

UNDERSTANDING THE MECHANISM OF TEXTURIZATION, AND THE  
RELATIONSHIP BETWEEN PROPERTIES OF WHEAT GLUTEN AND  
TEXTURIZED VEGETABLE PROTEIN

by

RYAN ROBERTS

B.S., Kansas State University, 2010

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Grain Science and Industry  
College of Agriculture

KANSAS STATE UNIVERSITY  
Manhattan, Kansas

2013

Approved by:

Major Professor  
Sajid Alavi

# **Copyright**

RYAN ROBERTS

2013

## Abstract

Texturized vegetable protein (TVP) based foods offer several advantages compared to animal protein, including lower costs and improved health benefits. Wheat gluten is often processed using extrusion to produce TVP. Processing aids, such as reducing agents (example, cysteine and sodium metabisulfite) and pH modifiers (example, tetra potassium phosphate) aid in texturization. Reduction of sulfhydryl groups, cleavage of disulfide bonds, and reformation of bonds between elongated protein molecules results in protein aggregation and texturization. This study focused on development of a fundamental understanding of these mechanisms for texturization using analytical tools such as the phase transition analyzer (PTA), in combination with lab- and pilot-scale extrusion. The abovementioned three chemicals were added to four varieties of gluten. The control treatment had no additives. PTA was used to understand the operative flow properties of gluten in an environment similar to an extrusion system. Addition of sulfite (0.18%) and cysteine (0.18%) lowered the thermal softening (Ts:36.6-44.1 °C) and thermal flow (Tf:79.6-105.6 °C) temperatures of all varieties of gluten as compared to the controls (Ts:38.8-48.2 °C; Tf:91.7-112.2 °C). Phosphate (3%) did not have the same lowering effect on Ts (40.2-47.0 °C) and Tf (96.2-108.2 °C), indicating a different mechanism.

Extrusion studies were conducted to gain an understanding of the reformation of disulfide bonds and texturization. Two of the varieties of gluten, a “superior” one that texturizes well and an “inferior” gluten requiring texturizing aids, were processed on a lab-scale extruder. Pilot scale extrusion was used to process the other two glutes (“superior” varieties) to obtain commercial quality products, which were evaluated for degree of texturization (hydration rate, absorption index and integrity). During lab-scale extrusion, texturization was observed only in the case of phosphate and corresponded with an increase in specific mechanical energy (SME) as compared to the control, indicating disulfide bond reformation. Phosphate also led to significantly ( $p < 0.05$ ) better texturization during pilot-scale extrusion, although SME trends were different due to

higher in-barrel moisture and a more ideal extrusion system. Fourier Transform Infrared Spectroscopy was used to examine protein structural changes and indicated a loss of  $\alpha$ -helix structure in TVP with an increase in  $\beta$ -sheet formation.

# Table of Contents

List of Figures .....	viii
List of Tables .....	x
Acknowledgements.....	xi
Chapter 1: Understanding the Mechanism of Texturization and the Relationship between Properties of Wheat Gluten and Textured Vegetable Protein .....	1
1: Background:.....	1
2: Gluten Structure:.....	3
3: Processing Aids: .....	5
3.1: Sulfite.....	5
3.2: Cysteine .....	5
3.3: Phosphate .....	6
Chapter 1 References .....	7
Chapter 2: Understanding Properties of Wheat Gluten and the Mechanism of Texturization – Lab Scale Studies .....	9
1. Introduction.....	9
2. Materials and Methods.....	12
2.1. Gluten characterization .....	12
2.2. Gluten physical properties .....	14
2.2.1: Gluten Index .....	14
2.2.2: Compression Test .....	14
2.2.3: Phase Transition Analysis.....	15
2.3. Blend preparation and extrusion processing .....	16
2.4. Statistical analysis .....	18
3. Results and discussion .....	18
3.1. Proximate analysis and amino acid profile .....	18
3.2. Rheological properties of wheat gluten .....	20
3.2.1: Gluten Index .....	20
3.2.2: Compression Test .....	21

3.2.3: Phase Transition Analysis.....	22
3.3. Extrusion processing.....	24
3.4. Post-Extrusion Phase Transition Analysis.....	25
4. Conclusion.....	28
Chapter 2 References.....	30
Chapter 3: Relationship between Properties of Wheat Gluten and Texturized Vegetable	
Protein processed using High Shear Extrusion.....	35
1: Introduction:.....	35
2. Material and methods.....	37
2.1. Material characterization.....	37
2.2. Formulation and Mixing.....	39
2.3. Gluten Characterization.....	39
2.3.1: Gluten Index.....	39
2.3.2: Phase Transition Analysis.....	39
2.4. Extrusion processing.....	40
2.4.1: Die Design.....	42
2.5. Final product analysis.....	43
2.5.1: Water Absorption Index.....	43
2.5.2: Textured Gluten Integrity Test.....	43
2.5.3: Hydration Time and Rate.....	44
2.5.4: Fourier Transform Infrared Spectroscopy.....	44
2.6. Statistical analysis.....	45
3. Results and discussion.....	46
3.1. Proximate analysis and amino acid profile.....	46
3.2. Rheological properties of wheat gluten.....	47
3.2.1: Gluten Index.....	47
3.2.2: Phase Transition Analysis.....	48
3.3. Extrusion processing.....	50
3.4. Post-extrusion quality analysis.....	51
3.4.1: Water Absorption Index.....	51
3.4.2: Textured Gluten Integrity Test.....	53

3.4.3. Bulk Density .....	54
3.4.4: Hydration Time and Rate.....	55
3.4.5: Fourier Transform Infrared Spectroscopy Results.....	58
4. Conclusion .....	62
Chapter 3 References .....	64
Chapter 4: Conclusion.....	68
Appendix A: SAS Output for Lab Scale Extrusion .....	70
Appendix B: SAS Output for Pilot Scale Extrusion .....	76

## List of Figures

Figure 2.1: Screw Configuration – Micro-18 Twin Co-Rotating Screw Extruder with accompanying head temperature profile. ....	17
Figure 2.2: Gluten Index Results for Superior and Inferior glutens (Control and Processing Aids). ....	20
Figure 2.3: Compression Tests of Superior and Inferior Glutens With and Without Processing Aids. ....	21
Figure 2.4: Average Softening Temperatures of Superior and Inferior Gluten (Control, 3.0% Phosphate, 0.18% Cysteine, 0.18% Sulfite). ....	22
Figure 2.5: Thermal Flow Temperatures of Superior and Inferior Glutens (Control, 3.0% Phosphate, 0.18% Cysteine, 0.18% Sulfite). ....	23
Figure 2.6: SME During the Extrusion of Superior and Inferior Glutens. ....	24
Figure 2.7: Extruded and Un-Extruded Glutens; Thermal Softening Temperature. ....	26
Figure 2.8: Extruded and Un-Extruded Glutens; Thermal Melting Temperature. ....	26
Figure 3.1: Temperature and screw profile for TX-52 Extruder ....	41
Figure 3.2: Die Configuration. ....	42
Figure 3.3: Gluten Index Results for U.S. and European Sourced Glutens. ....	47
Figure 3.4: Average Softening Temperatures of U.S. and European Sourced Gluten (Control, 3.0% Phosphate, 0.18% Cysteine, 0.18% Sulfite). ....	48
Figure 3.5: Thermal Melt Temperatures of U.S. and European Sourced Gluten (Control, 3.0% Phosphate, 0.18% Cysteine, 0.18% Sulfite) ....	49
Figure 3.6: SME during the Extrusion of U.S. and European Sourced Glutens. ....	50
Figure 3.7: Water Absorption Index for U.S. and European Sourced Glutens at Low and High In-Barrel-Moistures (32% and 36%, respectively) ....	51
Figure 3.8: Percent loss during Textured Gluten Integrity Testing ....	53
Figure 3.9: Hydration Rate of Textured Glutens at 32% and 36% IBM with Chemical Additives Added to Two Types of Gluten ....	57
Figure 3.10: FTIR spectra of control treatment, raw material and final product. ....	58
Figure 3.11: FTIR spectra of cysteine treatment, raw material and final product ....	59



Figure 3.12: FTIR spectra of sulfite treatment, raw material and final product .....60  
Figure 3.13: FTIR spectra of phosphate treatment, raw material and final product .....61

## List of Tables

Table 2.1: Proximate Analysis of Superior and Inferior Gluten Types .....	18
Table 2.2: Amino Acid Profile of Superior and Inferior Gluten types .....	19
Table 3.1: Wheat Gluten Formulation for Pilot Scale Extrusion.....	39
Table 3.2: Screw Configuration.....	41
Table 3.3: Proximate Analysis of US and European Glutens.....	46
Table 3.4: Amino Acid Profile of US and European Sourced Glutens .....	46
Table 3.5: Bulk densities of Textured Gluten at 32% and 36% IBM with Chemical.....	54
Table 3.6: Average Hydration Time for High and Low IBM with the Inclusion of Chemical Additives for Two Types of Wheat Gluten .....	55
Table A.1: Two-way comparison between gluten type and chemical treatment .....	71
Table A.2: Moisture effect on gluten types for thermal softening.....	73
Table A.3: Moisture effect on gluten types for thermal flow .....	73
Table B.1: Three-way comparison between source country, in-barrel moisture and chemical treatment for absorption index.....	78
Table B.2: Two-way comparison between source country and chemical treatment .....	81
Table B.3: Two-way comparison between source country and in-barrel moisture .....	83
Table B.4: Two-way comparison between source country and chemical treatment for TVP integrity .....	83
Table B.5: Two-way comparison between in-barrel moisture and chemical treatment for TVP integrity .....	84
Table B.6: Three-way comparison between source country, in-barrel moisture, and chemical treatment for TVP hydration time .....	87
Table B.7: Three-way comparison between source country, in-barrel moisture, and chemical treatment for TVP hydration rate.....	89
Table B.8: Effect of chemical treatment on thermal softening.....	91
Table B.9: Effect of chemical treatment on thermal flow .....	92

## **Acknowledgements**

I would like to thank MGP Ingredients, Inc. for the funding of this project and their continued support through out this research. Also, thank you to ConAgra Foods for the donation of the wheat flour used in the pilot scale portion of this research.

Thanks to my committee members, Sajid Alavi, Ody Maningat, and Jon Faubion for their useful comments, remarks and engagement through the learning process of this master thesis. Furthermore, I would like to express my gratitude to Brad Seabourn, Prini Gadgil, Mike Tilly, and their colleagues at USDA-ARS. Their input and expertise proved very valuable. Thank you to Becky Miller for running the gluten index's and compression tests.

Finally, thanks to Eric Maichel and his staff at the KSU Extrusion Lab. Without their help, this thesis would not have been possible.

# **Chapter 1: Understanding the Mechanism of Texturization and the Relationship between Properties of Wheat Gluten and Textured Vegetable Protein**

## **1: Background:**

The consumption of vegetable proteins as a food product has been increasing over the years because of animal diseases, global shortage of animal proteins, and economic reasons (Asgar et al, 2010). Perhaps the most common form of vegetable proteins being consumed is textured vegetable proteins (TVP). TVP is a vegetable protein that has a meat-like texture and taste after processing. TVP can be made from several grain proteins, with wheat gluten being one of the primary grains used. Soybean protein concentrate is also a large part of the TVP market. Another factor affecting vegetable protein consumption is lifestyle choice, which is especially true for consumers in the United States. One hundred vegans were polled and the majority answered health reasons as the major factor why they chose a vegan lifestyle (Dyett et al, 2013). Foods containing soy or other vegetable proteins may lead to a reduced risk from colon, breast, and prostate cancers (Kirk et al, 1999). Other factors are based on health beliefs and animal welfare opinions. Another benefit of textured vegetable proteins is the extended shelf life compared to animal proteins. TVP is also used to extend the use of animal proteins; TVP can be added to animal protein to reduce the costs of animal protein.

The two most common uses of TVP are as meat extenders and complete meat replacements. Meat extenders usually have a small piece size so they can be easily blended with ground or shredded cooked meat. This form of TVP is dried to storage moistures below 6% (dry basis) and hydrated prior to being mixed into a ground meat.

Meat replacers, however, are typically larger in size and are not dried to the same low moisture as are meat extenders. Meat replacers will resemble an actual cut of beef, pork, chicken, or fish. Both forms can be colored and flavored.

Before a vegetable protein can be consumed, it must go through several steps to reach the final product stage. First is the separation of the protein and starch components. This is especially important for grains whose protein component is used for TVP. For TVP comprised of soy protein, concentration of the protein is a key step. After the protein component is separated or concentrated, the protein will go through a process called extrusion. Extrusion is a continuous process where the addition of thermal and mechanical energy in a high pressure environment is the goal; manipulating the macromolecular structure and texture of the protein polymers occurs, as to achieve the desired final product.

During the extrusion process, material is fed at a predetermined rate to a preconditioner. There, steam and water are added to the material and mixed by two rotating shafts with mixing picks. The goal of preconditioning is to temper the material so the starch or protein begins to cook, which in turn leads to easier processing at extrusion. Once the material is preconditioned, it enters the extruder barrel. Water and steam can also be added to the barrel, but the main goal is to introduce shear into the system. The material is conveyed through the extruder barrel by either a single or twin screws. This process adds mechanical energy and heat, disrupting the starch's or protein's structure, thereby cooking each component. The end of the extruder barrel has a flow restricting and forming die. Dies come in many shapes and sizes, and die design can affect the structure and texture of the final product a great deal. The die also creates back pressure

in the extrusion system, resulting in lower starch gelatinization and protein denaturation temperatures. Depending on the desired final characteristics, all of the steps of the extrusion process, including preconditioning, can be manipulated to create the final product. Twin screw extrusion is perhaps the most common form of extrusion to make TVP. More efficient taste modifications and texture development have been made possible with twin screw extrusion (Akdogan, 1999).

## **2: Gluten Structure:**

The overall structure of the gluten polymer is very complex. Gluten proteins are made up of two families of proteins, glutenins and gliadins. Gliadins contain intra-chain disulfide bond linkages. Glutenin, the larger component of a gluten molecule, use both intra and inter-chain disulfide cross-linkages to maintain its structure. In addition to the disulfide bonds, gluten also contains non-covalent bonds. These include hydrogen, ionic and hydrophobic bonds. These three bonds are very important for glutenin and gliadin aggregation and impact the structure of the gluten molecule when it is introduced to a high moisture environment.

Breaking the covalent disulfide bonds in the glutens structure is the primary goal when extruding wheat gluten. It takes very little energy to break the non-covalent bonds (hydrogen, ionic, hydrophobic), so reduction of the disulfide bonds is the more energy intense step. Once these bonds are broken, an extruder utilizes retention time, moisture, heat, and die design to reform these bonds. As material travels down the extruder barrel, the disulfide bonds that connect the proteins are broken and, simultaneously, are being re-linked along the length of the extruder barrel (Shimada et al, 1988). Once the material leaves the die and encounters atmospheric pressures, the gluten

matrix will be further disrupted by the flashing off of superheated water vapor. This directly affects the porosity of the TVP and will affect its hydration rate and time, two key physical characteristics.

While many factors can impact the melt flow characteristics of wheat gluten, the main determining factor may be the environment in which the wheat was grown in. Manufacturers who use wheat gluten to create TVP share many things in common as the flour industry. The flour industry requires a very detailed analysis of the incoming crop to determine the best use for the wheat harvest. In a study completed in 2009, Pablaciones et al, (2009) used a chlorophyll meter to measure several parameters of growing wheat from two different years. They then created an algorithm to relate alveogram index, dough extensibility, tenacity-extensibility ratio and gluten content. It was found that there was substantial variability between the two crop years. Even more interesting is the fact that the protein content of wheat can be predicted with precision using the chlorophyll meter. However, more work is needed to prove that a model can be created when other factors, such as soil water content or soil nitrogen content, are added.

While the previous study mentioned examined a normal crop growing season, not all growing seasons are normal. More often than not, the wheat will be under some form of stress prior to harvest; water deficits and high temperatures, for example. Yang et al (2011) found that individual protein fractions of wheat, gliadins and glutenins, were affected by not only the type of stress, but also the length of the stress. This could, in turn, factor into changes in the quality of extruded wheat gluten.

### **3: Processing Aids:**

Because of variability between wheat crops, and the variability of the gluten extracted from the wheat, TVP manufacturers need to improve consistency at extrusion. The addition of so-called processing aids, such as phosphates, sulfites and cysteine, can improve the manufacturing of TVP.

#### **3.1: Sulfite**

In this work, metasodium bisulfite will be referred to as sulfite. Sulfite is a reducing agent. It goes through an oxidation-reduction reaction where an electron is either lost or gained from another molecule. The extrusion process breaks disulfide bonds through the inclusion of heat and shear forces, and when the gluten is extruded, the excess disulfide molecules aid in protein polymer reduction making the available sulfhydryl groups more readily available for new cross-links. The newly introduced sulfhydryl groups facilitate in creating new cross-linkages between protein polymers. Sulfite is an allergen, so use is limited.

#### **3.2: Cysteine**

Cysteine, the amino acid, is also a reducing agent. Because of its sulfhydryl group, two proteins can be linked by the creation of a disulfide bond. During the extrusion process, disulfide bonds are broken, allowing the protein polymer to extend and entangle with other polymeric chains. Since cysteine is a reducing agent, its presence will facilitate easier disulfide bond cleavage and create a higher potential for sulfhydryl oxidation, which reforms disulfide bonds. Bond reformation is taking place on extended and entangled protein polymers, resulting in a new structure formation. Like sulfite, cysteine is considered an allergen, so its use is limited in the industry.



### 3.3: Phosphate

Phosphates are not reducing agents. Instead, they are considered pH adjusters. Wheat gluten has no discernable iso-electric point. Therefore, it is difficult to determine at which point the positive and negative charges of the gluten molecule balance. However, since the gliadin portion of gluten is water soluble, gluten will reflect the iso-electric behavior of gliadin, giving gluten a pH between 5 and 6. Since the goal of the extrusion process is to break covalent disulfide bonds and sulfhydryl groups, it is necessary to increase the solubility of the gluten. This can be done by increasing or decreasing the pH of the gluten. By adding a phosphate, the gluten becomes more alkaline. This increases the solubility and makes the covalent disulfide bonds easier to cleave. Also, the non-covalent bonds (hydrogen, hydrophobic, ionic) also become easier to break.

## **Chapter 1 References**

Akdogan, H. (1999). High moisture food extrusion. *International Journal of Food Science and Technology*, 34, 195-207.

Asgar, M., Fazilah, A., Huda, N., Bhat, R., Karim, A. (2010). Nonmeat Protein Alternatives as Meat Extenders and Meat Analogs. *Comprehensive Reviews in Food Science and Food Safety, Institute of Food Technologists*, 9, 513-529.

Dyett, P., Sabate, J., Haddad, E., Rajaram, S., Shavlik, D. (2013). Vegan lifestyle behaviors. An exploration of congruence with health-related beliefs and assessed health indices. *Appetite*, 67, 119-124.

Kirk, P., Patterson, R., Lampe, J. (1999). Development of a soy food frequency questionnaire to estimate isoflavone consumption in US adults. *Journal of the American Dietetic Association*, (99)5, 558-563.

Poblaciones, M., Lopez-Bellido, L., Lopez-Bellido, R. (2009). Field estimation of technological bread-making quality in wheat. *Field Crops Research*, 112, 253-259

Yang, F., Jorgensen, A., Li, H., Sondergaard, I., Finnie, C., Svensson, B., Jiang, D., Wollenweber, B., Jacobsen, S. (2011). Implications of high-temperature events and water

deficits on protein profiles in wheat (*Triticum aestivum* L. Cv. Vinjett) grain. *Proteomics Journal*, 11, 1684-1695.

## **Chapter 2: Understanding Properties of Wheat Gluten and the Mechanism of Texturization – Lab Scale Studies**

### **1. Introduction**

The consumption of vegetable proteins as a food product has been increasing over the years because of animal diseases, global shortage of animal protein, and economic reasons (Asgar et al, 2010). Perhaps the most common form of vegetable proteins being consumed is textured vegetable proteins (TVP). TVP is a vegetable protein that has a meat like texture and taste after processing. TVP can be made from several grain proteins, with wheat gluten being one of the primary grains used.

Another factor affecting vegetable protein consumption is lifestyle choice, which is especially true for consumers in the United States. In a study where 100 vegans were polled, the majority answered health reasons as the major factor why they chose a vegan lifestyle (Dyett et al., 2013). This may be because foods containing vegetable proteins lead to a reduced risk from colon, breast, and prostate cancers (Kirk et al., 1999).

Gluten is a unique protein and plays an important role in determining the properties of bread dough. Water absorption, viscosity, and elasticity are just a few of the properties wheat gluten offers. Traditionally, gluten proteins have been classified as albumin, globulin, gliadin, and glutenin. All of these components exist as either monomers or as oligo- and polymers, which are linked by inter or intra-chain disulfide bonds (Wrigley and Bietz, 1988). Gliadin and glutenin have been found to directly impact the texture of an assortment of products, and a proper mixture of gliadin and

glutenin is very important to the viscoelastic properties of the gluten itself and also on the final product (Wieser, 2007).

