dissolved and not removed in pre-run filtration. SDS-PAGE is limited by the ability of large molecules to enter the gel. Lower percentage gels are able to better resolve higher weight molecules (at the expense of resolving smaller weight molecules) but are still limited to approximately 500 kDa, whereas capillary electrophoresis is theoretically able to inject and detect any molecular that can fit in the bore of the capillary (although, practically speaking, capillaries can become clogged and so samples are filtered to prevent this, as noted above).

Another benefit of capillary electrophoresis is quantitation and data analysis. With SDS-PAGE, any quantitation must be conducted with imaging software that is not

integrated into the gel system. Blurring or smearing may obscure bands, as well. However, with capillary electrophoresis, the resulting electropherogram can be analyzed for peak height, peak area, migration time, etc., and certain regions of interest can be magnified for closer inspection.

It should also be noted that SDS-PAGE requires the use of acrylamide which is a known neurotoxin.

5. Capillary Electrophoresis versus HPLC

Capillary electrophoresis and HPLC have several features in common which lend them both advantages in automation, quantitation, and improved resolution over methods such as SDS-PAGE (Baker 1995). However, a large variety of separations can be accomplished with one bare-fused silica capillary and one detector in capillary electrophoresis simply by varying the buffer composition and the direction of applied voltage. The same capillary can typically be used for 200 or more separations without showing a decline in performance. It can be argued that this performance parallels columns in HPLC and, indeed, many separations can be accomplished on a single column. More typically, though, columns are available in a variety of packing materials, particle sizes and shapes, and column dimensions; separations are optimized as much by changing columns as modifying elution solvents. Capillary electrophoresis offers a distinct advantage in cost here: coated bare fused silica can be purchased in spools for a fraction of the cost of an HPLC column.

Additionally, capillary electrophoresis requires only small quantities of sample (on the order of nanoliters to microliters) and buffers (on the order of a few milliliters).

Compare this to HPLC which requires several milliliters of sample and liters of buffer, resulting in high costs to purchase HPLC grade solvents and dispose of them properly.

Another benefit of capillary electrophoresis over HPLC is the wide pH range of potential buffers that are tolerable (Baker 1995); most HPLC columns cannot be used with mobile phases above pH 8.

III. RESEARCH GAPS

Extensive pharmaceutical research has been conducted in the area of proteins and capillary electrophoresis, where drug screening for drug therapies as well as quality control testing need to be conducted quickly, reproducibly, and quantitatively. By comparison, relatively little work has been carried out on food proteins with capillary electrophoresis. Published food protein research using capillary electrophoresis has primarily focused on identifying breeds of grains, and analysis has concentrated on the overall appearance of the electropherograms rather than the specific peak areas. This qualitative approach can be useful in visually distinguishing similarities and differences between samples at set points, but has not been applied to following changes over time in specific samples. Another important gap in the literature is the analysis of any actual food products; all samples evaluated to date are from the cereals themselves rather than in a processed food system.

One of the proposals for future work stemming from the research of Dong (2011) was that another method might be more suitable for analyzing zeins in the tortilla chip sample system. Protein patterns determined by SDS-PAGE were partially obscured by smearing and it was apparent that a portion of the samples was not entering the gel, most

likely due to extensive crosslinking. The research contained in this thesis seeks to address that recommendation.

IV. HYPOTHESIS

Capillary electrophoresis will prove more effective than SDS-PAGE in mapping changes that occur in proteins during processing and storage of tortilla chips. Specifically, capillary electrophoresis should be able to detect high molecular weight polymeric fractions and differentiate between proteins from baked versus fried tortilla chips.

V. SPECIFIC OBJECTIVES

1) Evaluate capillary electrophoresis SDS-MW kit and acetonitrile test methods for suitability in analyzing proteins extracted from tortilla chip samples using a P/ACE instrument (with UV detector);

2) Compare capillary electrophoresis results from both methods with those collected by Dong via SDS-PAGE.

VI. EQUIPMENT AND METHODS

A. Overview of Project

It is important to understand the underlying causes of protein damage from processing and lipid oxidation in order to protect the sensory, structural, and nutritional quality of processed foods. This study used two model systems—baked and fried tortilla chips—to compare protein damage from heat (baked) and oxidizing lipids (fried). Both of

these products have identical ingredients and handling up to baking or frying steps, and both are processed at comparable temperatures (180 °C). However, oil adsorbed during frying of tortilla chips can induce different damage during storage than the intrinsic lipids in the cornmeal. Thus, following protein changes in fresh products and after incubation at two elevated temperatures for several weeks should distinguish damage specific for thermal damage from lipid co-oxidation. Capillary electrophoresis is uniquely positioned to provide rapid, easy, and quantitative analysis of proteins extracted from incubated chips.

The design of this project is shown in Figure 10. Baked and fried tortilla chips from the same manufacturer were obtained from a local grocery store and divided into two batches for incubation at 40 °C and 60 °C for up to 8 weeks. Samples were withdrawn at zero (no incubation temperature delineation), one, two, three, four, six, and eight weeks for protein extraction and analysis by capillary electrophoresis.

The Bradford assay was used to determine the protein concentration of each sample so that the amount of protein injected into the capillary was comparable between runs. The SDS-MW kit method was run as the application of capillary electrophoresis that correlated most directly with SDS-PAGE. The acetonitrile method of Bean et al. (2000) provides an alternate, less costly means of testing proteins than does the SDS-MW kit method; this appears to be the first application of this method to a food system rather than a purified zein.

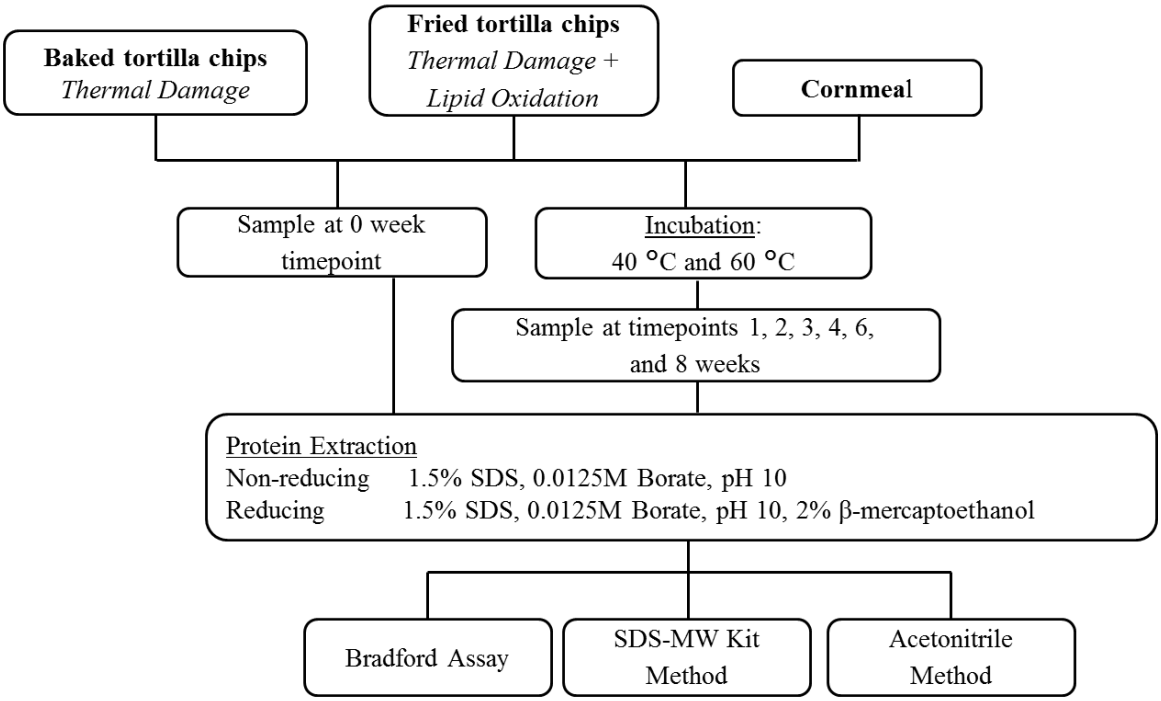

Figure 10: Project design for evaluating use of capillary electrophoresis to determine thermal and co-oxidation damage to proteins.

B. Instrumentation

Studies were conducted using a P/ACE MDQ Capillary Electrophoresis system (Beckman Coulter, Fullerton, CA) with a bare fused silica capillary (Polymicro Technologies, Phoenix, AZ). Protein concentrations by the Bradford analyses were determined using a Cary 50 UV/vis spectrophotometer (Varian Instruments, Walnut Creek, CA).

C. Materials

1. Tortilla Chips

Baked and fried tortilla chips from the same national manufacturer were procured from a local grocery store. Ingredients in both chips were listed as whole white corn, vegetable oil (corn, soybean, canola, and/or sunflower oil), and salt. The nutrition facts panels are shown in Figure 11. Neither type of chip had any preservatives, according to the package label, although it is common knowledge that frying oils generally contain tocopherols as antioxidants and a small amount of citric acid as a metal chelator.

Yellow cornmeal was also purchased at a local grocery store to serve as an unprocessed control. The brand was from the same company as the tortilla chips.

2. Extraction Reagents

Chloroform (J.T. Baker, Phillipsburg, NJ); methanol (J.T. Baker, Phillipsburg, NJ); sodium dodecyl sulfate, reagent grade (Fisher Scientific, Fairlawn, NJ); sodium hydroxide (Fisher Scientific, Fairlawn, NJ); β-mercaptoethanol, reagent grade (Sigma Chemical, St. Louis, MO); sodium tetraborate, analytical grade (J. T. Baker, Phillipsburg, NJ).

Nutrition Facts
Serving Size 1 oz. (28g/About 14 chips)

Amount Per Serving
Calories 120 Calories from Fat 25

	% Daily Value*
Total Fat 3g	**5%**
Saturated Fat 0.5g	**3%**
Trans Fat 0g	
Polyunsaturated Fat 1.5g	
Monounsaturated Fat 1g	
Cholesterol 0mg	**0%**
Sodium 140mg	**6%**
Total Carbohydrate 22g	**7%**
Dietary Fiber 2g	**7%**
Sugars 0g	
Protein 2g	

Vitamin A 0%	•	Vitamin C 0%
Calcium 2%	•	Iron 2%
Vitamin E 2%	•	Thiamin 2%
Phosphorus 6%	•	Magnesium 6%
Zinc 2%		

* Percent Daily Values are based on a 2,000 calorie diet. Your daily values may be higher or lower depending on your calorie needs:

	Calories:	2,000	2,500
Total Fat	Less than	65g	80g
Sat Fat	Less than	20g	25g
Cholesterol	Less than	300mg	300mg
Sodium	Less than	2,400mg	2,400mg
Total Carbohydrate		300g	375g
Dietary Fiber		25g	30g

Calories per gram:
Fat 9 • Carbohydrate 4 • Protein 4

Nutrition Facts
Serving Size 1 oz (28g/About 12 chips)

Amount Per Serving
Calories 140 Calories from Fat 60

	% Daily Value*
Total Fat 7g	**11%**
Saturated Fat 1g	**5%**
Trans Fat 0g	
Polyunsaturated Fat 3g	
Monounsaturated Fat 2g	
Cholesterol 0mg	**0%**
Sodium 120mg	**5%**
Total Carbohydrate 19g	**6%**
Dietary Fiber 2g	**7%**
Sugars 0g	
Protein 2g	

Vitamin A 0%	•	Vitamin C 0%
Calcium 2%	•	Iron 0%
Vitamin E 4%	•	Thiamin 2%
Riboflavin 2%	•	Vitamin B_6 4%
Phosphorus 6%	•	Magnesium 4%
Zinc 2%		

* Percent Daily Values are based on a 2,000 calorie diet. Your daily values may be higher or lower depending on your calorie needs:

	Calories:	2,000	2,500
Total Fat	Less than	65g	80g
Sat Fat	Less than	20g	25g
Cholesterol	Less than	300mg	300mg
Sodium	Less than	2,400mg	2,400mg
Total Carbohydrate		300g	375g
Dietary Fiber		25g	30g

Calories per gram:
Fat 9 • Carbohydrate 4 • Protein 4

Figure 11: Nutrition facts for baked (left) and fried (right) tortilla chips.

3. Bradford Reagents

Coomassie Blue G-250 (Sigma Chemical, St. Louis, MO); ethanol, 200 proof (ACROS Organic, NJ, USA); 85% phosphoric acid, HPLC grade (Fisher Scientific, Fairlawn, NJ); Whatman No. 1 filter paper (Whatman, Kent, England).

4. SDS-MW Kit Reagents

An SDS-MW Analysis Kit containing SDS-MW gel buffer, SDS sample buffer, SDS protein sizing standard (10 to 225 kDa), internal standard (10 kDa), acidic wash solution (0.1 N hydrochloric acid), and basic wash solution (0.1 N sodium hydroxide) was purchased from Beckman Coulter, Fullerton, CA.

5. Acetonitrile Method Reagents

Glycine, reagent grade (Fisher Scientific, Fairlawn, NJ); *o*-phosphoric acid, 85% (Fisher Scientific, Fairlawn, NJ); hydroxypropylmethylcellulose (Fisher Scientific, Fairlawn, NJ); acetonitrile, HPLC grade (J.T. Baker, Phillipsburg, NJ); acetic acid (J.T. Baker, Phillipsburg, NJ); hydrochloric acid (Fisher Scientific, Fairlawn, NJ).

6. Standards

For the Acetonitrile method: lyophilized bovine plasma gamma globulin (Bio-Rad, Hercules, CA); protein molecular weight standards, broad range MW 6.5 to 205 kDa (Amersheim, Otelfingen, Switzerland); ProSieve unstained protein markers, 5 to 225 kDa (Lonza, Basel, Switzerland).

For the SDS-MW kit method: the kit comes with a molecular weight standard that includes seven molecular weight markers ranging from 10 to 225 kDa. It is designed to be combined with the sample buffer and β-mercaptoethanol, heated at 100 °F for 3 minutes, and then analyzed within 24 hours.

D. Sample Preparation

235 g of baked chips, 260 g of fried chips, and 160 g of cornmeal were placed in aluminum trays, lightly covered with aluminum foil, and placed in 40 °C or 60 °C incubation chambers. 40 g of each sample were immediately frozen at -20 °F as controls. 40 g of each sample were withdrawn from the stability chambers and frozen at -20 °F at each of the following time points: one, two, three, four, six, and eight weeks. Samples were analyzed within four weeks.

The extraction method used by Dong (2011) was time-consuming. Therefore, attempts were made to shorten the required extraction time by using a Dionex ASE350 accelerated solvent extractor (Dionex, Sunnyvale, CA) which introduces the extraction solvent to the sample in a sealed and pressurized chamber. The method was as follows: a sample of tortilla chip was finely ground with a mortar and pestle and 1.0 g of chip was weighed. The sample was combined with Ottawa sand and this combination was transferred to a stainless steel ASE extraction cell. A filter paper frit at the bottom of the cell prevented fines from sifting through into the extract. The extraction chamber was placed into the instrument and run according to the method shown in Table 6.

Table 6: ASE 350 sample extraction method.

Temperature = 40 °C		Heat = 5 minutes
Static time = 15 minutes		Cycle = 1
Rinse Volume = 60 %		Purge = 60 seconds
Solvent A = 1	Cell type: SST	Solvent saver = Off

The extraction solvent was 70 % acetonitrile for compatibility with the acetonitrile capillary electrophoresis method. However, the samples were not concentrated enough (approximately 0.0367 mg/mL measured using the Bradford assay), even following evaporation. Substituting 70 % ethanol (known to be a good solvent for zeins) also resulted in very low protein concentration (approximately 0.02 mg/mL measured using the Bradford assay). With 100 % ethanol, the protein concentration in the extract increased to approximately 0.098 mg/mL but this was still too low. The highest protein concentrations (approximately 0.230 mg/mL, measured using the Bradford assay) obtained by increasing the amount of ground chip (2.50 g) and the static time (30 minutes) were still too dilute for capillary electrophoresis, so ASE was abandoned as a viable option for protein extraction from the tortilla chip samples.

Instead, protein was extracted from tortilla chip samples by a more traditional manual extraction process. Dong (2011) showed through analysis by SDS-PAGE that it is not necessary to subject the chips to exhaustive lipid extraction prior to protein extraction (e.g. using the ASE350), so a mild lipid extraction was conducted prior to protein extraction. Chips were ground with a mortar and pestle until they had a consistency resembling cornmeal. 0.520 g ± 10 mg were weighed into 15-mL plastic centrifuge tubes. 600 mL of chloroform that had been previously flushed with argon for 5 minutes was combined with 300 mL of methanol (2:1) and 10 mL of this resulting mixture was added to each tube. The solutions were flushed with argon for 1 minute each and incubated at 150 rpm for 24 hours at 37 °C. The bulk of the solution was decanted and the samples were then dried.

Two protein extraction buffers were prepared: reducing and non-reducing. For non-reducing buffer, 14.9939 g of SDS and 2.5248 g sodium tetraborate were dissolved in 1000 mL of purified water and adjusted to pH 10 with 1N sodium hydroxide. For the reducing buffer, 10 mL of β-mercaptoethanol was added to 500 mL of the non-reducing buffer. 10 mL of either reducing or non-reducing buffer was added to each of the samples and the tubes were then flushed with argon for 1 minute each. Samples were incubated at 150 rpm for 72 hours at 25 °C and then centrifuged at 8000 rpm for 10 minutes at 10 °C. The supernatant was decanted into separate tubes, and both the pellet and supernatant were flushed with argon and sealed.

E. Protein Concentration

Protein concentrations in extracts were determined by the Bradford assay (Dong 2011). The Bradford reagent was first prepared by dissolving 0.1 g Coomassie Blue G-250 in 50 mL of 95 % ethanol, adding 100 mL of 85 % phosphoric acid, diluting to 1 L with 18 MΩ pure water, and then filtering through Whatman No. 1 filter paper. A standard protein solution was made by dissolving 100 mg of Bovine Serum Albumin (BSA) in 10 mL of purified water from which a standard curve was generated by analyzing a series of solutions containing various concentrations of BSA (Table 7).

Each solution was vortexed and incubated at room temperature for 10 minutes and absorbance was measured at 595 nm. Absorbance data points were plotted as a function of BSA concentration. This process was repeated for a total of 5 curves for one batch of Bradford reagent and, once the data was fitted to a quadratic, the resulting quadratic

equation was applied to subsequent solutions of unknown concentration to determine protein content (Figure 12).

Table 7: Solution volumes for the Bradford assay standard curve.

	BSA Solution (µL)	Purified water (µL)	Bradford reagent (mL)
Blank	0	100	5
1	10	90	5
2	20	80	5
3	40	60	5
4	70	30	5
5	100	0	5

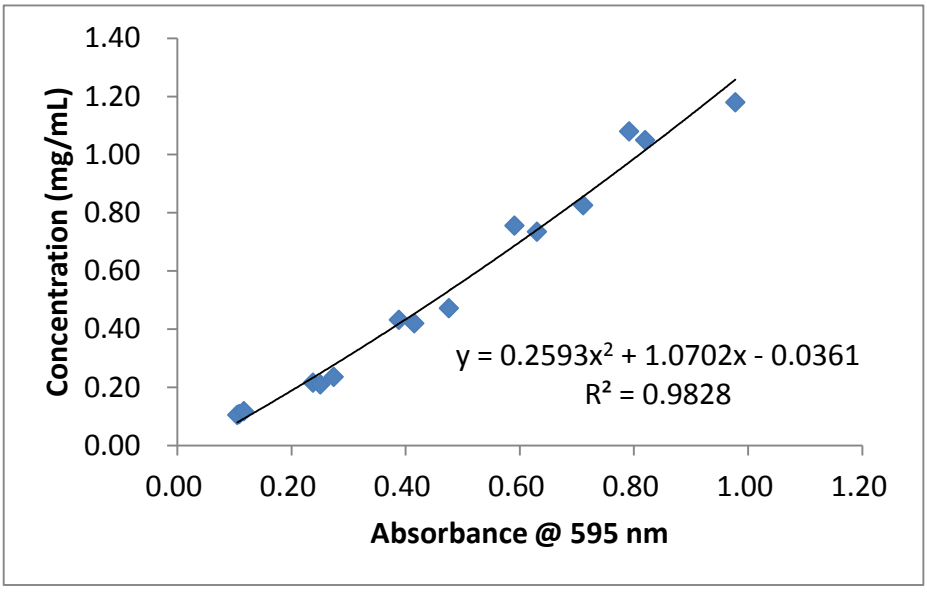

Figure 12: Example of Bradford assay standard curve.

To test incubated samples, a blank was prepared (100 µL of purified water added to 5 mL of Bradford reagent) concurrent with the unknown sample (100 µL of supernatant added to 5 mL of Bradford reagent). Blanks and samples were vortexed and incubated at room temperature for 10 minutes and then absorbance was measured at 595 nm. Each sample was measured in duplicate.

F. Capillary Electrophoresis of Maize Proteins by the SDS-MW Kit Method

Direct sample to sample comparisons in electropherograms require loading of identical protein concentrations. After determination of protein concentrations in extracts (Bradford method, as above), samples were diluted to yield 0.2 mg/mL (as per the instructions for the kit) using the SDS sample buffer provided in the kit. Aliquots of 150 µL were then filtered through a 0.45 µm PTFE membrane and collected in a PCR tube for use in Beckman Coulter capillary electrophoresis instruments. Samples were prepared in duplicate.

Instrument and method parameters were set as follows:

Instrument: 25 °C capillary and sample storage temperature

Collect current trace on all runs

Capillary: 40.2 cm length (30.2 cm to window)

50 µm inner diameter bare-fused silica

800 µm window aperture

Pre-Conditioning Method:

0.1 N sodium hydroxide wash, 5 minutes at 50 psi

0.1 N hydrochloric acid wash, 2 minutes at 50 psi

Water rinse, 2 minutes at 50 psi

SDS gel fill, 10 minutes at 40 psi

Separate with 15 kV for 10 minutes, 5 minute ramp time

Execute pre-conditioning method every 6 samples

Sample Analysis Method:

Water dip, 0.0 minutes

Sample injection, 20 seconds at 5 kV

Voltage separation with 15 kV (reverse polarity) for 60 minutes, 1 minute ramp, 20 psi at inlet and outlet, detect at 254 nm

End Run: 0.1 N sodium hydroxide wash, 10 minutes at 50 psi

0.1 N hydrochloric acid wash, 5 minutes at 50 psi

Water rinse, 2 minutes at 50 psi

Home position, lamp off

G. Capillary Electrophoresis of Maize Proteins by the Acetonitrile Method

An acetonitrile/β-mercaptoethanol mixture was prepared by adding 1500 µL of β-mercaptoethanol to 30 mL of acetonitrile. Each sample was diluted to 0.4 mg/mL with non-reducing extraction buffer to yield 600 µL to which was added 1875 µL of acetonitrile/β-mercaptoethanol solution; higher and lower concentrations of sample were investigated but this preparation resulted in a suitable balance between resolution and signal. Samples were filtered through a 0.45 µm PTFE membrane and collected in a 2 mL sample vial. Samples were prepared in duplicate.

Background electrolyte preparation was as follows:

Phos-gly: 50 mL water

2.0 g glycine

1600 µL o-phosphoric acid

Hydroxypropylmethylcellulose (HPMC):

50 mL water

0.6558 g HPMC

Background Electrolyte (BGE):

120 mL acetonitrile

16 mL phos-gly

8 mL HPMC

Dilute up to 200 mL with water

Instrument and method parameters were as follows:

Instrument: 25 °C capillary and sample storage temperature

Collect current trace on all runs

Capillary: 40.2 cm length (30.2 cm to window)

50 µm inner diameter bare-fused silica

800 µm window aperture

Method: 60 minute equilibration with 12.5 kV of BGE prior to first run

2 minute BGE rinse

Inject sample, 0.3 seconds at 0.5 psi

Separate with 12.5 kV for 60 minutes at 35 °C, 200 nm detection

0.1 M hydrochloric acid rinse, 1 minute

0.5 M acetic acid rinse, 0.5 minutes

End Run: 0.5 M acetic acid rinse, 10 minutes

Water rinse, 10 minutes

Home position, lamp off

VII. RESULTS

A. Protein Solubility (Extractability)

Figure 13 shows the protein concentration of the fresh samples before incubation (0 weeks). As can be seen, the sample protein concentrations are comparable indicating that the commercial processing methods do not have significant effect on protein solubility.

