12-2013

Investigation of Commercial Milk Enzyme-Linked Immunosorbent Assay (ELISA) Kits: Specificity and Utility for Residues of Foods Subjected to Proteolysis During Processing

Katherine O. Ivens
University of Nebraska – Lincoln, kivens2@unl.edu

INVESTIGATION OF COMMERCIAL MILK ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) KITS: SPECIFICITY AND UTILITY FOR RESIDUES OF FOODS SUBJECTED TO PROTEOLYSIS DURING PROCESSING

by

Katherine O. Ivens

A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Food Science and Technology

Under the Supervision of Professors Steven L. Taylor and Joseph L. Baumert

Lincoln, Nebraska

December, 2013

INVESTIGATION OF COMMERCIAL MILK ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) KITS: SPECIFICITY AND UTILITY FOR RESIDUES OF FOODS SUBJECTED TO PROTEOLYSIS DURING PROCESSING

Katherine O. Ivens, M.S.

University of Nebraska, 2013

Advisers: Stephen L. Taylor and Joseph L. Baumert

Analytical methods, such as enzyme-linked immunosorbent assays (ELISA), are used to detect and quantify residues from allergenic sources in food products. However, ELISAs have not been validated for use in foods that have been exposed to proteolysis. This thesis explores the specificities, sensitivities, and capabilities of commercially-available milk ELISA kits for detecting milk residues in cheeses that have undergone varying degrees of proteolysis.

The specificity, accuracy, and consistency of twelve commercially-available milk ELISA kits for individual milk proteins and commonly used milk-derived ingredients, including α-,β-, and κ-casein, β-lactoglobulin, α-lactalbumin, non-fat dry milk, sodium caseinate, and whey protein concentrate were evaluated. ELISA kits exhibited targeted specificities to milk proteins as opposed to broad-spectrum detection. Kits were able to detect milk residues in all derivative ingredients, although the kits were not quantitatively accurate.

Further, ELISA kits were used to investigate the effects of proteolysis in Cheddar cheese during aging. As cheese ripened and proteolysis continued, fewer milk residues were detected in samples using commercial milk ELISA kits. In a survey of retail cheeses produced with different degrees of proteolysis, the lowest concentration of milk residues were detected in Blue cheese, while Mozzarella cheese contained the highest milk residues. Emmentaler, Brie, and Limburger cheeses were also evaluated. Five samples of enzyme-modified cheese (EMC) were assessed for

their milk residue content; again, not all ELISA kits were able to detect milk residues in samples that had been subjected to extensive proteolysis.

The recommendation of specific ELISA kits for detecting milk residues is highly product-specific. Current commercially-available milk ELISA kits are capable of detecting milk residues in a variety of cheeses, including some that have been exposed to extensive proteolysis. However, the quantitative accuracy of commercial milk ELISA kits is jeopardized when proteolysis has occurred. Some ELISAs may have further application to monitor proteolysis and indicate cheese maturity during ripening because of their specificities.

To Alex and Dad, for approaching every situation with logic, reason, and humor.

Carpe diem

Horace, 23 BC, *Odes 1.11*

"You can't help respecting anybody who can spell TUESDAY, even if he doesn't spell it right; but spelling isn't everything. There are days when spelling Tuesday simply doesn't count."

-A.A. Milne, *Winnie-the-Pooh*

ACKNOWLEDGEMENTS

To begin, I would like to thank my advisers, Drs. Steve Taylor and Joe Baumert, for their constant dedication to students, research, and education. Working, learning, researching, and writing under your supervision continues to be a wonderful and rewarding experience. The environment within FARRP and the University of Nebraska that you have created is world-class and unparalleled. I am looking forward to working closely with you in the coming years of my graduate career and honored to have you as mentors.

My colleagues and coworkers, thank you for providing good ideas, examples, and distractions throughout my ongoing graduate work. I am especially thankful to Dr. Ben Remington for his friendship and opinions. To my friends Amy and Sonia; it is safe to say I know where I'd be without your dedication and passion for helping others. From the bottom of my heart: thank you for all you do.

I would especially like to thank Julie Nordlee for teaching me new methods and encouraging new ways of thinking. Working with you has been a great experience, and I hope it continues for years to come. I am proud to be your coworker and equally proud to be your friend.

To my dearest friend and honey bee, Alex: undoubtedly, this has been a difficult year for many reasons. You've been with me every moment, and I can't express my gratitude and appreciation sufficiently. In the years to come, I hope to echo the support, strength, and love you have shown me. Together, we will conquer anything.