While the gluten structure is primarily maintained by inter and intra-chain disulfide bonds, which are covalent bonds, non-covalent bonds do exist in gluten. Hydrogen bonds, hydrophobic bonds, and ionic bonds overlay the covalent disulfide bonds, contributing to the gluten structure. The non-covalent bonds do not offer the same strength as covalent bonds, but they are important when gluten goes through a mixing or shearing action (Wieser et al., 2006).

The extrusion process has proven to be very effective in the manufacturing of TVP. Because of the high shear, high temperature nature of an extrusion system, more efficient taste modifications and unique texture developments have been made possible when extruding vegetable protein (Akdogan, 1999). The structural development in regards to TVP is focused around texturization. Vegetable protein texturization means the extrudate will have a fibrous, meat like structure, similar to a piece of chicken, beef, or pork. While the extrusion process is a well established method for altering the structure of cereal polymers, there are still large amounts of inconsistencies between ingredients. On a lot to lot basis, the chemical composition and overall properties of an ingredient will change, usually due to environmental factors (Pablaciones et al, 2009). Yang et al. (2011) suggests stress events during growing play largely into gluten quality.

As stated above, there are inconsistencies between ingredient quality on a lot to lot basis. For this research, two lots of gluten were sourced that were of two different qualities. One gluten was of a superior quality and was known to texturize very well at extrusion. Very well texturizing gluten will result in a very fibrous structure that meets

certain final product standards set by the manufacturer. These standards include water absorption rate, hydration time, and TVP integrity. Poor texturizing gluten was also studied. This gluten did not meet final product quality standards and resulted in what the manufacture considered waste.

To counter these differences in gluten quality, processing aids are often added. Reducing agents like metasodium bisulfite and L-cysteine are used to aid in disulfide bond break down. As stated earlier, disulfide cross-linkages contribute greatly to gluten structure. Streker et al., (1995) and Ledward and Tester (1994) have reported that protein polymerization is driven by protein cross-linking during the extrusion process. This is due to the role of sulfhydryl groups having the potential to undergo disulfide – sulfhydryl interchanges (Li and Lee, 1998). This involves the cleavage and re-formation of disulfide bonds by internal components, like the protein itself, or external components (Dong and Hosney, 1995) like the before mentioned metasodium bisulfite or L-cysteine. It is believed that by adding L-cysteine to the gluten, there will be a higher potential for reactions with existing disulfide bonds due to the higher amount of sulfhydryl groups L-cysteine brings. On the other hand, metasodium bisulfite will introduce an excess of disulfide molecules to the gluten, creating a higher potential for reactions with the sulfhydryl groups native to the gluten.

Another processing aid commonly used is tetrasodium pyrophosphate. While not a reducing agent, phosphates will adjust the pH of the gluten. Mejri et al. (2005) found that a pH push to a more basic environment increased the solubility of partially hydrolyzed gluten. While the research outlined in this paper didn't use partially hydrolyzed gluten, a similar conclusion can be drawn as to the effect of phosphates on

gluten. By increasing the solubility of the gluten by adding phosphate and subjecting it to a high pressure and temperature extrusion system, the gluten polymers are reduced in overall size. This perhaps creates a higher potential for disulfide – sulfhydryl cross-linkages. This research is focused around the previously mentioned chemical additives.

## **2. Materials and Methods**

### **2.1. Gluten characterization**

Both lots of gluten used in this research were donated by MGP Ingredients (Atchison, KS). The “Superior” gluten is known to extrude very well without the aid of processing agents. The “Inferior” gluten, however, requires the addition of a processing aid to create a good texturized product. There were 4 treatments for each gluten type, a control treatment with no chemical additives, tetrasodium pyrophosphate at 3.00%, cysteine at 0.18%, sulfite at 0.18%. The inclusion rates for the chemical treatments are industry standards.

Protein, fat, ash, and total dietary fiber were found for the two gluten types. This was completed to gain a better understanding of the chemical composition of the glutes. The amino acid profile was completed to investigate primarily the cysteine content of the glutes. As stated previously, cysteine provides free thiol groups that aid in reformation of disulfide bonds. If one gluten type has a higher concentration of cysteine present prior to extrusion, it can be thought that the gluten will form a higher quality TVP.

Crude protein was determined using the AACC International Approved Method 46-30.01: Crude Protein – Combustion Method (1999). This combustion method, where nitrogen is freed by pyrolysis and combustion at high temperatures will determine crude

protein. The nitrogen is quantified by thermal conductivity detection. This method is applicable to all flours, cereal grains, oilseeds, and animal feeds.

The method to determine crude fat was AACC International Approved Method 30-25.01: Crude Fat in Wheat, Corn, and Soy Flour, Feeds, and Mixed Feeds (1999) A dried sample is exhaustively extracted by Soxhlet or continuous extraction, using petroleum ether as the solvent. When the solvent has evaporated the residue is dried to a constant weight at 100° Fahrenheit. The residue is expressed as percent crude fat or ether extract.

AACC International Approved Method 08-01-01 (1999), the basic method used to determine ash was used. A small amount (3-5 grams) of material is placed in an electric muffle furnace and incinerated at 550° - 590° Fahrenheit until light grey ash is obtained or a constant weight is acquired.

Total dietary fiber was found using AACC International Approved Method 32-07.01: Soluble, Insoluble, and Total Dietary Fiber in Foods and Food Products (1999). The insoluble dietary fiber is filtered and the residue is washed with warm distilled water. By combining the filtrate and water washings, a solution can be precipitated with 4 volumes of 95% EtOH to determine the soluble fiber portion of the sample.

Using a method from the Association of Official Analytical Chemists, cysteine, methionine, lysine and nine other amino acids were quantified (JAOAC 70:171-174, 1987). This method requires samples be hydrolyzed by 6 N HCL for 4 hours at 145°C. The amino acids were then determined by cation exchange chromatography in a Beckman 6300 amino acid analyzer (Beckman Instruments, San Ramon, CA).



## 2.2. Gluten physical properties

### 2.2.1: Gluten Index

Gluten index and wet gluten percent results were derived from the same test. Wet gluten percent did not result in useful data (data not shown), because the test procedure was designed to use wheat flour, hence the weight of the gluten that was isolated and the amount of water held by that gluten cannot be separated. However, the gluten index data was useful. Using the American Association of Cereal Chemists International (AACCI) Approved Method 38-12.02, the glutes were hydrated to form a dough and then placed on a special sieve and centrifuged. The gluten index is the “ratio of the wet gluten remaining on the sieve after centrifugation to the total wet gluten,” (AACC Method 38-12.02). When a substantial amount of wet gluten remains on the sieve, this translates as a more cohesive, stronger gluten.

### 2.2.2: Compression Test

Sufficient water was added to the gluten to form a continuous mass. After the dough was formed, the gluten was pressed between two metal plates with 5 kilograms of force placed on top so as to create a disk. After pressing, the gluten disk was removed and a 25 millimeter diameter section was cut out of the disk. The new gluten disks were positioned between two plates using a TA.XT2 Texture Analyzer (Texture Technologies, Scarsdale, NY) and pressed. The trigger force was 10 grams and compression was at 10 millimeters per second to reach a constant force of 100 grams. Once that force of 100 grams was maintained for 45 seconds, the distance the dough was compressed was measured by extrapolating the slope of the line back to the beginning of the test. Compression distance is directly related to the strength of the gluten. If a gluten is strong, it will offer more resistance, resulting in only a short distance needed until the 100 gram

constant force is reached. Weaker glutens will have less resistance, translating to a greater probe travel distance (Miller and Hosney, 1996).

### 2.2.3: Phase Transition Analysis

Prior to the addition of the chemical additives, the glutens were hydrated to a 14% (wet basis) moisture content. This was done by determining the amount of water needed to reach 14% (wet basis) moisture content and spraying with a spray bottle the amount of water required on the gluten as it was mixed in a Hobart table top mixer (Troy, OH). Previous experience was used to determine using 14% (wet basis) moisture content. After hydration, the chemical additives were added at the previously stated inclusion rates and mixed in the same Hobart table top mixer for 5 minutes to ensure a homogenous mix.

The 14% m.c. gluten samples, were analyzed on a Phase Transition Analyzer (PTA) (Wenger Manufacturing., Sabetha, KS), to determine softening and flow temperatures ( $T_s$  and  $T_f$ , respectively). Softening and flow temperatures are a measure of polymer deformation and flow behavior when under conditions similar to extrusion. The PTA utilizes pressure and heat to achieve softening and flow, so it is very similar to extrusion, but it does not impart any mechanical energy to the sample.

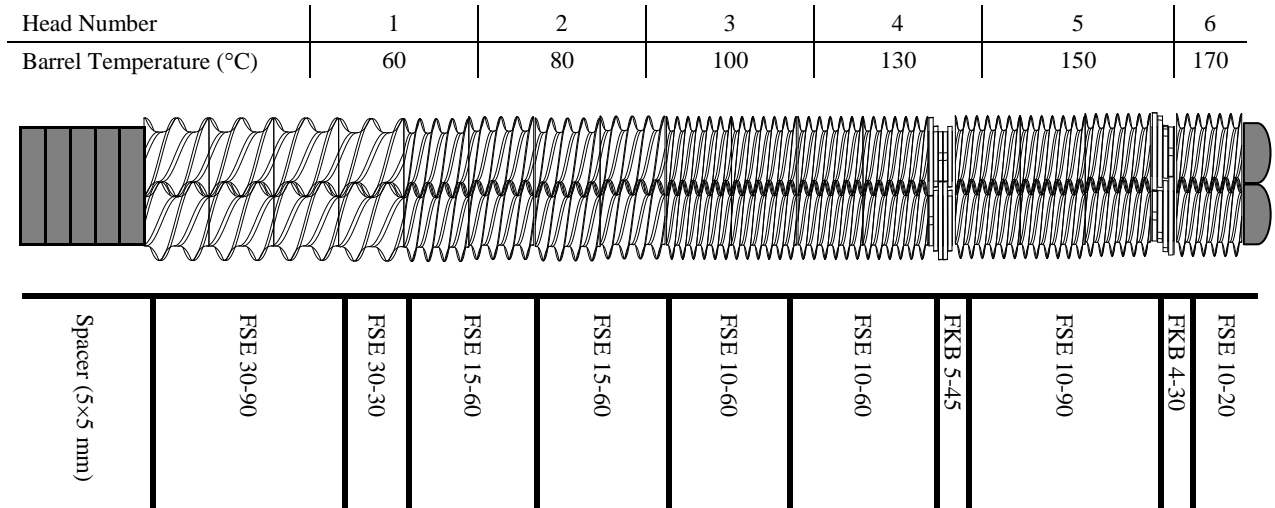
A 2-g sample was loaded into the PTA chamber with a closed die underneath. An initial compression of 12 MPa was applied for 15 seconds. The pressure was then fixed at 10 MPa and the sample was heated at 8°C/min, with a starting temperature of 25°C and  $T_s$  was obtained from the mid-point between the onset and end of softening. After the softening period, or when the material could not be compressed any further, the closed die was replaced with a 2 mm capillary die and heating was continued at the same rate and operating pressure.  $T_f$  was the temperature at which the material started to flow

through the capillary and was identified by a steep increase in displacement. (Karkle et al, 2012)

### 2.3. Blend preparation and extrusion processing

Prior to extrusion, the same procedure was used to hydrate the gluten as was used for hydration for PTA experimentation. However, for extrusion, three moisture levels were used. Prior to the addition of the chemical additives, the glutes were hydrated to three different moisture levels; 14%, 20% and 25% (all wet basis), by determining the amount of water needed to reach the three levels and spraying that amount of water on the gluten as it was mixed in a Hobart table top mixer (Troy, OH). These three moisture levels were chosen to give a wide range of responses at extrusion. After hydration, the chemical additives were mixed into 1.5 kg of the each gluten type in the same Hobart table top mixer for 5 minutes to ensure a homogenous mix.

Extrusion processing was carried out on a laboratory scale extruder (Micro-18, American-Leistritz, New Jersey). The screw profile (Figure 1) was designed to impart a great deal of shear to the samples. A 3.8 millimeter die opening was used.



FSE: forward conveying screw element (all double flighted, intermeshing)

FKB: forward kneading block; RKB: reverse kneading block

Numbers on screw elements: pitch (mm)-element length (mm)

Numbers on kneading blocks: number of disks-total block length (mm)-staggering angle of disks

Figure 2.1: Screw Configuration – Micro-18 Twin Co-Rotating Screw Extruder with accompanying head temperature profile.

Process conditions were kept constant through out the experiment with a feed rate of 3.15 kg/hr and a screw speed of 275 RPM.

The specific mechanical energy (SME) for each treatment was calculated using the following equation:

$$SME (kJ/kg) = \frac{\left( \frac{\tau - \tau_0}{100} \right) \times \frac{N}{N_r} \times P_r}{\dot{m}}$$

where  $\tau$  is the % torque,  $\tau_0$  is the no-load torque (17%),  $N$  is the measured screw speed in RPM,  $N_r$  is the rated screw speed (500 rpm),  $P_r$  is the rated motor power (2.2 kW) and  $\dot{m}$  is mass flow rate in kg/s (Zhu et. al., 2010).

#### 2.4. Statistical analysis

Data were analyzed using the GLM procedure in SAS (Cory, NY). The GLM procedure uses the method of least squares to fit general linear models. By doing so, 2-way and 3-way ANOVA can be completed; the level of significance was a p-value of 0.05. Interactions were examined to measure for significance between the gluten type, hydration level and the chemical treatment.

### **3. Results and discussion**

#### 3.1. Proximate analysis and amino acid profile

	<b>Superior Gluten</b>	<b>Inferior Gluten</b>
<b>Protein (%)</b>	86.26	77.26
<b>Carbohydrates (%)</b>	7.66	17.04
<b>Fat (%)</b>	1.31	1.3
<b>Ash (%)</b>	0.82	1.06
<b>Total Dietary Fiber (%)</b>	3.95	3.34
<b>Total</b>	100	100

Table 2.1: Proximate Analysis of Superior and Inferior Gluten Types

As Table 1 displays, the superior gluten had a higher protein content (86.26%) than the inferior gluten (77.26%). This is an early indication there may be a difference in the quality of these two glutes. Because of the superior glutes higher protein content, it can be thought it will extrude to make a higher quality TVP.

<b>Amino Acid</b>	<b>Superior Gluten</b>	<b>Inferior Gluten</b>
<b>Aspartic Acid</b>	2.92	2.56
<b>Threonine</b>	1.91	1.68
<b>Glutamic Acid</b>	28.93	26.79
<b>Proline</b>	9.76	9.20
<b>Glycine</b>	2.95	2.61
<b>Alanine</b>	2.24	1.99
<b>Cysteine</b>	1.59	1.41
<b>Valine</b>	3.59	3.25
<b>Methionine</b>	1.39	1.22
<b>Isoleucine</b>	3.15	2.90
<b>Leucine</b>	5.84	5.29
<b>Lysine</b>	1.56	1.29
<b>Total</b>	65.83	60.19

Table 2.2: Amino Acid Profile of Superior and Inferior Gluten types

As expected, the superior gluten had a higher amino acid amount (65.83 grams per 100 grams of gluten) than the inferior gluten (60.19 grams per 100 grams of gluten). This is because the higher protein content of the superior gluten (86.26%) will bring in more total amino acids.

## 3.2. Rheological properties of wheat gluten

### 3.2.1: Gluten Index

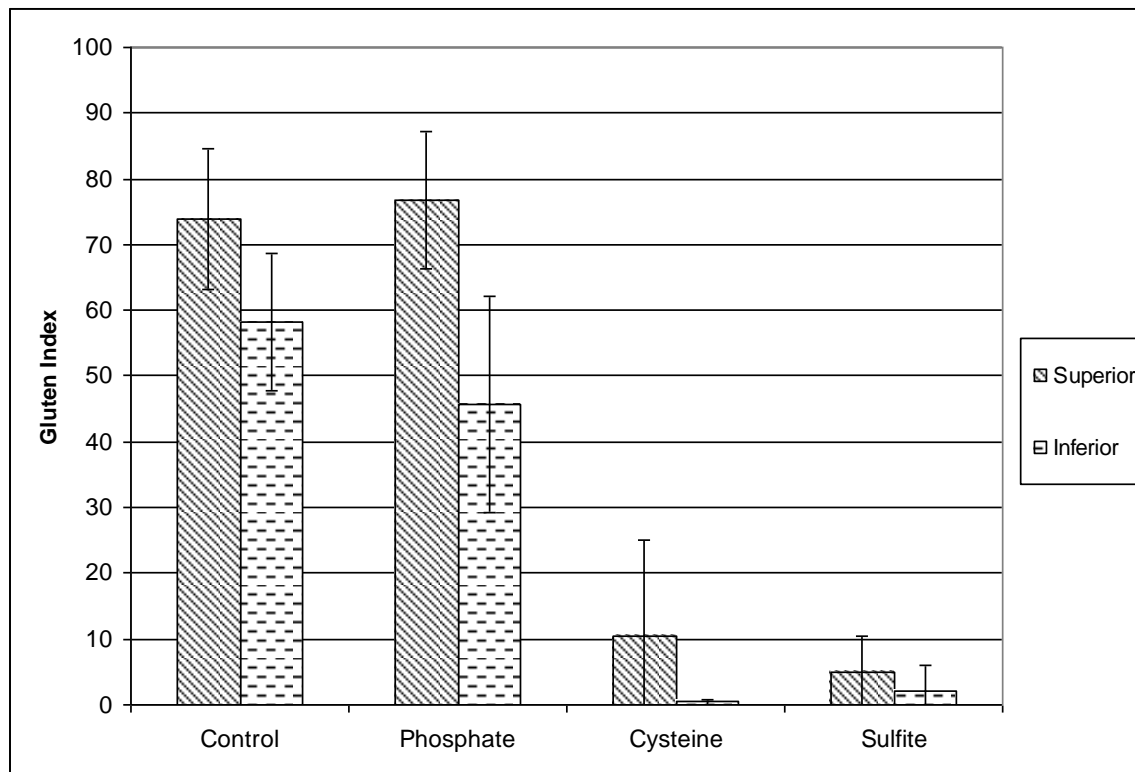


Figure 2.2: Gluten Index Results for Superior and Inferior glutes (Control and Processing Aids).

As Figure 2 shows, the strength of the raw vital gluten is significantly reduced by sulfite and cysteine for both the Superior and Inferior gluten types. This was expected, given the reducing action of cysteine and sulfite. With no additives, the inferior gluten was weaker than the superior gluten. This makes sense because the inferior gluten is known to have difficulties at extrusion, and those differences are because it is poorer quality gluten. Interestingly, the presence of phosphate reduced the strength of the inferior gluten but not the superior gluten. Statistically significant differences ( $p$ -value  $< 0.0001$ ) were observed between the gluten types (superior vs. inferior) and the chemical

treatments (control, phosphate, cysteine, sulfite). Furthermore, the interaction between gluten type and chemical treatment was also found to be significant, indicating there are strong trends when comparing the factors of chemical treatment to the gluten type.

### 3.2.2: Compression Test

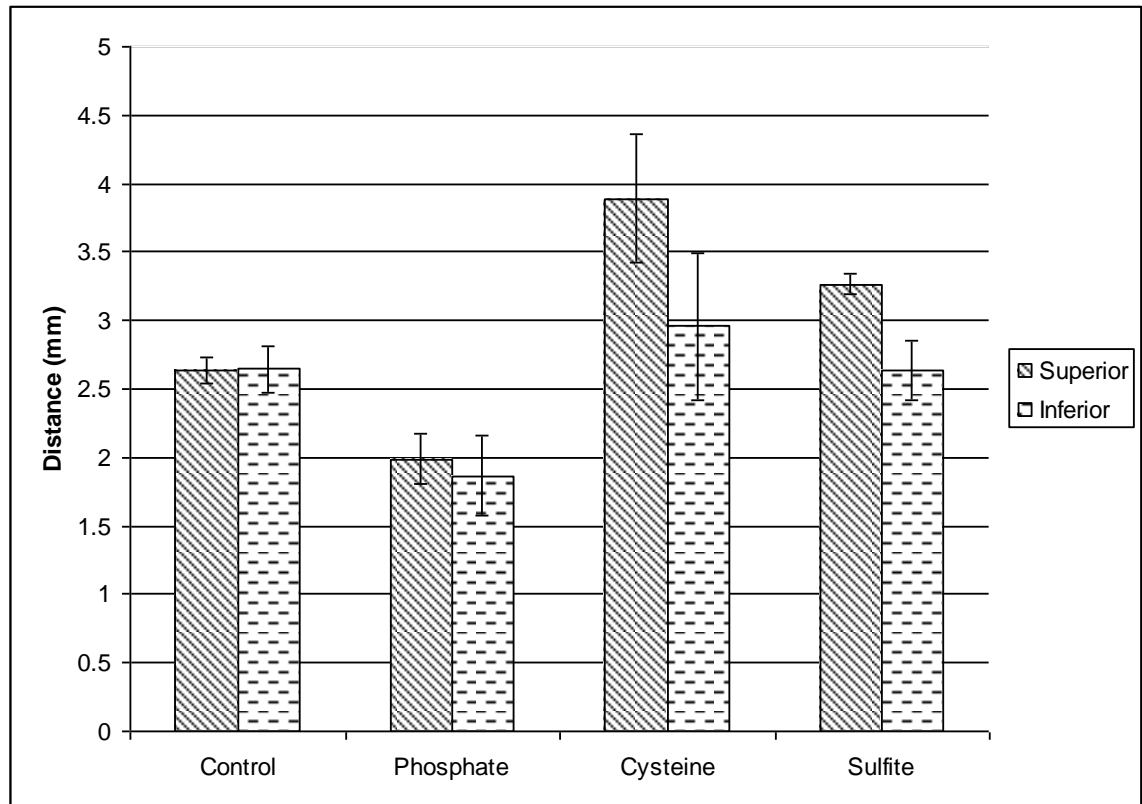


Figure 2.3: Compression Tests of Superior and Inferior Glutes With and Without Processing Aids.