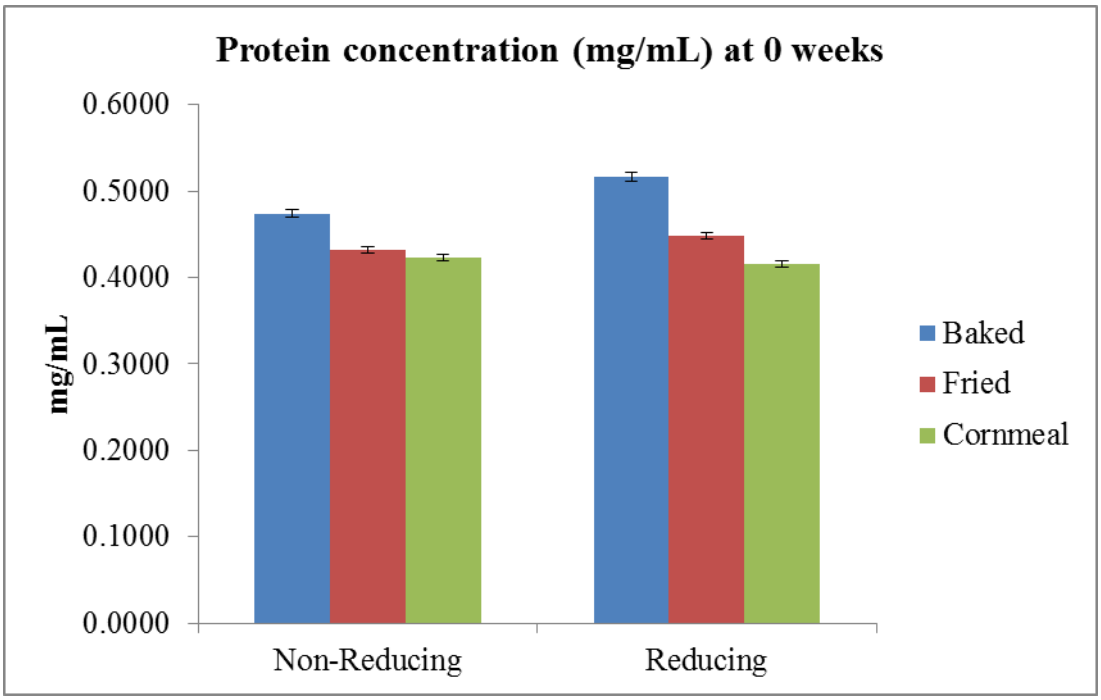

Figure 13: Protein concentration (mg/mL) for fresh samples (0 weeks incubation).

Figures 14 and 15 show changes in protein concentrations of the various samples over the course of incubation. Dong's work showed a general trend wherein protein concentration increased and decreased a small amount in the first three weeks and then rose to a greater concentration and again fell leading up to 8 weeks in the 40 °C samples; there was no discernable trend for the 60 °C samples (Dong, 2011). The data from this research does not show a similar trend. Overall, the protein concentration diminishes over the incubation period for the 40 °C samples except for the non-reducing cornmeal and non-reducing baked samples that increase transiently at 3 weeks. At 60 °C, protein solubility decreases in baked samples but increases in fried samples and cornmeal during the 8 week incubation.

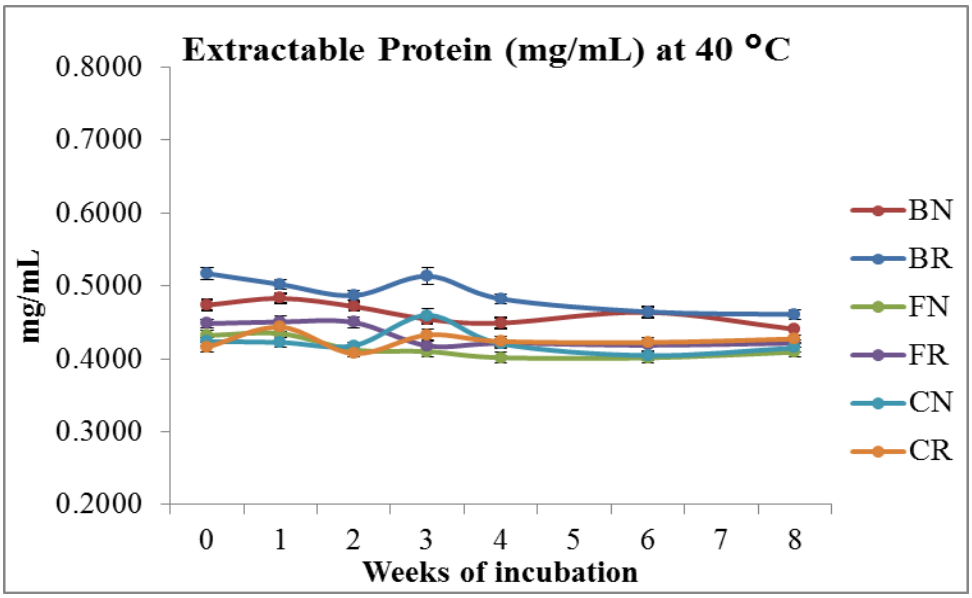

Figure 14: Changes in extractable protein after incubation at 40 °C. (BN = baked non-reducing, BR = baked reducing, FN = fried non-reducing, FR = fried reducing, CN = cornmeal non-reducing, CR = cornmeal reducing).

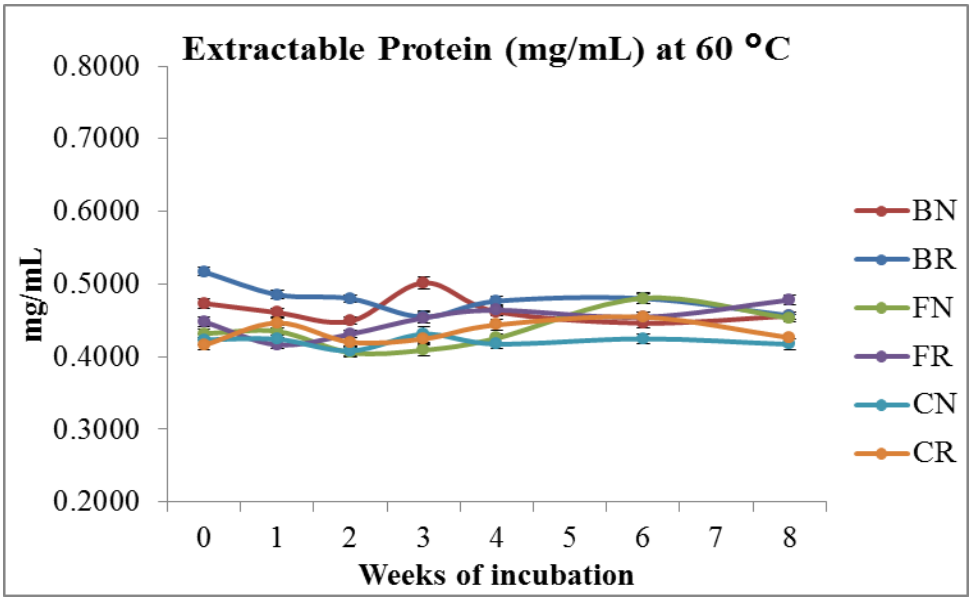

Figure 15: Changes in extractable protein after incubation at 60 °C. (BN = baked non-reducing, BR = baked reducing, FN = fried non-reducing, FR = fried reducing, CN = cornmeal non-reducing, CR = cornmeal reducing).

B. Capillary Electrophoresis

1. Reproducibility and Sensitivity of the Acetonitrile Method

Initially, the intent was to analyze the samples that had been extracted in the studies of Dong (2011). Using the Bradford method described above, the protein concentration was determined for all samples which were then diluted to the same concentration (see Methods section) and analyzed using the Acetonitrile method for capillary electrophoresis. Two initial tests were performed to determine suitability.

a. Reproducibility of Capillary Electrophoresis Measurements

To verify that capillary electrophoresis analyses of protein extracts were reproducible, two repetition experiments were performed. First, a zein F4000 standard solution was run on two successive days. Results (Figure 16) show only minor variability between the larger molecular weight peaks (toward the left side of the electropherogram) and some variability between the smaller molecular weight peaks (toward the right side of the electropherogram).

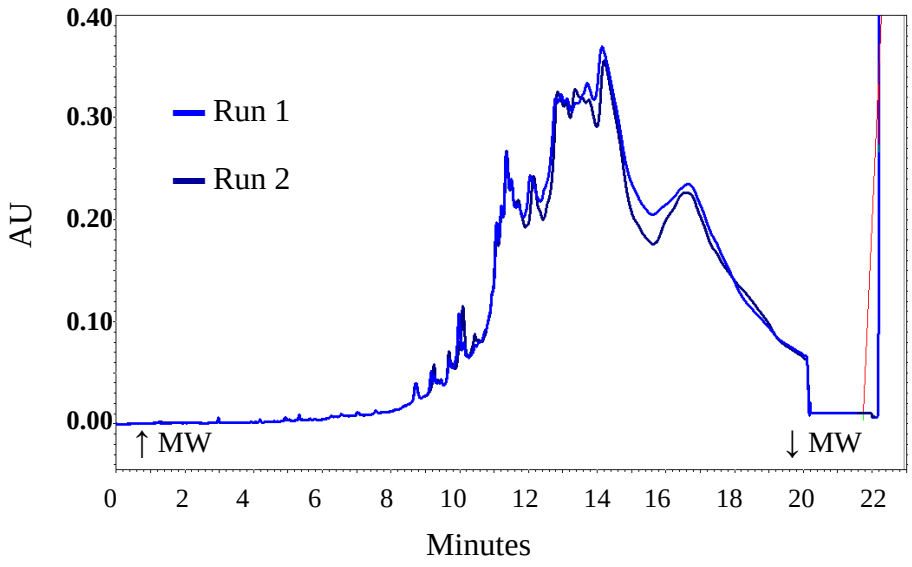

Figure 16: Zein F4000 analyzed in duplicate to show acceptable reproducibility (24 hour lag between run times), Acetonitrile method.

Second, fresh samples were analyzed in duplicate at week 0 and after six months storage in the freezer. Figure 17 shows that the form, shape, and size of the peaks are the same but there is a shift in migration time because a new capillary had to be constructed between the two sets of analyses. Although the capillaries were measured and an attempt

was made to keep them the same size, realistically there are slight differences in length and so there will be slight differences in migration time as well.

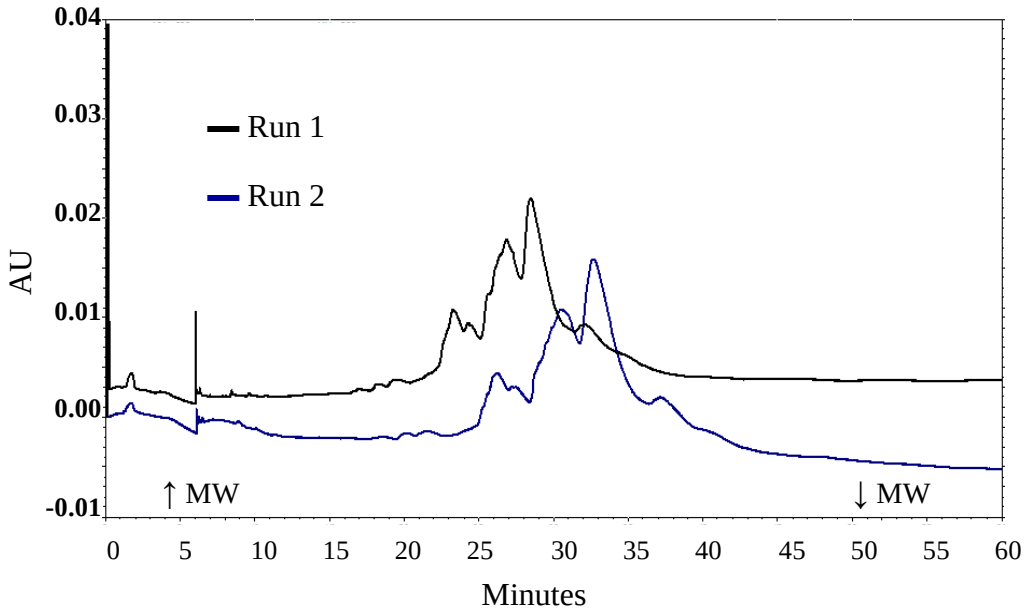

Figure 17: 0 week baked non-reducing protein extract (sample from Dong 2011) run in duplicate to show acceptable reproducibility (6 months of storage between Run 1 and Run 2) by Acetonitrile method.

These two sets of data indicate that differences in peak patterns observed in electropherograms of proteins from incubated samples are due to actual changes in the chemistry rather than inconsistency of the instrumentation and running conditions.

b. Ability of Capillary Electrophoresis to Distinguish Differences in Proteins from Baked versus Fried Extracts

One baked sample and one fried sample (both 0 week time point) were selected from an early preparation of Dong (2011) incubated samples to test method conditions. Although Bean et al. (2000) indicated that a range of acetonitrile concentrations could be used to analyze zeins by capillary electrophoresis, attempts to use 70 % acetonitrile as a sample diluent in this research yielded no usable results. However, with 100 % acetonitrile (for a final concentration of 75 %), it was possible to detect peaks. Figure 18 shows peptide patterns from fresh tortilla chips (0 weeks incubation). Consistent with SDS-PAGE (Figure 19), capillary electrophoresis clearly documents that baking versus frying alone is sufficient to induce differences in protein damage. Proteins from fried chips show major loss of several peptide bands (indicated by red arrows) and gain of only a few smaller molecular weight bands (indicated by green arrows) relative to baked chips.

As will be discussed later, the peaks in Figure 18 come to a sharper point than those of other sample runs. Although these samples were early suitability samples and were not reported in her thesis, these samples were prepared in the same manner as the later samples reported by Dong as well as the samples evaluated in this research. However, it is not known why they have a different overall appearance compared to subsequent samples. It is unlikely that any degradation from storage would result in better-defined peaks and so this would point to some difference in the preparation method that was not noted.

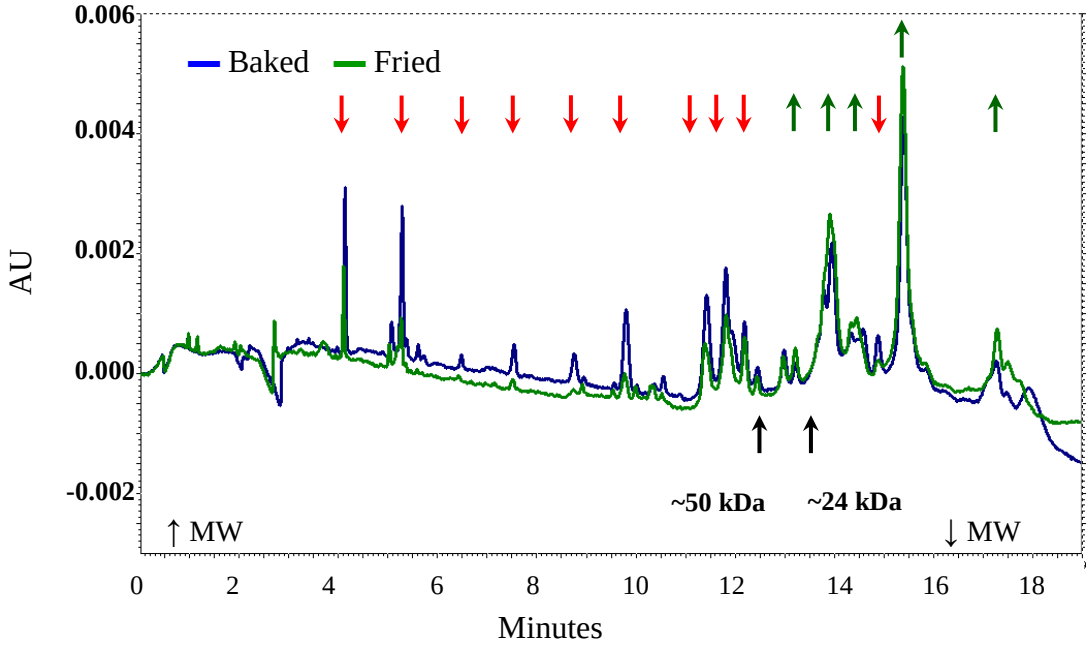

Figure 18: 0 week samples analyzed 24 hours apart (samples from Dong 2011) by Acetonitrile method.

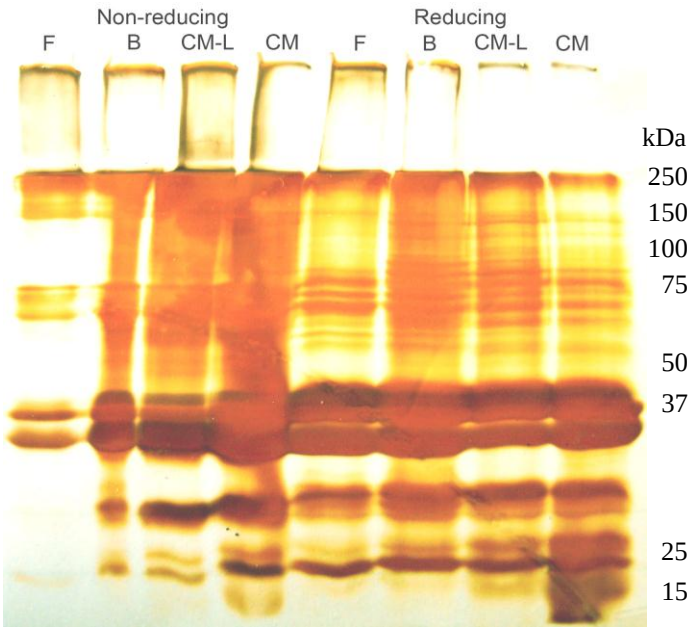

Figure 19: SDS-PAGE (Dong 2011). F = fried, B = baked, CM = cornmeal, CM-L = cornmeal with lipid removal before protein extraction.

2. Analysis of Samples from Dong (2011) Experiments

Given these promising results, the Acetonitrile method was applied to the second set of incubated samples that Dong generated. Results are shown in Figures 20 through 24. Additionally, by comparing Dong's SDS-PAGE results with those of capillary electrophoresis it is possible to correlate band regions with peak regions, as seen in Figure 25.

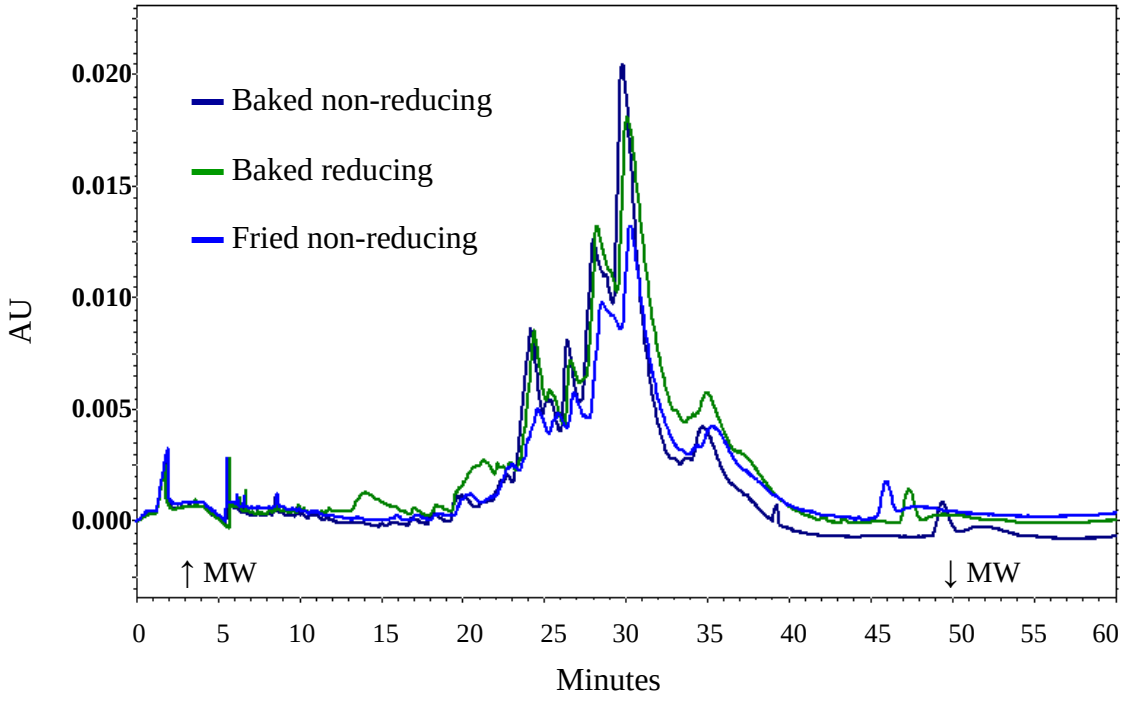

Figure 20: Protein extracts from 0 week baked non-reducing and reducing and fried non-reducing protein extracts (samples from Dong 2011), Acetonitrile method.

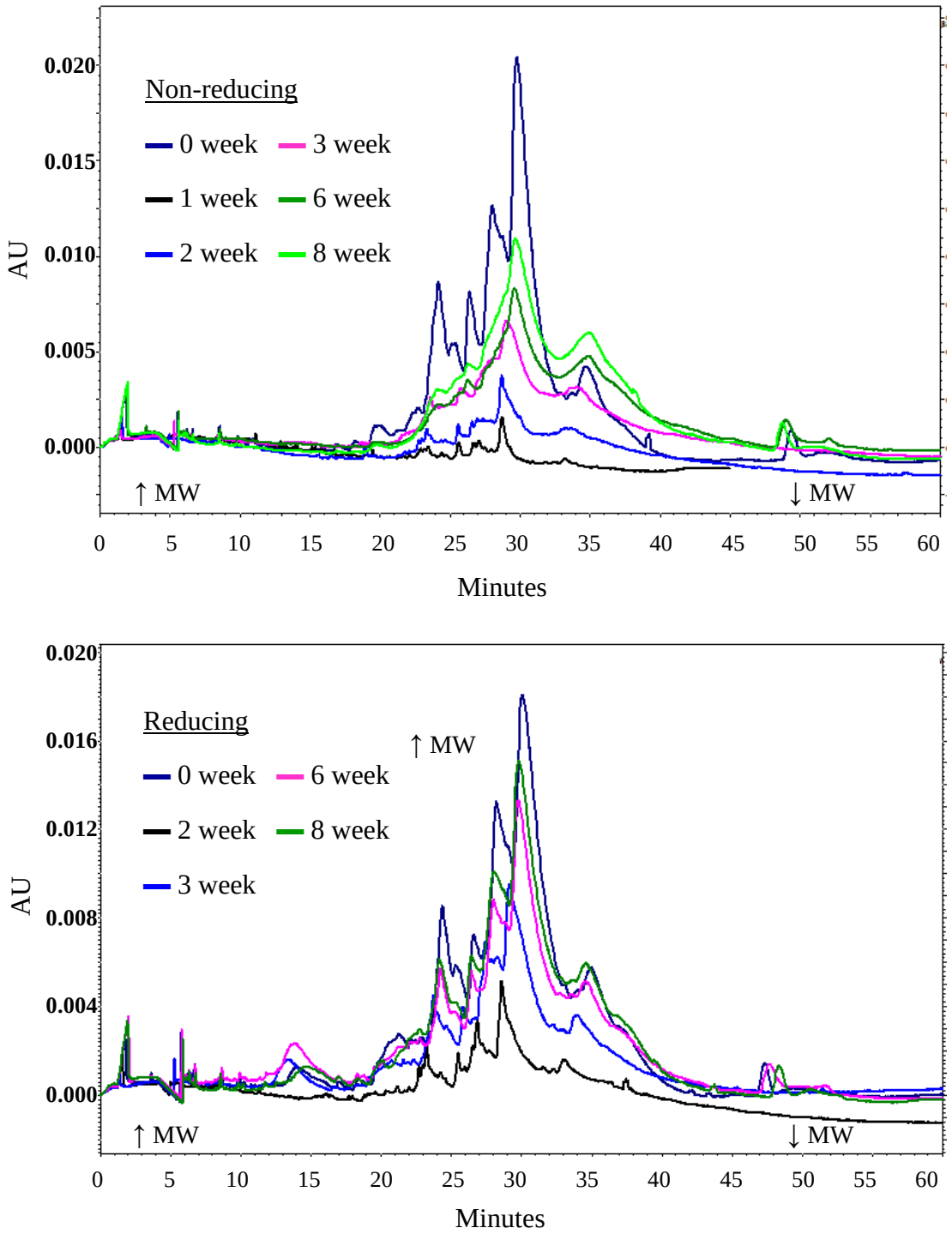

Figure 21: Protein extracts from baked tortilla chips incubated for up to 8 weeks at 40 °C (samples from Dong 2011), Acetonitrile method.