Mom and Dad, I am eternally grateful for your passion and dedication to education, family, and faith. I am so proud to be your daughter, and I am fortunate to have your love and support as I continue to grow. I often ponder about how different life might be if you had made different decisions in your lives, and I am thankful to you for setting such meaningful examples by choosing the paths that honor your family and faith.

Although I will always be a loyal alumna of Michigan State University, I am more and more honored by my experiences and education at the University of Nebraska.

GRANT INFORMATION

Financial support for this research was generously provided by USDA-NIFA-AFRI Grant 2011-67017-20080 and the Food Allergy Research and Resource Program at the University of Nebraska-Lincoln.

TABLE OF CONTENTS

GRANT INFORMATION ..... **vii**
LIST OF TABLES ..... **xii**
LIST OF FIGURES ..... **xv**
LIST OF EQUATIONS ..... **xix**
CHAPTER 1. LITERATURE REVIEW **1**
1. Introduction 1
2. Food Allergens and Allergy 2
3. Adverse Reactions to Food 3
a. Food Intolerances 3
b. IgE-mediated Food Allergy 4
c. Non-IgE-mediated Food Allergy 7
d. Diagnosis of Food Allergy 8
e. Treatment and Therapy for Food Allergy 10
4. Bovine Milk 10
a. Composition 11
b. Cow's Milk Allergy (CMA) 16
c. Major Allergenic Epitopes of Bovine Milk 18
5. Detection of Food Allergens 24
a. ELISAs 25
b. Commercially-available ELISAs: Interpretation of Results and Limitations 28
6. Effects of Processing on Allergens 32
a. Thermal Denaturation of Proteins 35

b. Processing-induced changes in allergenicity and immunoreactivity 36

c. Enzymatic Hydrolysis and Fermentation 39

7. Cheese Manufacture 41

a. Milk Selection and Pre-treatment 42

b. Starter Culture Addition 42

c. Post-acidification 43

8. Proteolysis during Cheese Manufacture and Ripening 44

a. Chymosin 45

b. Indigenous Milk Enzymes 47

c. Primary Starter Cultures 48

d. Non-starter Lactic Acid Bacteria 49

e. Secondary Cultures and Adjunct Microflora 50

9. Monitoring Proteolysis in Dairy Products 52

a. Non-specific Methods 52

b. Specific Methods 53

10. Conclusions 54

11. References 56

CHAPTER 2. EVALUATION AND VALIDATION OF COMMERCIAL MILK ELISA KITS 67

1. Abstract 67

2. Introduction 67

3. Materials and Methods 73

Kit Selection and Procurement 73

ELISA Analysis 75

4. Results 76

5. Discussion 83

6. References 86

CHAPTER 3: COMMERCIAL MILK ELISA KIT SENSITIVITES TO PURIFIED MILK PROTEINS AND MILK-DERIVED INGREDIENTS 101

1. Abstract 101

2. Introduction 102

3. Materials and Methods 106

Materials, Equipment, and Kits 106

Spike Preparation 109

Sample Evaluation: Protein Concentration and Electrophoresis 109

Kit Selection 110

Sample Preparation and Extraction for ELISA ktis 111

ELISA Analysis 112

Criteria for acceptable ELISA results 113

Data Analysis 113

Kit Comparison Approaches 113

4. Results 115

SDS-PAGE 115

Casein Kits and Detection of α-, β-, and κ-casein 121

BLG Kits and Detection of Whey Proteins 124

Total Milk Kits and Detection of Milk Proteins 126

Detection of Milk-Derived Ingredients with ELISA kits 128

Detection of NFDM using milk ELISA kits 135

5. Discussion 138

6. References 141

CHAPTER 4. EFFECT OF PROTEOLYSIS DURING CHEDDAR CHEESE AGING ON THE DETECTION OF MILK PROTEIN RESIDUES BY ENZYME-LINKED IMMUNOSORBENT ASSAY 144

1. Abstract 144

2. Introduction 145

3. Materials and Methods 150

Cheese manufacture 150

Sampling during manufacture and aging 151

Sample Preparation for SDS-PAGE and ELISA Analyses 151

Protein Concentration of Extracts 152

Electrophoresis 155

ELISA Analysis 156

4. Results/Discussion 157

Protein Concentration of Extracts 157

Electrophoresis 162

ELISA Analysis 165

5. Acknowledgements 177

6. References 178

CHAPTER 5. SURVEY OF RETAIL CHEESES: THE EFFECT OF DIFFERENT METHODS OF FERMENTATION ON THE DETECTION OF MILK PROTEIN RESIDUES BY ENZYME-LINKED IMMUNOSORBENT ASSAY 181