As explained previously, stronger and more elastic gluten offers more resistance to compression while weaker, less elastic glutes will flow more readily under the force of the probe allowing the probe to travel a longer distance before the constant force is reached. The results (Figure 2.5.2) are consistent with this model. Sulfite and cysteine presence resulted in greater probe travel than was seen for control and phosphate treatments. This can be interpreted as the addition of these two chemicals resulted in both



gluten types becoming weaker. It appears that the addition of phosphate made both the superior and inferior gluten less viscous/more elastic. This may be attributed to phosphate's ability to increase the water holding capacity of gluten and making for a more entangled gluten matrix. Statistically significant differences ( $p$ -value  $< 0.0001$ ) were observed between the gluten types (superior vs. inferior) and the chemical treatments (control, phosphate, cysteine, sulfite). However, the interaction between gluten type and chemical treatment was found to be not significant ( $p$ -value  $> 0.05$ ), indicating there were no trends for those interactions.

### 3.2.3: Phase Transition Analysis

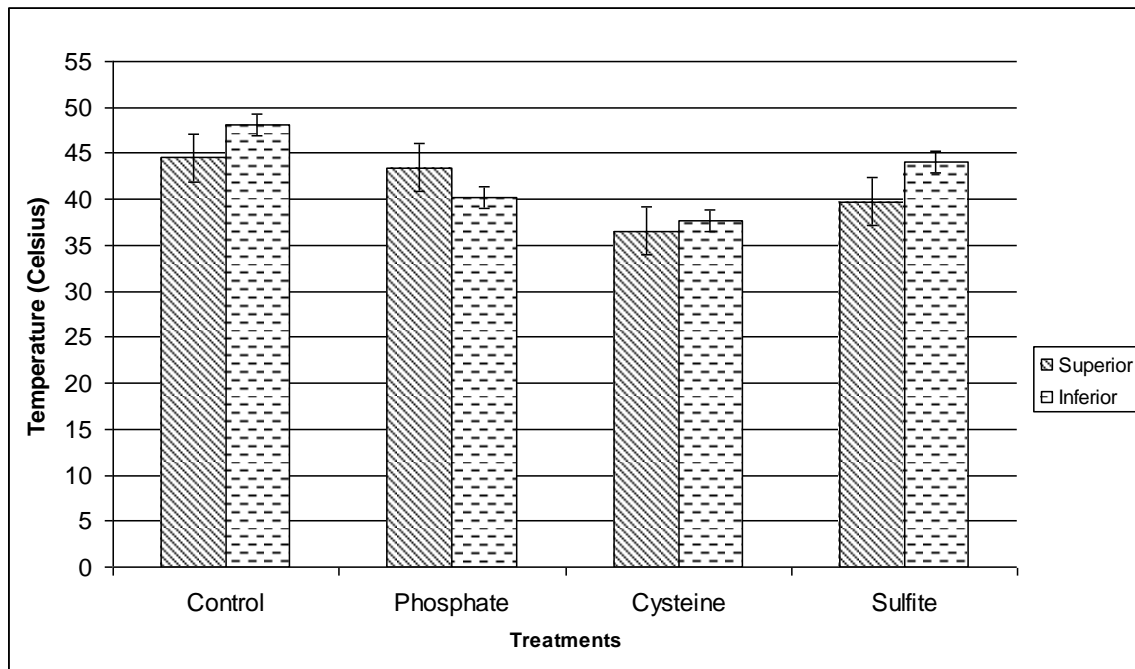


Figure 2.4: Average Softening Temperatures of Superior and Inferior Gluten (Control, 3.0% Phosphate, 0.18% Cysteine, 0.18% Sulfite).

Processing aids, especially the sulfite and cysteine, resulted in a decrease in the average softening temperature. The phosphate treatment displayed very little decrease when compared to the control treatments (Control, Superior – 44.5° C and 3.0%

Phosphate, Superior – 44.45° C). Sulfite and cysteine are reducing agents, so a decrease in average softening temperature wasn't surprising. When heat and pressure were added during the PTA test, disulfide bonds and hydrogen bonds were broken easier with the presence of cysteine and sulfite, resulting in a lower softening and thermal flow. For the control treatment, it is seen that the inferior gluten had a slightly higher thermal softening temperature (44.5° C for superior as opposed to 48.15° C for inferior). This is possibly due to the lower quality of the inferior gluten where more thermal energy and pressure is needed to reach not only thermal softening, but also thermal melting, which was also observed.

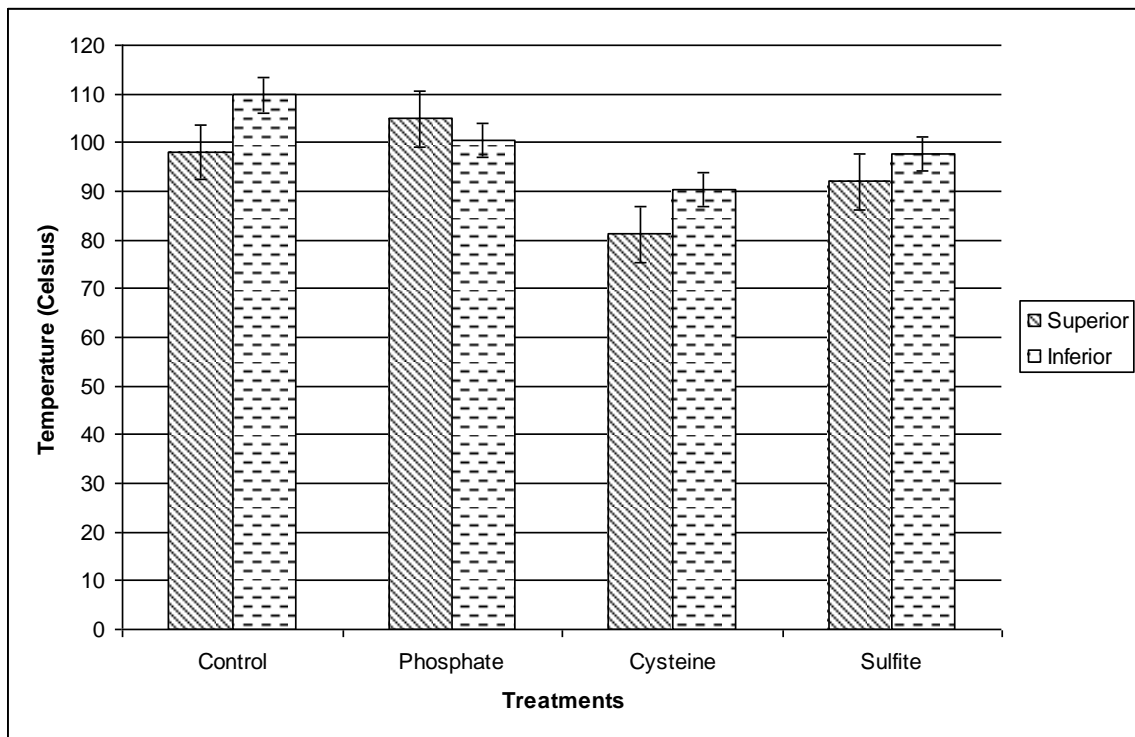


Figure 2.5: Thermal Flow Temperatures of Superior and Inferior Glutens (Control, 3.0% Phosphate, 0.18% Cysteine, 0.18% Sulfite).

Similar results were observed for thermal flow temperature (Figure 5) as for the average softening temperatures. As expected, the sulfite and cysteine treatments experienced a decrease in flow temperature when compared to the control treatments of the superior and inferior gluten. Another similarity was observed between the average softening and flow temperature. For the control, sulfite, and cysteine treatments, the superior gluten had a lower average softening and flow temperature. However, the phosphate treatments displayed the opposite of this; the superior gluten had a higher average softening and flow temperature than that of the inferior gluten.

### 3.3. Extrusion processing

Specific Mechanical Energy (SME) is calculated to show how much mechanical energy is being imparted on a material during the extrusion process.

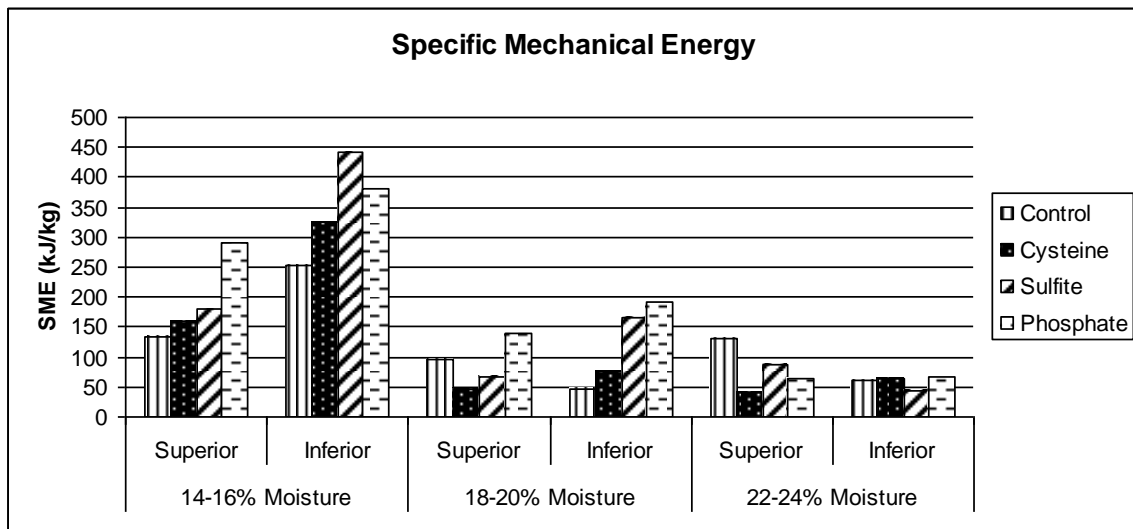


Figure 2.6: SME During the Extrusion of Superior and Inferior Glutens.

Typically during extrusion, a lower SME is encountered when the extrudate has a high in-barrel moisture (30-40% w.b.). This is because water acts as a plasticizer, lowering the viscosity of the melt and making it easier for the extruder to force the melt through the die. This was true in this work; (Figure 6) where the 22-24% w.b. moisture

gluten treatments had lower SME's. Conversely, for the 18-20% w.b. moisture and 14-16% w.b. moisture treatments, a substantial response was observed because the melt viscosity was increased to a point where differences between treatments were truly observed. As displayed by the Figure 6, an increase in SME is observed for both gluten types when observing each of the treatments; control, cysteine, sulfite, and phosphate (this is the order in which the treatments were processed). When compared to the gluten quality tests (Gluten Index and Compression Tests), it could be theorized that with the addition of cysteine and sulfite, a decrease in SME would be experienced. However, this was not the case. The reasoning behind this is that in the presence of reducing agents (cysteine and sulfite), disulfide molecules became readily available for cross-linkages with the gluten protein strands. As these disulfide bonds reformed, the viscosity of the melt increased, causing the extruder to work harder to force material through the die opening. It is hypothesized that like reducing agents, the phosphate is forming cross-linkages with protein molecules. Phosphate is commonly used to adjust the pH of gluten, and by creating an optimal pH range, disulfide bond reduction and sulfhydryl group oxidation to reform disulfide bonds has a higher potential. By creating a higher potential of disulfide reformation, an increase in SME is observed.

#### 3.4. Post-Extrusion Phase Transition Analysis

This step was done to analyze if there were any differences in thermal softening and melt when raw vital gluten was compared to finished extrudate. It was thought that the complete disulfide reduction may have not been achieved during extrusion. However, it appears when looking at the below data that the extrusion process may have completely reduced and reformed disulfide bonds in the gluten, implying texturization did occur.

However, the physical appearance of the extrudate was not that of a textured vegetable protein; no fibrous structure. It is believed that the extruder did texturize the glutes, but since the Micro-18 has a low volume to surface area ratio, both glutes went past texturization and on to protein deformation.

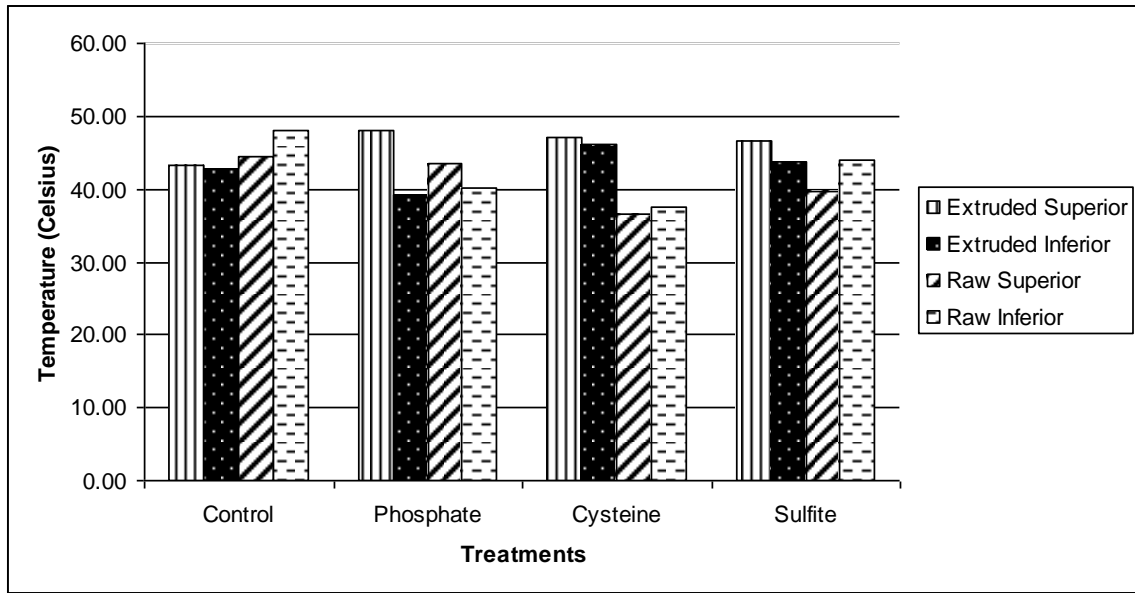


Figure 2.7: Extruded and Un-Extruded Glutens; Thermal Softening Temperature

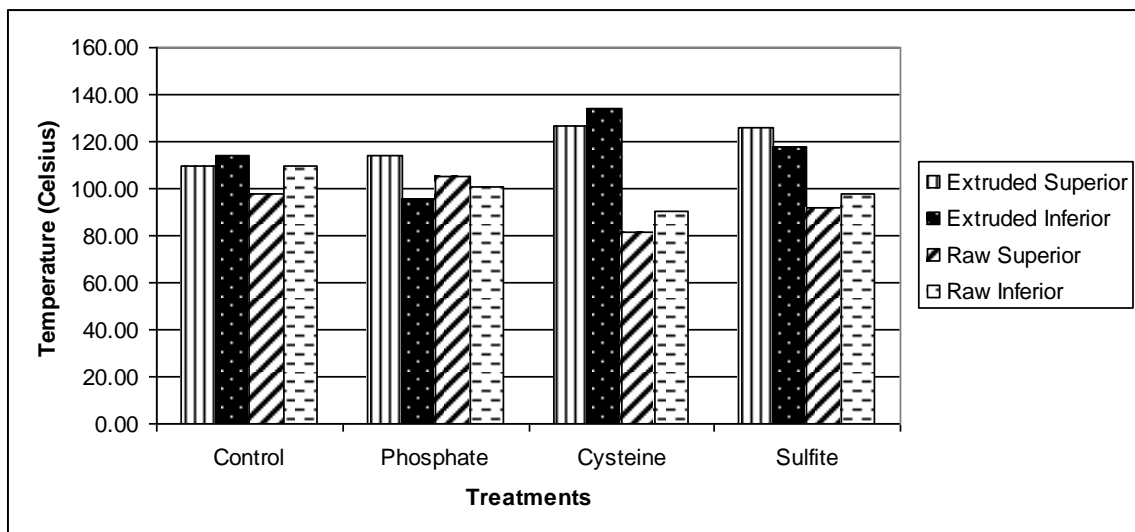


Figure 2.8: Extruded and Un-Extruded Glutens; Thermal Melting Temperature.

It is noteworthy to mention that the moisture these PTA's were completed at 13-16% w.b.; this moisture content provided the best response during testing. Both the superior and inferior glutes have similar softening temperatures when looking at the extruded and raw samples, with the exception of the cysteine treatment. For the cysteine treatment, the extruded samples, both superior and inferior, have a thermal softening that are  $\sim 10^{\circ}\text{C}$  higher than the raw gluten samples. This is expounded when looking at the thermal melt results. There is nearly a  $50^{\circ}\text{C}$  difference between the extruded glutes and the raw glutes. Similarly, the control and sulfite treatments also have a higher thermal melt for the extruded samples. The only exception is the phosphate treatment. The thermal melt temperature for the extruded samples is very similar to the thermal melt temperature of the raw glutes.

The possible explanation for the lower thermal melt temperature for the raw glutes compared to the extruded treatments, especially for the cysteine and sulfite treatments, is the complete reduction of the disulfide bonds. During extrusion, the disulfide bonds are reduced and the newly available bonds are used to cross link the elongated protein strands inside the extruder barrel. Since the functionality of the sulfite and cysteine is fully taken advantage of, the benefits they offer in lowering thermal softening and melt will not be observed when testing for those properties after the extrusion process has been utilized.

The reason differences are not seen between the extruded phosphate and raw phosphate treatments is due to phosphate not being a reducing agent. Instead, phosphate will increase the water holding capacity of protein.

#### **4. Conclusion**

Pre-extrusion testing confirmed the difference in quality of the two types of gluten. This is especially true when looking at the Gluten Index data. The inferior gluten, which required processing aids, was much weaker than the superior gluten when looking at the control treatments. The weakness of the inferior gluten was expounded when cysteine and sulfite is added. When phosphate was added, very similar results were observed as for the control treatments.

The initial hypotheses were confirmed with this research. Cysteine and sulfite weaken the gluten structure by reducing disulfide bonds. This was apparent with PTA testing. Both types of gluten saw a reduction in thermal softening and thermal melting with these two chemicals present. However, since the PTA does not add mechanical energy, no texturization was achieved. When mechanical energy is added, reduction fully occurs and reforms disulfide bonds to complete texturization. While not all treatments at extrusion appeared to be texturized, it is believed that due to the high volume to surface area ratio of the Micro-18 twin screw extruder not only texturized the gluten, but also caused the glutes to reach deformation temperatures. The phosphate treatments of both gluten types did, however, have a textured form. This is due to the unique water holding properties of phosphate. The water in the fine meal was used more efficiently by remaining available to lower the energy requirements to break disulfide bonds.

While cysteine and sulfite do lower the melt viscosity of gluten, an increase in SME was observed. This increase in SME is also observed for the phosphate treatments. This is due to the extrusion processes unique capabilities to not only break down disulfide bonds, but to also use those intact bonds to cross link the loose protein strands. The

reformation of these bonds will make a gluten melt form a defined structure, and conversely, make the extruder work harder to push the melt through the die opening.



## **Chapter 2 References**

AACC International. Approved Methods of Analysis, 11th Ed. Method 08-01.01. Ash – Basic Method. Approved November 3, 1999. AACC International, St. Paul, MN, U.S.A. <http://dx.doi.org/10.1094/AACCIntMethod-08-01.01>.

AACC International. Approved Methods of Analysis, 11th Ed. Method 46-30.01. Crude Protein – Combustion Method. Approved November, 3 1999. AACC International, St. Paul, MN, U.S.A. <http://dx.doi.org/10.1094/AACCIntMethod-46-30.01>.

AACC International. Approved Methods of Analysis, 11th Ed. Method 30-25.01. Crude Fat in Wheat, Corn, Soy Flour, Feeds, and Mixed Feeds. Approved November 3, 1999. AACC International, St. Paul, MN, U.S.A. <http://dx.doi.org/10.1094/AACCIntMethod-30-25.01>.

AACC International. Approved Methods of Analysis, 11th Ed. Method 32-07.01. Soluble, Insoluble, and Total Dietary Fiber in Foods and Food Products. Approved November 3, 1999. AACC International, St. Paul, MN, U.S.A. <http://dx.doi.org/10.1094/AACCIntMethod-32-07.01>.