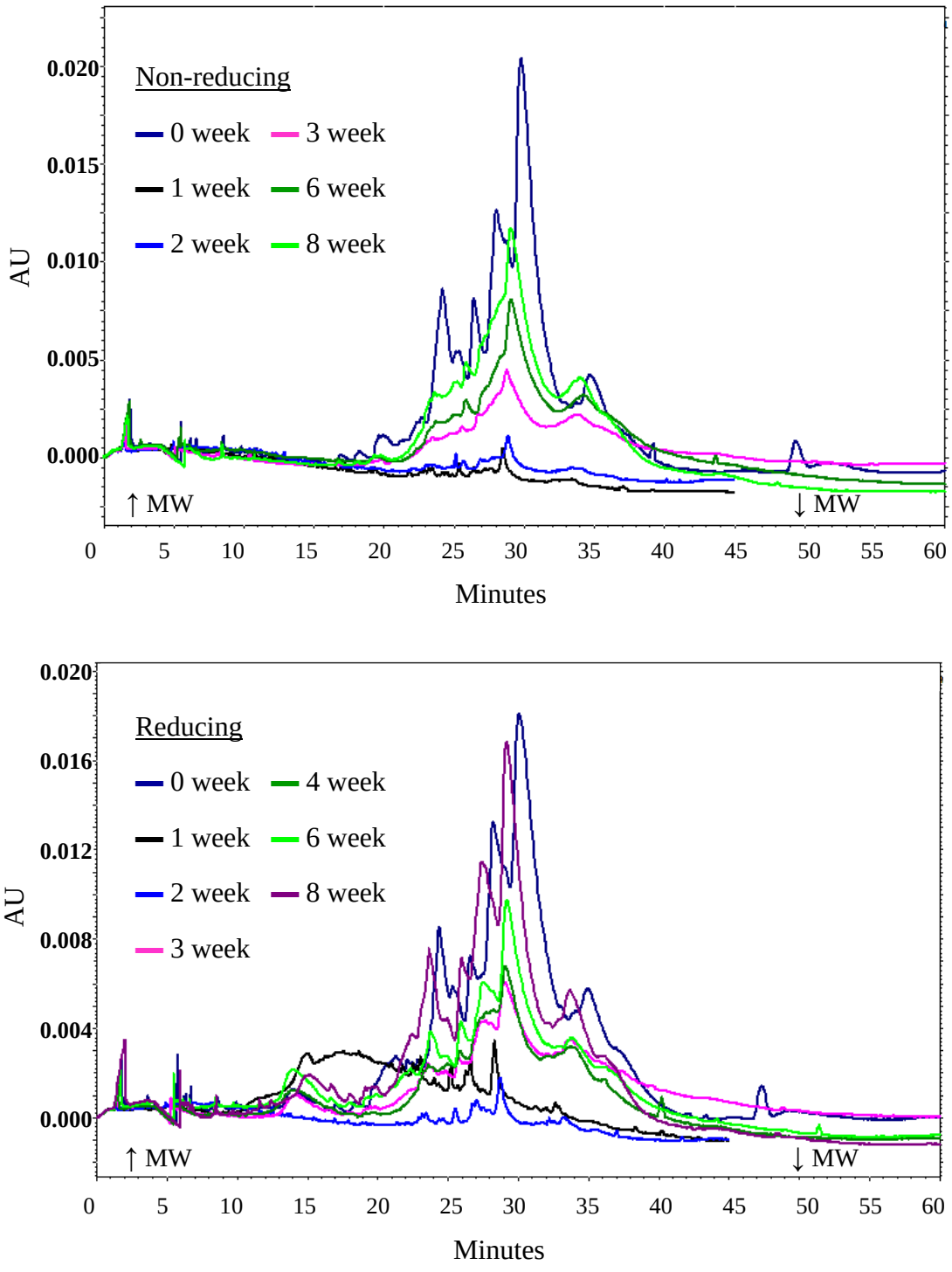

Figure 22: Protein extracts from baked tortilla chips incubated for up to 8 weeks at 60 °C (samples from Dong 2011), Acetonitrile method.

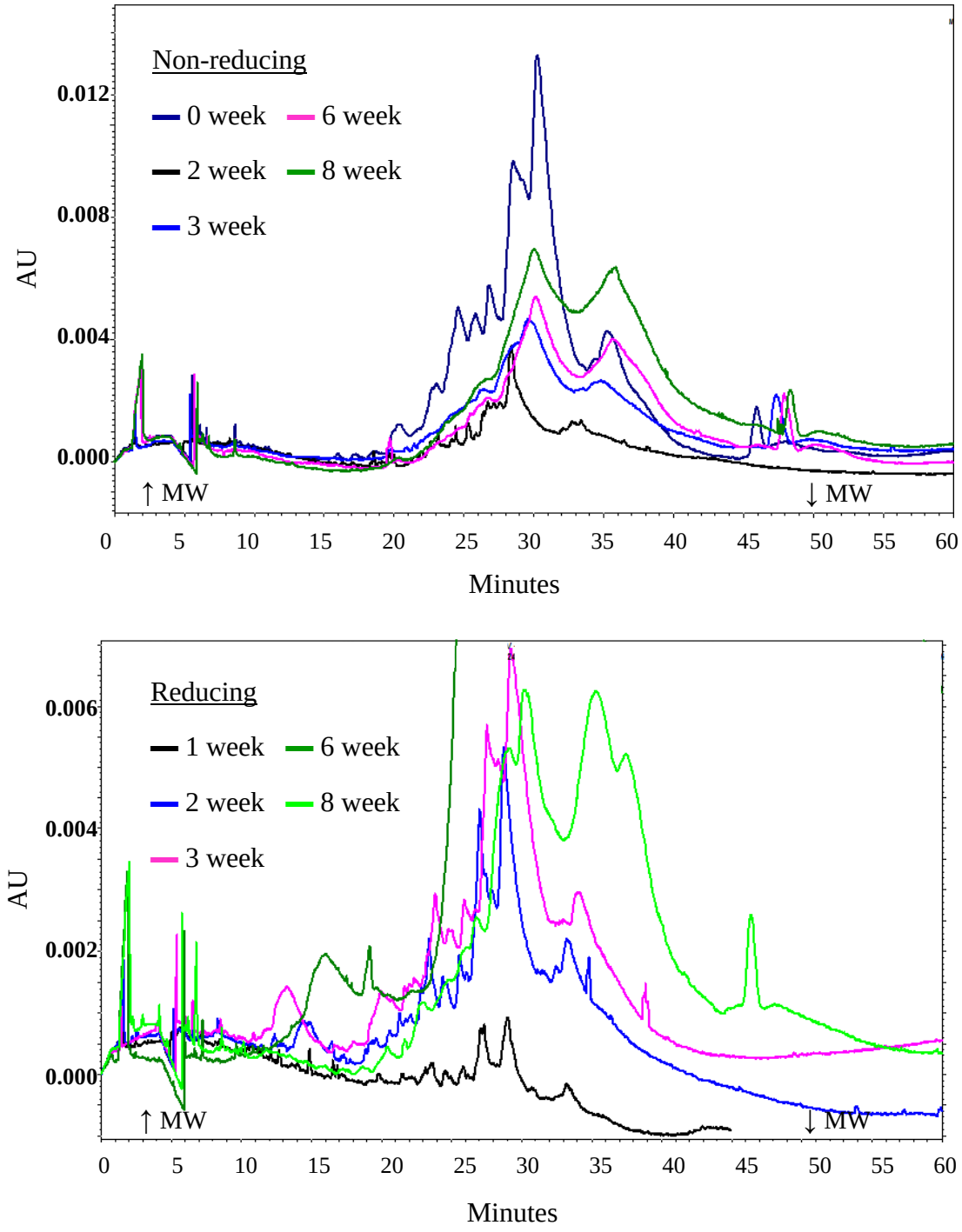

Figure 23: Protein extracts from fried tortilla chips incubated for up to 8 weeks at 40 °C (samples from Dong 2011), Acetonitrile method.

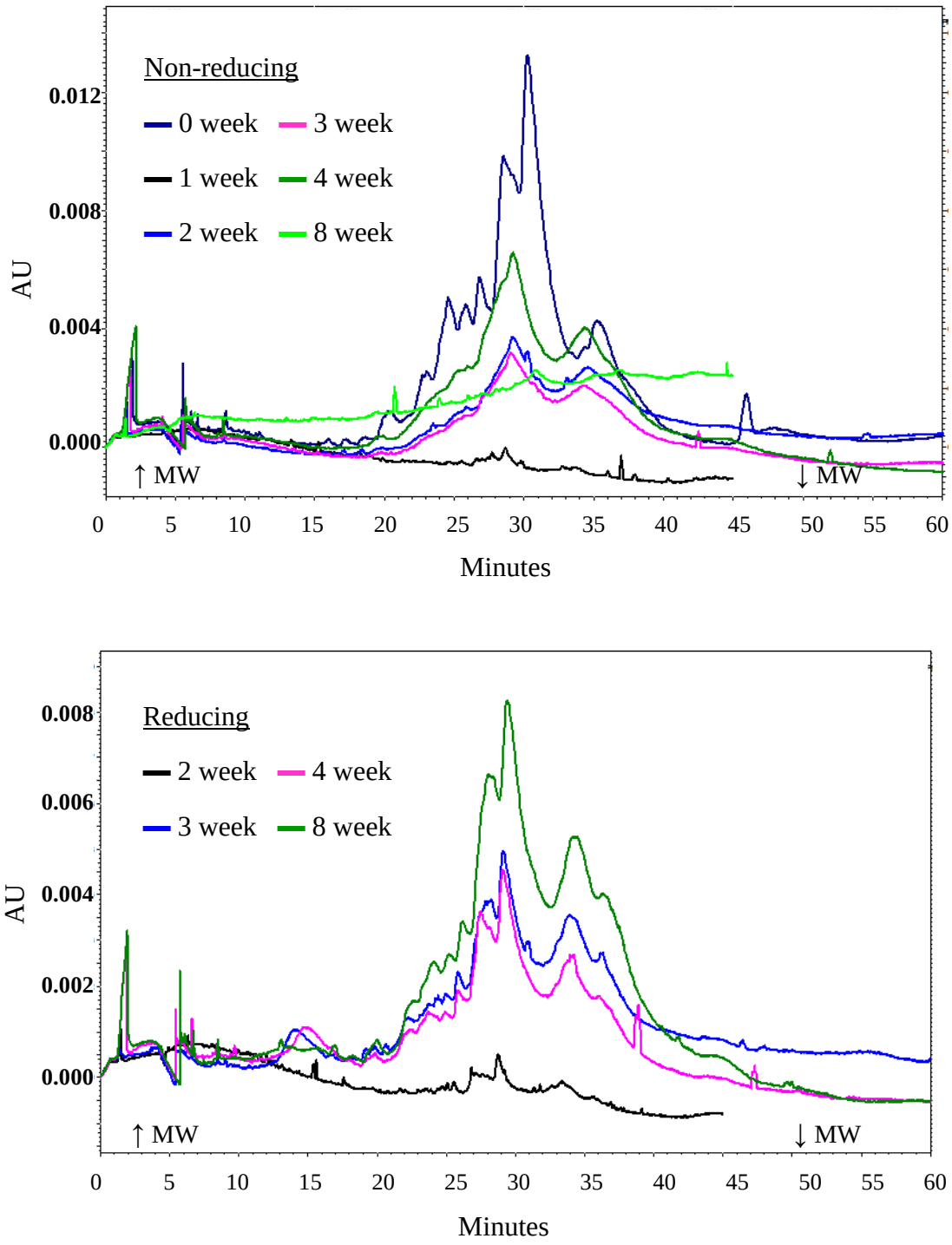

Figure 24: Protein extracts from fried tortilla chips incubated for up to 8 weeks at 60 °C (samples from Dong 2011), Acetonitrile method.

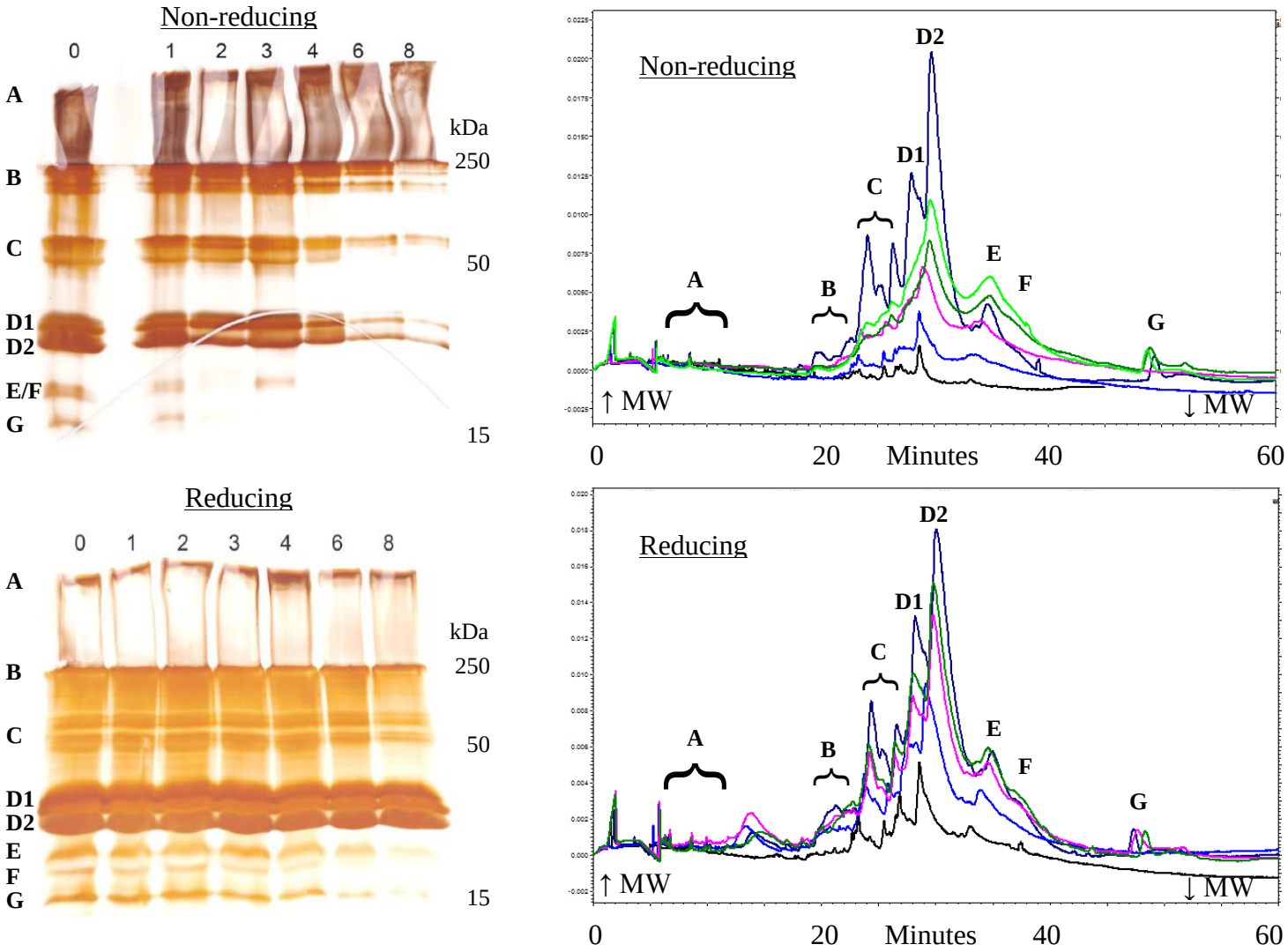

Figure 25: Comparison of SDS-PAGE (Dong 2011) and capillary electrophoresis regions for 40 °C baked tortilla chip samples.

Although it is clear that there are changes in peak heights (and thus relative concentration) of components of each of the samples over time, it is difficult to attribute differences to incubation because there does not appear to be a definite trend. A pattern can be seen between the non-reducing and reducing samples: reducing samples show more prevalent peaks at approximately 15 and 35 minutes as compared to the same region in non-reducing samples. This indicates the presence of crosslinking (based on the appearance of smaller molecular weight peaks) and also increased solubility of larger proteins (based on the appearance of larger molecular weight proteins). Given that this method has been shown by preliminary zein measurements (Figures 17 and 18) and Bean et al. (2000) as well as other research groups to be reproducible, it is not likely that out of trend results such as the very small peak heights for the 2 week fried reducing 60 °C sample (Figure 23) are the result of inadequacies of the test method. It is more likely that this should be attributed to degradation of the samples during storage in the freezer. Combined with the fact that some of the samples were unavailable, it became clear that accurate evaluation required fresh materials. To eliminate the possibility that storage degradation rather than co-oxidation was responsible for observed changes in the electropherogram, a new set of samples was incubated, extracted, and analyzed.

3. Capillary Electrophoresis Analysis of Samples from Repeat Incubation Experiment: Acetonitrile Method

Despite initial promising results, there were some concerns about protein changes during frozen storage of the extracts. Thus, to validate the initial results, the experiment

was repeated with fresh samples so that uncompromised extracts could be obtained for analysis.

Figure 26 shows peptide patterns in fresh samples (0 weeks incubation) for baked and fried tortilla chips as well as cornmeal as a control. Despite the fact that its processing steps differ from tortilla chips, cornmeal should contain similar zein patterns for comparison of potential instrument bias throughout runs.

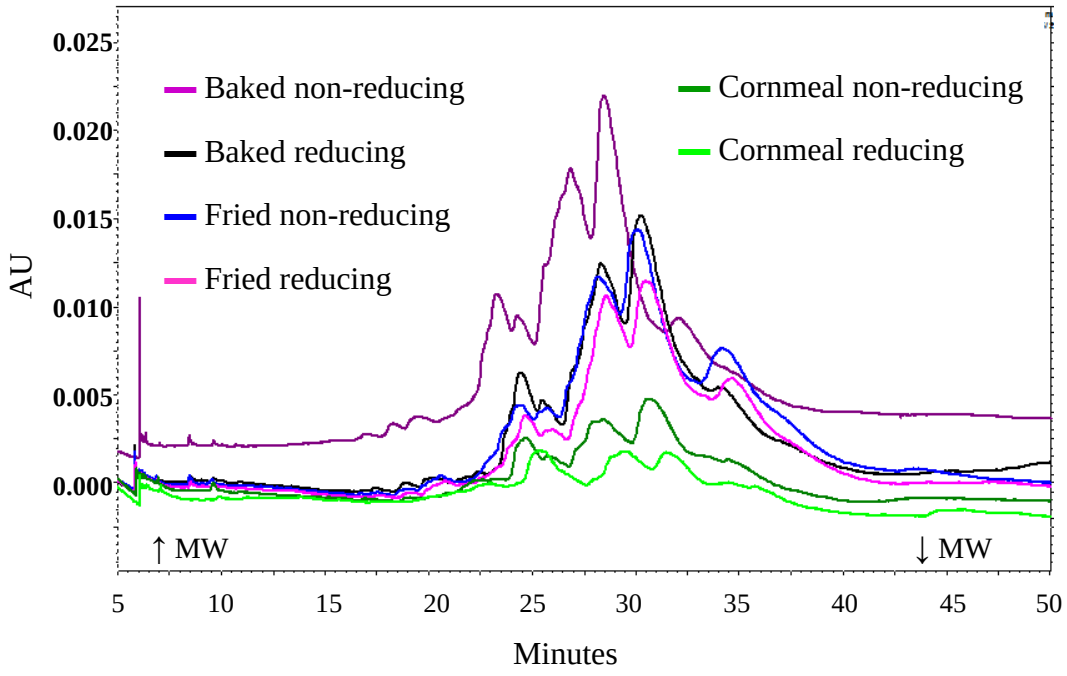

Figure 26: Protein extracts from fresh (0 week) cornmeal controls and baked and fried tortilla chips, Acetonitrile method.

Peptide patterns mapped with capillary electrophoresis are surprisingly different than expected based on SDS-PAGE gels. In SDS-PAGE, cornmeal extracts showed more detailed band patterns than tortilla chip proteins. Monomers released from disulfide crosslinked dimers and polymers increased in SDS-PAGE after reduction, being evident

in more material accumulation in lower molecular weight peptides (Figure 19). The electropherograms show patterns opposite from SDS-PAGE. Cornmeal shows the lowest response and least definition, non-reducing samples show higher absorption (indicating more material) than reducing, and shifts to lower molecular weight material are not evident after reduction. Similar to SDS-PAGE, it is interesting that protein levels detected for the various extracts by capillary electrophoresis appear to be quite different, despite identical concentrations of protein in loading the solutions. This is an important issue that will be discussed in more detail later.

One final observation must be made about peak shapes in the electropherograms. In these fresh samples, peaks were broad and diffuse rather than sharp and distinctive as is the hallmark for capillary electrophoresis. One possible explanation is that the broadening results from overloading. To test this, a zein sample with overlapping peaks (poor baseline resolution) was also analyzed at $1/10^{th}$ the concentration. As can be seen in Figure 27, dilution did not improve peak resolution with this method. Therefore, it can be said that these peaks are genuinely very broad, which most likely results from a continuum of interactions between peptides in a variety of configurations with very small molecular weight and surface differences, hence a broad distribution of migration times. This is consistent with the smearing or fuzziness of bands in SDS-PAGE, due to random protein modifications.

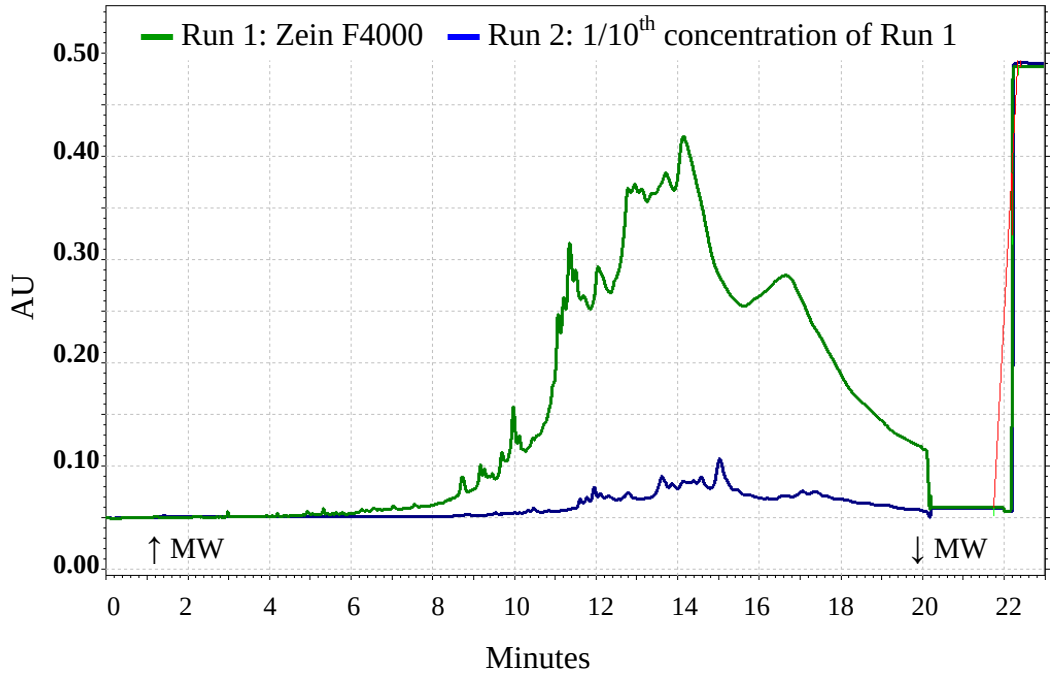

Figure 27: Zein F4000 at full concentration and 1/10th dilution, Acetonitrile method.

Electropherograms of proteins extracted from baked and fried tortilla chips incubated at 40 °C and 60 °C for up to 8 weeks are shown in Figures 28 through 30. The electropherograms show slight shifts in migration times of major peaks (in the 20 to 35 minute region) from the tortilla chip extracts over time. Without molecular weight standards to calibrate daily runs (see Discussion section), the possibility that shifts arise from instrument variation from day to day cannot be eliminated. However, the shifts indicate changes in molecular weight, which are to be expected based on SDS-PAGE results, so at the present time it can be judged that the shifts reflect actual protein modifications.

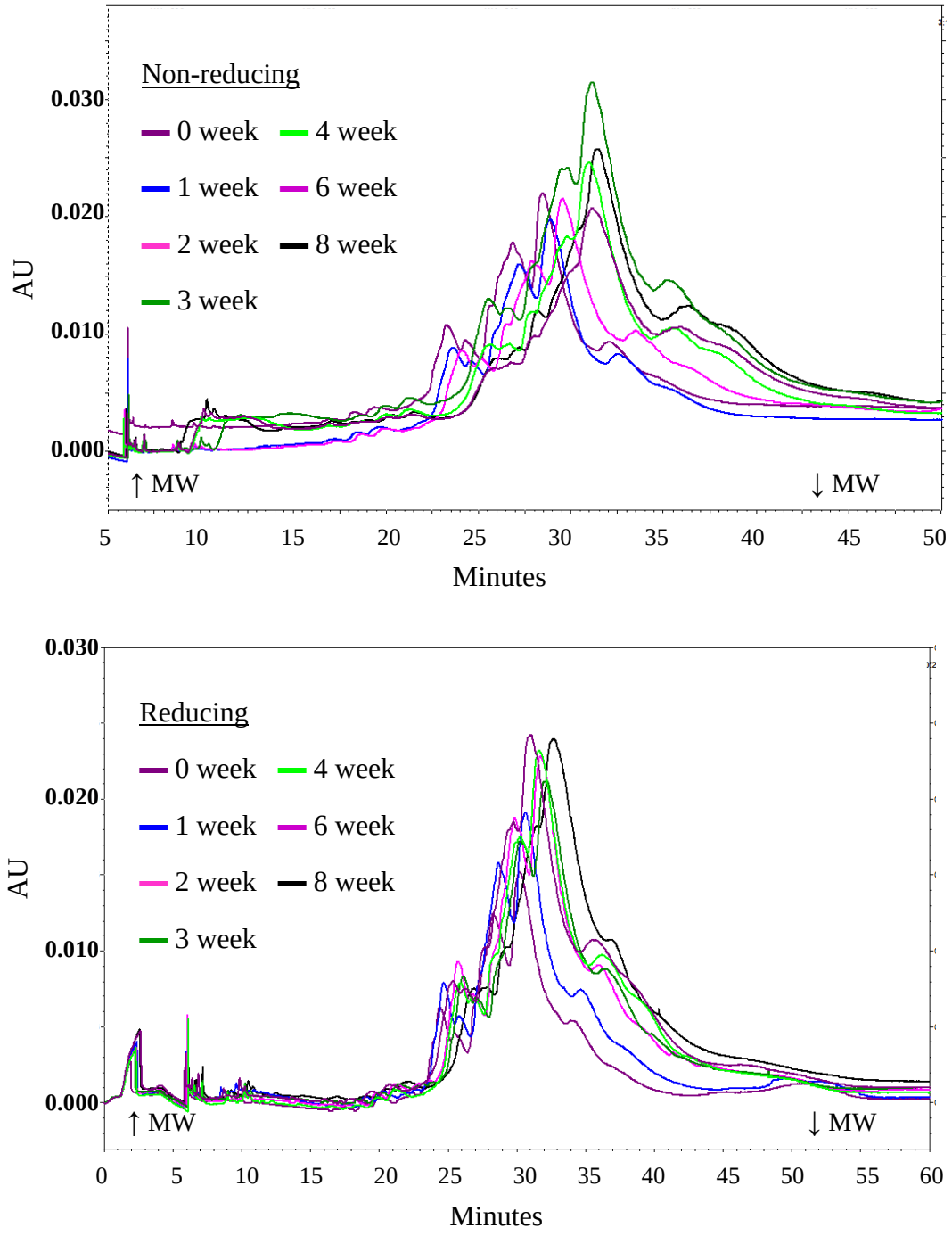

Figure 28: Protein extracts from baked tortilla chips incubated for up to 8 weeks at 40 °C, Acetonitrile method.