1. Abstract 181

2. Introduction 181

3. Materials and Methods 183

Cheese Procurement and Sample Preparation 183

Preparation of Extracts for SDS-PAGE and ELISA Analysis 184

Protein Concentration of Extracts 185

Electrophoresis 187

ELISA Analysis 188

Data Analysis 190

4. Results 191

Protein Concentration Determination Assays 191

Electrophoresis 193

ELISA Analysis 195

5. Discussion 199

6. References 204

CHAPTER 6. THE DETECTION OF MILK RESIDUES IN ENZYME-MODIFIED CHEESES USING COMMERCIAL ENZYME-LINKED IMMUNOSORBENT ASSAY KITS 206

1. Abstract 206

2. Introduction 207

3. Materials and Methods 209

Sample Procurement and Preparation 209

Determination of Protein Concentration 211

Electrophoresis 212

Enzyme-Linked Immunosorbent Assays 213

Data Analysis and Criteria for Acceptable Performance of ELISA kits 215

4. Results 216

Determination of Protein Concentration 216

Electrophoresis 218

ELISA Analysis 221

5. Discussion 224

6. References 230

7. Acknowledgments 231

APPENDIX A. ADDITIONAL MATHEMATICAL EQUATIONS AND CONVERSIONS 232

APPENDIX B. COMMERCIAL MILK ELISA KIT OPERATING INFORMATION 235

APPENDIX C. RESEARCH SUMMARY 236

LIST OF TABLES

CHAPTER 1 ..... Page

Table 1.1. Sizes of Major Allergenic Milk Proteins 11

Table 1.2. Food Processing Methods and Classification 33

CHAPTER 2

Table 2.1 Commercial milk ELISA kits and lot numbers used 74

Table 2.2. Results from standard solution analysis of commercial ELISA kits..... 77

Table 2.3. Kit performance sorted by common calibrator 81

Table 2.4. Information provided in kit inserts regarding calibrators and reporting units 82

CHAPTER 3

Table 3.1. Sensitivity, range, calibration, and reporting units of commercial milk ELISA kits 108

Table 3.2. LECO Dumas Protein Analysis of Milk-Derived Ingredients 129

Table 3.3. Conversion Factors for ppm spike to ppm reporting unit 130

Table 3.4. Sensitivities of milk ELISA kits to purified milk proteins and milk ingredients..... 137

CHAPTER 4

Table 4.1. Cumulative Protein Concentration Assay data for Cheddar cheese aged 0.5-24 months 161

Table 4.2. Sensitivities of selected milk ELISA kits as described in Chapter 3 166

Table 4.3. Milk residues in aged Cheddar cheese as detected by the Neogen Veratox® Total Milk kit 166

Table 4.4. Detection of milk residues in aged Cheddar cheese with the Neogen Veratox® Casein kit. 168

Table 4.5. Detection of casein by the r-Biopharm® Fast Casein kit in aged Cheddar cheese..... 170

Table 4.6. Detection of casein residues in aged Cheddar cheese using the ELISA Systems™ Casein kit 172

Table 4.7. Cumulative ELISA analysis of milk residues in aged Cheddar cheese 174

CHAPTER 5

Table 5.1. Commercial Milk ELISA kit sensitivities..... 189

Table 5.2. Micro-Lowry Protein Assay of Retail Cheeses..... 191

Table 5.3. Microtannin Protein Assay of Retail Cheeses..... 192

Table 5.4 Absorbance at 280 nm of Retail Cheeses with Warburg-Christian Correction. 192

Table 5.5. r-Biopharm RIDASCREEN® Fast Casein ELISA analysis of retail cheeses 196

Table 5.6. Neogen Veratox® Total Milk ELISA analysis of retail cheeses 196

Table 5.7. Neogen Veratox® Casein ELISA Analysis of Retail Cheeses. 197

Table 5.8. ELISA Systems™ Casein results of retail cheese analyses. 197

Table 5.9. Sensitivities of ELISA kits to purified casein fragments..... 199

CHAPTER 6

Table 6.1. Enzyme-Modified Cheese Samples 209

Table 6.2. Micro-Lowry Analysis of EMC Extracts 217

Table 6.3. Microtannin Analysis of EMC Extracts 217

Table 6.4. A_{280} Results from EMC Extracts 218

Table 6.5. Neogen Veratox® Total Milk ELISA Results for EMCs 221

Table 6.6. Neogen Veratox® Casein Analysis of EMC Samples 222

Table 6.7. r-Biopharm RIDASCREEN® Fast Casein Analysis of EMCs 223

Table 6.8. ELISA Systems™ Casein Analysis of EMCs 223

Table 6.9. Specificities of Commercial ELISA Kits for Detection of Purified Milk Proteins .. 225