AACC International. Approved Methods of Analysis, 11th Ed. Method 38-12.02. Wet Gluten, Dry Gluten, Water-Binding Capacity, and Gluten Index. Approved November 8,

2000. AACC International, St. Paul, MN, U.S.A.

<http://dx.doi.org/10.1094/AACCIIntMethod-38-12.02>.

Akdogan, H. (1999). High moisture food extrusion. *International Journal of Food Science and Technology*, 34, 195-207.

Asgar, M., Fazilah, A., Huda, N., Bhat, R., Karim, A. (2010). Nonmeat Protein Alternatives as Meat Extenders and Meat Analogs. *Comprehensive Reviews in Food Science and Food Safety, Institute of Food Technologists*, 9, 513-529.

Dong, W., Hosney, R. C., (1995). Effects of certain breakmaking oxidants and reducing agents on dough rheological properties. *Cereal Chemistry*. Vol. 72, 58-64.

Dyett, P., Sabate, J., Haddad, E., Rajaram, S., Shavlik, D. (2013). Vegan lifestyle behaviors. An exploration of congruence with health-related beliefs and assessed health indices. *Appetite*, 67, 119-124.

Gehrke, C.W., Rexroad, P.R., Schisla, R.M., Absheer, J.S., Zumwalt, R.W., (1987) Quatative Analysis of Cystine, Methionine, Lysine, and Nine Other Amino Acids by a Single Oxidation-4 h Hydrolysis Method, *J. Assoc. Off. Anal. Chem.* 70:717-174.

Karkle, E., Alavi, S., Dogan, H. (2012). Cellular architecture and its relationship with mechanical properties in expanded extrudates containing apple pomace. *Food Research Interational*, 46, 10-21.

Kirk, P., Patterson, R., Lampe, J. (1999). Development of a soy food frequency questionnaire to estimate isoflavone consumption in US adults. *Journal of the American Dietetic Association*, (99)5, 558-563.

Ledward, D. A., Tester, R. F., (1994). Molecular transformations of proteinaceous foods during extrusion processing. *Trends in Food Science Technology*. Vol. 5, 117-120.

Li, M., Lee, T.C., (1998). Effect of cysteine on the molecular weight distribution and the disulfide cross-link of wheat flour proteins in extrudates. *Journal of Agricultural and Food Chemistry*. Vol. 46, 846-853.

Mejri, M., Roge, B., BenSouissi, A., Michels, F., Mathlouthi, M., (2005). Effects of some additives on wheat gluten solubility : A structural approach. *Journal of Food Chemistry*. Vol. 92, 7-15.

Miller, R., Hoseney, R. (1996). Evaluating Vital Wheat Gluten Quality. *Cereal Foods World*, (41)5, 412-416.

Poblaciones, M., Lopez-Bellido, L., Lopez-Bellido, R. (2009). Field estimation of technological bread-making quality in wheat. *Field Crops Research*, 112, 253-259.

Streker, T. D., Cavalieri, R. P., Zollars, R. L., Pomeranz, Y. (1995) Polymerization and mechanical degradation kinetics of gluten and glutenin at extruder melt-section temperatures and shear rates. *Journal of Food Science*. Vol. 60, 532-537, 557.

Weiser, H., Bushuk, W., MacRitchie, F., (2006). The polymeric glutenins. In: Wrigley, C., Bekes, F., Bushuk, W., (Eds.), *Gliadin and Glutenin: the Unique Balance of Wheat Quality*. St. Paul American Association of Cereal Chemists, 213-240.

Weiser, H., (2007). *Wheat Chemistry*. *Journal of Food Microbiology*.

Wrigley, C. W., Bietz, J. A., (1988), Proteins and amino acids. In: Pomeranz, Y. (Ed), *Wheat – Chemistry and Technology*, vol. 1. St. Paul, American Association of Cereal Chemists, 159-275.

Yang, F., Jorgensen, A., Li, H., Sondergaard, I., Finnie, C., Svensson, B., Jiang, D., Wollenweber, B., Jacobsen, S. (2001). Implications of high-temperature events and water deficits on protein profiles in wheat (*Triticum aestivum* L. Cv. Vinjett) grain. *Proteomics Journal*, 11, 1684-1695.

Zhu, L.J., Shukri, R., de Mesa-Stonestreet, N.J., Alavi, S., Dogan, H., Shi, Y.C., (2010) Mechanical and microstructural properties of soy protein – high amylose corn starch

extrudates in relation to physiochemical changes of starch during extrusion. *Journal of Food Engineering*, 100, 2, 232-238.

## **Chapter 3:** **Relationship between Properties of Wheat Gluten and Texturized Vegetable Protein processed using High Shear Extrusion**

### **1: Introduction:**

Consumption of vegetable proteins as a food product has been increasing over the years because of animal diseases, global shortage of animal protein, and economic reasons (Asgar et al, 2010). Texturized vegetable protein, or TVP, is perhaps the most common form of vegetable proteins being consumed. TVP is a vegetable protein that is made via an extrusion process. This process will result in the TVP having a meat like texture. After processing, meat flavors can be introduced to the TVP to give it the desired taste. TVP can be made from several grain proteins, with wheat gluten, the focus of this research, being one of the primary grains used.

Extrusion is a continuous process where a material will undergo mixing, kneading, cooking, and shaping. It is not a new process, and is widely used in the food industry. This is due to the wide range of products that can be made. Extrusion is also a low cost process that is very energy efficient (Harper, 1981). For creating TVP, a twin screw extruder is primarily used. This is because more efficient taste modifications and texture developments have been made possible with twin screw extrusion (Akdogan, 1999). Texturization results from the shear an extrusion system will impart on the proteins, whether it is soy protein or wheat gluten. By introducing shear, high temperatures, and high pressure, the gluten polymers are disrupted (Akdogan, 1999). It is

even theorized that the extrusion process will create covalent cross-linkages between protein polymers (Areas, 1992). This is supported by Levine and Slade (1990). They suggest that by increasing the mobility of the gluten molecules, which occurs at high temperatures, a gluten protein will create intermolecular disulfide covalent bonds. This makes the extrusion process an ideal system to texturize protein because high temperatures and shear will be introduced. This will create the three-dimensional fibrous structure that is desired when making a TVP.

As material travels down the extruder barrel as is introduced to heat, shear, and moisture, the disulfide bonds that connect the protein polymers are broken and are simultaneously being re-linked along the length of the extruder barrel (Shimada et al, 1988). Once the material leaves the die and encounters atmospheric pressures, the gluten matrix will be further disrupted by the flashing off of superheated water vapor. This can directly affect the porosity of the TVP, which will affect its hydration rate and time, two key physical characteristics.

One factor that may impact gluten quality is the environment in which it was grown. More often than not, the wheat will be under some form of stress prior to harvest; water deficits and high temperatures for example. Yang et al. (2011) found that individual protein fractions of wheat, gliadins and glutenins, were affected by not only the type of stress, but also the length of the stress. This could, in turn, factor into changes in the quality of extruded wheat gluten.

## **2. Material and methods**

### **2.1. Material characterization**

Two lots of gluten were used; one from the U.S. and one from Europe. The U.S. gluten was Heartland 75 (White Energy, Russell KS USA). The European gluten was Drei Hasen Vital Wheat Gluten (Crespel and Deiters, Ibbenburen, Germany). Both types of gluten had a protein content of at least 75%. Each gluten type was analyzed as a control treatment (no chemical additives), tetrasodium pyrophosphate at 3.00%, cysteine treatment at an inclusion rate of 0.18%, and finally, sulfite, also at a 0.18% inclusion rate. These inclusion levels were chosen because they are industry standards. At extrusion, the study was carried out at two in-barrel-moistures (IBM); 32% and 36%. This translates to a 2 x 4 x 2 factorial design.

Protein, fat, ash, and total dietary fiber were found for the two gluten types. This was completed to gain a better understanding of the chemical composition of the glutes. The amino acid profile was completed to investigate primarily the cysteine content of the glutes. As stated previously, cysteine provides free thiol groups that aid in reformation of disulfide bonds. If one gluten type has a higher concentration of cysteine present prior to extrusion, it can be thought that the gluten will form a higher quality TVP.

Crude protein was determined using the AACC International Approved Method 46-30.01: Crude Protein – Combustion Method (1999). This is a combustion method, where nitrogen is freed by pyrolysis and combustion at high temperatures. The nitrogen is quantified by thermal conductivity detection. This method is applicable to all flours, cereal grains, oilseeds, and animal feeds.



The method to determine crude fat was AACC International Approved Method 30-25.01: Crude Fat in Wheat, Corn, and Soy Flour, Feeds, and Mixed Feeds (1999). A dried sample is exhaustively extracted by Soxhlet or continuous extraction, using petroleum ether as the solvent. When the solvent has evaporated the residue is dried to a constant weight at 100° Fahrenheit. The residue is expressed as percent crude fat or ether extract.

AACC International Approved Method 08-01.01 (1999), the basic method used to determine ash was used. A small amount (3-5 grams) of material is placed in an electric muffle furnace and incinerated at 550° - 590° Fahrenheit until light grey ash is obtained or a constant weight is acquired.

Total dietary fiber was found using AACC International Approved Method 32-07.01: Soluble, Insoluble, and Total Dietary Fiber in Foods and Food Products (1999). The insoluble dietary fiber is filtered and the residue is washed with warm distilled water. By combining the filtrate and water washings, a solution can be precipitated with 4 volumes of 95% EtOH to determine the soluble fiber portion of the sample.

Using a method from the Association of Official Analytical Chemists, cysteine, methionine, lysine and nine other amino acids were quantified (JAOAC 70:171-174, 1987). This method requires samples be hydrolyzed by 6 N HCL for 4 hours at 145°C. The amino acids were then determined by cation exchange chromatography in a Beckman 6300 amino acid analyzer (Beckman Instruments, San Ramon, CA).

## 2.2. Formulation and Mixing

<b>Batch</b>	<b>Wheat Gluten</b>	<b>Wheat Flour</b>	<b>Soda Ash</b>	<b>Chemical Additive</b>	<b>Total</b>
<b>Control</b>	76.04%	23.85%	0.11%	0%	100%
<b>Phosphate</b>	76.04%	23.85%	0.11%	3.00%	103%
<b>Cysteine</b>	76.04%	23.85%	0.11%	0.18%	100.18%
<b>Sulfite</b>	76.04%	23.85%	0.11%	0.18%	100.18%

Table 3.1: Wheat Gluten Formulation for Pilot Scale Extrusion

Batch sizes were 200 lbs and all were mixed in a ribbon mixer (Wenger Manufacturing, Sabetha, KS) for 5 minutes to ensure homogeneity.

## 2.3. Gluten Characterization

### 2.3.1: Gluten Index

The Gluten Index and Wet Gluten Percent are the results from the same test. Using the American Association of Cereal Chemists International (AACCI) Approved Method 38-12.02, the glutes were hydrated to form a dough and then placed on a special sieve and centrifuged. The Gluten Index is the “ratio of the wet gluten remaining on the sieve after centrifugation to the total wet gluten,” (AACCI Method 38-12.02). This means that the more gluten remaining on the sieve translates to a more cohesive, stronger gluten.

### 2.3.2: Phase Transition Analysis

The two types of gluten were hydrated to 14% moisture (wet basis) and were analyzed on a Phase Transition Analyzer (PTA) (Wenger Manufacturing., Sabetha, KS), to determine the softening and flow temperatures ( $T_s$  and  $T_f$ , respectively). Softening and flow temperatures are a measure of polymer deformation and flow behavior when under

conditions similar to extrusion. The PTA utilizes pressure and heat to reach softening and flow, so it is very similar to extrusion. However, it does not impart any mechanical energy on the sample.

A 2-g sample was loaded into the chamber with a closed die underneath, and an initial compression of 12 MPa was applied for 15 seconds. The pressure was then fixed at 10 MPa and the sample was heated at 8°C/min, with a starting temperature of 25°C.  $T_s$  was obtained from the mid-point between onset and end of softening. After the softening period, the closed die was replaced with a 2 mm capillary die and heating was continued at the same rate and operating pressure.  $T_f$  was the temperature at which the material started to flow through the capillary and is identified by a steep increase in displacement. (Karkle et al, 2012)

#### 2.4. Extrusion processing

Extrusion processing was carried out on a pilot scale twin-screw extruder (TX-52, Wenger Manufacturing, Sabetha, KS) with a 9 head configuration. The TX-52 is equipped with a differential diameter cylinder preconditioner with a volumetric capacity of 0.056 m<sup>3</sup> (DDC2, Wenger Manufacturing, Sabetha, KS). The preconditioner paddles were set to forward pitch for the first third of the preconditioner, followed by a neutral pitch for the second third, and finally a reverse pitch segment at the preconditioner outlet. The preconditioner speed was set at 350 RPM. Due to differences in the properties of the two gluten types, slightly different processing conditions were used between the two. For the U.S. gluten, a constant feed rate of 100 kg/hr was used. However, to maintain good product quality, the European gluten required a feed rate of 90 kg/hr. Other than this, all of the other processing conditions remained the same.

	Temperature and Screw Profile							
Head Number	2	3	4	5	6	7	8	9
Temperature (° C)	40	40	90	105	120	120	120	120

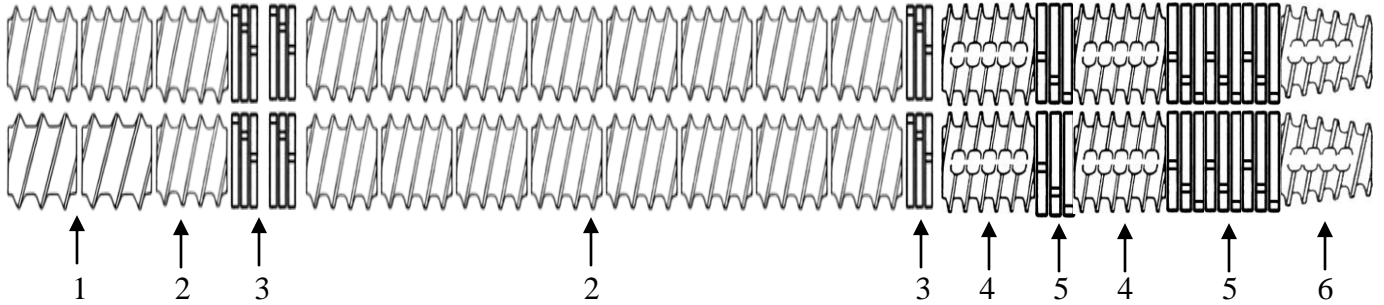


Figure 3.1: Temperature and screw profile for TX-52 Extruder

Number	Corresponding Elements
1	Full Pitch, Forward, 9 unit
2	3/4 Pitch, Forward, 9 unit
3	Kneading Lobe, Forward, 3 unit
4	1/2 Pitch, Forward, Cut Flight, 9 unit
5	Kneading Lobe, Reverse, 3 unit
6	1/2 Pitch Cone, Forward, 9 unit

Table 3.2: Screw Configuration

The screw profile (Figure 1) was designed to impart a high amount of mechanical energy on the wheat gluten melt. A high amount of shear is required to physically break down the gluten polymers and to expose thiol sites to disulfide reformation. Screw speed was held constant at 356 RPM for both types of gluten. Table 2 displays the corresponding element names for each number from Figure 1.

The specific mechanical energy (SME) for each treatment was calculated using the following equation:

$$SME (kJ/kg) = \frac{\left( \frac{\tau - \tau_0}{100} \right) \times \frac{N}{N_r} \times P_r}{\dot{m}}$$

where  $\tau$  is the % torque,  $\tau_0$  is the no-load torque (7%),  $N$  is the measured screw speed in RPM,  $N_r$  is the rated screw speed (336 rpm),  $P_r$  is the rated motor power (22.37 kW) and  $\dot{m}$  is mass flow rate in kg/s (Zhu et. al., 2010).

#### 2.4.1: Die Design

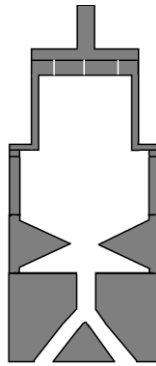


Figure 3.2: Die Configuration

As previously stated, die design can have a drastic impact on the final product characteristics. To create this TVP, a very unique die design was used. From the bottom up, there was a 2-to-1 adaptor. This will direct flow from the two screw shafts into a single flow. Because there is very little open area in this part of the configuration, shear and pressure builds, aiding in gluten depolymerization and in forming new cross-linkages. The next component was a Venturi die that has a 5 millimeter insert. Again, flow is being directed while also creating a pressure gradient. Following the Venturi die, there was 69.85 millimeters of spacers. This creates a longer retention time which allows the melted gluten mass to agglomerate and complete cross-linkages. After this group of spacers, there was a die holder that contained a capacity ring with four 1 millimeter by 14 millimeter slits. This was where product came out of the die, and where product was cut

by a rotating knife. Furthermore, the capacity ring directed the flow to make a 90 degree turn to exit the die. This also aids in final texturization.

## 2.5. Final product analysis

### 2.5.1: Water Absorption Index

The water absorption index testing procedure was adapted from an American Soybean Association technical bulletin (1988) by Kearns et al., Wenger Manufacturing. This test displays the amount of water a TVP will absorb at a set weight of product and set time. Twenty grams of textured wheat gluten was soaked in 100 mL of room temperature water for 20 minutes. After soaking, the hydrated product was drained on a screen for 5 minutes. The final weight was recorded. To calculate the Water Absorption Index, this equation was used:  $(\text{Rehydrated wt.} - \text{Original Wt.}) / \text{Original Wt.}$

### 2.5.2: Textured Gluten Integrity Test

Like the water absorption test, this testing procedure was adapted from an American Soybean Association technical bulletin (1988) by Kearns et al., Wenger Manufacturing. Measuring the integrity and strength of textured vegetable proteins is very important. Not only does it gauge degree of texturization, but it will also display how well the final product will integrate into an animal protein when TVP is mixed into the animal protein. Four hundred grams of final product was soaked in 1.5 L of room temperature water for 30 minutes. Once hydrated, the textured gluten was drained on a screen for 5 minutes. Utilizing a Hobart bench top mixer (Troy, OH) with a meat grinding attachment, the hydrated texturized gluten was ground through a die plate with 6.35 mm holes. One hundred grams of ground material was then placed on a U.S. 20 mesh sieve screen (850 microns) and washed with water at 15 p.s.i for 1 minute. After washing, the ground product was pressed slightly by hand to remove any excess water.

After reweighing, the percent loss was calculated:  $(\text{Initial wt.} - \text{Final wt.}) / \text{Initial wt.} * 100\%$ .

### 2.5.3: Hydration Time and Rate

Hydration time and rate were found using the testing procedure used by MGP Ingredients, Inc. Both hydration time and rate are very important to determine the quality of textured vegetable protein. These tests help determine preparation time and ideal hydration procedures. Seventy-five grams of final product was soaked in 900 mL of room temperature water. During hydration, the textured gluten was checked every 2 minutes to see if full hydration had been accomplished. When the textured gluten contained no hard areas, it was considered fully hydrated. The time was recorded and the hydrated sample was weighed to calculate hydration rate. Hydration rate is calculated as the following:  $(\text{Final wt.} - \text{Initial wt.}) / (\text{Hydration Time})$ . This displays the amount of water the sample gained per minute of hydration.

### 2.5.4: Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) is a unique way of studying the infrared signatures of proteins. FTIR allows for examination of the composition and structure of a protein. Because the extrusion process is such an aggressive system, it was believed that the native structure of the gluten would be drastically altered, resulting in new structure formation. Proteins have a structural repeat unit, the peptide groups, which have nine distinct mid-infrared bands, or amides. These amide groups are called A, B, and I-VII. The area of interest for this research was the amide III region. Previous work by Singh et. al. (1993) has shown the usefulness of the amide III region for estimation of secondary structure in protein. The amide III region offers several advantages, with perhaps the most important being water vibrations do not interfere with the infrared

spectra. Furthermore, the secondary structures, like the  $\alpha$ -helix,  $\beta$ -sheets and turns, and also random coils are all confined to the amide III region (Seabourn et. al., 2008). Further work has also recognized band assignments of secondary structures in the amide III region;  $\alpha$ -helix ( $1330\text{-}1295\text{ cm}^{-1}$ ),  $\beta$ -sheets ( $1245\text{-}1220\text{ cm}^{-1}$ ),  $\beta$ -turns ( $1295\text{-}1270\text{ cm}^{-1}$ ) and random coils ( $1270\text{-}1255\text{ cm}^{-1}$ ) (Cai and Singh, 1999).

FTIR work for this research was completed at the USDA-ARS facility in Manhattan, KS. The raw material and the final products were analyzed. Prior to testing, a dough was made of the raw material on a 1 to 1 basis of dry raw material to water. Making a dough was an important step; it ensured the glutens interfaced well with the testing equipment. The final product was ground to a small particle size, but it required more water (1 to 2 ratio) to create a dough due to the hydrophilic properties of the TVP. After a background test was completed, the gluten (raw or final) was positioned on a ZnSe FTIR cell and then scanned with the interferometer. After scanning, the sample is removed from the cell and discarded. Then, the cell is cleaned and prepared for the next test.