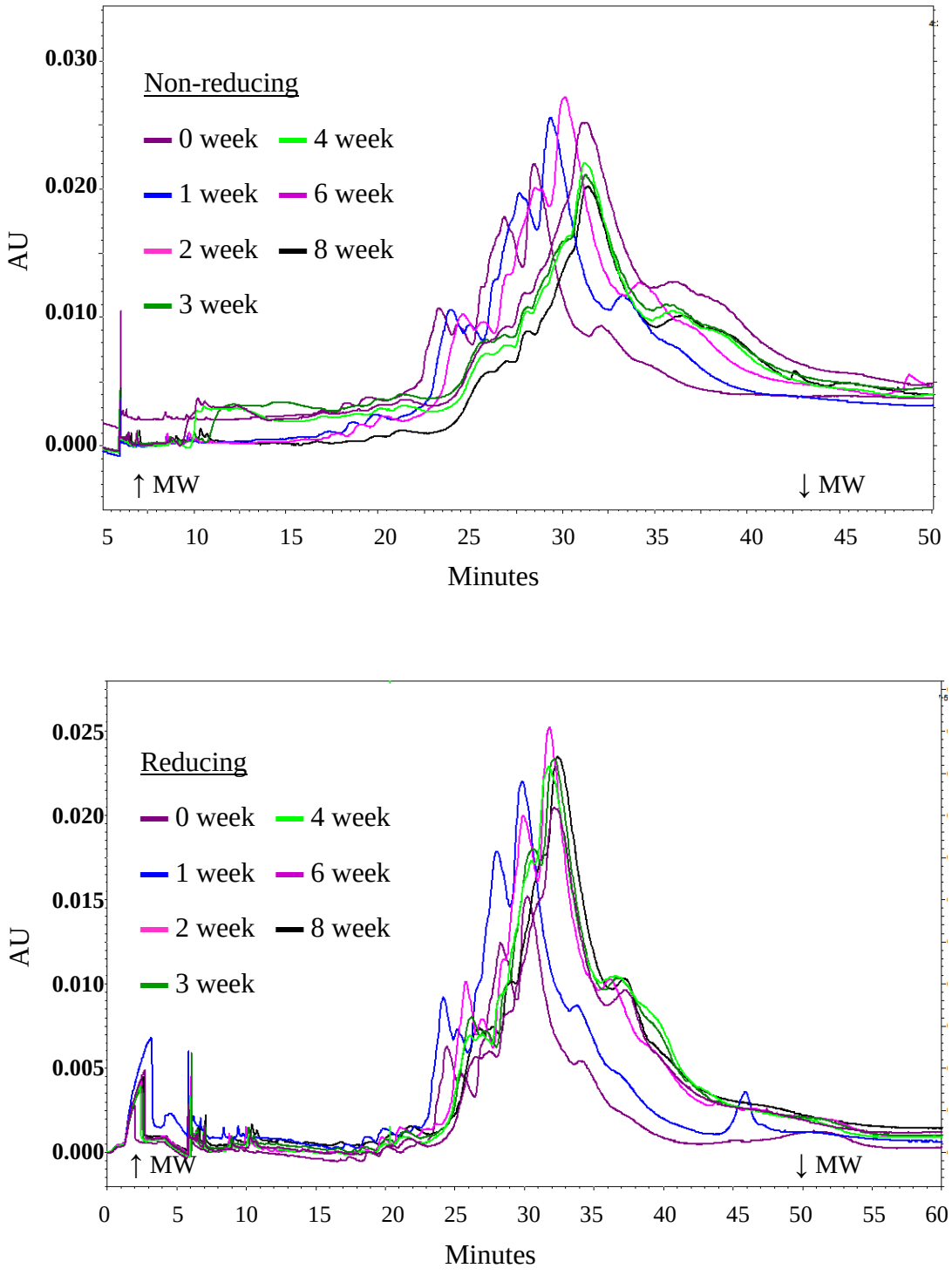

Figure 29: Protein extracts from baked tortilla chips incubated for up to 8 weeks at 60 °C, Acetonitrile method.

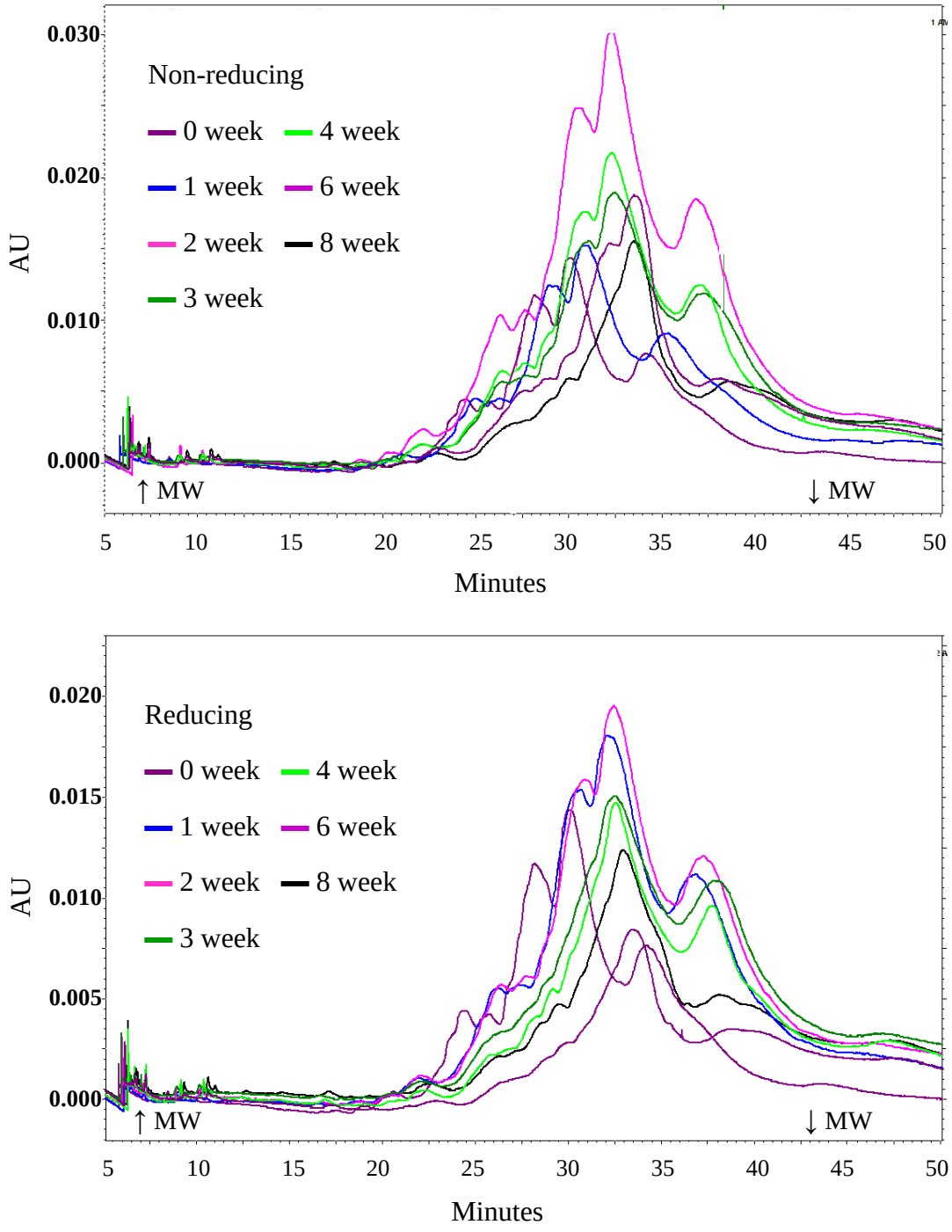

Figure 30: Protein extracts from fried tortilla chips incubated for up to 8 weeks at 40 °C, Acetonitrile method.

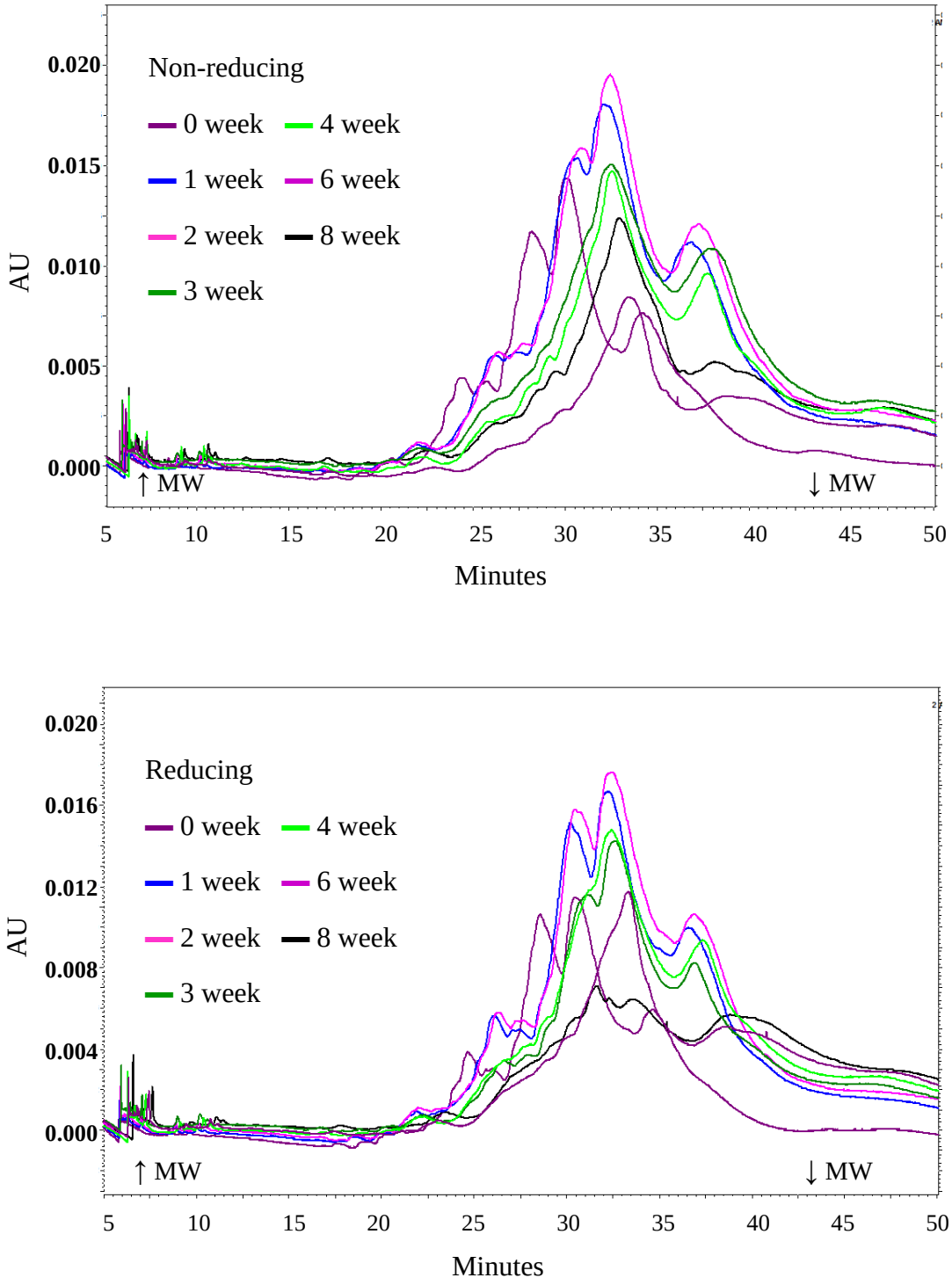

Figure 31: Protein extracts from fried tortilla chips incubated for up to 8 weeks at 60 °C, Acetonitrile method.

Peaks in the 25 to 35 minute region show a gradual decrease in molecular weight. Peptides in baked samples seem to increase over incubation time while peptides in fried samples are highest early and then decrease with incubation. Fried samples show a distinct loss of peak resolution. However, while this overlaid format is a standard way to view capillary electrophoresis data, the sheer number of traces makes it difficult to distinguish anything other than general patterns of peak shifts in this format. It is easier to look for molecular weight shifts in the stacked view of the electropherograms, shown in Figures 32 through 35.

In the stacked view of data, it is possible to see a general trend in the samples from 0 weeks to 8 weeks. As incubation duration increases, the main peaks become conflated and less defined from one another and the migration time increases slightly. Such change is consistent with increased fragmentation of proteins in the fried chips, a change that was not easily distinguishable in the SDS-PAGE gels. These low molecular weight fractions are not prevalent in the cornmeal samples (as seen in Figure 26) and are present to a lesser degree in the baked samples than in the fried samples.

Three standards that were available in the lab and previously used to calibrate SDS-PAGE gels were analyzed by the capillary electrophoresis Acetonitrile method. Only one yielded any usable information (Figure 36). Additionally, the molecular weight standard that was included in the SDS-MW kit was applied to the Acetonitrile method and resulted in no discernable peak pattern. It is not understood why this is the case since the standards were prepared in the same manner as the extracted protein samples from the tortilla chips. However, it is interesting to note that none of the applications of this method in the literature indicate the use of any molecular weight standards.

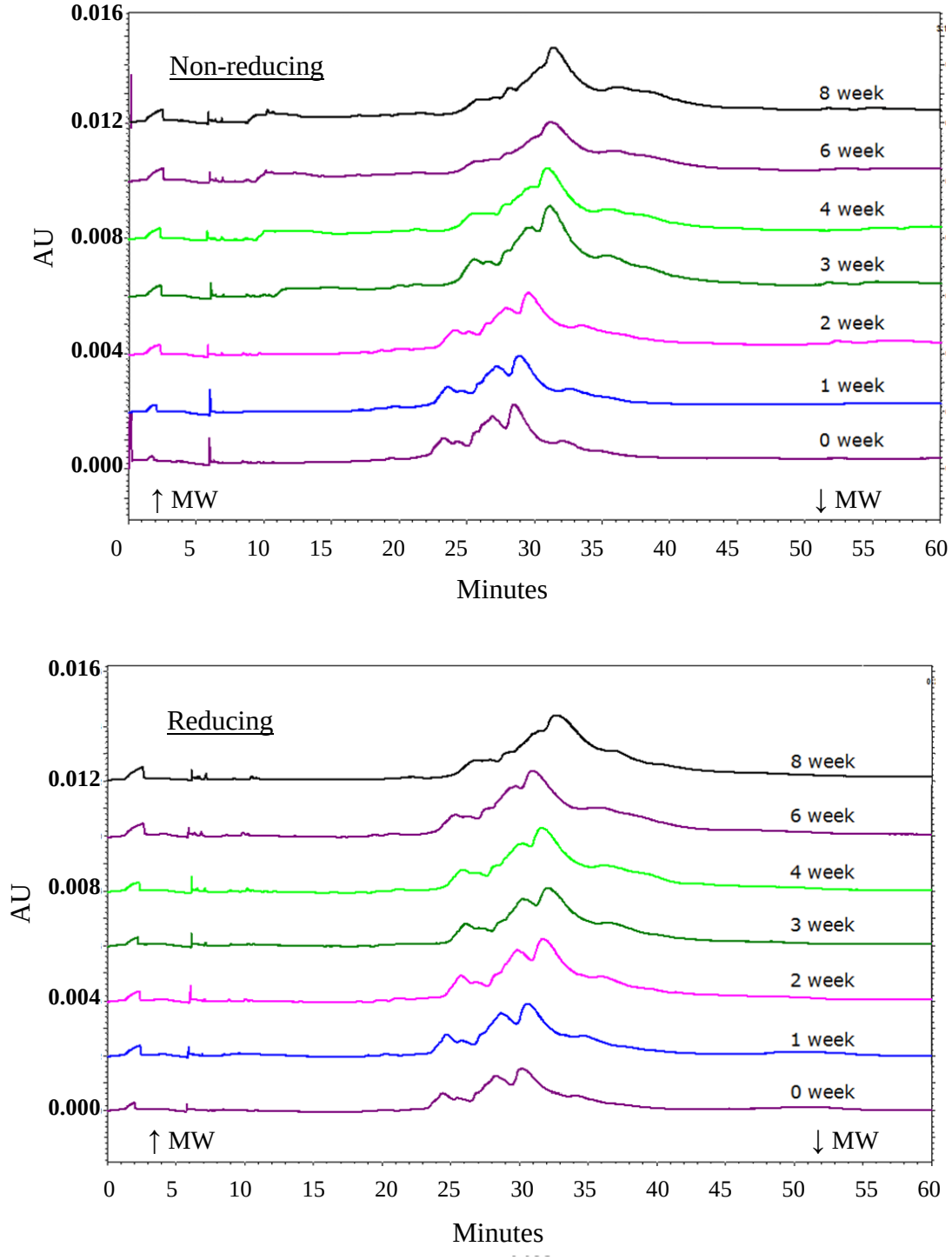

Figure 32: Protein extracts from baked tortilla chips incubated for up to 8 weeks at 40 °C, Acetonitrile method (stacked traces).

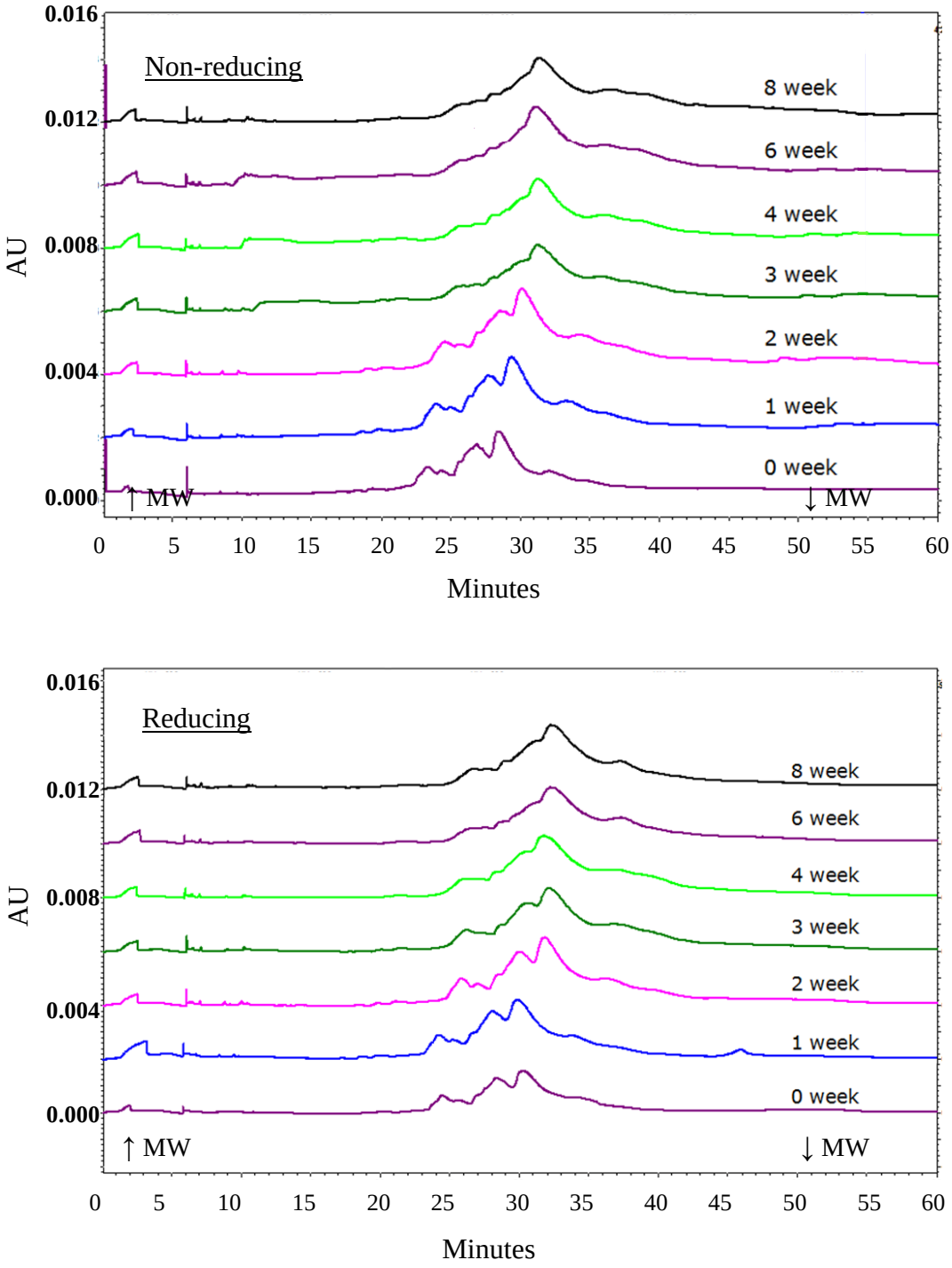

Figure 33: Protein extracts from baked tortilla chips incubated for up to 8 weeks at 60 °C, Acetonitrile method (stacked traces).

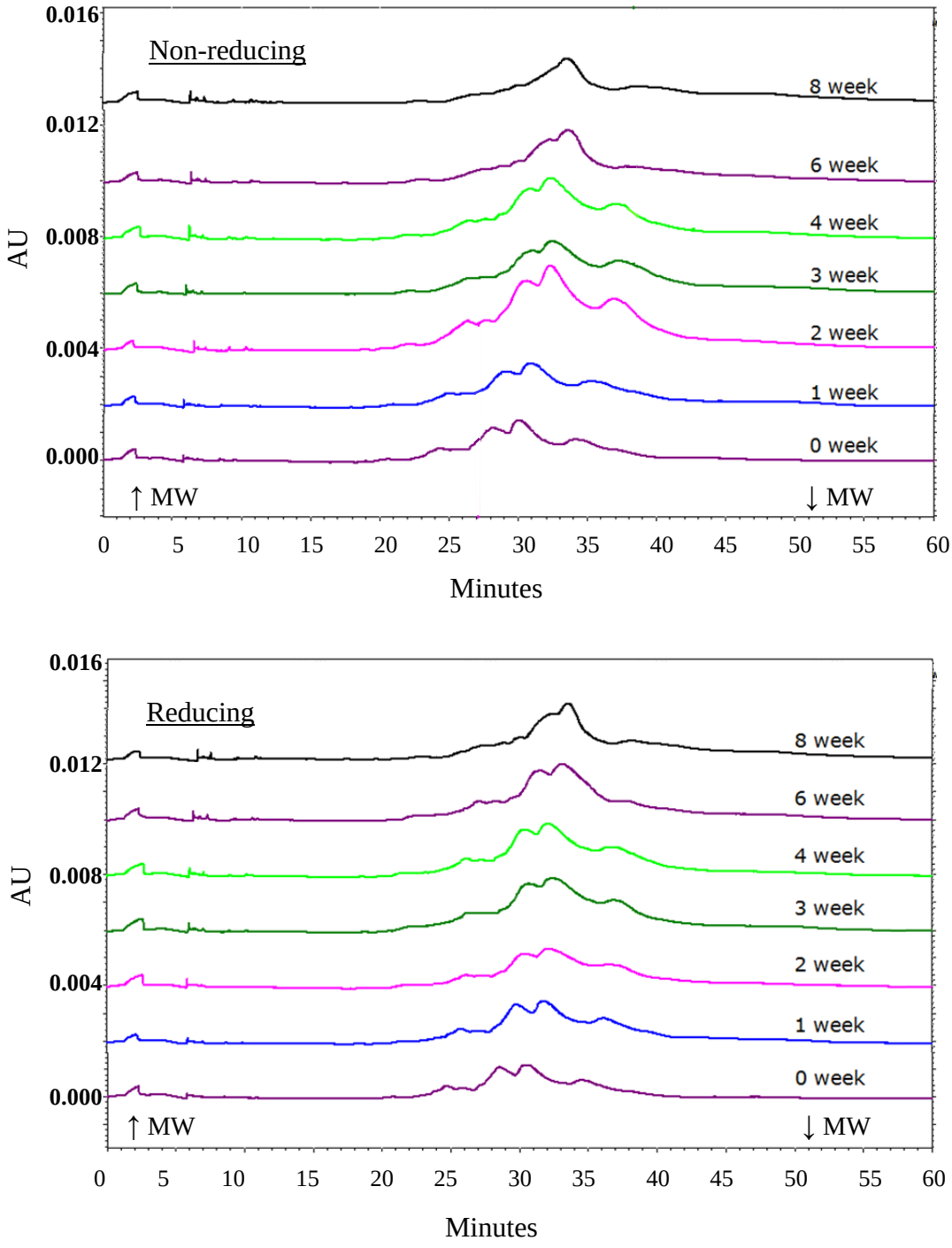

Figure 34: Protein extracts from fried tortilla chips incubated for up to 8 weeks at 40 °C, Acetonitrile method (stacked traces).