APPENDIX B

Table B.1. Sensitivity, range, calibration, and reporting units of commercial milk ELISA kits 235

LIST OF FIGURES

CHAPTER 1 ..... Page

Figure 1.1. Sensitization and Degranulation of IgE- mediated food allergy.....6

Figure 1.2. Casein micelle structure as occurs in bovine milk.....12

Figure 1.3. Potential effects of processing on protein structure and epitopes.....17

Figure 1.4. α_{s1}-casein sequence with IgE and IgG binding epitopes.....19

Figure 1.5. α_{s2}-casein amino acid sequence and sequential IgE binding epitopes 20

Figure 1.6. β-casein amino acid sequence and IgE and IgG binding epitopes.....21

Figure 1.7. κ-casein IgE and IgG binding epitopes.....22

Figure 1.8. IgE and IgG binding epitopes on ALA.....23

Figure 1.9. IgE and IgG binding epitopes on BLG.....24

CHAPTER 2

Figure 2.1. Analysis of manufacturer-provided standard solutions. 78

Figure 2.2. Coefficient of Determination data for Commercial ELISA kits.....80

Figure 2.3. Performance of kit standards by common calibrator.....81

CHAPTER 3

Figure 3.1. SDS-PAGE image of α-casein; ≥70% purity 116

Figure 3.2. SDS-PAGE image of β-casein; ≥98% purity 117

Figure 3.3. SDS-PAGE Image of κ-casein; ≥70% purity 118

Figure 3.4. SDS-PAGE image of β-lactoglobulin: ≥90% purity 119

Figure 3.5. SDS-PAGE of Milk-Derived ingredients 120

Figure 3.6. Neogen Veratox® Casein Proportional ELISA Results 122

Figure 3.7. ELISA Systems™ Casein Proportional ELISA Analysis.....123

Figure 3.8. r-Biopharm RIDASCREEN® Fast Casein Proportional ELISA Analysis.....124

Figure 3.9. Results of BLG ELISA Kits 125

Figure 3.10. Neogen Veratox® Total Milk Proportional Spikes 127

Figure 3.11. Proportional detection with r-Biopharm RIDASCREEN® Fast Milk ELISA 128

Figure 3.12. Neogen Veratox® Total Milk Analysis of Milk Ingredient Spikes.....129

Figure 3.13. Proportional Neogen Veratox® Total Milk Analysis of Milk Ingredients Spikes .....
..... 131-132

Figure 3.14. r-Biopharm RIDASCREEN® Fast Milk Analysis of Milk Ingredient Spikes. 133

Figure 3.15. Proportional r-Biopharm RIDASCREEN® Fast Milk Analysis of Milk Ingredient Spikes.....134

Figure 3.16. Detection of NFDM spikes by commercial milk ELISA kits.....136

CHAPTER 4

Figure 4.1. Protein Concentration of Cheddar cheese aged 0.5-24 months as determined by the Lowry Protein Assay.....158

Figure 4.2. Protein concentration of Cheddar cheese aged 0.5-24 months as measured by absorbance at 280nm.....159

Figure 4.3. Protein concentration of Cheddar cheese aged 0.5-24 months, as measured using the microtannin protein assay. 160

Figure 4.4. Protein concentration of Cheddar cheese aged 0.5-24 months as determined by the Lowry Protein Assay, Absorbance at 280nm, and the Microtannin Protein Assay. 161

Figure 4.5. Profiles of aged Cheddar cheese (A) 163

Figure 4.6. Profiles of aged Cheddar cheese. (B) 164

Figure 4.7. Detection of milk residues in aged Cheddar cheese with the Neogen Veratox® Total Milk kit 167

Figure 4.8. Detection of ppm NFDM in aged Cheddar cheese using the Neogen Veratox® Casein kit. 169

Figure 4.9. Detection of casein residues in aged Cheddar cheese using the r-Biopharm RIDASCREEN® Fast Casein kit. 171

Figure 4.10. Detection of casein residues in aged Cheddar cheese using the ELISA Systems™ Casein kit. 173