## 2.6. Statistical analysis

Data were analyzed using the GLM procedure in SAS (Cary, NC). GLM procedure uses the method of least squares to fit general linear models. By doing so, 2-way and 3-way ANOVA can be completed; the level of significance was a p-value of 0.05. Interactions were examined for to measure for significance between the gluten type, hydration level and the chemical treatment.



### **3. Results and discussion**

#### **3.1. Proximate analysis and amino acid profile**

	<b>U.S. Gluten</b>	<b>European Gluten</b>
<b>Protein (%)</b>	76.34	77.26
<b>Carbohydrates (%)</b>	18.0395	15.363
<b>Fat (%)</b>	1.22	1.7
<b>Ash (%)</b>	0.9405	0.707
<b>Total Dietary Fiber (%)</b>	3.46	4.97
<b>Total</b>	100	100

Table 3.3: Proximate Analysis of US and European Glutens

Table 3 indicates that the two gluten types were very similar in their chemical composition.

<b>Amino Acid</b>	<b>US Gluten</b>	<b>EURO Gluten</b>
<b>Aspartic Acid</b>	2.55	2.67
<b>Threonine</b>	1.56	1.64
<b>Glutamic Acid</b>	27.14	27.39
<b>Proline</b>	9.41	9.06
<b>Glycine</b>	2.63	2.63
<b>Alanine</b>	1.98	2.02
<b>Cysteine</b>	1.42	1.40
<b>Valine</b>	3.27	3.29
<b>Methionine</b>	1.21	1.21
<b>Isoleucine</b>	2.90	2.92
<b>Leucine</b>	5.29	5.39
<b>Lysine</b>	1.37	1.36
<b>Total</b>	60.73	60.98

Table 3.4: Amino Acid Profile of US and European Sourced Glutens

Similar to the proximate analysis, the amino acid profiles of these two types of gluten were very comparable. This indicates there was not a large difference in the chemical composition of the two gluten types.

### 3.2. Rheological properties of wheat gluten

#### 3.2.1: Gluten Index

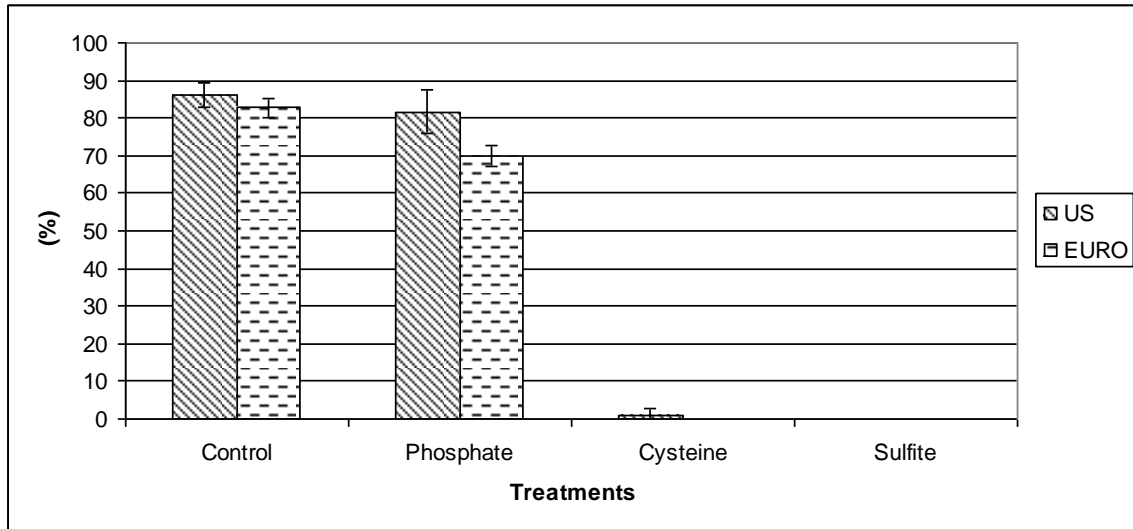


Figure 3.3: Gluten Index Results for U.S. and European Sourced Glutens.

The strength of both the U.S. and European glutes were drastically reduced for the sulfite and cysteine treatments. This was expected, since the reducing action of cysteine and sulfite are so efficient, even when heat and pressure are not added. For the control treatment, the European gluten appeared to be weaker than the U.S. gluten. However, through statistical analysis, no significant differences were observed between the two types of gluten ( $p\text{-value} > 0.05$ ). When observing the interaction between source country and chemical treatment, there were significant differences ( $p\text{-value} < 0.0001$ ).

### 3.2.2: Phase Transition Analysis

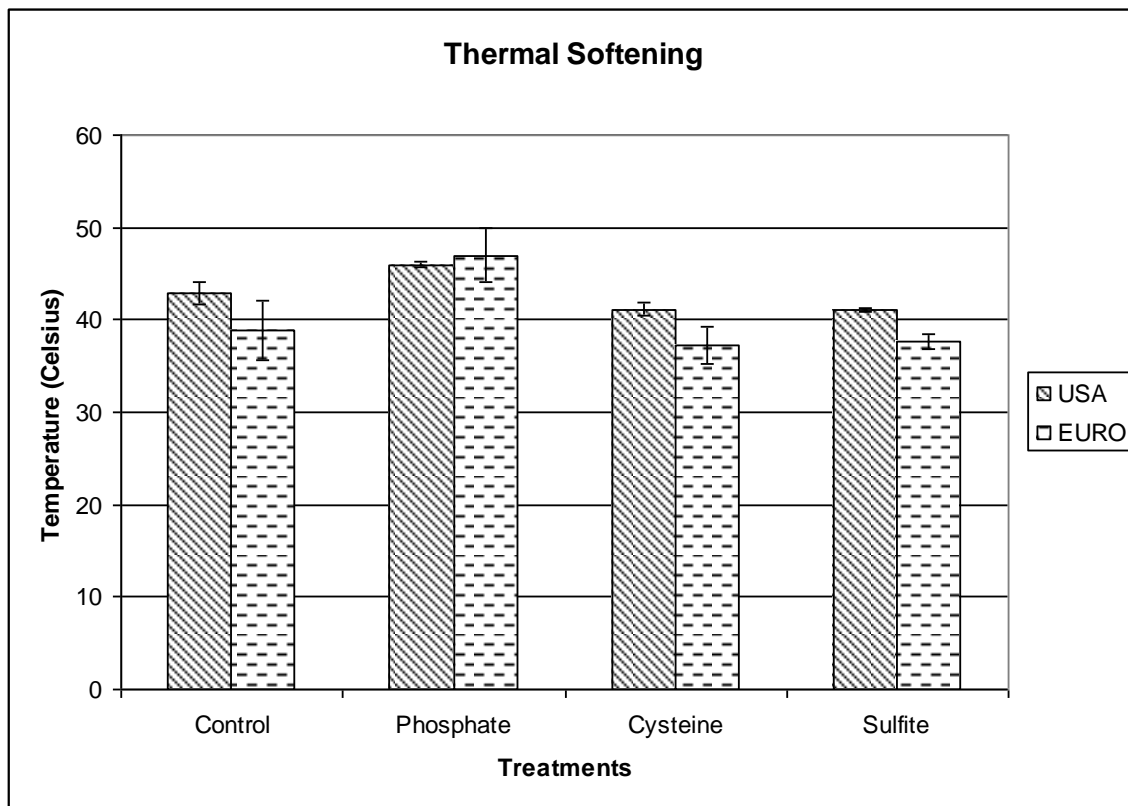


Figure 3.4: Average Softening Temperatures of U.S. and European Sourced Gluten (Control, 3.0% Phosphate, 0.18% Cysteine, 0.18% Sulfite)

When processing aids were added to both gluten types, especially the sulfite and cysteine, a decrease in average softening temperature was observed and there was a statistical difference in softening temperature between the treatments (control, phosphate, cysteine, sulfite) with a p-value of  $< 0.05$ . There was also a statistical difference between the two gluten source countries, regardless of the treatment (p-value  $< 0.05$ ). However, the interaction of source country and the treatments have the same trends respective to the country and chemical treatment.

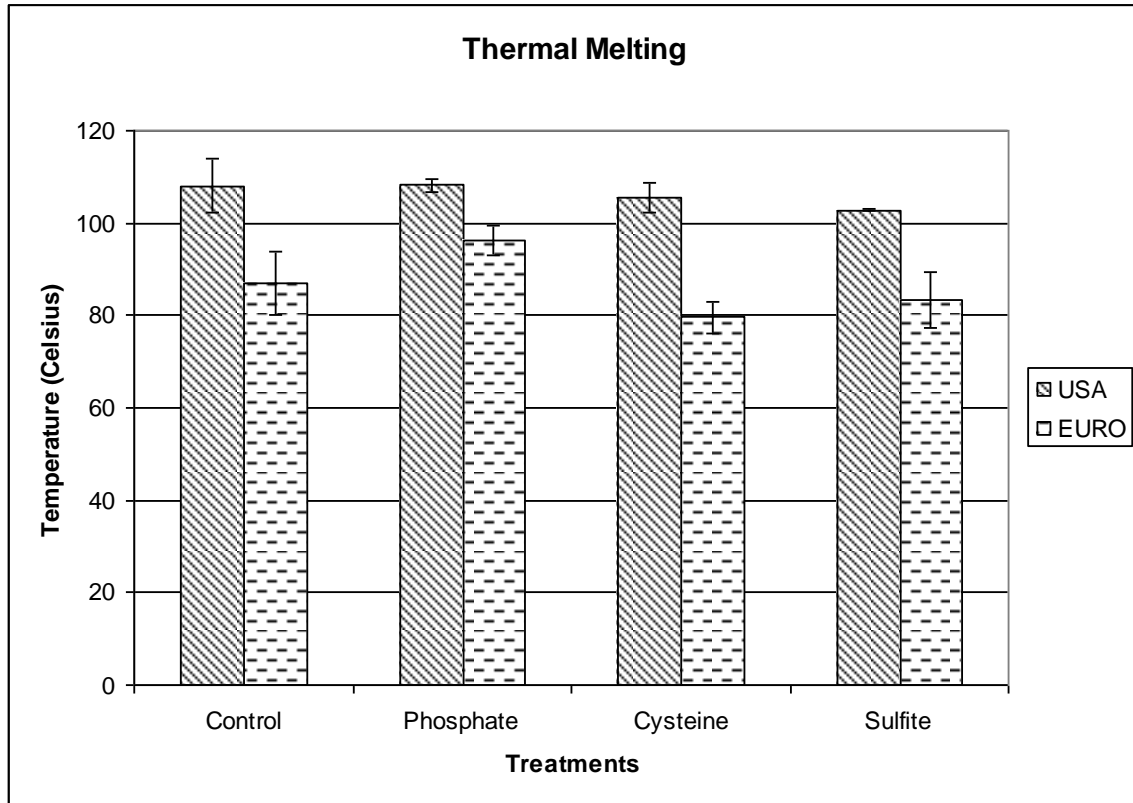


Figure 3.5: Thermal Melt Temperatures of U.S. and European Sourced Gluten (Control, 3.0% Phosphate, 0.18% Cysteine, 0.18% Sulfite)

When processing aids were added to both gluten types, especially the sulfite and cysteine, a decrease in average softening temperature was observed and there was a statistical difference in softening temperature between the treatments (control, phosphate, cysteine, sulfite) with a p-value of  $< 0.05$ . There was also a statistical difference between the two gluten source countries, regardless of the treatment (p-value  $< 0.05$ ). However, the interaction of source country and the treatments have the same trends respective to the country and chemical treatment.

### 3.3. Extrusion processing

Specific Mechanical Energy (SME) is calculated to show how much mechanical energy is being imparted on a material during the extrusion process.

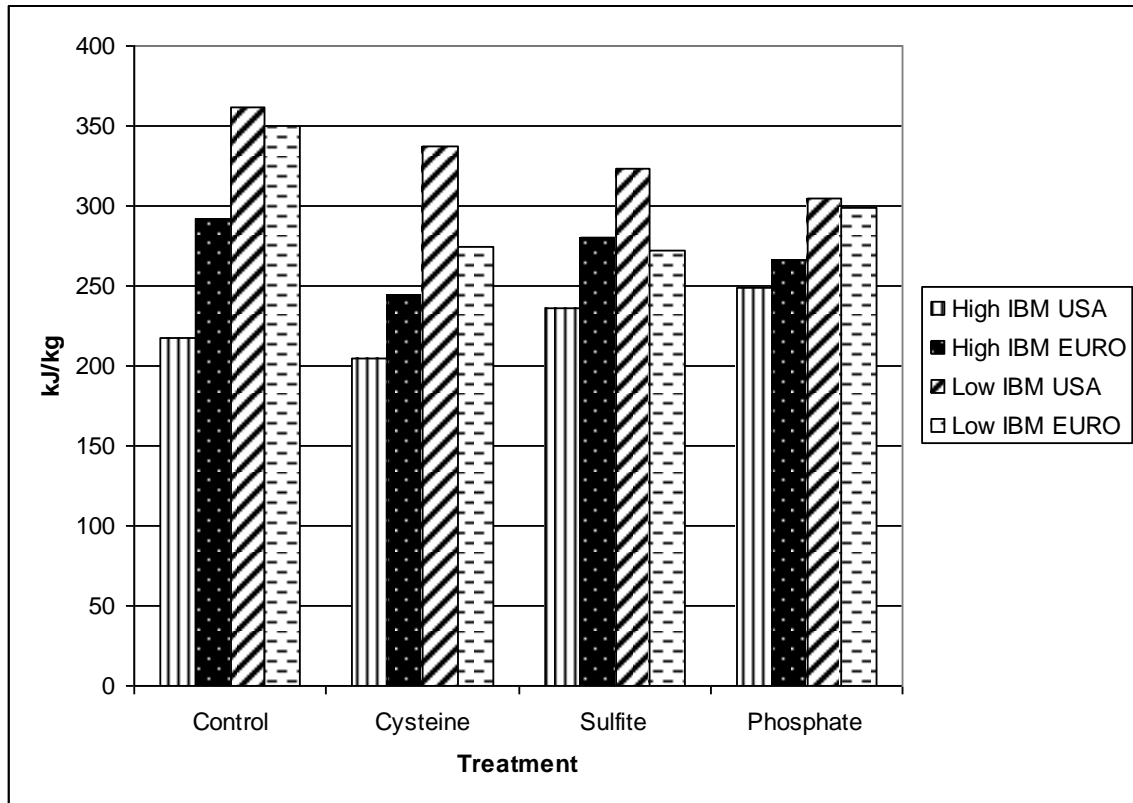


Figure 3.6: SME during the Extrusion of U.S. and European Sourced Glutens.

Typically during the extrusion of material, a lower SME is experienced when the extrudate has a high in-barrel moisture (IBM). This is because water acts as a plasticizer; the melt viscosity is lowered to a point where the extruder does not have to work as hard to force the melt through the die openings. In this research, that remains true; the higher IBM level (36%) have a lower SME than the lower IBM level (32%). When compared to the gluten quality tests (Gluten Index and Compression Tests), it could be theorized that with the addition of cysteine and sulfite, a decrease in SME would be experienced.

However, this was not the case. The reasoning is that in the presence of reducing agents

(cysteine and sulfite), disulfide molecules and the sulfhydryl groups oxidized and were reduced more defiantly, making the newly created disulfide molecules readily available for cross-linkages with the gluten protein molecules. As these disulfide bonds formed and cross-linked the protein molecules, the viscosity of the melt increased, causing the extruder to work harder to force material through the die opening.

### 3.4. Post-extrusion quality analysis

#### 3.4.1: Water Absorption Index

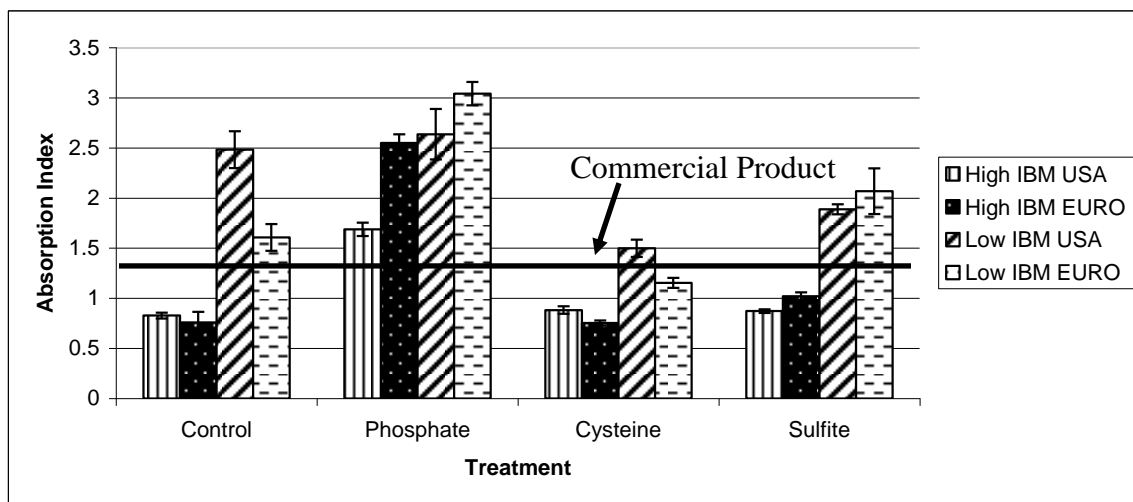


Figure 3.7: Water Absorption Index for U.S. and European Sourced Glutens at Low and High In-Barrel-Moistures (32% and 36%, respectively)

Measuring the water absorption of textured vegetable protein is very important, regardless of protein source. Not only does it give an indication of the degree of texturization, but also the quality of texturization. If a TVP is poorly texturized, it will have a low absorption index which will impact other characteristics like hydration rate and time.

For this research, it is seen that the high IBM level of 36% all had a lower absorption index than the low IBM level of 32% with the exception being the phosphate treatments. This is due to the increased protein solubility the phosphate brings. Because of the increased protein solubility, the phosphate treatments for both U.S. and European sourced gluten have the highest absorption index, for both IBM levels. For comparison, the absorption index for the control US and EURO low IBM level was 2.5 and 1.6, respectively. At the same IBM level the absorption index for US and EURO glutes with 3.00% phosphate present was 2.6 and 3.04, respectively. While the difference between the US gluten with and without phosphate may not seem large, there is a significant difference between the combination of the three factors (source country, IBM level, chemical treatment) with a of p-value  $< 0.0001$ . In fact, all of the combinations between source country, IBM level, and chemical treatments were significantly different (p-value  $< 0.0001$ ). However, some of the individual interaction groups were the same. For example, the US gluten processed at 36% IBM with the addition of 0.018% cysteine was the same as the US gluten processed at 36% IBM with no chemical additives present.

### 3.4.2: Textured Gluten Integrity Test

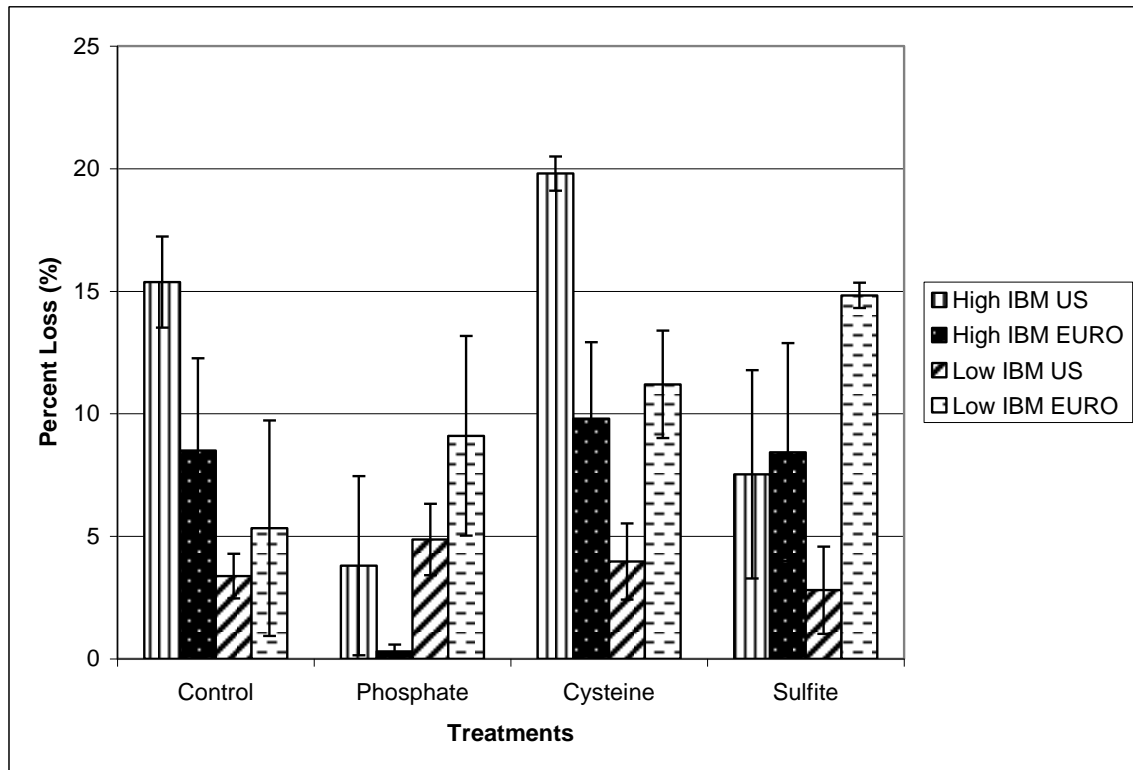


Figure 3.8: Percent loss during Textured Gluten Integrity Testing

When TVP is mixed into an animal protein, ensuring a uniform mix is very important. This is where the integrity of the textured gluten plays a significant role. If the gluten is poorly texturized, it will not create a homogenous mix with animal protein and the overall texture of the animal protein will suffer. It has previously been stated that the high IBM level resulted in a poorly texturized product. However, this is not reflected very well when looking at the integrity test results. This is because of two factors. First, the high IBM treatments did not hydrate as well as the lower IBM treatments. This resulted in a hard pieces of product when ground through the meat grinder. Second, these hard pieces had a larger particle size than the ground textured gluten processed at the lower IBM. Because the ground textured gluten had a larger particle size as a result of poor hydration, more material remained on the sieve screen when the ground material was



washed with water. So, even though the degree of texturization was poorer, the high IBM level treatments displayed a better integrity.