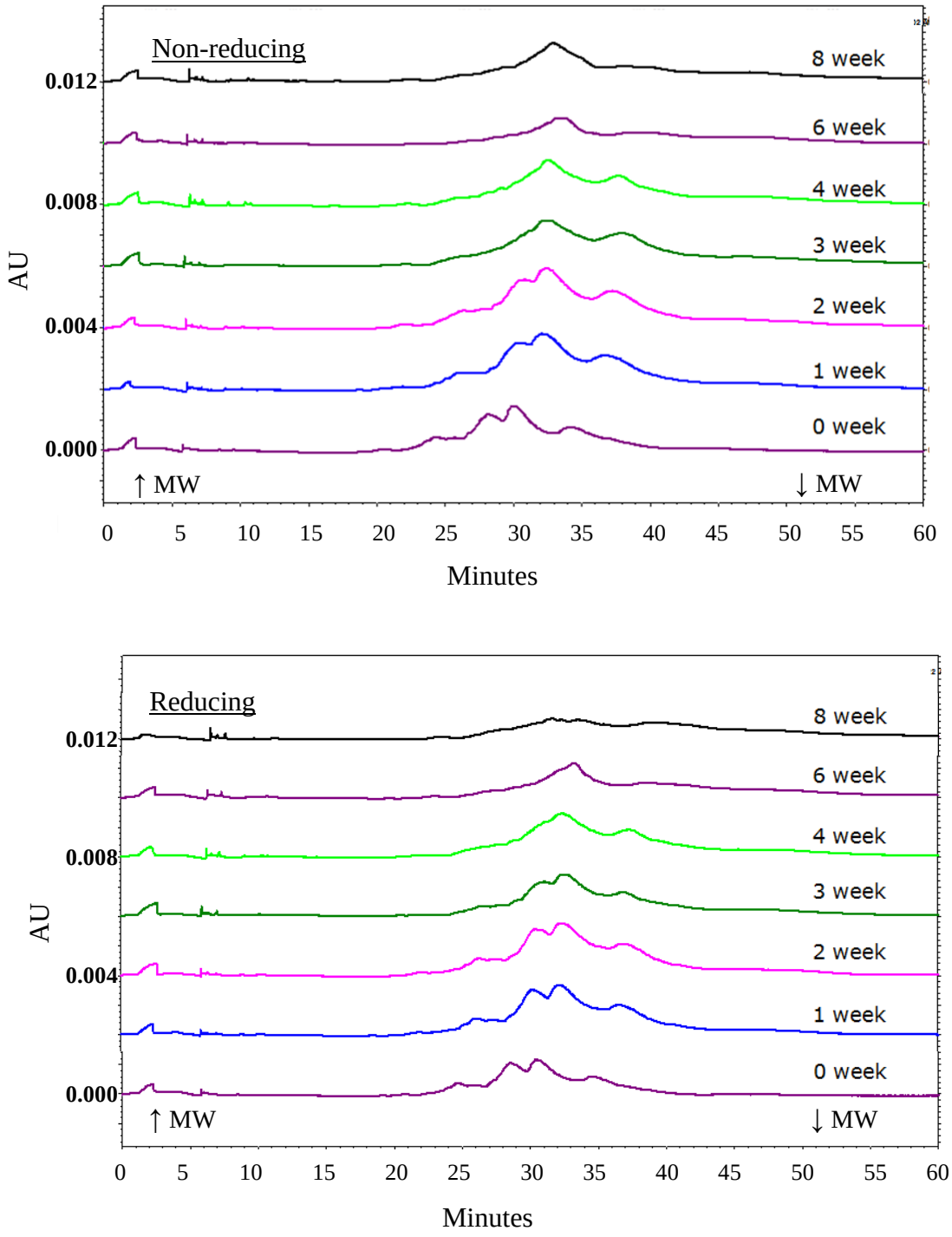

Figure 35: Protein extracts from fried tortilla chips incubated for up to 8 weeks at 60 °C, Acetonitrile method (stacked traces).

By comparing migration times of Bio-Rad 50 and 24 kDa molecular weight marker peaks (12.5 and 13.5 minutes, respectively) with those of a sample analyzed under the same conditions (such as the Zein F4000, Figure 16), it can be determined that the largest peaks in the protein extracts are in this molecular weight range and, therefore, most likely α-zeins. This also indicates that the peaks on the left of the main peak area are in the greater than 50 kDa size range and may be dimers of α-zeins, multimers of the other zein fractions, or even possible monomers of the smallest glutelins.

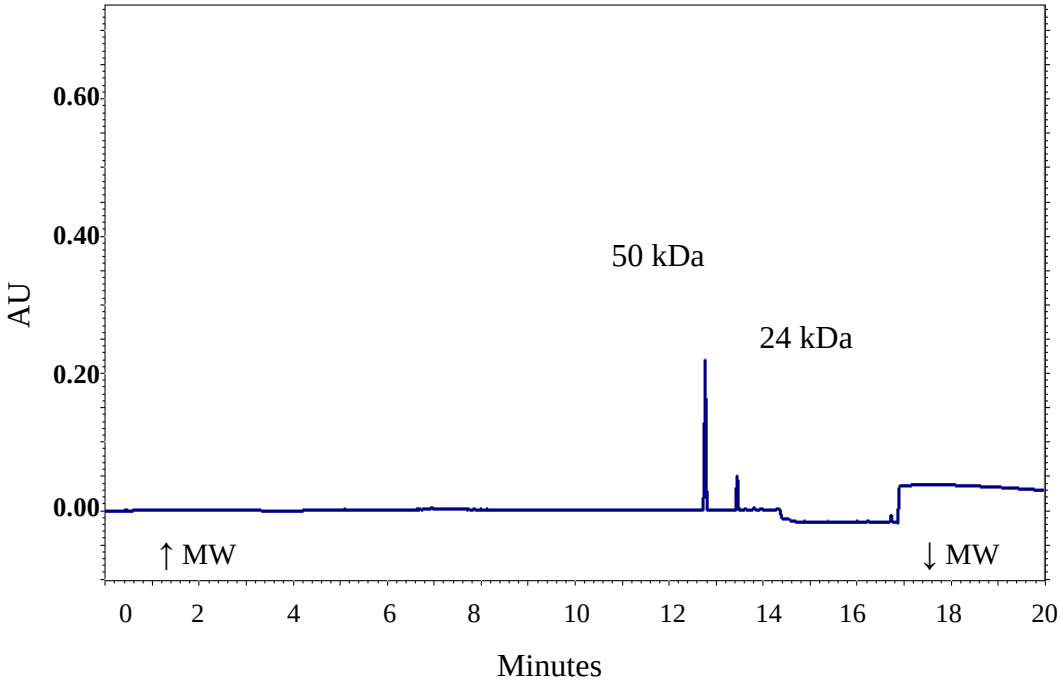

Figure 36: Bio-Rad molecular weight marker (Bovine γ-Globulin), Acetonitrile method.

4. Capillary Electrophoresis Analysis of Samples from Repeat Incubation Experiment: SDS-MW Kit Method

The second method attempted was the SDS-MW kit method, albeit without the use of a dual wavelength detector. It offers the advantage of separating peptides by molecular weight in the same manner as SDS-PAGE, so that comparable patterns should be obtained. The electropherogram of molecular weight markers supplied with the SDS-MW kit (Figure 37) shows that the full range of molecular weights expected for zeins and glutelins plus extended high molecular weights can be separated and identified using a UV detector. Peak assignments are made based on migration time and relative peak heights. The apparent peak at approximately 11 minutes is a system peak (circled in Figure 37). The peaks before and after the labeled peaks are baseline artifacts of the test system that a dual wavelength detector would normally cancelled out.

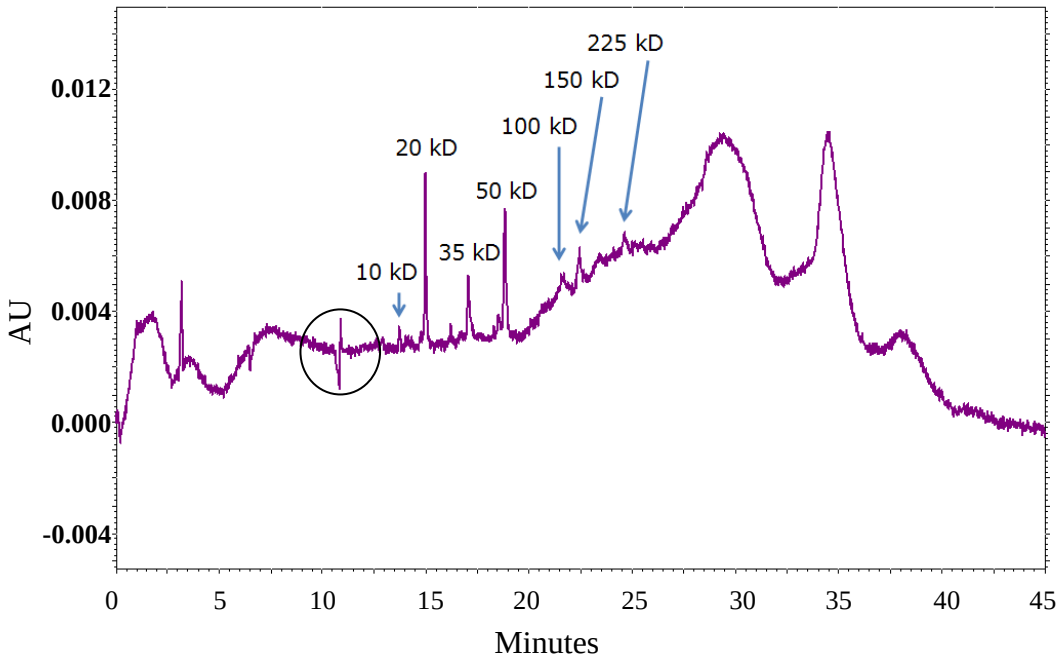

Figure 37: Molecular Weight Marker by SDS-MW kit method.

Overlaid electropherograms for proteins extracted from tortilla chips incubated at 40 °C and 60 °C for up to 8 weeks are shown in Figures 38 through Figure 42. Surprisingly, most peptides detected migrate in the 11 to 16 minute region, less than 30 kDa in size according to the standard markers. As with the Acetonitrile method samples, voltage was increased and run time was extended to several hours in order to ensure that no late-migrating peaks were missed in the samples (all peaks were captured within 40 minutes and so run time was set accordingly).

Stacked electropherograms for the same samples are presented in Figures 43 through 47.

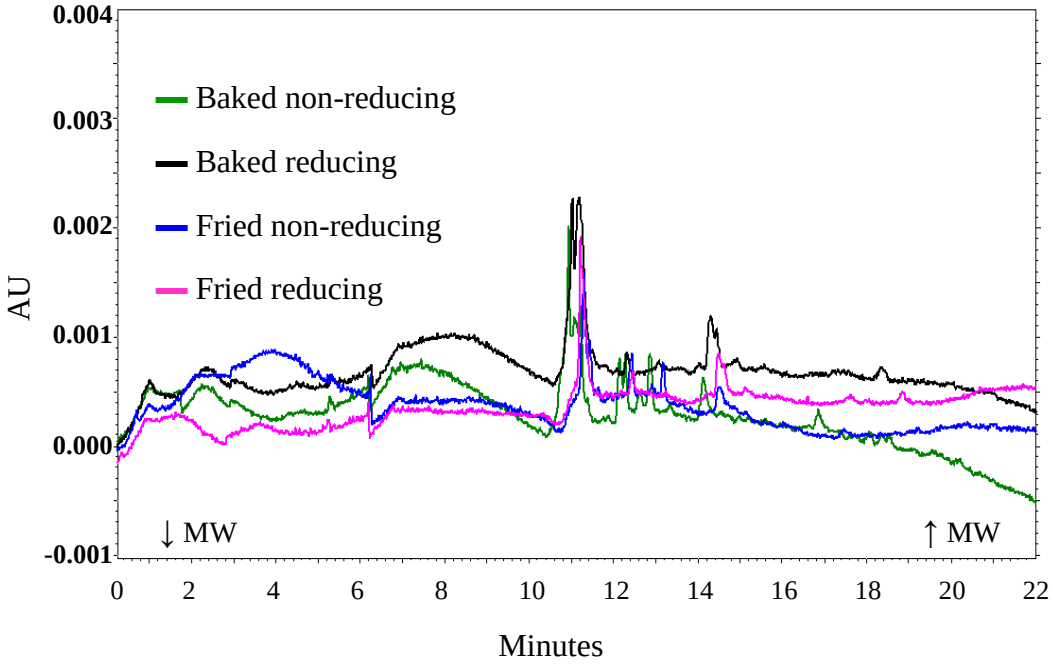

Figure 38: Protein extracts from fresh (0 week) baked and fried tortilla chips, SDS-MW kit method.

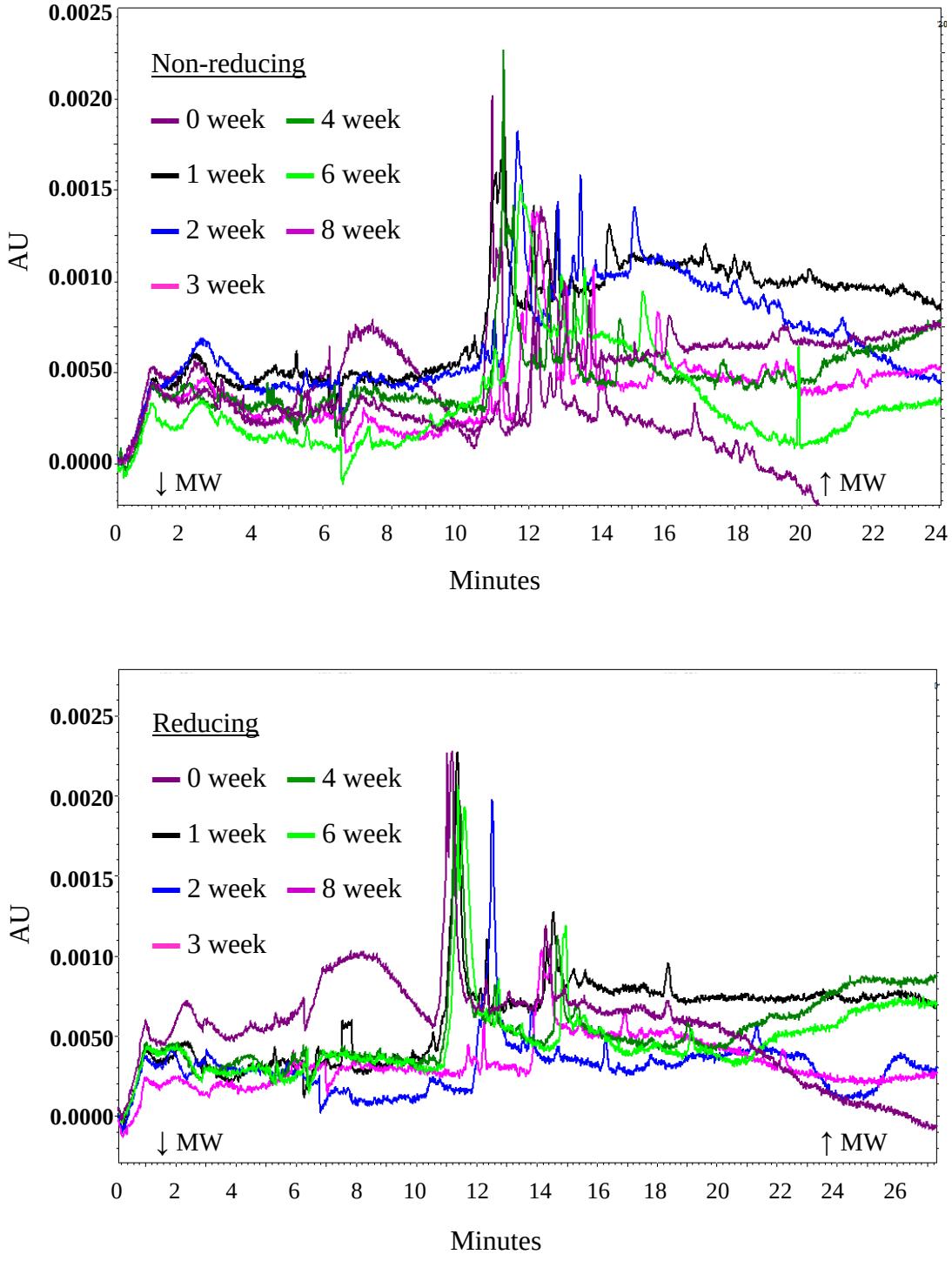

Figure 39: Protein extracts from baked tortilla chips incubated for up to 8 weeks at 40 °C, SDS-MW kit method.

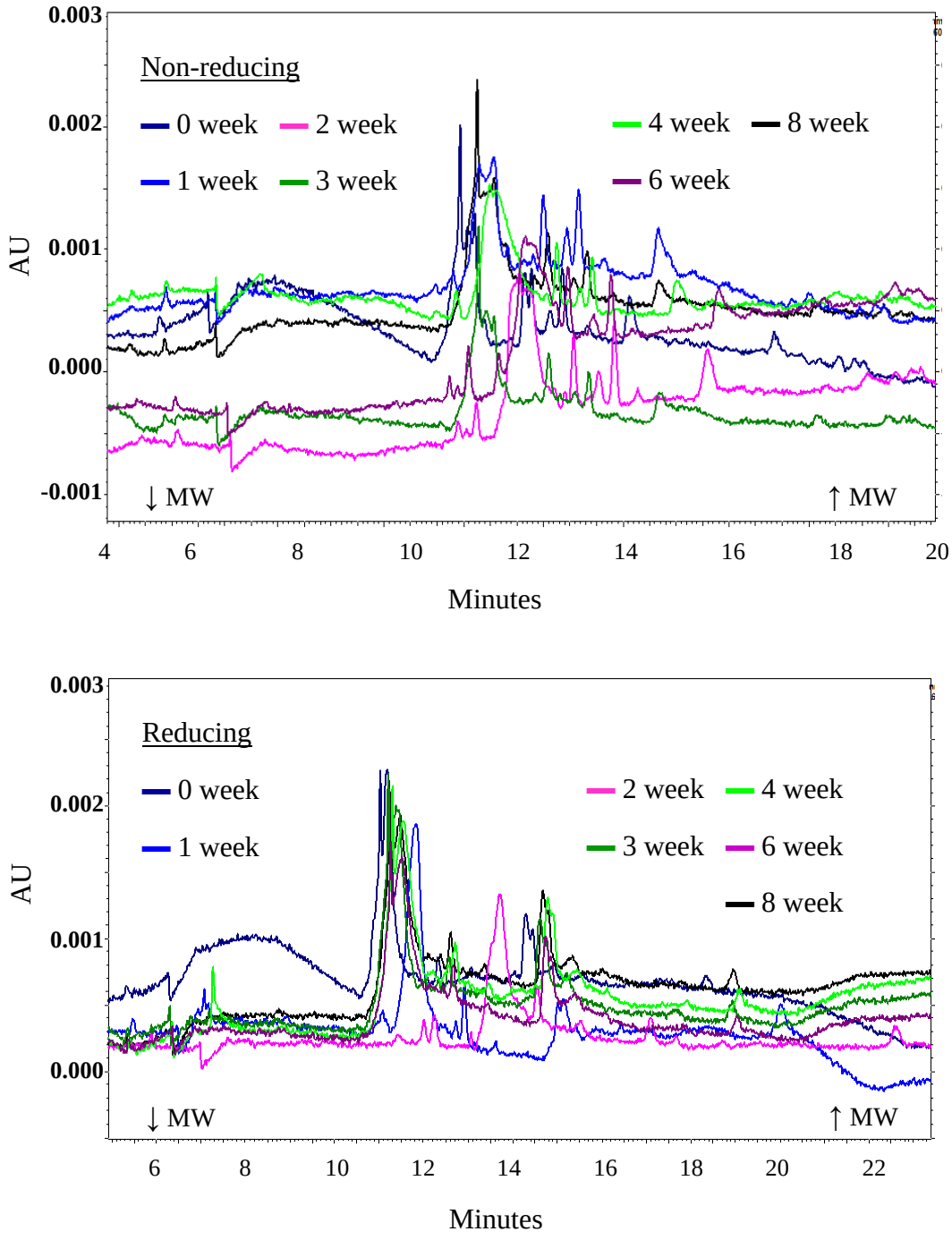

Figure 40: Protein extracts from baked tortilla chips incubated for up to 8 weeks at 60 °C, SDS-MW kit method.

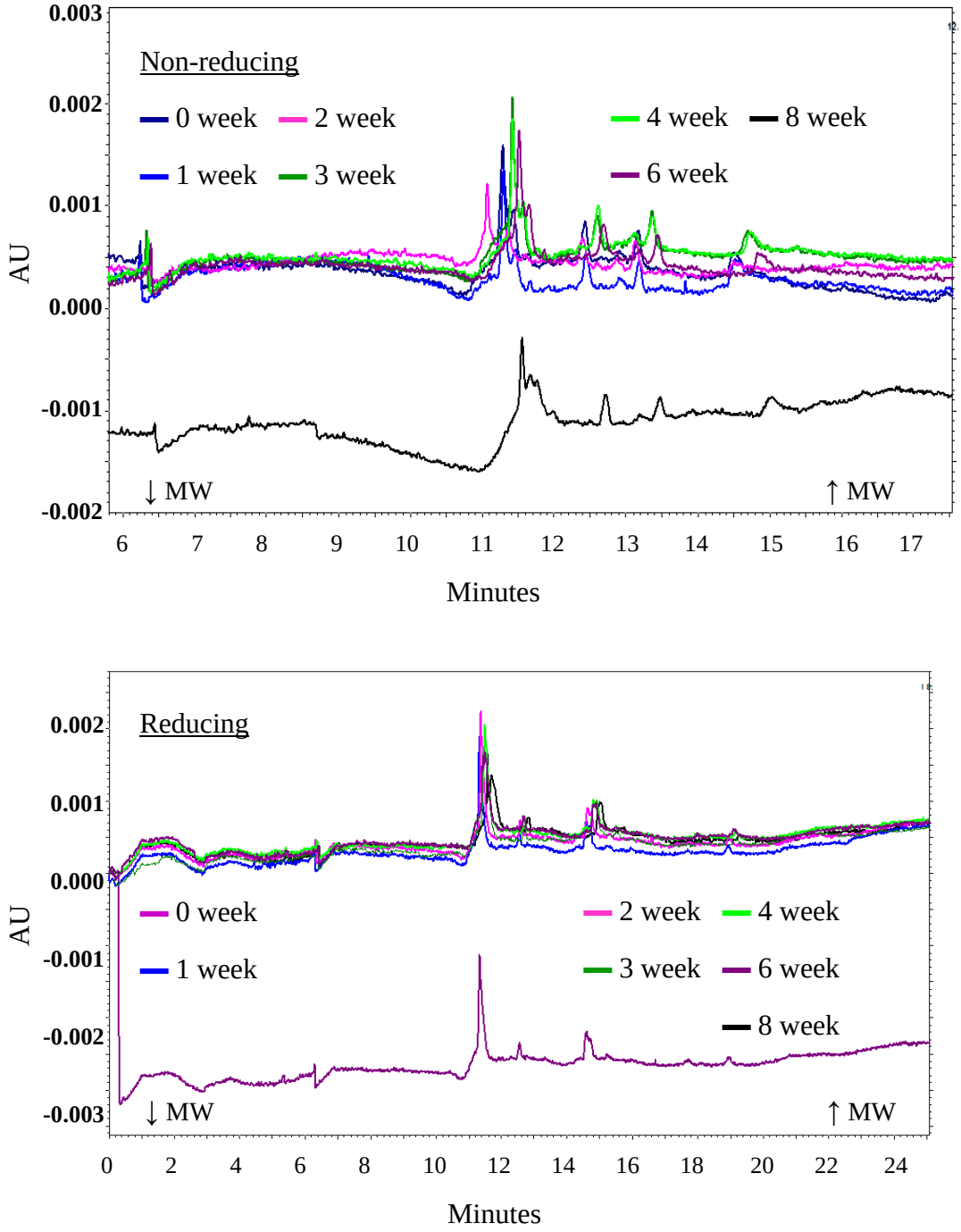

Figure 41: Protein extracts from fried tortilla chips incubated for up to 8 weeks at 40 °C, SDS-MW kit method.