When analyzing the statistics for the integrity test, it was found that there were significant differences when analyzing the combination of source country and treatment. There were also significant differences between the treatments as well as the IBM level, regardless of gluten source and IBM level. Additionally, there are significant differences between the combination of source country and IBM level. However, it was found that there was no significant difference between the glutens source country. The commercial product did not have any loss during integrity testing.

### 3.4.3. Bulk Density

	<i>High In-Barrel-Moisture (36%)</i>			
	<b>Control</b>	<b>Phosphate</b>	<b>Cysteine</b>	<b>Sulfite</b>
<b>USA</b>	245.27 +/- 3.62	228.8 +/- 3.5	273.77 +/- 3.16	254.57 +/- 3.66
<b>EURO</b>	258 +/- 3.14	214.17 +/- 0.30	272.77 +/- 6.07	215.13 +/- 9.88
	<i>Low In-Barrel-Moisture (32%)</i>			
	<b>Control</b>	<b>Phosphate</b>	<b>Cysteine</b>	<b>Sulfite</b>
<b>USA</b>	158.96 +/- 0.75	179.12 +/- 8.57	193.76 +/- 0.81	196.76 +/- 7.44
<b>EURO</b>	196.1 +/- 9.06	231.26 +/- 10.72	226.7 +/- 3.9	192.33 +/- 5.16

Table 3.5: Bulk densities of Textured Gluten at 32% and 36% IBM with Chemical Additives Added to Two Types of Gluten

While no statistics were completed for bulk densities, there were observational differences between the final product bulk densities at the two IBM levels. At the higher IBM (36%) there was very little observed differences between the US and EURO glutens. Since the gluten was processed at a higher IBM, there was not a large response when comparing the two types of gluten; the high moisture in the barrel appears to equalize the bulk densities. However, when examining the bulk densities of the low IBM (32%)

material, an observational difference was seen, with the exception of the sulfite treatment; US gluten with the inclusion of sulfite had a bulk density of 196.76 g/L while its European counterpart had a bulk density of 192.33 g/L. For the other three treatments (control, phosphate, and cysteine) the European gluten had what appeared to be a much higher bulk density than the US sourced gluten. This suggests the European gluten had a lower vapor pressure capacity and did not expand as much as the US sourced gluten.

Bulk density will also have an effect on time to full hydration, hydration rate, and absorption index. If a textured gluten has a lower bulk density, it can be thought that the gluten has more expansion. This means the individual textured gluten pieces will have more porosity, allowing for faster water uptake. The commercial product had a density of 245.33 g/L. This is very similar to the high in-barrel moisture treatments. While still an important factor, the commercial product was made on a much larger extruder with slightly different process settings, so its density compared to the treatments may not hold much validity.

#### 3.4.4: Hydration Time and Rate

	<i>High In-Barrel-Moisture (36%)</i>				
	<b>Control</b>		<b>Phosphate</b>	<b>Cysteine</b>	<b>Sulfite</b>
<b>USA</b>	28.35		21.77	31.36	34.19
<b>EURO</b>	28.59		8.45	38.35	34.05
	<i>Low In-Barrel-Moisture (32%)</i>				
	<b>Control</b>		<b>Phosphate</b>	<b>Cysteine</b>	<b>Sulfite</b>
<b>USA</b>	13.05		11.32	19.59	20.35
<b>EURO</b>	15.02		6.07	37.15	15.11

Table 3.6: Average Hydration Time for High and Low IBM with the Inclusion of Chemical Additives for Two Types of Wheat Gluten

The amount of time it takes a textured vegetable protein to reach full hydration is a very important factor. Not only does it affect the preparation time for the TVP, but it also has an impact on the amount of water a TVP will absorb. If a TVP absorbs too much water, it may have a negative effect on the animal protein the TVP is being added to. Statistically, there were significant differences between each result, regardless of the source country, IBM level, and chemical treatment (p-value < 0.0001).

As stated earlier, phosphate increases the solubility of gluten. Because of this, better texturization is achieved. Because there is better texturization, there may be more porosity, leading to faster water uptake. This was confirmed; the phosphate treatments of both US and European sourced gluten have the lowest fully hydrated times. While the addition of cysteine, sulfite, as well as the lack of chemical treatment (control), appeared to be very similar observationally, there were significant differences when looking at the combination of source country and chemical treatment (< 0.0001). For the hydration time of commercial product, a value of 22.76 minutes was found. This is very similar to the high in-barrel moisture treatments. However, as stated earlier, the commercial product was made on a much larger extruder than the TX-52, so this data may not be valid for comparison to the treatments studied in this research. This is also true for the hydration rate data.

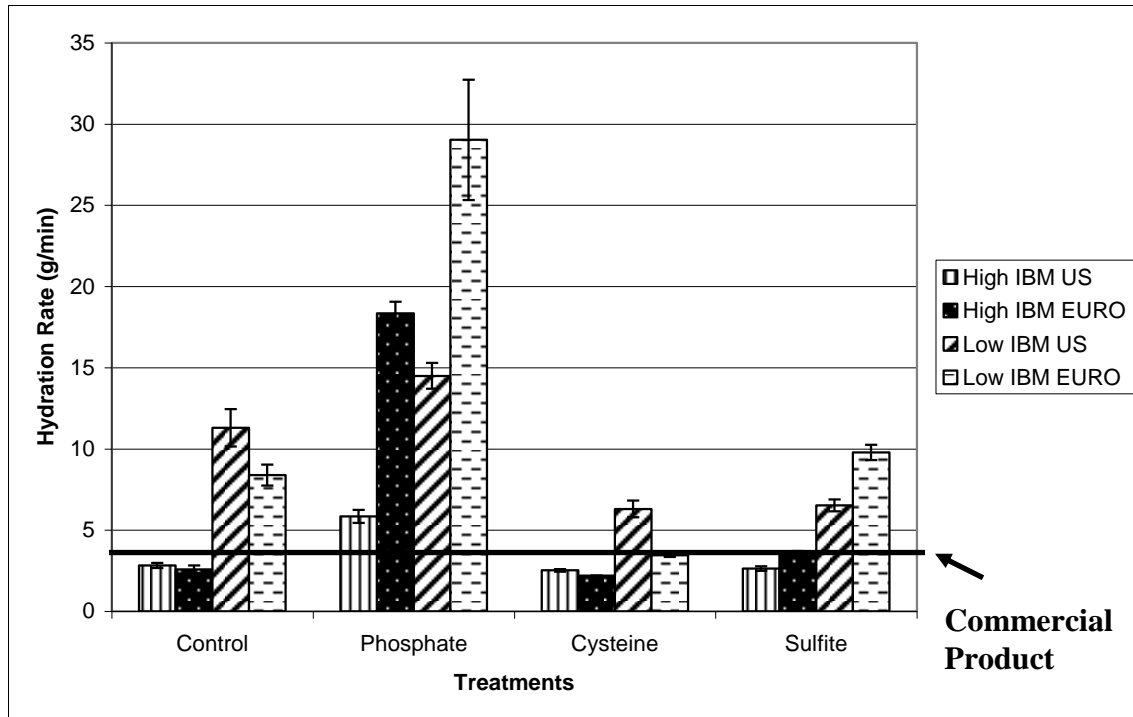


Figure 3.9: Hydration Rate of Textured Glutens at 32% and 36% IBM with Chemical Additives Added to Two Types of Gluten

As stated previously, the amount of water uptake will have an impact on the animal protein the TVP is being added to. If there is too much or too little water, the overall texture of the animal protein when the TVP is added may suffer. The above bar chart displays that the phosphate treatments, for both US and European sourced glutens, had the highest amount of water uptake. They also had the lowest time to full hydration. While this suggests the phosphate treatments are more texturized than the other treatments, it could lead to negative impacts on the animal protein.

Statistically, there are significant differences between the combination of source country, IBM level, and chemical treatment ( $< 0.0001$ ). The interactions suggest that the trends are the same, respective to source country, IBM level and chemical treatment.

### 3.4.5: Fourier Transform Infrared Spectroscopy Results

As stated previously, FTIR is a novel way to examine the secondary structures of gluten proteins after shear is applied. Due to time constraints at the USDA-ARS, only the U.S. sourced gluten was examined.

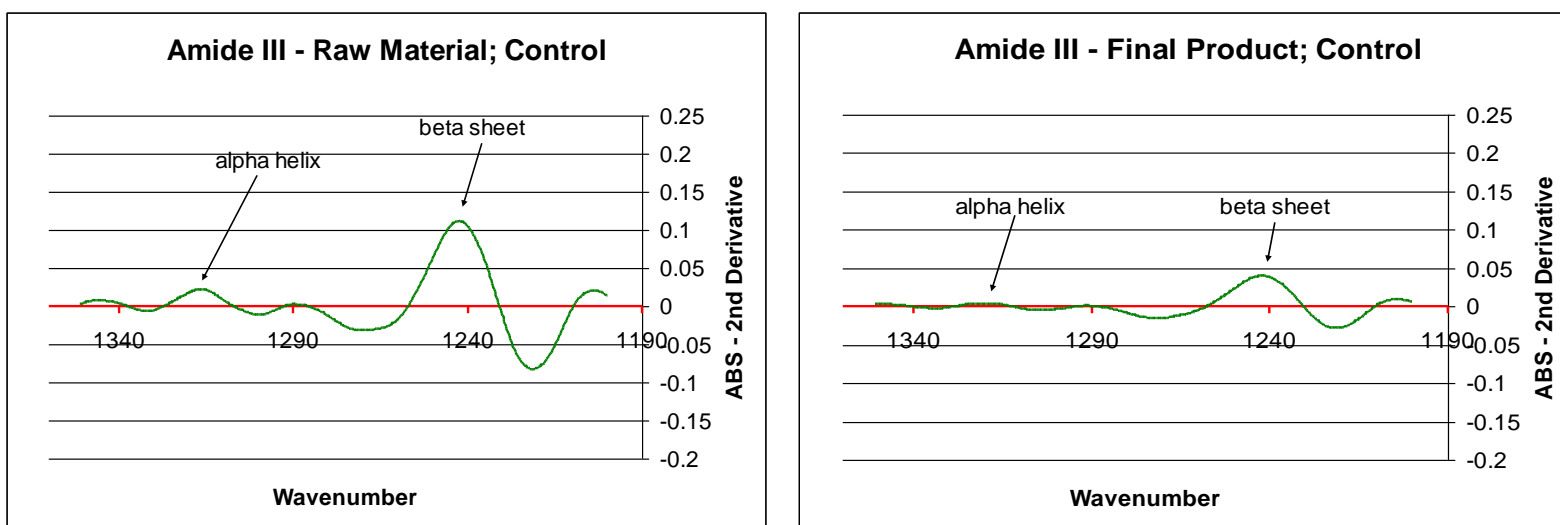


Figure 3.10: FTIR spectra of control treatment, raw material and final product

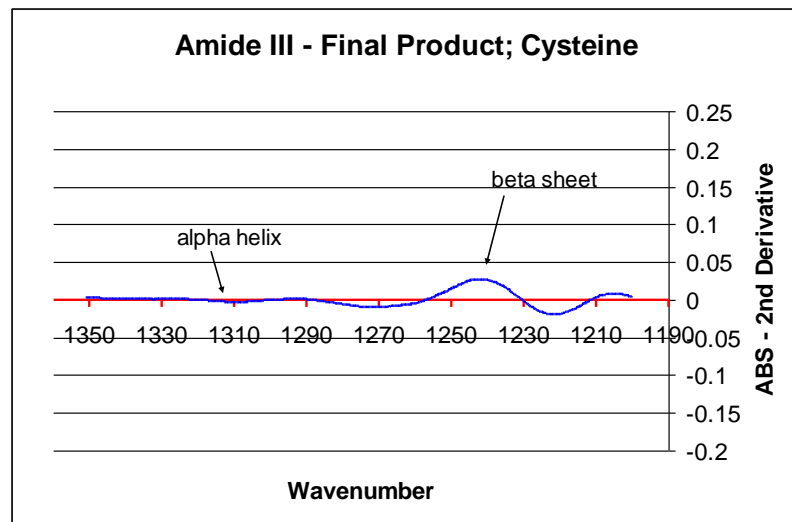
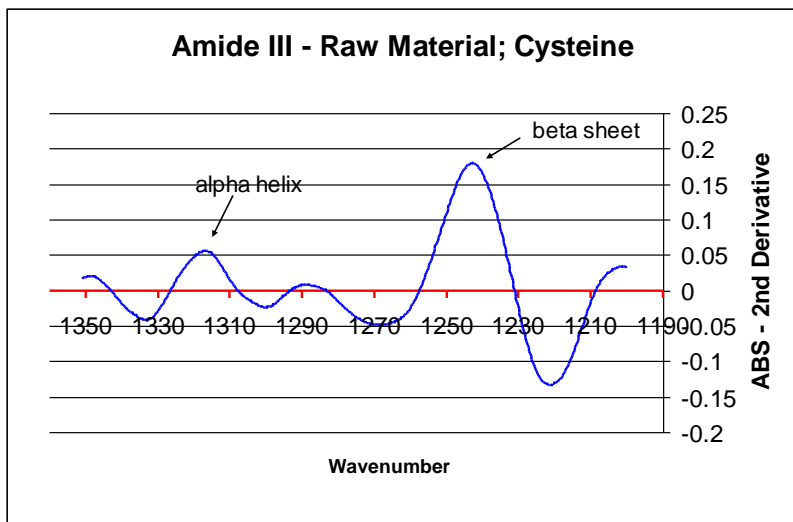


Figure 3.11: FTIR spectra of cysteine treatment, raw material and final product

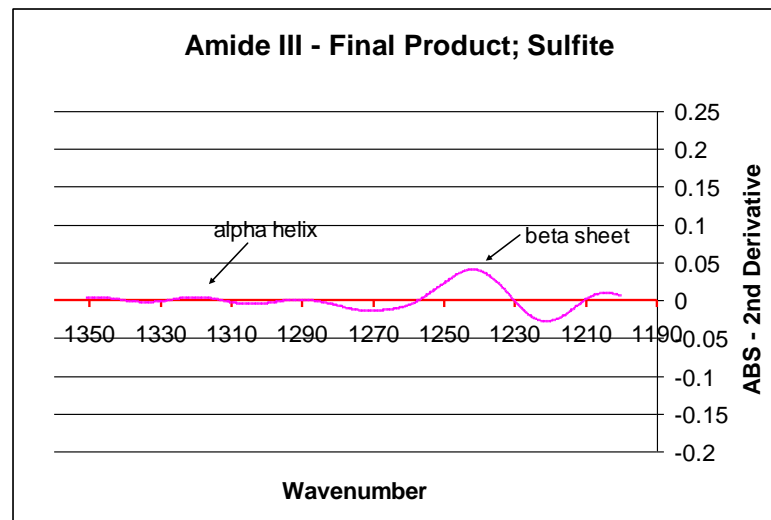
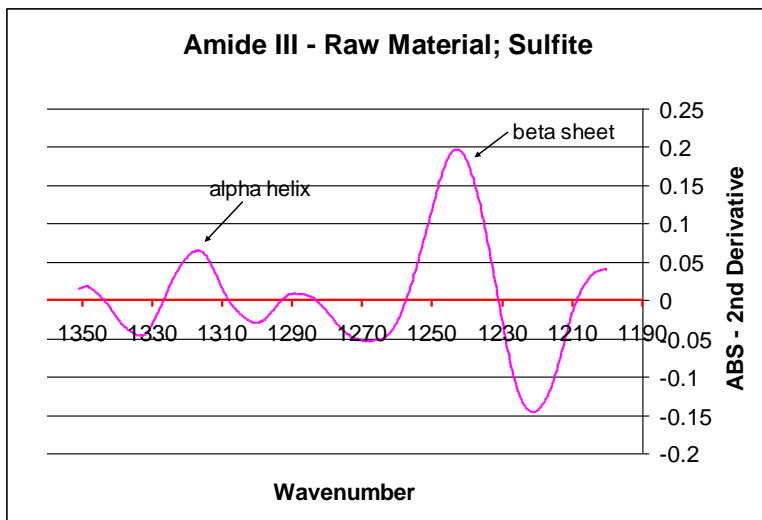


Figure 3.12: FTIR spectra of sulfite treatment, raw material and final product

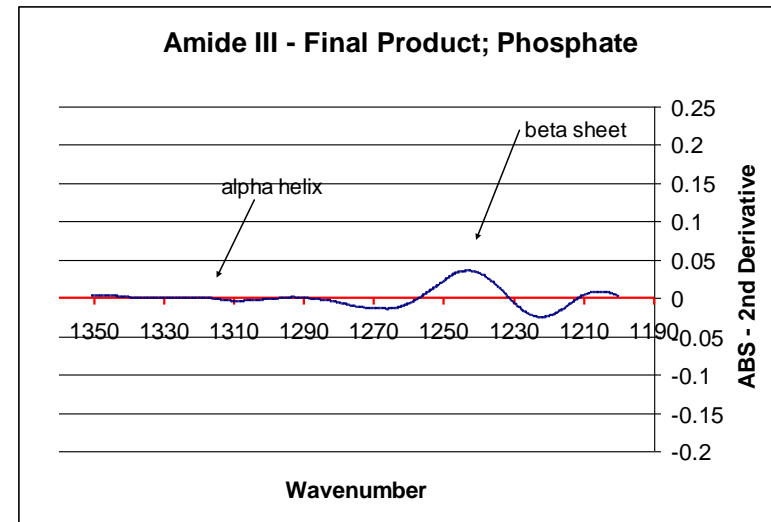
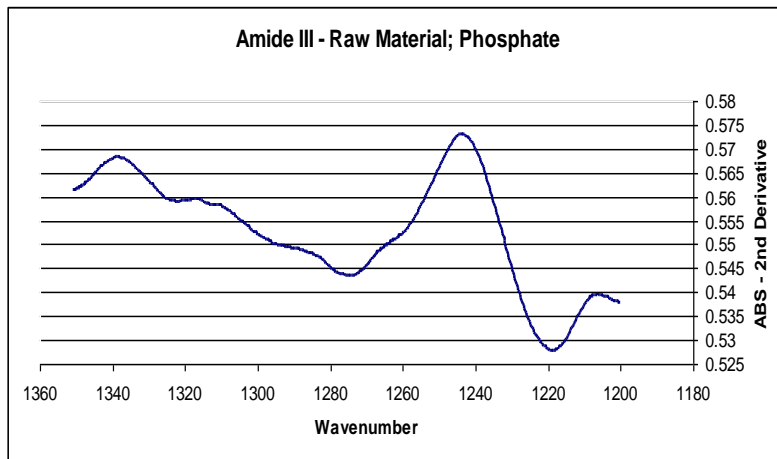


Figure 3.13: FTIR spectra of phosphate treatment, raw material and final product



When looking at this FTIR data, the amide III region is the most important. This is because water vibrations do not interfere with the protein spectrum (Seabourn et al., 2008). In each pairing of graphs, the raw gluten is found on the left with the final product on the right. It can be clearly seen that the final product displays a very different spectra than the raw material. Both the alpha helix and beta sheet peaks decrease in height for the final product. This indicates that the gluten proteins are having their secondary structures completely altered. This is due to the extrusion system being such a high heat and high shear environment.

#### **4. Conclusion**

Pre-extrusion testing showed that there were very little differences between US and European sourced gluten. This was especially true for the Gluten Index results. As for phase transition analysis, differences were observed between each of the four treatments. However, the interaction between source country and chemical treatment had the same trends. While there was a clear difference between the combination of source country and treatment, the trends were similar.

At processing, a very aggressive screw profile was used to oxidize and reduce the disulfide bonds and thiol sulfhydryl groups that link gluten molecules. From SME data, it can be determined that reformation of disulfide bonds did occur. There was an increase in SME when comparing the control treatment to all three of the chemical treatments. The extruder had to work harder to overcome these new cross-linkages and force the newly formed gluten matrix through the die.

Final product characteristics are the most important part of this research since they will directly impact the consumers. There were significant differences between all of

the treatments, with some being more significant than others. However, the interactions between some treatments were very similar.

Textured vegetable proteins have a clear need worldwide. From a health and lifestyle perspective, a growth in the textured vegetable protein industry will occur. More research is needed to better understand the mechanisms behind protein texturization as well as process optimization to create better textured vegetable proteins.

### **Chapter 3 References**

AACC International. Approved Methods of Analysis, 11th Ed. Method 08-01.01. Ash – Basic Method. Approved November 3, 1999. AACC International, St. Paul, MN, U.S.A. <http://dx.doi.org/10.1094/AACCIntMethod-08-01.01>.

AACC International. Approved Methods of Analysis, 11th Ed. Method 46-30.01. Crude Protein – Combustion Method. Approved November, 3 1999. AACC International, St. Paul, MN, U.S.A. <http://dx.doi.org/10.1094/AACCIntMethod-46-30.01>.

AACC International. Approved Methods of Analysis, 11th Ed. Method 30-25.01. Crude Fat in Wheat, Corn, Soy Flour, Feeds, and Mixed Feeds. Approved November 3, 1999. AACC International, St. Paul, MN, U.S.A. <http://dx.doi.org/10.1094/AACCIntMethod-30-25.01>.

AACC International. Approved Methods of Analysis, 11th Ed. Method 32-07.01. Soluble, Insoluble, and Total Dietary Fiber in Foods and Food Products. Approved November 3, 1999. AACC International, St. Paul, MN, U.S.A. <http://dx.doi.org/10.1094/AACCIntMethod-32-07.01>.

AACC International. Approved Methods of Analysis, 11th Ed. Method 38-12.02. Wet Gluten, Dry Gluten, Water-Binding Capacity, and Gluten Index. Approved November 8,

2000. AACC International, St. Paul, MN, U.S.A.

<http://dx.doi.org/10.1094/AACCIntMethod-38-12.02>.

Akdogan, H. (1999). High moisture food extrusion. *International Journal of Food Science and Technology*, 34, 195-207.

Areas, J.A.G., (1992). Extrusion of Food Proteins. *Critical Reviews in Food Science and Nutrition*. 32, 365-392.

Asgar, M., Fazilah, A., Huda, N., Bhat, R., Karim, A. (2010). Nonmeat Protein Alternatives as Meat Extenders and Meat Analogs. *Comprehensive Reviews in Food Science and Food Safety, Institute of Food Technologists*, 9, 513-529.

Cai, S., Singh, B.R., (1999) Identification of beta-turn and random coil amide III infrared bands for secondary structure estimation of proteins. *Biophys. Chem.*, 80, 7-20.

Gehrke, C.W., Rexroad, P.R., Schisla, R.M., Absheer, J.S., Zumwalt, R.W., (1987). Quatitative Analysis of Cystine, Methionine, Lysine, and Nine Other Amino Acids by a Single Oxidation-4 h Hydrolysis Method, *J. Assoc. Off. Anal. Chem.* 70:717-174.

Harper, J.M., (1981). Extrusion of Foods, Vol. 1, 21-45. Boca Raton; CRC Press, Inc.

Karkle, E., Alavi, S., Dogan, H. (2012). Cellular architecture and its relationship with mechanical properties in expanded extrudates containing apple pomace. *Food Research Interational*, 46, 10-21.

Kearns, J., Rokey, G., Huber, G. (1988) Extrusion of Textured Proteins. *American Soybean Association Technical Bulletin*.

Levine, H., Slade, L., (1990). Influence of the glassy and rubbery states on thermal, mechanical, and structural properties of dough and baked products. *Dough Rheology and Baked Product Texture: Theory and Practice*.

Singh, B.R., DeOliveira, D.B., Fu, F.N., Fuller, M.P., (1993) Fourier transform infrared analysis of amide III bands of proteins for the secondary structure estimation. *Proceedings of Bimolecular Spectroscopy III*, 47-55.

Yang, F., Jorgensen, A., Li, H., Sondergaard, I., Finnie, C., Svensson, B., Jiang, D., Wollenweber, B., Jacobsen, S. (2001). Implications of high-temperature events and water deficits on protein profiles in wheat (*Triticum aestivum* L. Cv. Vinjett) grain. *Proteomics Journal*, 11, 1684-1695.

Zhu, L.J., Shukri, R., de Mesa-Stonestreet, N.J., Alavi, S., Dogan, H., Shi, Y.C., (2010) Mechanical and microstructural properties of soy protein – high amylose corn starch

extrudates in relation to physiochemical changes of starch during extrusion. *Journal of Food Engineering*, 100, 2, 232-238.

## Chapter 4: Conclusion

This research yielded interesting results. While we were unable to extruded superior and inferior quality gluten through a pilot scale extruder, very distinct trends are found when comparing the European and U.S. sourced gluten. Previous research was unclear as to the mechanisms behind texturization. In theory, disulfide bonds were being reduced and then through the extrusion process, free thiol groups were forming new covalent bonds with those reduced disulfide bonds (Levine and Slade , 1990; Akdogan, 1999; Areas, 1992). The research outlined in this manuscript takes the understanding behind texturization to the next level. By introducing reducing agents and a pH adjusting chemical, we found the flow behavior change with all four gluten types (superior, inferior, European, U.S.).

To further the understanding of how texturization occurs, a very systematic approach was designed. First, it was important to qualify the gluten types using simple physical tests. Gluten index and compression testing did this and showed that the addition of sulfite and cysteine drastically reduced the strength and cohesiveness of the gluten, regardless of the type. Phosphate appeared to have little effect on gluten strength, but this was somewhat expected, because there is no application of heat or high shear during testing. The effect of phosphate was more apparent during the next phases of the research.

After gaining an understanding of the effect of these chemicals on a physical basis, it was important to examine the physicochemical interactions the chemical additives had on the gluten types. This was done using Phase Transition Analysis (PTA). A PTA simulates an extrusion system without mechanical energy. They are typically

used to understand melt flow behaviors, and once those behaviors are found, the extrusion system can be more accurately built to make the best possible product. Like with the gluten index and compression tests, the reducing agents appeared to have a substantial impact on both thermal softening and flow of all four types of gluten. This was expected, since the glutes lost strength when sulfite and cysteine were added for gluten index and compression testing. Since the PTA adds heat and pressure, the effect of the reducing agents was seen more prominently. Literature supports that the gluten polymers become more mobile with the application of heat and pressure. This increased mobility leads to disruption of the protein sub-units and reduction of the disulfide bonds that link them together. It was known that texturization would not occur during PTA testing since there is no application of mechanical energy, which was an overall conclusion of the PTA tests. However, the reduction in thermal softening and flow indicates that when introduced to an extrusion system, the melt viscosity, when the reducing agents are present, will be lowered, creating a higher potential for new disulfide cross-linkages, or texturization



## Appendix A: SAS Output for Lab Scale Extrusion

```

1
                                The SAS System

                                The GLM Procedure

                                Class Level Information

Class          Levels   Values
Gluten         2       Bad Good
Treatment      4       Control Cysteine Phosphat Sulfite

                                Number of Observations Read      24
                                Number of Observations Used      24

2
                                The SAS System

                                The GLM Procedure

Dependent Variable: RespDist

Source          DF          Sum of Squares      Mean Square      F Value      Pr > F
Model          7          9.00886000         1.28698000      14.34      <.0001
Error         16          1.43558933         0.08972433
Corrected Total 23          10.44444933

                                R-Square      Coeff Var      Root MSE      RespDist Mean
                                0.862550      10.95344      0.299540      2.734667

Source          DF          Type III SS      Mean Square      F Value      Pr > F
Gluten          1          1.05336600         1.05336600      11.74      0.0035
Treatment       3          7.07989500         2.35996500      26.30      <.0001
Gluten*Treatment 3          0.87559900         0.29186633      3.25      0.0494

3
                                The SAS System

                                The GLM Procedure
                                Least Squares Means
                                Adjustment for Multiple Comparisons: Tukey

                                RespDist      LSMEAN
                                LSMEAN      Number
Gluten  Treatment
Bad      Control      2.64333333      1
Bad      Cysteine      2.95733333      2
Bad      Phosphat      1.86766667      3
Bad      Sulfite       2.63233333      4
Good     Control      2.63166667      5
Good     Cysteine      3.89066667      6
Good     Phosphat      1.98966667      7
Good     Sulfite       3.26466667      8

```

Least Squares Means for effect Gluten\*Treatment  
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: RespDist

Compression Test: Gluten*Treatment								
i/j	1	2	3	4	5	6	7	8
1	x	0.8922	0.0853	1	1	0.0021	0.2005	0.2467
2	0.8922	x	0.0074	0.8749	0.8738	0.0255	0.0195	0.9019
3	0.0853	0.0074	x	0.0925	0.093	< 0.001	0.9995	0.0007
4	1.00	0.8749	0.0925	x	1.00	0.0019	0.2154	0.2301
5	1.00	0.8738	0.093	1.00	x	0.0019	0.2163	0.2292
6	0.0021	0.0255	< 0.001	0.0019	0.0019	x	< 0.0001	0.2396
7	0.2005	0.0195	0.2154	0.2154	0.2163	< 0.0001	x	0.0017
8	0.2467	0.9019	0.2301	0.2301	0.2292	0.2396	0.2396	x

Table A.1: Two-way comparison between gluten type and chemical treatment

The SAS System						4		
Obs	Gluten	Moisture	Treatment	_TYPE_	_FREQ_	m Soft Temp	mMelt Temp	
1	Bad	High	Control	0	2	42.70	116.15	
2	Bad	High	Cysteine	0	2	46.15	125.05	
3	Bad	High	Phosphat	0	2	39.25	95.65	
4	Bad	High	Sulfite	0	2	43.80	117.70	
5	Bad	Low	Control	0	2	60.00	155.90	
6	Bad	Low	Cysteine	0	2	62.55	156.75	
7	Bad	Low	Phosphat	0	2	65.10	152.15	
8	Bad	Low	Sulfite	0	2	67.05	165.00	
9	Bad	Medium	Control	0	2	45.30	118.55	
10	Bad	Medium	Cysteine	0	2	46.45	127.75	
11	Bad	Medium	Phosphat	0	2	51.25	122.15	
12	Bad	Medium	Sulfite	0	2	53.25	139.80	
13	Good	High	Control	0	2	43.25	127.30	
14	Good	High	Cysteine	0	2	47.00	126.80	
15	Good	High	Phosphat	0	2	48.15	114.30	
16	Good	High	Sulfite	0	2	46.65	125.75	
17	Good	Low	Control	0	2	50.20	109.55	
18	Good	Low	Cysteine	0	2	54.55	145.80	
19	Good	Low	Phosphat	0	2	58.90	140.20	
20	Good	Low	Sulfite	0	2	60.90	151.15	
21	Good	Medium	Control	0	2	53.10	143.55	
22	Good	Medium	Cysteine	0	2	50.10	136.65	
23	Good	Medium	Phosphat	0	2	49.05	117.95	
24	Good	Medium	Sulfite	0	2	47.80	133.50	

The SAS System

5

The GLM Procedure

Class Level Information

Class	Levels	Values
-------	--------	--------

Gluten	2	Bad Good
Moisture	3	High Low Medium
Treatment	4	Control Cysteine Phosphat Sulfite

Number of Observations Read	24
Number of Observations Used	24

6

The SAS System

The GLM Procedure

Dependent Variable: mSoftTemp

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	1036.206042	172.701007	10.94	<.0001
Error	17	268.263542	15.780208		
Corrected Total	23	1304.469583			

R-Square	Coeff Var	Root MSE	mSoftTemp Mean
0.794350	7.735363	3.972431	51.35417

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Gluten	1	7.2600000	7.2600000	0.46	0.5067
Moisture	2	974.4339583	487.2169792	30.88	<.0001
Treatment	3	54.5120833	18.1706944	1.15	0.3570

7

The SAS System

The GLM Procedure

Dependent Variable: mMeltTemp

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	4184.761667	697.460278	4.63	0.0058
Error	17	2560.412917	150.612525		
Corrected Total	23	6745.174583			

R-Square	Coeff Var	Root MSE	mMeltTemp Mean
0.620408	9.305814	12.27243	131.8792

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Gluten	1	16.833750	16.833750	0.11	0.7422
Moisture	2	3286.243333	1643.121667	10.91	0.0009
Treatment	3	881.684583	293.894861	1.95	0.1597

8

The SAS System

The GLM Procedure  
Least Squares Means

Moisture	mSoftTemp LSMEAN	LSMEAN Number
High	44.6187500	1
Low	59.9062500	2
Medium	49.5375000	3

Least Squares Means for effect Moisture  
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: mSoftTemp

Thermal Softening: Moisture Effect			
i/j	1	2	3
1	x	< 0.0001	0.0241
2	< 0.0001	x	< 0.0001
3	0.0241	< 0.0001	x

Table A.2: Moisture effect on gluten types for thermal softening

Moisture	mMeltTemp LSMEAN	LSMEAN Number
High	118.587500	1
Low	147.062500	2
Medium	129.987500	3

Least Squares Means for effect Moisture  
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: mMeltTemp

Thermal Flow: Moisture Effect			
i/j	1	2	3
1	x	0.0002	0.0806
2	0.0002	x	0.0128
3	0.0806	0.0128	x

Table A.3: Moisture effect on gluten types for thermal flow

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Class Level Information

Class	Levels	Values
Gluten	2	Bad Good
Treatment	4	Control Cysteine Phosphat Sulfite

Number of Observations Read 44  
 Number of Observations Used 44

10

The SAS System

The GLM Procedure

Dependent Variable: GlutenIndex

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	43968.62386	6281.23198	46.62	<.0001
Error	36	4849.95500	134.72097		
Corrected Total	43	48818.57886			

R-Square 0.900653  
 Coeff Var 44.49814  
 Root MSE 11.60694  
 GlutenIndex Mean 26.08409

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Gluten	1	3180.13630	3180.13630	23.61	<.0001
Treatment	3	34916.80803	11638.93601	86.39	<.0001
Gluten*Treatment	3	5719.71561	1906.57187	14.15	<.0001

11

The SAS System

The GLM Procedure

Dependent Variable: WetGluten

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	1371.707955	195.958279	2.31	0.0474
Error	36	3057.660000	84.935000		
Corrected Total	43	4429.367955			

R-Square 0.309685  
 Coeff Var 31.03749  
 Root MSE 9.216019  
 WetGluten Mean 29.69318

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Gluten	1	479.4224074	479.4224074	5.64	0.0230
Treatment	3	376.6854545	125.5618182	1.48	0.2368
Gluten*Treatment	3	444.0568182	148.0189394	1.74	0.1756

12

The SAS System

Obs	Gluten	Treatment	_TYPE_	_FREQ_	mResp Dist	m Gluten Index	mWet Gluten
1	Bad	Control	0	4	2.64333	58.1500	32.0750
2	Bad	Cysteine	0	6	2.95733	0.3167	22.2167
3	Bad	Phosphat	0	6	1.86767	23.2667	33.0167
4	Bad	Sulfite	0	6	2.63233	1.3833	19.2833
5	Good	Control	0	4	2.63167	73.9000	34.6750
6	Good	Cysteine	0	6	3.89067	0.6833	32.7333
7	Good	Phosphat	0	6	1.98967	76.6667	32.0333
8	Good	Sulfite	0	6	3.26467	0.9333	33.9667

13

The SAS System

The CORR Procedure

3 Variables: mRespDist mGlutenIndex mWetGluten

Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum
mRespDist	8	2.73467	0.65498	21.87733	1.86767
mGlutenIndex	8	29.41250	34.52605	235.30000	0.31667
mWetGluten	8	30.00000	5.83130	240.00000	19.28333

Pearson Correlation Coefficients, N = 8  
Prob > |r| under H0: Rho=0

	mResp Dist	mGluten Index	mWet Gluten
mRespDist	1.00000	-0.56223 0.1469	-0.00657 0.9877
mGlutenIndex	-0.56223 0.1469	1.00000	0.48286 0.2255
mWetGluten	-0.00657 0.9877	0.48286 0.2255	1.00000

## Appendix B: SAS Output for Pilot Scale Extrusion

1

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
Country	2	EURO US
Moisture	2	High Low
Treatment	4	Control Cysteine Phosphat Sulfite

Number of Observations Read	80
Number of Observations Used	80

2

The SAS System

The GLM Procedure

Dependent Variable: AbsIndex

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	15	43.76114645	2.91740976	211.46	<.0001
Error	64	0.88297190	0.01379644		
Corrected Total	79	44.64411835			

R-Square	Coeff Var	Root MSE	AbsIndex Mean
0.980222	7.307371	0.117458	1.607394

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Country	1	0.01036263	0.01036263	0.75	0.3894
Moisture	1	15.43568425	15.43568425	1118.82	<.0001
Country*Moisture	1	0.65675940	0.65675940	47.60	<.0001
Treatment	3	22.01649431	7.33883144	531.94	<.0001
Country*Treatment	3	3.54496373	1.18165458	85.65	<.0001
Moisture*Treatment	3	1.61705386	0.53901795	39.07	<.0001
Countr*Moistu*Treatm	3	0.47982826	0.15994275	11.59	<.0001

3

The SAS System

The GLM Procedure  
Least Squares Means  
Adjustment for Multiple Comparisons: Tukey

Country	Moisture	Treatment	AbsIndex LSMEAN	LSMEAN Number
EURO	High	Control	0.75800000	1
EURO	High	Cysteine	0.75230000	2
EURO	High	Phosphat	2.54920000	3
EURO	High	Sulfite	1.02100000	4
EURO	Low	Control	1.60670000	5
EURO	Low	Cysteine	1.15300000	6
EURO	Low	Phosphat	3.04170000	7
EURO	Low	Sulfite	2.06830000	8
US	High	Control	0.82740000	9
US	High	Cysteine	0.88260000	10
US	High	Phosphat	1.68190000	11
US	High	Sulfite	0.87270000	12
US	Low	Control	2.48130000	13
US	Low	Cysteine	1.49810000	14
US	Low	Phosphat	2.63670000	15
US	Low	Sulfite	1.88740000	16

Least Squares Means for effect Countr\*Moistu\*Treatm  
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: AbsIndex



Absorption Index: Country*Moisture*Treatment																
i/j	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	x	1	<.0001	0.0535	<.0001	0.0002	<.0001	<.0001	0.9999	0.9453	<.0001	0.9724	<.0001	<.0001	<.0001	<.0001
2	1	x	<.0001	0.0434	<.0001	0.0001	<.0001	<.0001	0.9997	0.9231	<.0001	0.9584	<.0001	<.0001	<.0001	<.0001
3	<.0001	<.0001	x	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.9999	<.0001	0.9981	<.0001
4	0.0535	0.0434	<.0001	x	<.0001	0.9155	<.0001	<.0001	0.4129	0.8824	<.0001	0.8181	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	<.0001	x	<.0001	<.0001	<.0001	<.0001	<.0001	0.9997	<.0001	<.0001	0.983	<.0001	0.0274
6	0.0002	0.0001	<.0001	0.9155	<.0001	x	<.0001	<.0001	0.0042	0.0407	<.0001	0.0279	<.0001	<.0001	<.0001	<.0001
7	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	x	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
8	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	x	<.0001	<.0001	0.0002	<.0001	<.0001	<.0001	<.0001	0.5297
9	0.9999	0.9997	<.0001	0.4129	<.0001	0.0042	<.0001	<.0001	x	1	<.0001	1	<.0001	<.0001	<.0001	<.0001
10	0.9453	0.9231	<.0001	0.8824	<.0001	0.0407	<.0001	<.0001	1	x	<.0001	1	<.0001	<.0001	<.0001	<.0001
11	<.0001	<.0001	<.0001	<.0001	0.9997	<.0001	<.0001	0.0002	<.0001	<.0001	x	<.0001	<.0001	0.5024	<.0001	0.3144
12	0.9724	0.9584	<.0001	0.8181	<.0001	0.0279	<.0001	<.0001	1	1	<.0001	x	<.0001	<.0001	<.0001	<.0001
13	<.0001	<.0001	0.9999	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	x	<.0001	0.763	<.0001
14	<.0001	<.0001	<.0001	<.0001	0.983	0.0017	<.0001	<.0001	<.0001	<.0001	0.5024	<.0001	<.0001	x	<.0001	0.0002
15	<.0001	<.0001	0.9981	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.763	<.0001	x
16	<.0001	<.0001	<.0001	<.0001	0.0274	<.0001	<.0001	0.5297	<.0001	<.0001	0.3144	<.0001	<.0001	0.0002	<.0001	x

Table B.1: Three-way comparison between source country, in-barrel moisture and chemical treatment for absorption index

5

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
Country	2	Euro US
Treatment	4	Control Cysteine Phosphat Sulfite

Number of Observations Read 16  
 Number of Observations Used 16

6

The SAS System

The GLM Procedure

Dependent Variable: GlutenIndex

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	25785.44938	3683.63563	476.73	<.0001
Error	8	61.81500	7.72687		
Corrected Total	15	25847.26438			