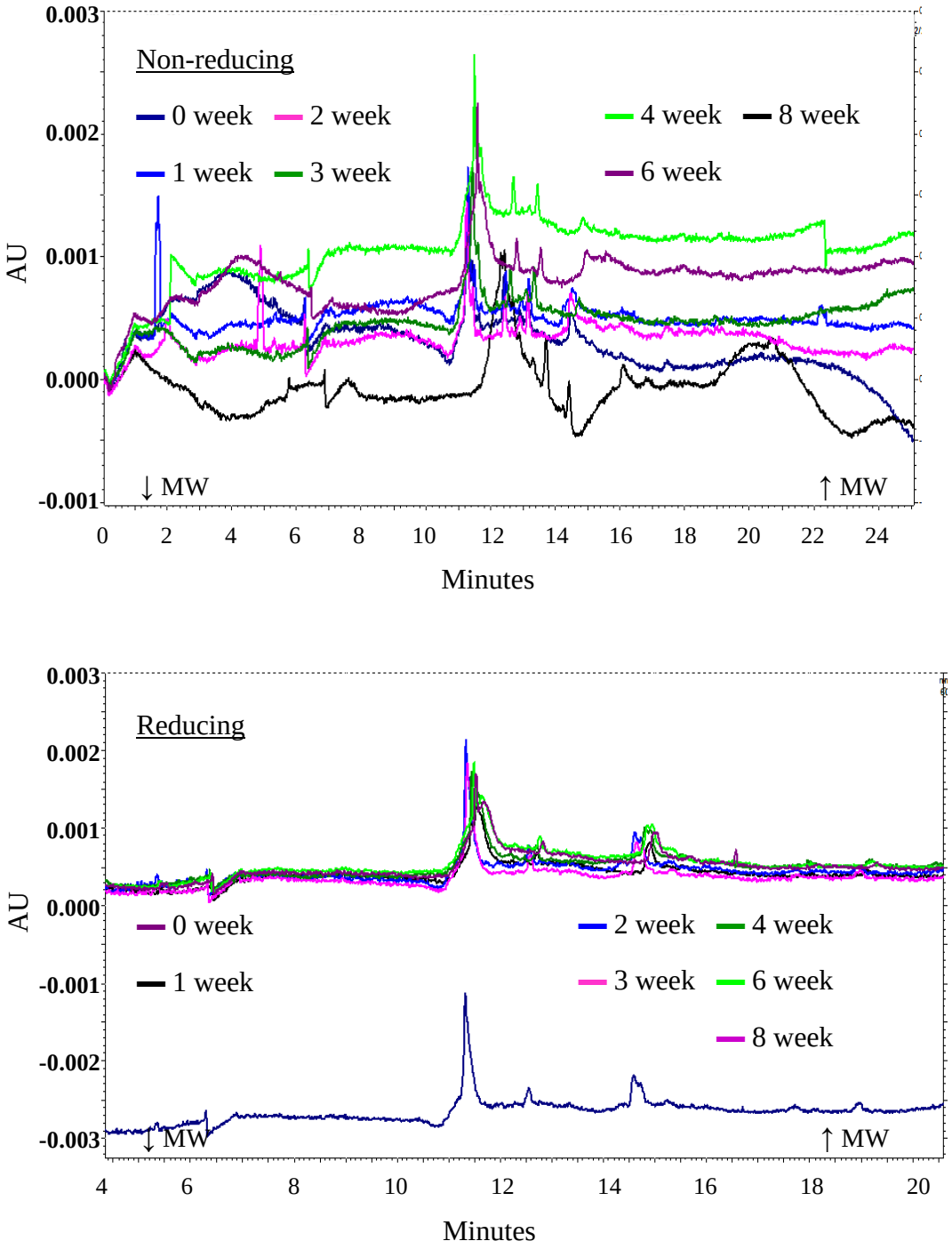

Figure 42: Protein extracts from fried tortilla chips incubated for up to 8 weeks at 60 °C, SDS-MW kit method.

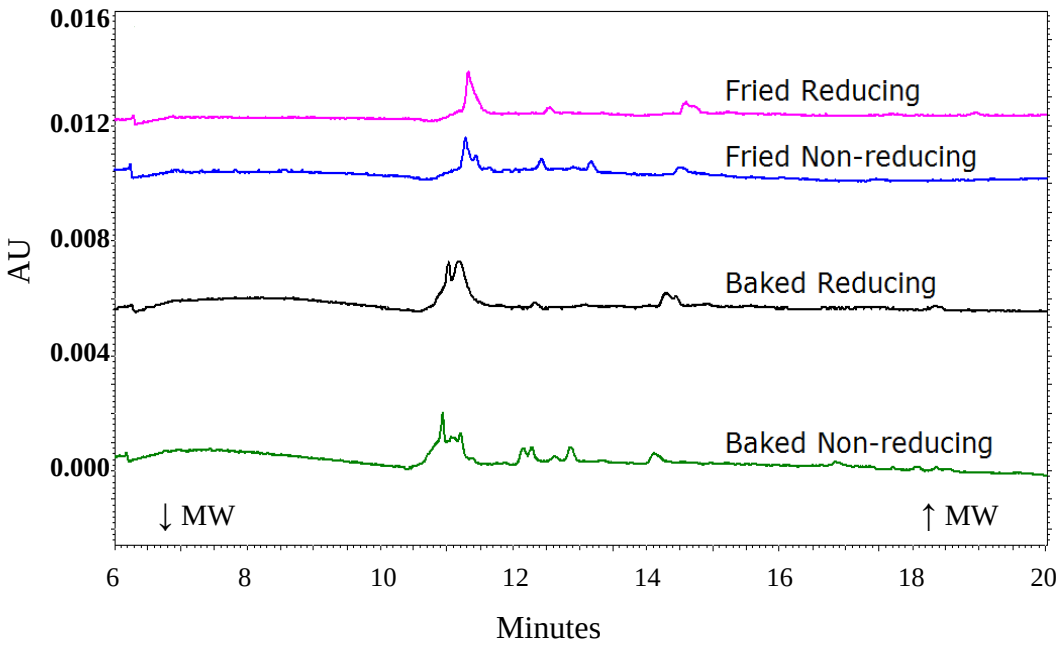

Figure 43: Protein extracts from fresh (0 week) baked and fried tortilla chips, SDS-MW kit method.

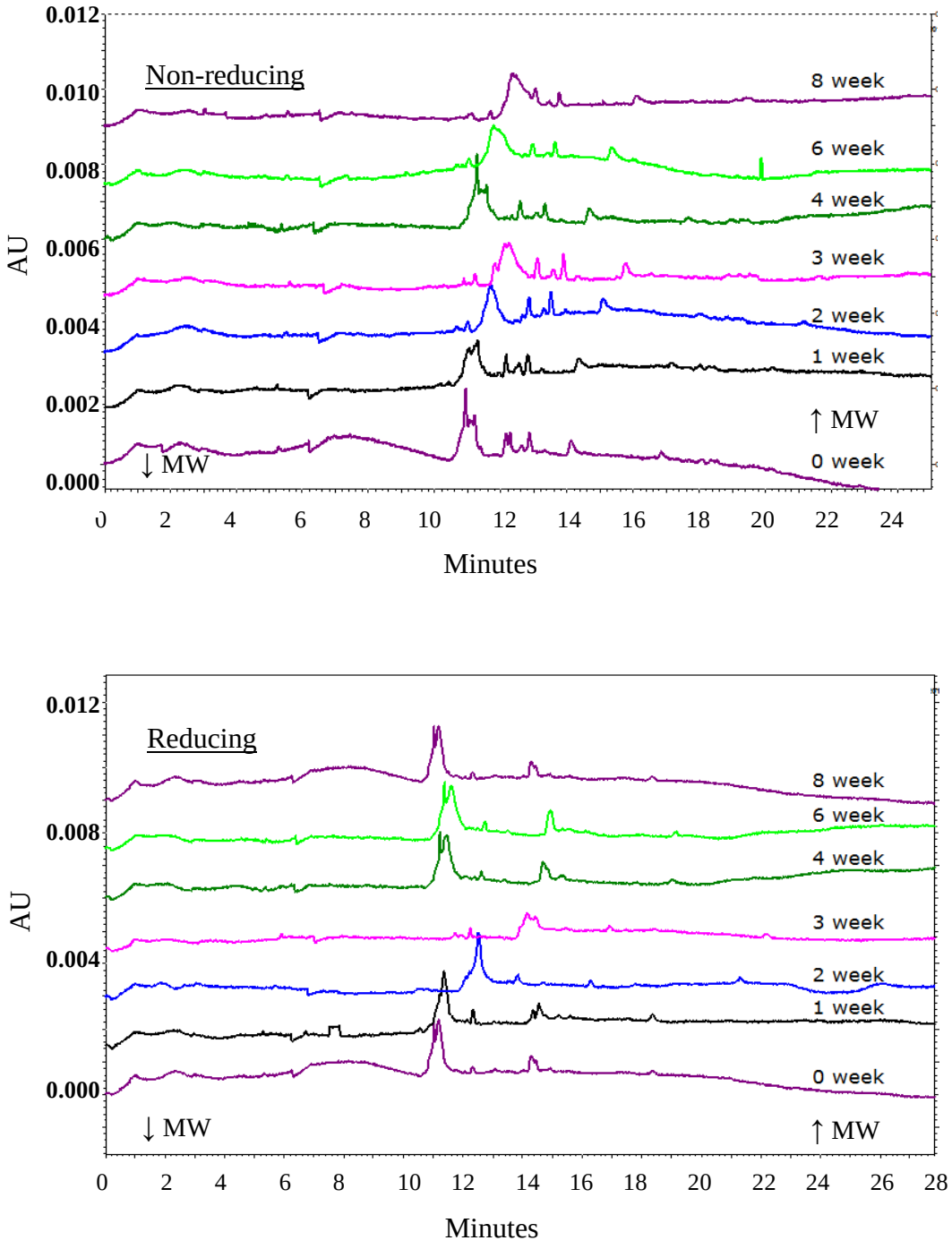

Figure 44: Protein extracts from baked tortilla chips incubated for up to 8 weeks at 40 °C, SDS-MW kit method.

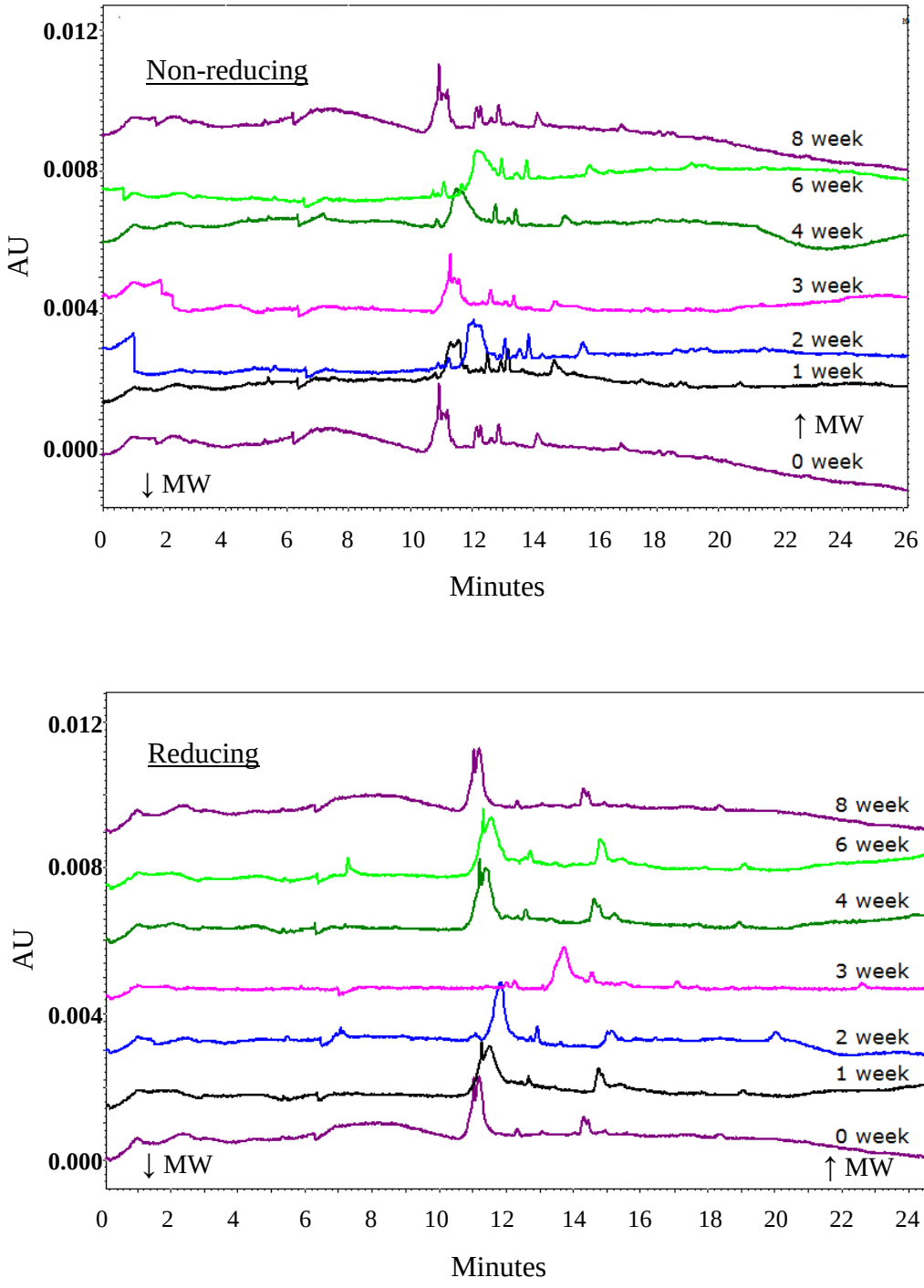

Figure 45: Protein extracts from baked tortilla chips incubated for up to 8 weeks at 60 °C, SDS-MW kit method.

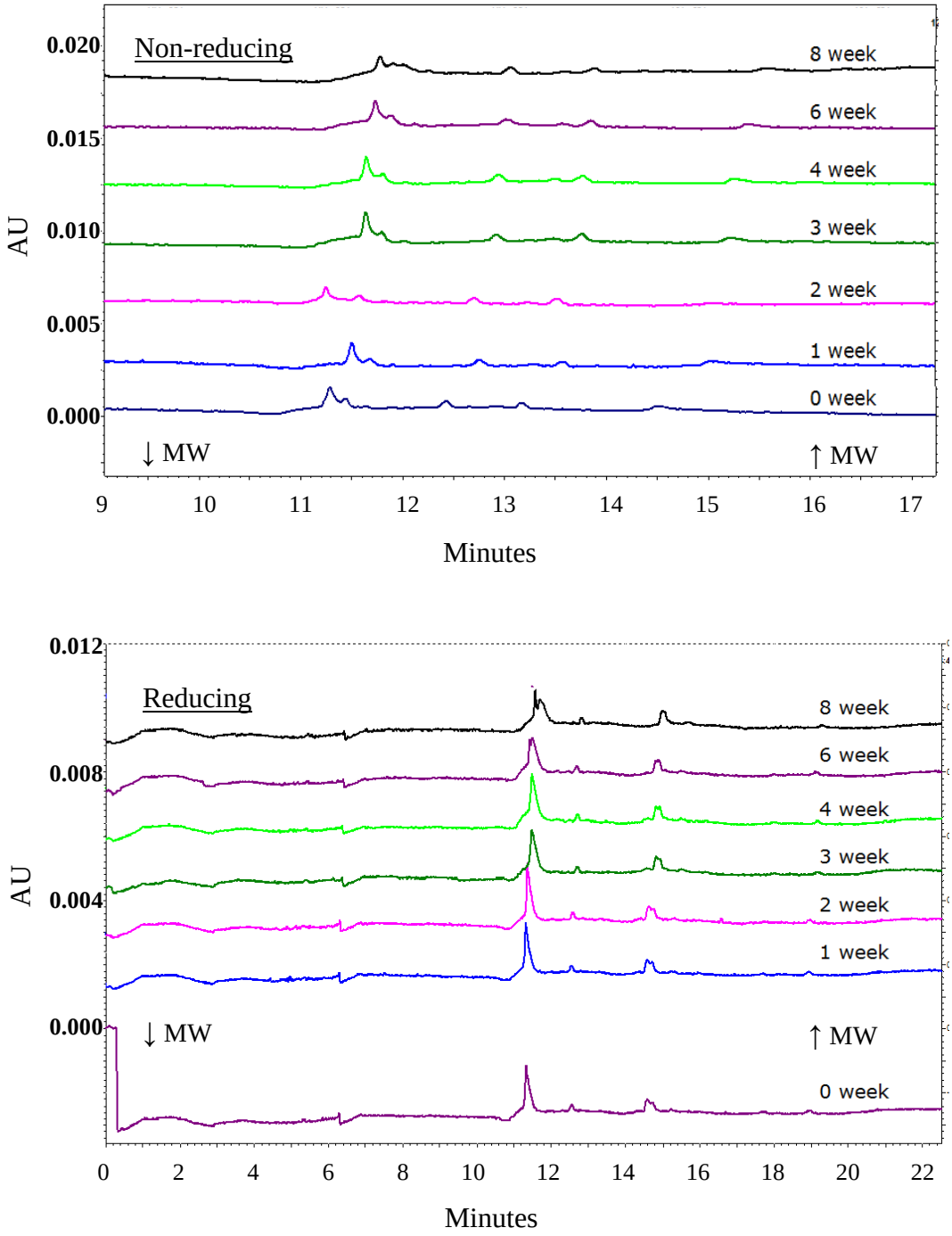

Figure 46: Protein extracts from fried tortilla chips incubated for up to 8 weeks at 40 °C, SDS-MW kit method.

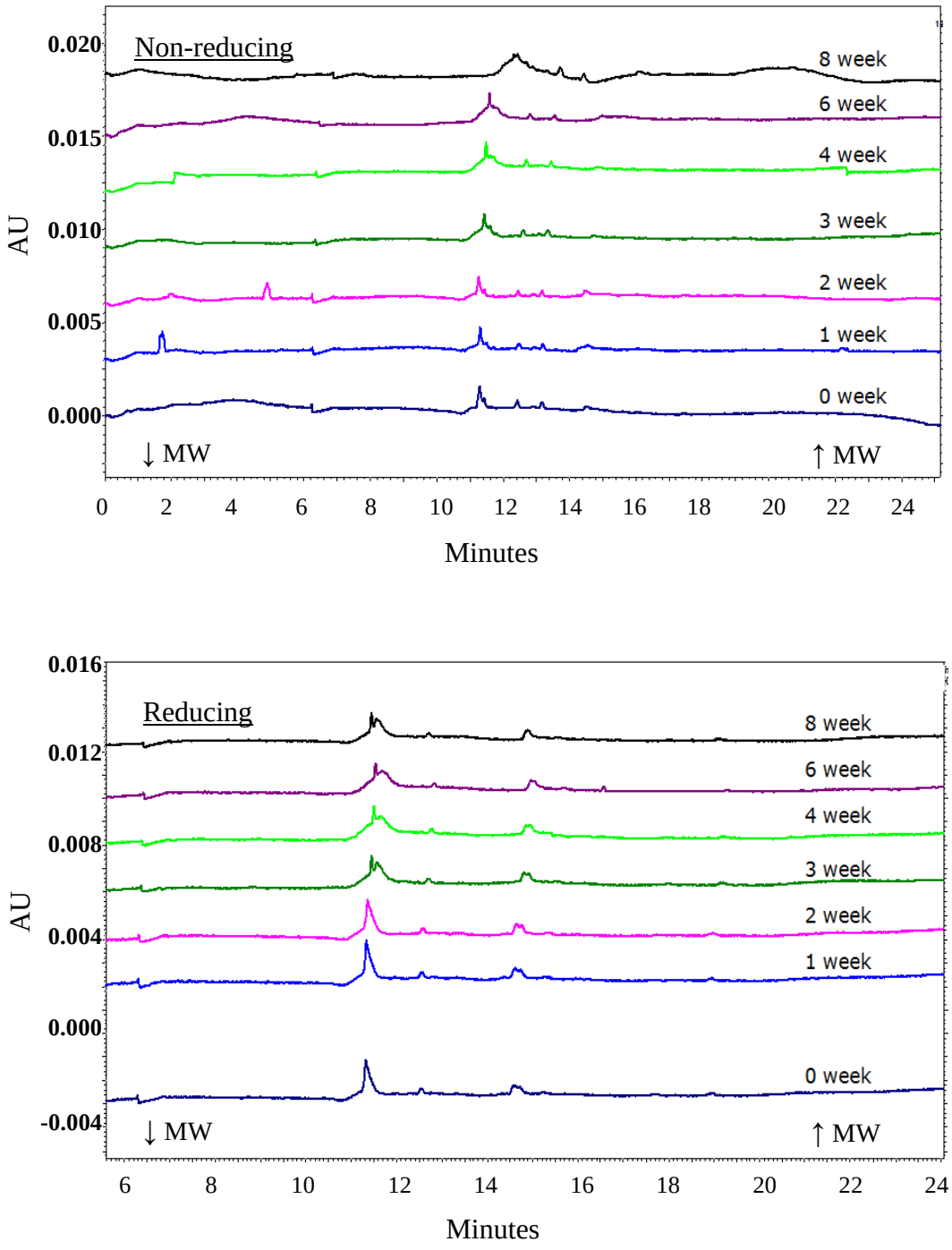

Figure 47: Protein extracts from fried tortilla chips incubated for up to 8 weeks at 60 °C, SDS-MW kit method.

VIII. DISCUSSION

A. Comparison to Literature

It can be seen that the baselines for the SDS-MW kit method meander more than those in the Acetonitrile method. This kit is designed to be a standard method that requires very little sample preparation. However, it is clear that there is a fairly high level of unsuitability with regards to these particular samples. There are two possible reasons. The first is that the kit is designed to be used with a dual wavelength detector, as mentioned above, which would use one wavelength to detect the sample and another that is in a region in which the analytes are not expected to be detected and these values are subtracted out as noise. This project did not have access to that type of detector and so a large amount of noise and drift is present. The second problem could be the sample matrix. It is possible that the residual salt on the tortilla chips post-extraction is enough to cause an increase in baseline noise and variability. Either way, it can be seen that this data is unsuitable for routine testing and only the broadest generalizations can be made from electropherograms obtained without prior dialysis. Therefore, the acetonitrile method appears to be the preferred method for analyzing zeins in a food system. This is fortunate since the cost of the acetonitrile reagents is far less than the SDS-MW kit.

One of the primary benefits of capillary electrophoresis is its quantitative capacity. Theoretically, the reproducibility between runs is sufficient for the software to be set to automatically process the data as new results are generated, searching for and enumerating peaks within pre-set windows. It is clear from the complicated peak patterns and lack of baseline resolution in many of the samples that this is not always a viable option. Attempts were made to quantify the peaks in the above samples but the software

was often confounded by the proximity of peaks to one another or very small peaks that were nearly at the threshold of being noise. Manual integration can be forced on the system but it is a tedious and time-consuming task. In sample matrices such as those in the published literature (pure zein samples), quantitation is not reported although it should be fairly straightforward due to decent baseline resolution; however, in complicated sample matrices such as those extracted from foods, the limits of the software are too great to make it practical.

Although there is a general resemblance of the Acetonitrile method results in this research to the zein patterns reported in the literature, the quality of the results for the Acetonitrile method are quite different. Literature electropherograms show very flat baselines with clustered but independent peaks references. The main difference between those studies and this one is the sample being analyzed. As mentioned previously, no studies have investigated zeins that have been extracted from food products; rather all of the zein samples come directly from corn samples which yield cleaner sample patterns. Nor have any of the published studies subjected the samples to heat stress or co-oxidation from lipids. Thus, the multi-faceted sample matrix extracted from the tortilla chips is definitely a complicating factor, but this alone cannot account for the lack of peak resolution since the reducing samples do not have significantly better resolution than the non-reducing samples. In tortilla chip proteins, the presence of extensive cross-linking and some other undetermined factors modify how peptides migrate in capillary electrophoresis. Also, as in HPLC, mixed mode separation makes it difficult to decipher causes of peak movement.

Another notable difference is in run time. Literature retention times are touted as

being in the 10 to 15 minute range. However, in order to capture all of the peaks in these samples, it was necessary to run the samples for 40 minutes (exploratory samples were analyzed for several hours to ensure that no peaks were migrating outside the analysis window). This is considerably longer but attempts to run at higher voltages resulted in excessive Joule heating and so the run time could not be shortened. The Joule heating could be because of salt in the tortilla chips samples, as mentioned previously.

Another limitation on the ability to shorten the run time was related to instrument temperature. Because resistance of the buffer decreases with an increase in temperature, attempts were made to decrease migration time by raising the temperature of the capillary. However, this resulted in unsteady current traces and, thus, unacceptably high Joule heating leading to complete obscuration of the sample peaks. Once again, it is possible that this is, in part, related to the salt concentration of the sample. It is also possible that this is an instrument-related issue. Whatever the reason, the inability to increase temperature was a major obstacle to decreasing migration time, but even with separation times over 30 minutes, capillary electrophoresis still provided a time savings over analysis by SDS-PAGE.