R-Square 0.997608  
 Coeff Var 6.917967  
 Root MSE 2.779726  
 GlutenIndex Mean 40.18125

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Country	1	66.83062	66.83062	8.65	0.0187
Treatment	3	25634.74688	8544.91563	1105.87	<.0001
Country*Treatment	3	83.87188	27.95729	3.62	0.0647

7

The SAS System

The GLM Procedure  
 Least Squares Means

Country	GlutenIndex LSMEAN
Euro	38.1375000
US	42.2250000

Treatment	GlutenIndex LSMEAN
Control	84.4750000
Cysteine	0.5500000
Phosphat	75.7000000
Sulfite	-0.0000000

8

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
Country	2	Euro US
Treatment	4	Control Cysteine Phosphat Sulfite

Number of Observations Read 16  
 Number of Observations Used 16

9

The SAS System

The GLM Procedure

Dependent Variable: WetGluten

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	3456.959375	493.851339	136.35	<.0001
Error	8	28.975000	3.621875		
Corrected Total	15	3485.934375			

R-Square 0.991688    Coeff Var 7.512943    Root MSE 1.903122    WetGluten Mean 25.33125

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Country	1	13.505625	13.505625	3.73	0.0896
Treatment	3	3438.711875	1146.237292	316.48	<.0001
Country*Treatment	3	4.741875	1.580625	0.44	0.7330

10

The SAS System

The GLM Procedure

Least Squares Means

Adjustment for Multiple Comparisons: Tukey

Treatment	WetGluten LSMEAN	LSMEAN Number
Control	33.0750000	1
Cysteine	35.4250000	2
Phosphat	32.8250000	3
Sulfite	-0.0000000	4

Least Squares Means for effect Treatment  
 Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: WetGluten

Wet Gluten Percent: Country*Treatment				
i/j	1	2	3	4
1	x	0.3625	0.9975	< 0.0001
2	0.3625	x	0.2881	< 0.0001
3	0.9975	0.2281	x	< 0.0001
4	< 0.0001	< 0.0001	< 0.0001	x

Table B.2: Two-way comparison between source country and chemical treatment

The SAS System

11

Obs	Country	Moisture	Treatment	Loss	Loss2
1	US	High	Phosphat	0.00%	0.0
2	US	High	Phosphat	7.30%	7.3
3	US	High	Phosphat	4.10%	4.1
4	US	Low	Phosphat	5.90%	5.9
5	US	Low	Phosphat	5.50%	5.5
6	US	Low	Phosphat	3.20%	3.2
7	US	High	Cysteine	19.30%	19.3
8	US	High	Cysteine	19.50%	19.5
9	US	High	Cysteine	20.60%	20.6
10	US	Low	Cysteine	2.70%	2.7
11	US	Low	Cysteine	3.50%	3.5
12	US	Low	Cysteine	5.70%	5.7
13	US	High	Sulfite	11.80%	11.8
14	US	High	Sulfite	7.50%	7.5
15	US	High	Sulfite	3.30%	3.3
16	US	Low	Sulfite	3.40%	3.4
17	US	Low	Sulfite	4.20%	4.2
18	US	Low	Sulfite	0.80%	0.8
19	US	High	Control	17.10%	17.1
20	US	High	Control	13.40%	13.4
21	US	High	Control	15.60%	15.6
22	US	Low	Control	3.50%	3.5
23	US	Low	Control	2.40%	2.4
24	US	Low	Control	4.20%	4.2
25	Euro	High	Phosphat	0.10%	0.1
26	Euro	High	Phosphat	0.00%	0.0
27	Euro	High	Phosphat	0.50%	0.5
28	Euro	Low	Phosphat	13.80%	13.8
29	Euro	Low	Phosphat	6.90%	6.9
30	Euro	Low	Phosphat	6.60%	6.6
31	Euro	High	Cysteine	13.40%	13.4
32	Euro	High	Cysteine	7.90%	7.9
33	Euro	High	Cysteine	8.10%	8.1
34	Euro	Low	Cysteine	13.60%	13.6
35	Euro	Low	Cysteine	10.70%	10.7
36	Euro	Low	Cysteine	9.30%	9.3
37	Euro	High	Sulfite	8.20%	8.2
38	Euro	High	Sulfite	13.00%	13.0
39	Euro	High	Sulfite	4.10%	4.1
40	Euro	Low	Sulfite	15.00%	15.0
41	Euro	Low	Sulfite	15.30%	15.3
42	Euro	Low	Sulfite	14.20%	14.2
43	Euro	High	Control	11.20%	11.2
44	Euro	High	Control	4.20%	4.2
45	Euro	High	Control	10.10%	10.1
46	Euro	Low	Control	5.20%	5.2
47	Euro	Low	Control	1.00%	1.0
48	Euro	Low	Control	9.80%	9.8

12

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
Country	2	Euro US
Moisture	2	High Low
Treatment	4	Control Cysteine Phosphat Sulfite

Number of Observations Read 48  
 Number of Observations Used 48

13

The SAS System

The GLM Procedure

Dependent Variable: Loss2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	15	1247.044792	83.136319	10.32	<.0001
Error	32	257.673333	8.052292		
Corrected Total	47	1504.718125			

R-Square 0.828756  
 Coeff Var 35.22304  
 Root MSE 2.837656  
 Loss2 Mean 8.056250

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Country	1	6.5268750	6.5268750	0.81	0.3747
Moisture	1	60.5252083	60.5252083	7.52	0.0099
Country*Moisture	1	380.2502083	380.2502083	47.22	<.0001
Treatment	3	271.9506250	90.6502083	11.26	<.0001
Country*Treatment	3	142.9756250	47.6585417	5.92	0.0025
Moisture*Treatment	3	344.8206250	114.9402083	14.27	<.0001
Countr*Moistu*Treatm	3	39.9956250	13.3318750	1.66	0.1962

14

The SAS System

The GLM Procedure

Least Squares Means

Adjustment for Multiple Comparisons: Tukey

Country	Moisture	Loss2 LSMEAN	LSMEAN Number
Euro	High	6.7333333	1
Euro	Low	10.1166667	2
US	High	11.6250000	3
US	Low	3.7500000	4

Least Squares Means for effect Country\*Moisture

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: Loss2

Gluten Integrity: Country*Moisture				
i/j	1	2	3	4
1	x	0.0307	0.001	0.0673
2	0.0307	x	0.5684	< 0.0001
3	0.001	0.5684	x	< 0.0001
4	0.0673	< 0.0001	< 0.0001	x

Table B.3: Two-way comparison between source country and in-barrel moisture

The SAS System

15

The GLM Procedure  
Least Squares Means  
Adjustment for Multiple Comparisons: Tukey

Country	Treatment	Loss2 LSMEAN	LSMEAN Number
Euro	Control	6.9166667	1
Euro	Cysteine	10.5000000	2
Euro	Phosphat	4.6500000	3
Euro	Sulfite	11.6333333	4
US	Control	9.3666667	5
US	Cysteine	11.8833333	6
US	Phosphat	4.3333333	7
US	Sulfite	5.1666667	8

Least Squares Means for effect Country\*Treatment  
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: Loss2

Gluten Integrity: Country*Treatment								
i/j	1	2	3	4	5	6	7	8
1	x	0.3855	0.8579	0.111	0.8041	0.08	0.76	0.9589
2	0.3855	x	0.0225	0.9967	0.9967	0.9889	0.0138	0.0482
3	0.8579	0.0225	x	0.0037	0.111	0.0024	1	1
4	0.111	0.9967	0.0037	x	0.8579	1	0.0022	0.0086
5	0.8041	0.9967	0.111	0.8579	x	0.7825	0.0731	0.2065
6	0.08	0.9889	0.0024	1	0.7825	x	0.0014	0.0057
7	0.76	0.0138	1	0.0022	0.0731	0.0014	x	0.9995
8	0.9589	0.0482	1	0.0086	0.2065	0.0057	0.9995	x

Table B.4: Two-way comparison between source country and chemical treatment for

TVP integrity

The GLM Procedure  
 Least Squares Means  
 Adjustment for Multiple Comparisons: Tukey

Moisture	Treatment	Loss2	LSMEAN	LSMEAN Number
High	Control	11.9333333		1
High	Cysteine	14.8000000		2
High	Phospat	2.0000000		3
High	Sulfite	7.9833333		4
Low	Control	4.3500000		5
Low	Cysteine	7.5833333		6
Low	Phospat	6.9833333		7
Low	Sulfite	8.8166667		8

Least Squares Means for effect Moisture\*Treatment  
 Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: Loss2

Gluten Integrity: Moisture*Treatment								
i/j	1	2	3	4	5	6	7	8
1	x	0.6563	<.0001	0.2704	0.0013	0.1738	0.0818	0.5593
2	0.6563	x	<.0001	0.0049	<.0001	0.0025	0.0009	0.0184
3	<.0001	<.0001	x	0.0184	0.8346	0.0335	0.0782	0.0049
4	0.2704	0.0049	0.0184	x	0.3684	1	0.9985	0.9995
5	0.0013	<.0001	0.8346	0.3684	x	0.5141	0.7426	0.1513
6	0.1738	0.0025	0.0335	1	0.5141	x	0.9999	0.9944
7	0.0818	0.0009	0.0782	0.9985	0.7426	0.9999	x	0.9477
8	0.5593	0.0184	0.0049	0.9995	0.1513	0.9944	0.9477	x

Table B.5: Two-way comparison between in-barrel moisture and chemical treatment for

TVP integrity

The GLM Procedure  
 Class Level Information

Class	Levels	Values
Country	2	Euro US
Moisture	2	High Low
Treatment	4	Control Cysteine Phospat Sulfite

Number of Observations Read 48  
 Number of Observations Used 48

18

The SAS System

The GLM Procedure

Dependent Variable: Time

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	15	5074.126315	338.275088	527.16	<.0001
Error	32	20.534067	0.641690		
Corrected Total	47	5094.660381			

R-Square	Coeff Var	Root MSE	Time Mean
0.995969	3.532671	0.801055	22.67563

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Country	1	1.459519	1.459519	2.27	0.1413
Moisture	1	1434.125352	1434.125352	2234.92	<.0001
Country*Moisture	1	43.643602	43.643602	68.01	<.0001
Treatment	3	2503.001556	834.333852	1300.21	<.0001
Country*Treatment	3	734.239290	244.746430	381.41	<.0001
Moisture*Treatment	3	246.909023	82.303008	128.26	<.0001
Countr*Moistu*Treatm	3	110.747973	36.915991	57.53	<.0001

19

The SAS System

The GLM Procedure

Dependent Variable: Rate

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	15	2413.895501	160.926367	147.03	<.0001
Error	32	35.023712	1.094491		
Corrected Total	47	2448.919213			

R-Square	Coeff Var	Root MSE	Rate Mean
0.985698	12.90287	1.046179	8.108116

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Country	1	116.192105	116.192105	106.16	<.0001
Moisture	1	446.778223	446.778223	408.21	<.0001
Country*Moisture	1	0.105784	0.105784	0.10	0.7579
Treatment	3	1289.728511	429.909504	392.79	<.0001
Country*Treatment	3	460.834932	153.611644	140.35	<.0001
Moisture*Treatment	3	82.829285	27.609762	25.23	<.0001
Countr*Moistu*Treatm	3	17.426662	5.808887	5.31	0.0044

20

The SAS System

The GLM Procedure

Least Squares Means

Adjustment for Multiple Comparisons: Tukey



Country	Moisture	Treatment	Time LSMEAN	LSMEAN Number
Euro	High	Control	28.3500000	1
Euro	High	Cysteine	31.3666667	2
Euro	High	Phosphat	21.7700000	3
Euro	High	Sulfite	34.1966667	4
Euro	Low	Control	13.0500000	5
Euro	Low	Cysteine	19.5966667	6
Euro	Low	Phosphat	11.3233333	7
Euro	Low	Sulfite	20.3566667	8
US	High	Control	28.5933333	9
US	High	Cysteine	38.3500000	10
US	High	Phosphat	8.4500000	11
US	High	Sulfite	34.0566667	12
US	Low	Control	15.0200000	13
US	Low	Cysteine	37.1500000	14
US	Low	Phosphat	6.0700000	15
US	Low	Sulfite	15.1100000	16

Least Squares Means for effect Countr\*Moistu\*Treatm  
Pr > |t| for H0: LSMean(i)=LSMean(j)

Hydration Time: Country*Moisture*Treatment																	
i/j	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
1	x	0.005	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	
2	0.005	x	<.0001	0.0107	<.0001	<.0001	<.0001	<.0001	0.0134	<.0001	<.0001	0.0186	<.0001	<.0001	<.0001	<.0001	
3	<.0001	<.0001	x	<.0001	<.0001	0.1184	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	
4	<.0001	0.0107	<.0001	x	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1	<.0001	0.0065	<.0001	<.0001	
5	<.0001	<.0001	<.0001	<.0001	x	<.0001	0.408	0.1686	<.0001	<.0001	<.0001	<.0001	0.2194	<.0001	<.0001	0.1686	
6	<.0001	<.0001	0.1184	<.0001	<.0001	x	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	
7	<.0001	<.0001	<.0001	<.0001	0.408	<.0001	x	<.0001	<.0001	<.0001	0.009	<.0001	0.0003	<.0001	<.0001	0.0002	
8	<.0001	<.0001	0.7154	<.0001	<.0001	0.9978	<.0001	x	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	
9	1	0.0134	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	x	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	
10	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	x	<.0001	<.0001	<.0001	0.8854	<.0001	<.0001	
11	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.009	<.0001	<.0001	<.0001	x	<.0001	<.0001	<.0001	0.0588	<.0001	
12	<.0001	0.0186	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	x	<.0001	0.0037	<.0001	<.0001	
13	<.0001	<.0001	<.0001	<.0001	0.2194	<.0001	0.0003	<.0001	<.0001	<.0001	<.0001	<.0001	x	<.0001	<.0001	1	
14	<.0001	<.0001	<.0001	0.0065	<.0001	<.0001	<.0001	<.0001	<.0001	0.8854	<.0001	0.0037	<.0001	x	<.0001	<.0001	
15	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0588	<.0001	<.0001	<.0001	x	<.0001	
16	<.0001	<.0001	<.0001	<.0001	0.1686	<.0001	0.0002	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1	<.0001	<.0001	x

Table B.6: Three-way comparison between source country, in-barrel moisture, and chemical treatment for TVP hydration time

Country	Moisture	Treatment	Rate LSMEAN	LSMEAN Number
Euro	High	Control	2.8206715	1
Euro	High	Cysteine	2.5155117	2
Euro	High	Phosphat	5.8389144	3
Euro	High	Sulfite	2.6426587	4
Euro	Low	Control	11.2935227	5
Euro	Low	Cysteine	6.3058486	6
Euro	Low	Phosphat	14.4807090	7
Euro	Low	Sulfite	6.5202901	8
US	High	Control	2.5814692	9
US	High	Cysteine	2.1963494	10
US	High	Phosphat	18.3475345	11
US	High	Sulfite	3.5147620	12
US	Low	Control	8.3922510	13
US	Low	Cysteine	3.4600722	14
US	Low	Phosphat	29.0345964	15
US	Low	Sulfite	9.7846900	16

The SAS System

22

The GLM Procedure

Least Squares Means

Adjustment for Multiple Comparisons: Tukey

Least Squares Means for effect Countr\*Moistu\*Treatm

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: Rate

Hydration Rate: Country*Moisture*Treatment																	
i/j	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
1	x	1	0.0748	1	<.0001	0.0202	<.0001	0.0106	1	1	<.0001	1	<.0001	1	<.0001	<.0001	
2	1	x	0.0323	1	<.0001	0.008	<.0001	0.0041	1	1	<.0001	0.9976	<.0001	0.9987	<.0001	<.0001	
3	0.0748	0.0323	x	0.0462	<.0001	1	<.0001	1	0.0389	0.0126	<.0001	0.361	0.2288	0.326	<.0001	0.0049	
4	1	1	0.0462	x	<.0001	0.0118	<.0001	0.0061	1	1	<.0001	0.9995	<.0001	0.9997	<.0001	<.0001	
5	<.0001	<.0001	<.0001	<.0001	x	0.0002	0.0473	0.0003	<.0001	<.0001	<.0001	<.0001	0.1011	<.0001	<.0001	0.9109	
6	0.0202	0.008	1	0.0118	0.0002	x	<.0001	1	0.0098	0.0029	<.0001	0.1329	0.5325	0.1162	<.0001	0.0205	
7	<.0001	<.0001	<.0001	<.0001	0.0473	<.0001	x	<.0001	<.0001	<.0001	0.0063	<.0001	<.0001	<.0001	<.0001	0.0004	
8	0.0106	0.0041	1	0.0061	0.0003	1	<.0001	0.005	0.005	0.0015	<.0001	0.0773	0.6963	0.0669	<.0001	0.0382	
9	1	1	0.0389	1	<.0001	0.0098	<.0001	0.0015	x	1	<.0001	0.9988	<.0001	0.9994	<.0001	<.0001	
10	1	1	0.0126	1	<.0001	0.0029	<.0001	<.0001	1	x	<.0001	0.9676	<.0001	0.9773	<.0001	<.0001	
11	<.0001	<.0001	0.361	<.0001	<.0001	<.0001	0.0063	<.0001	<.0001	<.0001	x	<.0001	<.0001	<.0001	<.0001	<.0001	
12	1	0.9976	0.2288	0.9995	<.0001	0.1329	<.0001	0.0773	0.9988	0.9676	<.0001	x	0.0002	1	<.0001	<.0001	
13	<.0001	<.0001	<.0001	<.0001	0.1011	0.5325	<.0001	0.6963	<.0001	<.0001	<.0001	0.0002	x	0.0002	<.0001	0.9501	
14	1	0.9987	0.326	0.9997	<.0001	0.1162	<.0001	0.0669	0.9994	0.9773	<.0001	1	0.0002	x	<.0001	<.0001	
15	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	x	<.0001
16	<.0001	<.0001	0.0049	<.0001	0.9109	0.0205	0.0004	0.0382	<.0001	<.0001	<.0001	<.0001	0.9501	<.0001	<.0001	<.0001	x

Table B.7: Three-way comparison between source country, in-barrel moisture, and chemical treatment for TVP hydration rate

23

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
Country	2	EURO US
Treatment	4	Control Cysteine Phosphat Sulfite

Number of Observations Read	16
Number of Observations Used	16

24

The SAS System

The GLM Procedure

Dependent Variable: SoftTemp

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	184.4493750	26.3499107	8.19	0.0041
Error	8	25.7450000	3.2181250		
Corrected Total	15	210.1943750			

R-Square	Coeff Var	Root MSE	SoftTemp Mean
0.877518	4.325940	1.793913	41.46875

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Country	1	27.3006250	27.3006250	8.48	0.0195
Treatment	3	140.0318750	46.6772917	14.50	0.0013
Country*Treatment	3	17.1168750	5.7056250	1.77	0.2299

25

The SAS System

The GLM Procedure

Dependent Variable: MeltTemp

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	1892.109375	270.301339	14.33	0.0006
Error	8	150.895000	18.861875		
Corrected Total	15	2043.004375			

R-Square	Coeff Var	Root MSE	MeltTemp Mean
0.926141	4.509014	4.343026	96.31875

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Country	1	1550.390625	1550.390625	82.20	<.0001
Treatment	3	243.076875	81.025625	4.30	0.0441
Country*Treatment	3	98.641875	32.880625	1.74	0.2354

26

The SAS System

The GLM Procedure  
Least Squares Means  
Adjustment for Multiple Comparisons: Tukey

Country	SoftTemp	H0:LSMean1=
	LSMEAN	LSMean2 Pr >  t
EURO	40.1625000	0.0195
US	42.7750000	

Country	MeltTemp	H0:LSMean1=
	LSMEAN	LSMean2 Pr >  t
EURO	86.475000	<.0001
US	106.162500	

27

The SAS System

The GLM Procedure  
Least Squares Means  
Adjustment for Multiple Comparisons: Tukey

Treatment	SoftTemp	LSMEAN
	LSMEAN	Number
Control	40.8250000	1
Cysteine	39.2000000	2
Phosphat	46.4750000	3
Sulfite	39.3750000	4

Least Squares Means for effect Treatment  
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: SoftTemp

Thermal Softening				
i/j	1	2	3	4
1	x	0.5983	0.0092	0.6755
2	0.5983	x	0.002	0.999
3	0.0092	0.002	x	0.0023
4	0.6755	0.999	0.0023	x

Table B.8: Effect of chemical treatment on thermal softening

Treatment	MeltTemp LSMEAN	LSMEAN Number
Control	97.475000	1
Cysteine	92.600000	2
Phosphat	102.200000	3
Sulfite	93.000000	4

Least Squares Means for effect Treatment  
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: MeltTemp

Thermal Flow				
i/j	1	2	3	4
1	x	0.4361	0.4604	0.5024
2	0.4361	x	0.0557	0.9991
3	0.4604	0.0557	x	0.0669
4	0.5024	0.9991	0.0669	x

Table B.9: Effect of chemical treatment on thermal flow