B. Fresh (0 week) Samples

By comparing the 0 week samples (Figure 26), it is possible to note differences in crosslinking and other features. There are few differences between non-reducing and reducing for either baked or fried tortilla chip extracts. At 0 week time point for baked samples, fragmentation has already occurred, or at least there are fewer multimers of non α-zeins. In general, the fried samples have less resolution. They also have a more

pronounced shoulder at around 35 minutes and a less pronounced peak at 25 minutes than the baked samples. It can also be seen that, while they all have similar migration times the fried reducing sample appears to have higher signal intensity (despite the fact that all samples were measured for protein concentration and adjusted accordingly). The abundance of protein material is in the 24 kDa range (approximately where zeins would be expected to resolve) but the region where Dong's research was showing notable banding, the glutelin region, is populated by defined but very small peaks that do not have nearly the same intensity shown by Dong's gels.

Figure 43 shows the 0 week samples tested by the SDS-MW kit method for all four tortilla chip sample configurations: fried reducing, fried non-reducing, baked reducing, and baked non-reducing. The SDS-MW kit method shows difference more readily in the 0 week samples. When comparing the reducing to the non-reducing samples, the peaks in the 12 to 13 minute region (20 to 35 kDa) differ. The non-reducing samples show several peaks in that region; the reducing samples do not and have a slight shift in the size and quality of the major peaks at approximately 11 minutes. Peaks that disappeared with reduction are most likely disulfide-crosslinked versions of the zein migrating at 11 minutes.

The Acetonitrile and SDS-MW kit methods appear quite different. One reason is the mode of injection: pressure versus voltage. Voltage is selective in its uptake for anions while pressure injects all molecules. Another reason could be related to the sample buffer chemistry. It is possible that there is interference from the sample buffer that comes with the SDS-MW kit. Given the proprietary nature of the kit, it is very difficult

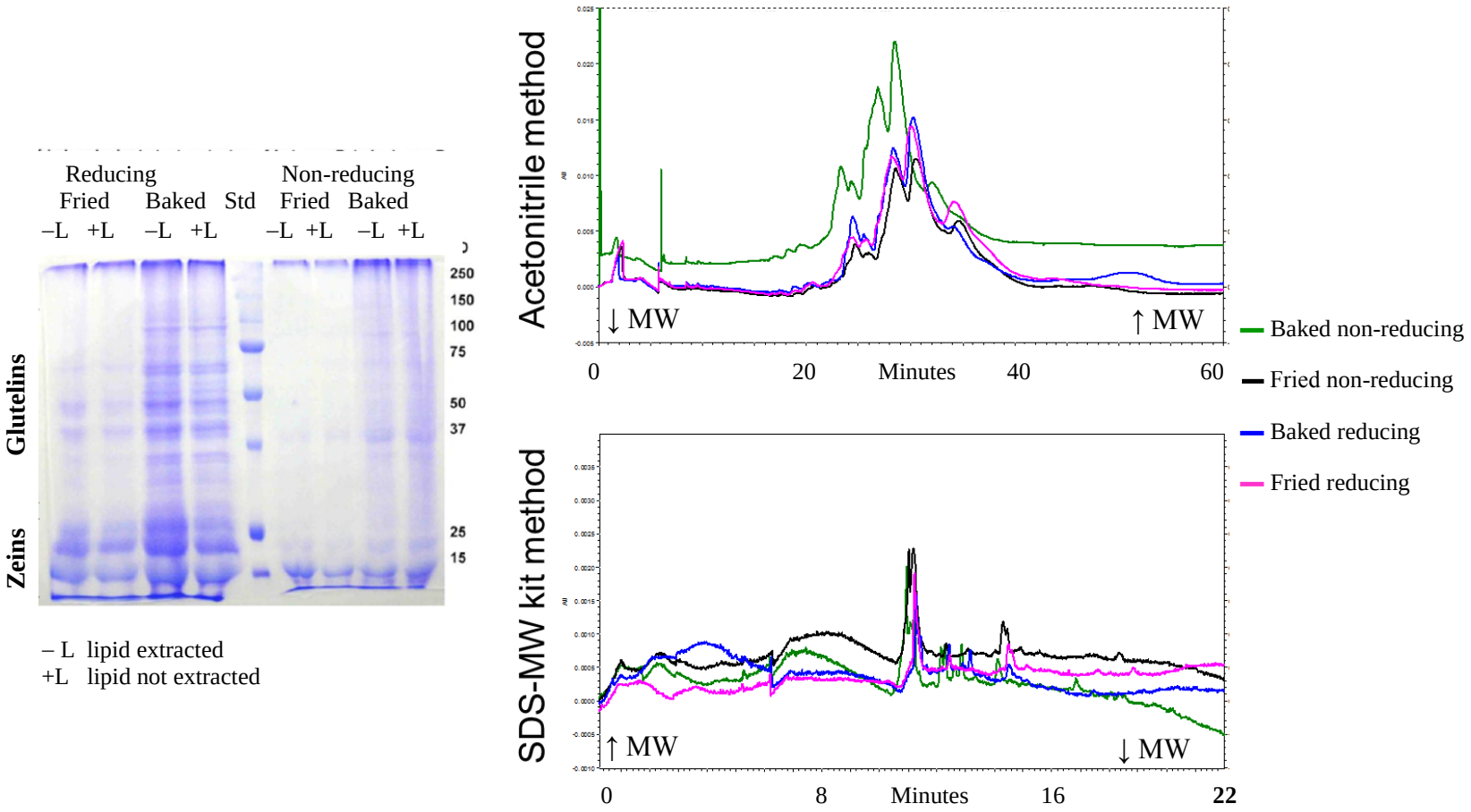

Figure 48: 0 week samples by SDS-PAGE (Dong 2011) and capillary electrophoresis (Acetonitrile and SDS-MW kit methods).

to understand the chemistry behind the outcomes. An attempt was made to analyze the samples using the gel matrix without diluting the samples in the buffer, but there was too much baseline fluctuation and noise to detect any useful peaks. Therefore, interaction with the sample buffer cannot be ruled out.

There are several points that can be compared and contrasted between the results in this research and those of Dong (2011). Figure 48 shows the analyses of 0 week samples by SDS-PAGE, SDS-MW kit method capillary electrophoresis, and Acetonitrile method capillary electrophoresis. The large amount of glutelins detected in the SDS-PAGE method are not detected by capillary electrophoresis. In the Acetonitrile method, it is possible that glutelins are present in the very early part of the electropherogram (immediately following the system peak). In the SDS-MW kit method, there are no glutelins and even zeins appear to be condensed. Absence of high molecular weight peptides cannot be attributed to dye-binding, duration of separation, or instrument detectability. One theory is that it is due to the filtration step that each sample underwent before being injected into the capillary as a safe-guard against clogging the capillary. This research required 0.45 μm filters because the tortilla chip extracts did not pass through the 0.2 μm filters typically used in capillary electrophoresis research. Filter retention is likely where the very high molecular weight proteins (the proteins that did not enter the SDS-PAGE gel represented by the dark bands at the very top of the image) were lost. It is also possible that the bands that should have been seen in the glutelin region were then trapped amongst the very high molecular weight protein mass on the filter. Additionally, Dong's data suggested that surface chemistry changes affected the binding

of Coomassie dye to the proteins. Decarboxylation, deamination, or alkylation (lipid addition) of glutelin side chains would prevent these proteins from being loaded into the capillary using the voltage method. It was hoped that capillary electrophoresis would avoid the polymer and surface issues that created problems for SDS-PAGE, but it looks as though voltage injection and filtration resulted in an unfavorable outcome and surface effects still have ramifications in this methodology.

C. Incubated Samples

All samples at all of the time points were evaluated by both the Acetonitrile and SDS-MW kit capillary electrophoresis methods. As shown in the figures above, comparing all the weeks simultaneously in one graph makes it difficult to discern trends, but stacking electropherograms reduces resolution. Thus, to simplify discussion, it is useful to examine only the 0 week and 8 week time points for comparison. Figure 49 focuses on the SDS-MW kit method results and Figure 50 focuses on the Acetonitrile method results.

As noted in previous sections, crosslinking is evident when comparing the reducing and non-reducing 0 week samples analyzed by the SDS-MW kit method. Beyond this, degradation is not readily apparent in the baked samples. However, in the fried samples, the peaks that fall in the region of less than 10 kDa show degradation over the course of the 8 weeks and also that there is more severe degradation in the 60 °C samples than in the 40 °C samples. Additionally, it looks as though the crosslinking that was initially destroyed by the reducing sample preparation is re-initiated over the 8 weeks. These observations are consistent with results of Dong (2011).

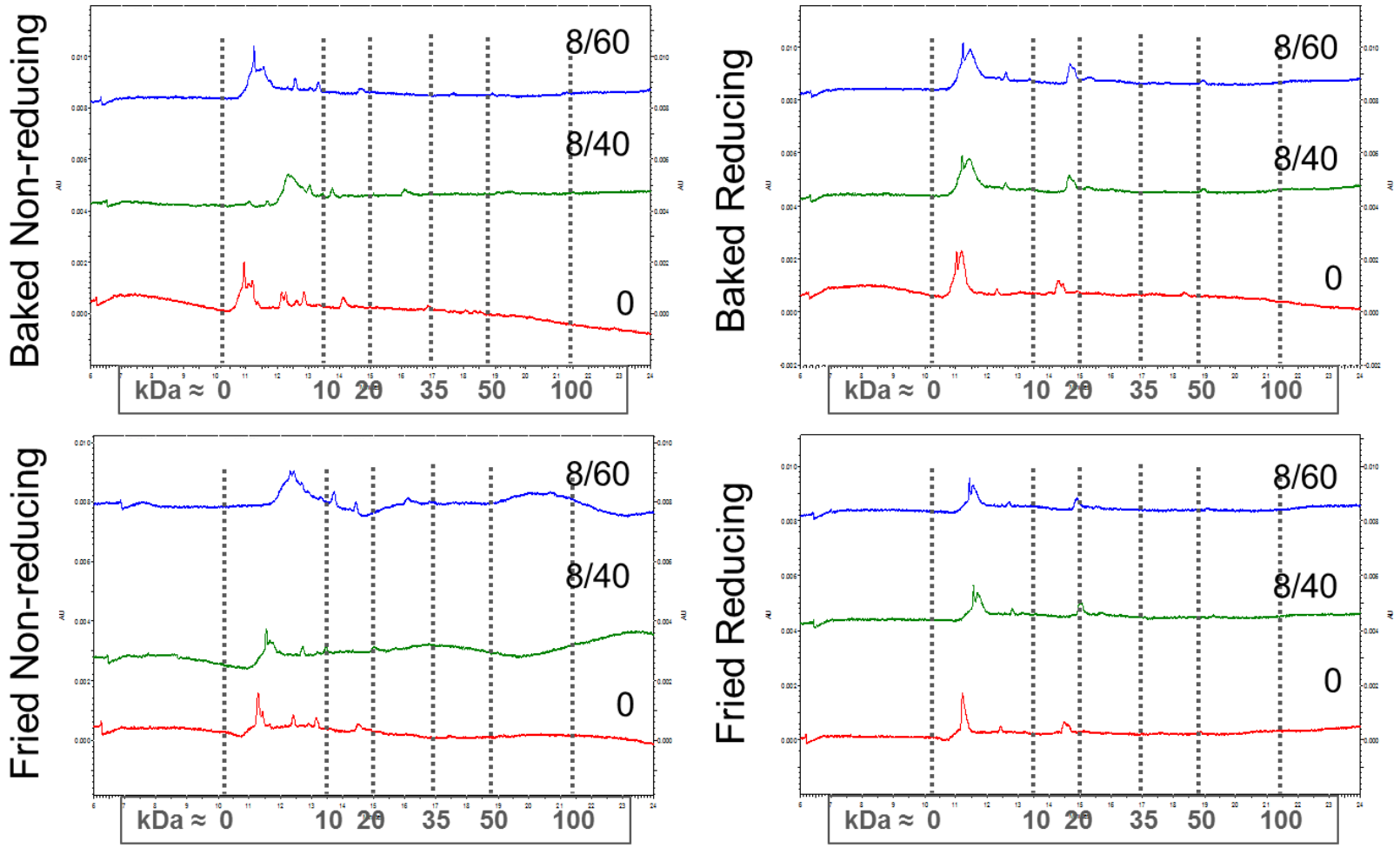

Figure 49: Comparison of 0 weeks and 8 weeks samples by reducing status, SDS-MW kit method (Trace labels: 0 = 0 weeks, 8/40 = 8 weeks at 40 °C, 8/60 = 8 weeks at 60 °C).

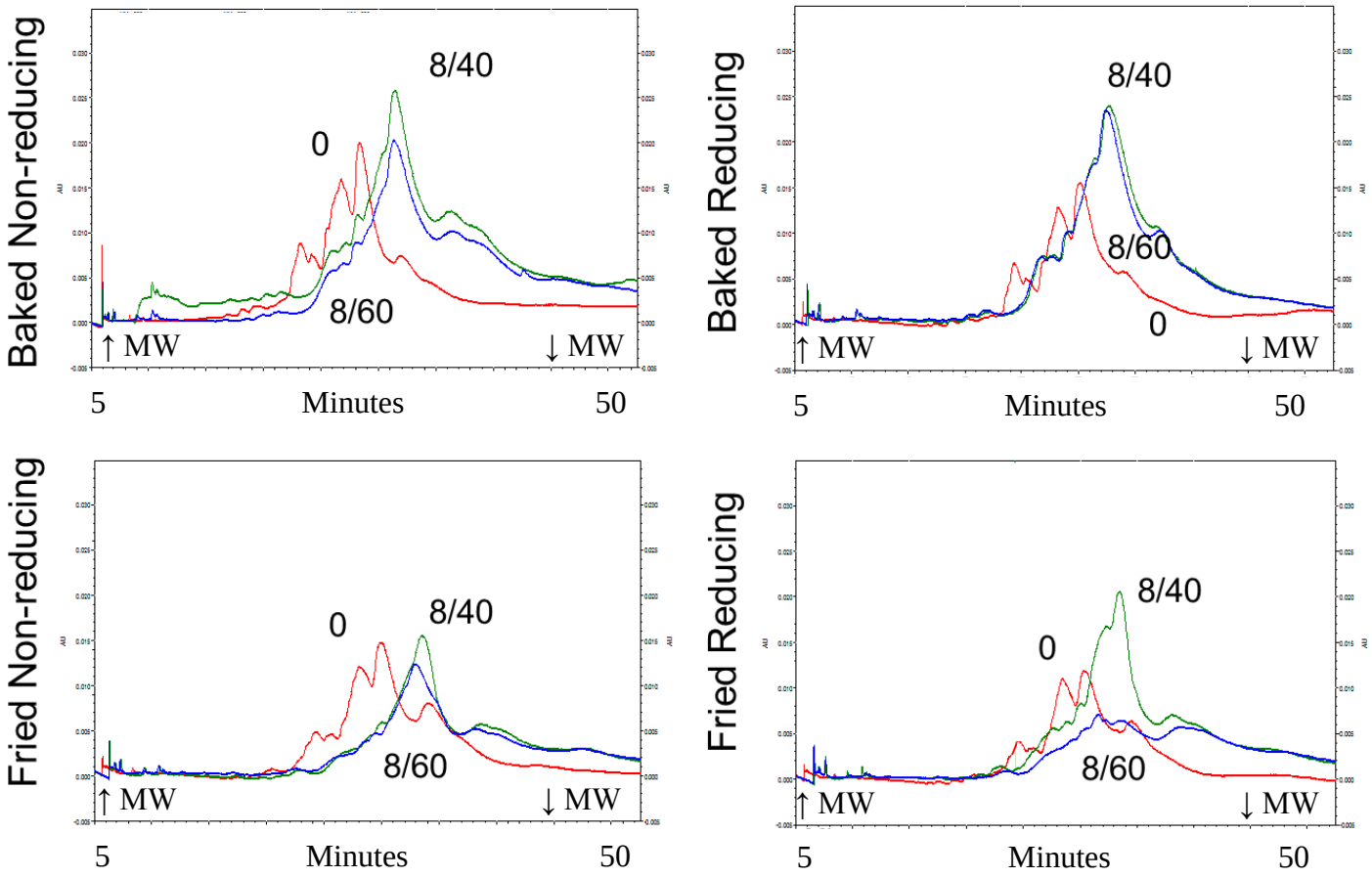

Figure 50: Comparison of 0 weeks and 8 weeks samples by type of tortilla chip, Acetonitrile method (Trace labels: 0 = 0 weeks, 8/40 = 8 weeks at 40 °C, 8/60 = 8 weeks at 60 °C).

The Acetonitrile method presents a somewhat different picture. Crosslinking may be evident in a few small peaks in the 6 to 7 minute region, although there does not appear to be much difference in the very high molecular weight peaks, whether comparing baked to fried or reducing to non-reducing. The most notable effects appear to be fragmentation and loss of peak definition: the central cluster of peaks show shifts among relative peak intensities and to longer retention times (lower molecular weight) with incubation.

Dong's data showed pronounced disulfide crosslinking, so electropherograms of non-reducing and reducing samples were specifically compared to determine whether capillary electrophoresis detected these changes (Figure 51). Overall, reduction with β-mercaptoethanol does not result in much change.

No notable differences are apparent in baked samples but in fried samples it appears that there is disulfide crosslinking by 8 weeks. In part, these observations support Dong's conclusions reached with SDS-PAGE: the observed cross-linking is not all based on disulfide bonding. However, the disulfide crosslinks in Dong's data were in high molecular weight fractions which were apparently excluded by filtration in capillary electrophoresis. This lack of expected differences between the non-reducing and the reducing samples can also be explained if zeins being detected are the C and D fractions. These low molecular weight monomers have lower thiol contents and so are likely less susceptible to disulfide crosslinking, hence they will not change with reduction.

Temperature effects are more notable than reducing effects on peak profiles in electropherograms (Figure 52). Extensive degradation is particularly obvious in reduced

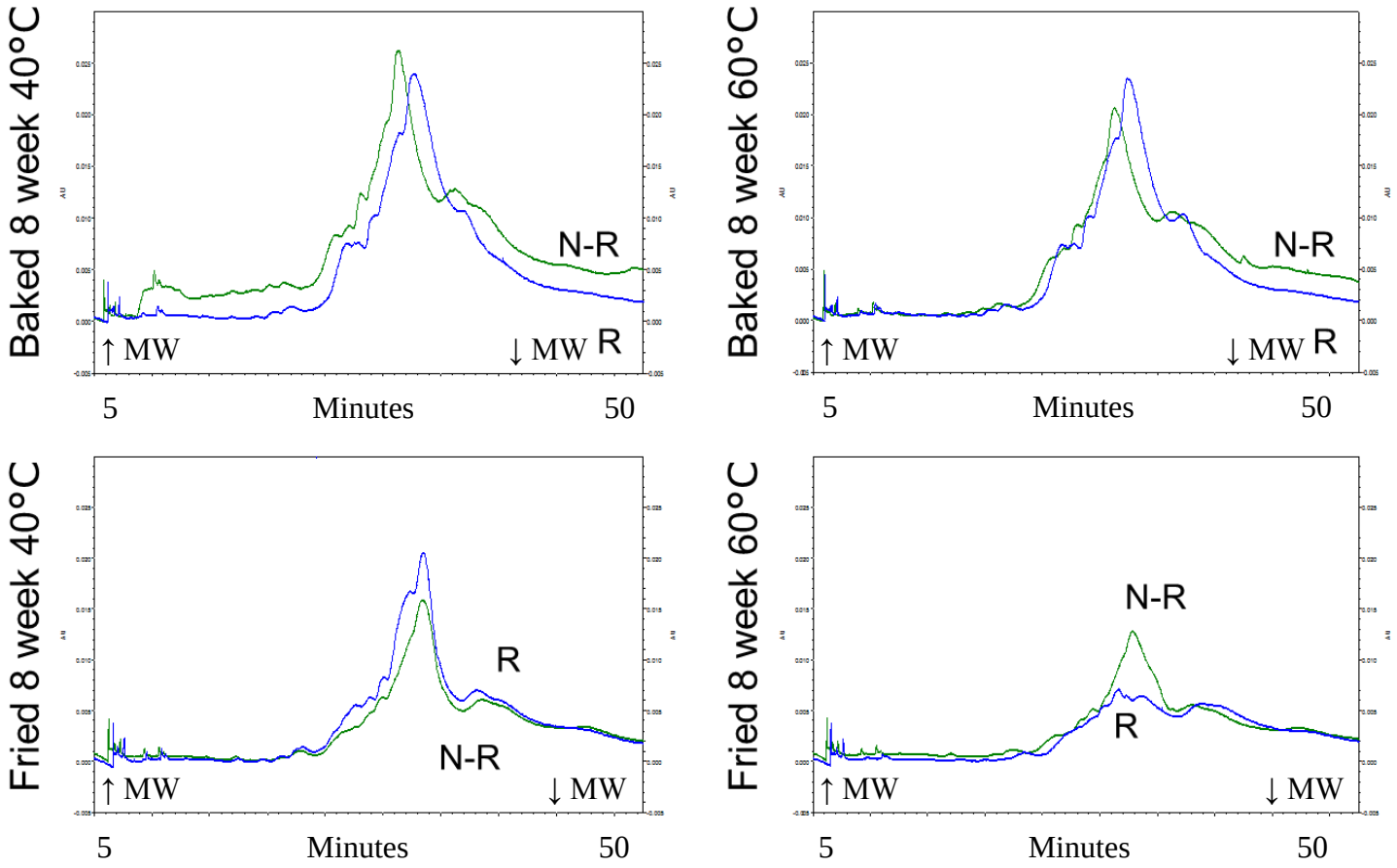

Figure 51: Comparison of non-reducing and reducing samples by incubation temperature, Acetonitrile method (Trace labels: N-R = non-reducing, R = reducing).

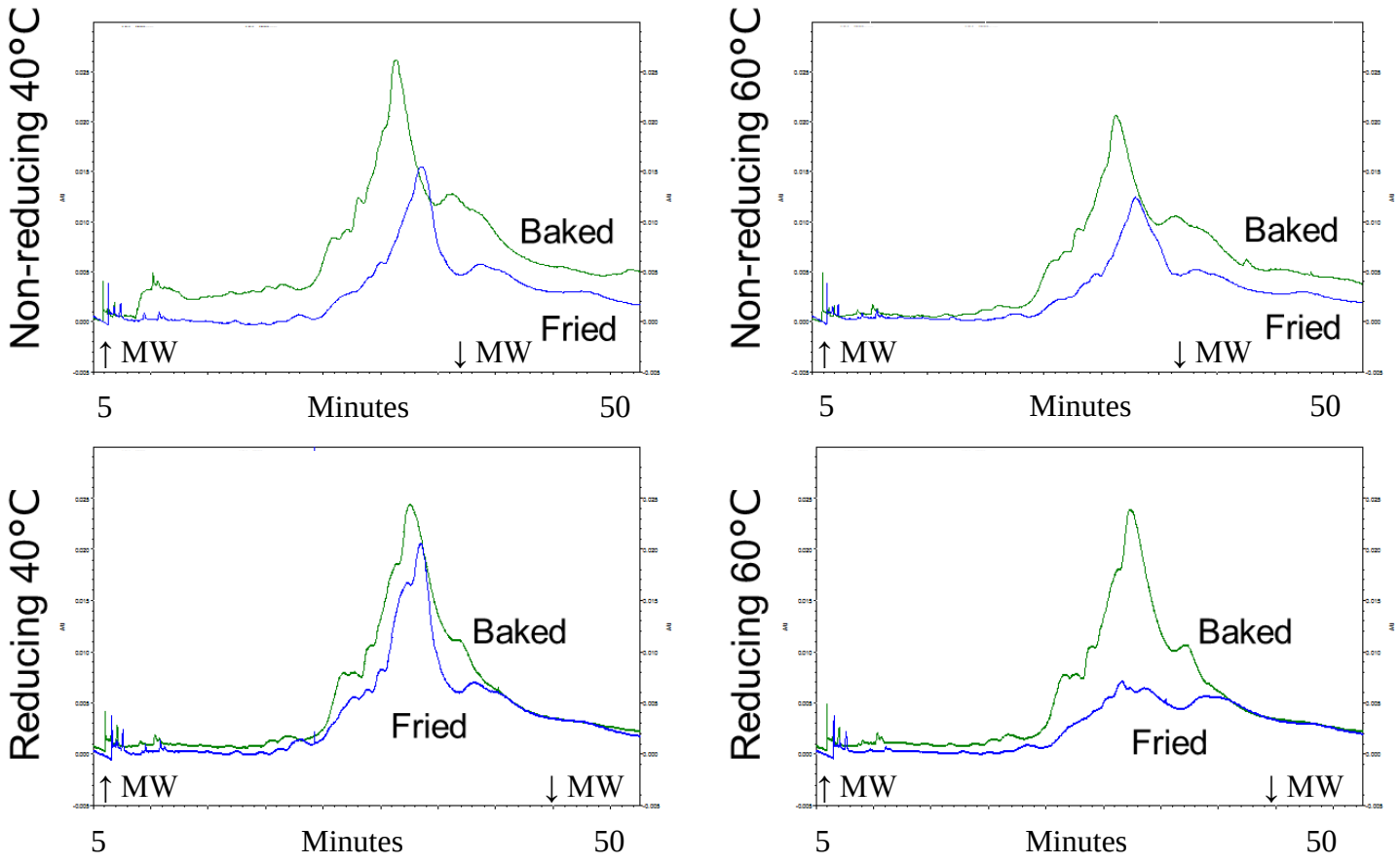

Figure 52: Comparison of fried and baked tortilla chips by reducing status, Acetonitrile method

proteins from fried chips. These observations corroborate Dong's conclusions that the presence of lipids during chip processing and higher storage temperatures result in more damage, although in this study fragmentation as well as crosslinking is clearly documented.

At both 40 °C and 60 °C, the non-reducing samples show a shift to smaller-sized proteins or protein fragments: a new large peak appears at approximately 32 to 33 minutes while the α-zein peak at approximately 30 minutes is reduced to a shoulder peak. The differences between baked and fried protein extracts are the same at both temperatures, implying that the scissions involved are not thermally-driven, but rather result from chemical reactions such as those caused by radicals. There are no marked changes in the very high molecular weight fractions (to the far left of the electropherogram), probably due to removal of these fractions by filtration.

Temperature effects of 40 °C versus 60 °C are more pronounced in reduced samples. At 40 °C, the reduced fried sample show almost the same amount of protein as the baked sample, albeit with more of the smaller molecular weight proteins. However, at 60 °C, the fried sample all but disappears in comparison to the baked sample even though samples were normalized for protein concentration during sample preparation. As with the non-reducing samples, the very high molecular weight proteins at the far left do not seem to change significantly, and no other substantial new peaks appear elsewhere in the electropherogram. These changes certainly indicate extensive protein modification but the reasons for the poor performance in capillary electrophoresis cannot be determined at the present time. It can be speculated that several factors may be involved,

including protein insolubility in the acetonitrile, failure to load onto the capillary, removal of high molecular weight fractions in the filtration, or other unknown reasons.

It is possible to observe the progression of degradation over the incubation period by comparing weekly changes in samples (Figures 53 through 56). Dong found that at 40 °C, most detectable crosslinking had occurred by 4 weeks for fried and 6 weeks for baked samples (reducing and non-reducing); after that, structures grew to be too large to enter the gel. SDS-MW kit method electropherograms (Figures 44 through 47) show a similar degradation pattern—loss of peak resolution in the peaks in the center of the electropherogram concurrent with generation of peaks in the high molecular weight region in the left portion of the electropherograms, although the degradation is less pronounced than with SDS-PAGE. Because the molecular weight increases are small, it is possible that peptide fragments are crosslinking to parent zeins. In contrast, the Acetonitrile method (Figures 32 through 35) appears to show opposite effects from SDS-PAGE. There is an unexpected shift towards smaller molecular weights (longer migration times; peaks E, F, and G in Figure 25) as incubation progresses, suggesting that scission rather than polymerization is occurring,

Why the Acetonitrile and SDS-MW kit methods show different overall trends in the shift of molecular weight is not fully understood but may be related to the injection mode. The SDS-MW kit method relies on charge-based injection, while the Acetonitrile method uses pressure injection of a fixed sample plug. Therefore, the two methods will inherently have different peak profiles and it is possible that this bias combined with the differing modes of separation and sample environments causes the conflicting results.

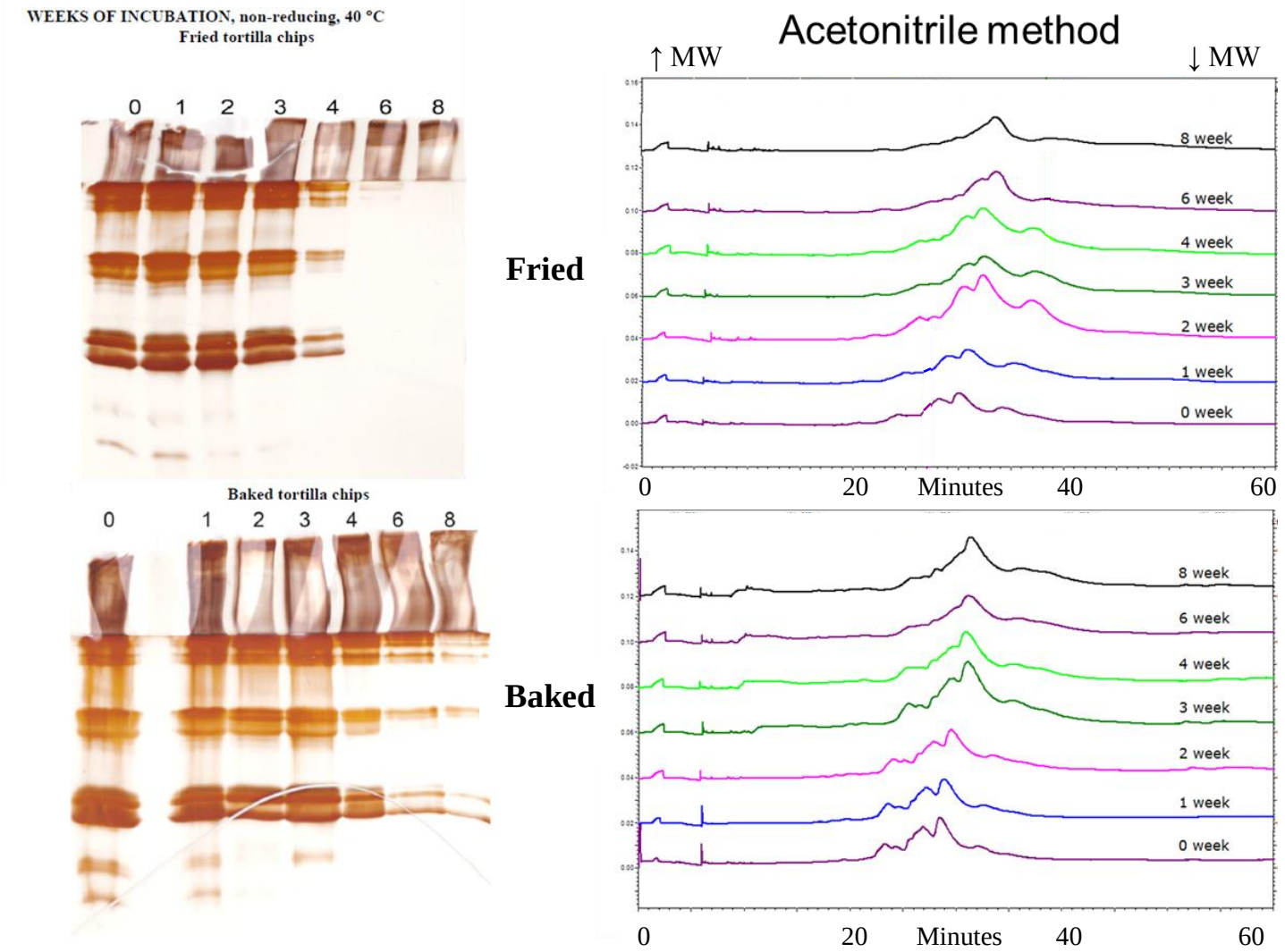

Figure 53: 40 °C non-reducing samples by SDS-PAGE (Dong 2011) and capillary electrophoresis (Acetonitrile method).

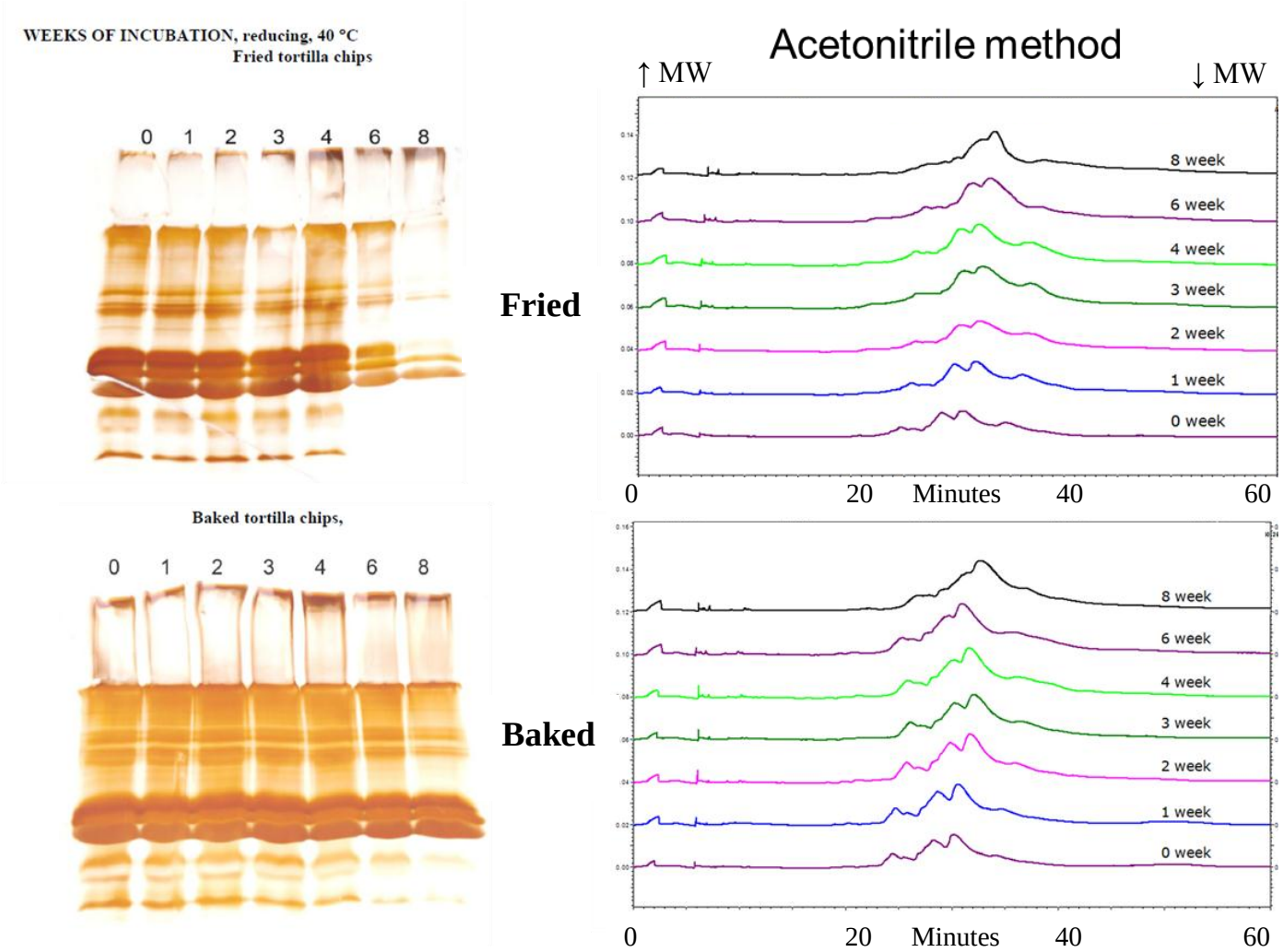

Figure 54: 40 °C reducing samples by SDS-PAGE (Dong 2011) and capillary electrophoresis (Acetonitrile method).

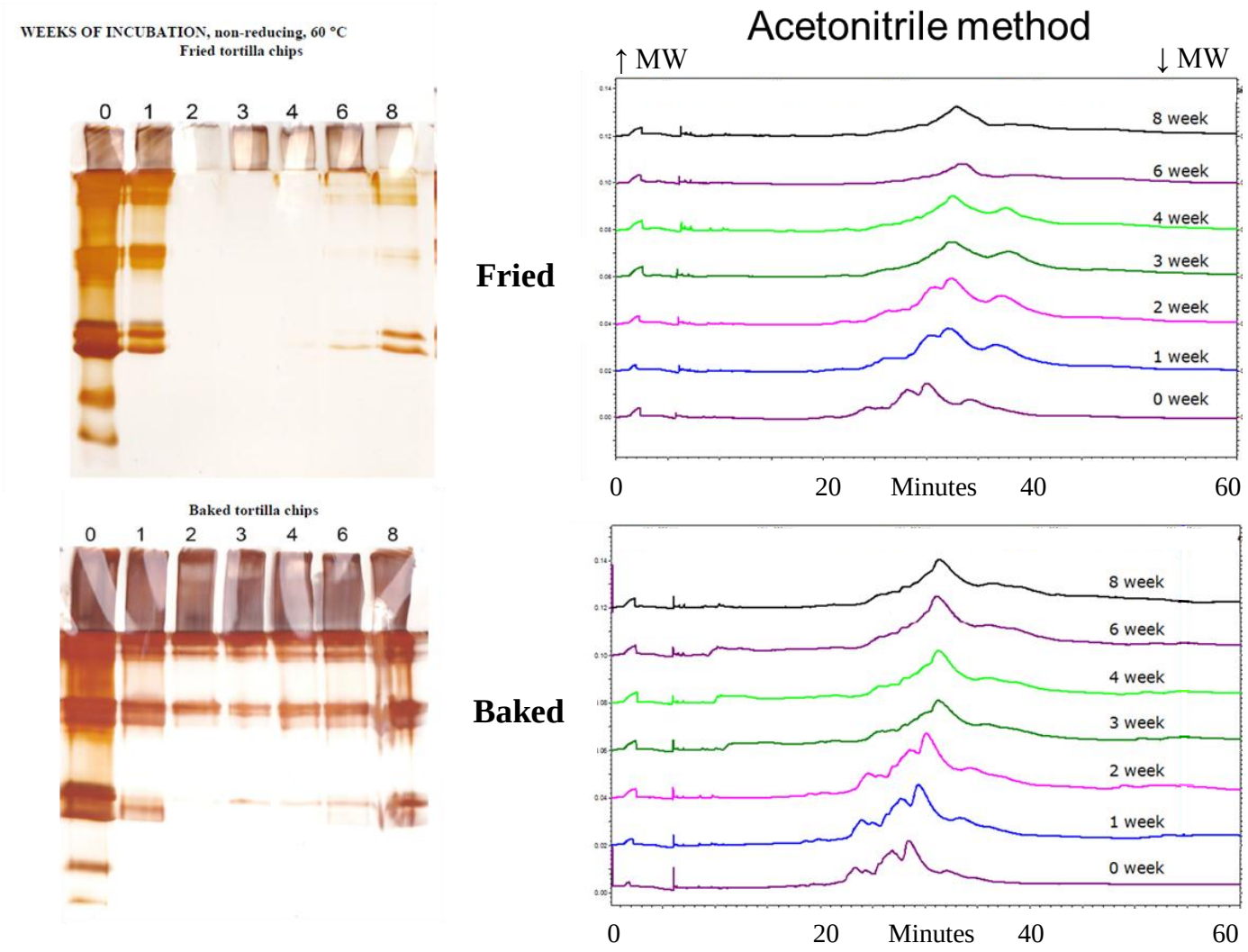

Figure 55: 60 °C non-reducing samples by SDS-PAGE (Dong 2011) and capillary electrophoresis (Acetonitrile method).

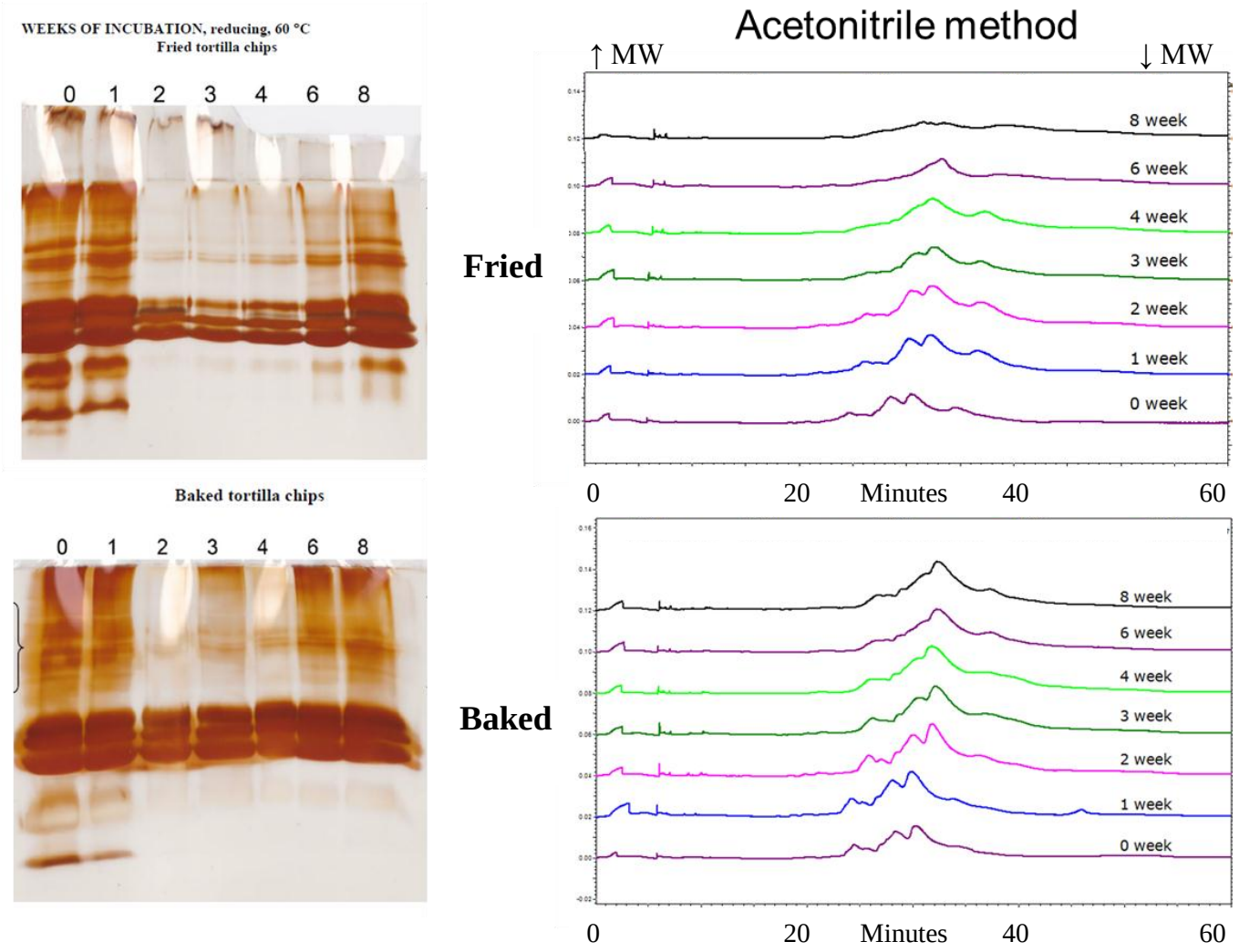

Figure 56: 60 °C reducing samples by SDS-PAGE (Dong 2011) and capillary electrophoresis (Acetonitrile method).

Dong determined that the extensive crosslinking shown in samples after 4 weeks of incubation at 60 °C (fried and baked, reducing and non-reducing) was not due to disulfide bonds, although disulfide crosslinking developed after that. Capillary electrophoresis generally supports this observation but the detected degradation occurs earlier in the incubation period: after 3 weeks for non-reducing samples and 4 weeks for reducing samples. This is earlier than crosslinking appears in the 40 °C samples and so it is apparent that crosslinking is accelerated at higher temperatures. Dong's data also showed that high molecular weight proteins appeared and then decreased as the incubation time progressed. In contrast, capillary electrophoresis by Acetonitrile method shows a steady loss of high molecular weight molecules with nearly complete degradation. It also shows an increase and then decrease in the lower molecular weight region. Again, this seems to support a conclusion that scission is occurring.

Notably, the extensive smearing of the bands from random degradation and crosslinking observed in Dong's experiments was corroborated by capillary electrophoresis in the widespread lack of baseline resolution, especially in the areas of greatest analyte concentration. These are not overloaded samples but rather species that have molecular weights very close to each other. Such smearing is a cost of analyzing samples in a food system as complex as tortilla chips wherein proteins are oxidized and otherwise modified. In comparison, much of the literature has focused on model systems extracted directly from corn kernels or highly refined zeins samples purchased from manufacturers. The smearing or lack of resolution may also be explained by the wide variety of structures resulting from protein crosslinking. Rather than exhibiting one or

two peaks that can be defined as co-oxidants, it is more likely that the co-oxidation species are represented by a continuum of molecular weights and charge distributions.

IX. SUMMARY AND CONCLUSIONS

This research sought to determine whether capillary electrophoresis is a viable method for distinguishing modified from native zein proteins in complex food systems such as tortilla chips. The hope was that this methodology would provide a means to assess changes in protein patterns over time in an automated and quantifiable manner without the size limitations of SDS-PAGE. To parallel previous research with SDS-PAGE, baked and fried tortilla chip samples were incubated at two temperatures and samples were analyzed over the course of 8 weeks.

Following extraction, protein concentrations were measured by the Bradford assay and extracts were diluted as needed to equalize concentrations in samples prior to capillary electrophoresis analysis. Samples then underwent capillary electrophoresis by two methods: SDS-MW kit and Acetonitrile. The benefits of the SDS-MW kit method are that it is a commercially available kit, does not require preparation of background electrolyte, and provides the closest correlation to SDS-PAGE. The detractions are cost, lack of the detector for which the kit is specifically intended, and lack of resolution. The benefits of the Acetonitrile method are that the materials are low cost and readily available. Disadvantages are separation of peptides by mixed modes so that molecular weights of fractions could not be determined unambiguously.

Results of the study do not support the hypothesis that capillary electrophoresis can map modified peptide fractions, particularly high molecular weight fractions, more

effectively than SDS-PAGE. Many limitations were encountered, the most serious of which are inability to clearly detect protein fractions other than low molecular weight zeins and possible strong effects of protein modifications on their loading and migration in the capillary. Difficulty in identifying fractions in the Acetonitrile method due to mixed-mode separation may have been tolerable if high molecular weight fractions were detected because at least then patterns of peptide change could have been followed.

It is likely that all of these problems are related to uncertainty in the chemistry that drives modified proteins into and through the capillary. In the pressure loading of the Acetonitrile method, all proteins in the sample solution are forced as a plug into the capillary and should be separated by electroosmotic flow, charged and neutral molecules alike. Four to five peptide fractions were detected but their molecular weight appeared to be low and resolution was poor—all fractions overlapped considerably within a 20 to 25 minute migration time. The lack of resolution most likely results from a combination of mixed mode separation (combination of molecular weight, effective size, and chemical interactions), lack of charged groups to effect separation, and very similar molecular weights. Lack of detection of peptides with molecular weights greater than about 30 kDa is a potentially fatal flaw with this method. At the present time, it is speculated that filtration of the extract through 0.45 μm filters before capillary electrophoresis removes the larger proteins, either directly by size exclusion from the filter or indirectly by entrainment in the protein film. However, the possibility that surface modifications in glutelins and other higher molecular weight fractions interferes with electrical properties and hence migration in capillary electrophoresis cannot be eliminated at the present time.

Loading peptides by voltage in the SDS-MW kit method yields even worse results. The method verifies detection of only low molecular weight zeins or modified peptides (typically less than 20 kDa). However, resolution of peaks within this range is much improved over the Acetonitrile method: several fractions are clearly distinguished, some of which involved disulfide bonds and changed after reduction and some of which are disulfide independent.

Within the limitations of molecular weight fractions detected, both the Acetonitrile and SDS-MW kit methods distinguish progressive changes in extracted proteins with incubation, resulting in longer migration times. With the SDS-MW kit method, longer migration times indicate higher molecular weights, verifying the polymerization observed with the tortilla chip systems in a previous study (Dong, 2011). With the Acetonitrile method, longer retention times should result from smaller proteins. Whether results of this study reflect scission processes not detected by the SDS-PAGE procedure or, alternatively, protein surface modifications that alter migration in the capillary remains to be determined.

Overall, results of this study suggest that capillary electrophoresis has some intriguing possibilities for supplementing SDS-PAGE and other protein analyses of modified proteins, particularly in verifying the presence of surface modifications. However, significant hurdles—the reasons for lack of high molecular weight peptide loading and migration, and understanding of the molecular mechanisms driving migration—remain to be overcome before capillary electrophoresis can become a primary method for analysis of modified proteins.

X. FUTURE WORK

There are unresolved questions stemming from these methods and the complicated nature of separation within the capillary. In order for definitive conclusions to be drawn from any protein analysis by capillary electrophoresis data, it is critical that the behavior of the proteins be understood to their fullest. First and foremost, effects of filtration on the concentrations of protein fractions as well as their migration pattern must be determined to understand why fractions present in SDS-PAGE analyses are not detected in capillary electrophoresis. By measuring the size distribution of all molecules in the diluted but unfiltered sample, it could be determined if there are aggregates present. Examining the molecules that remain on the filter would help to identify the extent to which proteins are being trapped among large non-proteins. If proteins are, indeed, being filtered to a high degree then, a more suitable sample diluent should be identified to aid in the solubilization of the proteins. As was noted in the future work section of Dong (2011), Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrometry is particularly attractive as an alternative means of analyzing these samples. This method is sensitive and allows for analysis of a wide range of molecular weight molecules.

Surface charge modification must also be explored since separation of fractions is driven by those charges alone, as in the case of the Acetonitrile method, or as a result of SDS binding properties, as in the case of the SDS-MW kit method. Although the purpose of this research project was to evaluate capillary electrophoresis as an improvement over traditional protein methods, acid or neutral PAGE methods could be explored as well as HPLC methods since acetonitrile is a compatible chromatographic mobile phase.

Application to charged microporous filters and subsequent protein concentration determination could help to understand the relative proportions of charged and uncharged moieties in the samples.

Finally, given the problems with detecting polymers, capillary electrophoresis may be more useful in analyzing peptides generated by protease digestion of modified proteins. The patterns of peptides released after different proteases can reveal much about sites of original protein modifications.

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