Figure 4.11. Detected ppm NFDM in aged Cheddar cheese as measured by commercial milk ELISA kits 176

CHAPTER 5

Figure 5.1. Cumulative results of protein assays for retail cheese samples. 193

Figure 5.2. SDS-PAGE gel of 5% sodium chloride soluble cheese extracts; 15 μg protein per lane 195

Figure 5.3. Commercial Milk ELISA detection of retail cheeses. 198

CHAPTER 6

Figure 6.1. Cumulative Results of Protein Concentration for EMCs. 218

Figure 6.2. Enzyme-Modified Cheese 15 μg of protein loaded per lane. 219

Figure 6.3. Enzyme-Modified Cheese, 100 μg of protein loaded per lane 220

Figure 6.4. Cumulative ELISA Analysis of Enzyme Modified Cheese (EMC) Extracts 224

LIST OF EQUATIONS

CHAPTER 2 ..... Page

Equation 2.1. Calculation of % Difference from expected values (%DFE). 77

CHAPTER 3

Equation 3.1. Converting ppm casein to ppm milk protein 114

Equation 3.2. Converting ppm BLG to ppm milk protein 114

Equation 3.3. Converting ppm milk protein to ppm NFDM 114

Equation 3.4. Converting ppm NFDM to ppm milk protein 114

Equation 3.5. Applying conversion factors to spike concentrations for equivalent casein estimate 114

Equation 3.6. Applying conversion factors to kit results for whey protein spikes 114

Equation 3.7. Calculating ratios of quantitation for ELISA kits using milk proteins 115

Equation 3.8. Converting spike concentration to ppm milk protein 130

Equation 3.9. Converting spike concentrations to ppm NFDM 131

CHAPTER 5

Equation 5.1. Warburg-Christian Method for determining protein concentration from A280 187

Equation 5.2. Conversion detected concentration of ppm NFDM to ppm casein 190

Equation 5.3. Conversion of detected ppm casein to ppm NFDM 191

CHAPTER 6

Equation 6.1. Converting ppm NFDM to ppm casein 215

Equation 6.2. Converting ppm casein to ppm NFDM 216

APPENDIX A

Equation A.1. Converting ppm α-casein spikes to equivalent ppm casein 232

Equation A.2. Converting ppm β-casein spikes to equivalent ppm casein 232

Equation A.3. Converting ppm κ-casein spikes to equivalent ppm casein 232

Equation A.4. Calculating proportions: What proportion of casein is α-casein? 232

Equation A.5. Calculating proportions: What proportion of milk protein is α-casein? 232

Equation A.6. Calculating proportions: What proportion of NFDM is α-casein? 232

Equation A.7. Calculating proportions: What proportion of milk protein is BLG? 233

Equation A.8. Calculating proportions: What proportion of NFDM is BLG? 233

Equation A.9. Calculating proportions: What proportion of milk protein is ALA? 233

Equation A.10. Calculating proportions: What proportion of NFDM is ALA? 233

Equation A.11. Calculating conversion factors: WPC34 to ppm milk protein. 233

Equation A.12. Calculating conversion factors: WPC34 to ppm NFDM 233

Equation A.13. Calculating conversion factors: WPC80 to ppm milk protein 233

Equation A.14. Calculating conversion factors: WPC80 to ppm NFDM 233

Equation A.15. Calculating conversion factors: Sodium Caseinate to ppm milk protein.....233

Equation A.16. Calculating conversion factors: Sodium Caseinate to ppm NFDM.....233

CHAPTER 1. LITERATURE REVIEW

1. Introduction

Food allergy is an increasing problem around the world. Not only are food allergies inconvenient, they can also be life-threatening and deprive afflicted individuals of certain nutritional requirements and quality of life. With increased demand for readily-available and shelf-stable foods by growing populations, food processing technology has developed and advanced in the past century. While beneficial for storage and safety, processing also may affect nutritional, organoleptic, structural, chemical and allergenic properties of the food product. While processing may decrease the allergenicity of foods, it may also reveal previously hidden epitopes and alter immunoreactivity. The relationships between structure, function, and allergenicity for the major allergenic food proteins are mostly unclear at this time.

Milk allergy is quite prevalent, affecting between 2-3% of children in the United States (Sicherer, 2011b). While many children diagnosed with milk allergy will outgrow their sensitivity prior to adolescence, milk still presents a risk for many who ingest it. Resolution of milk allergy has recently been reported near 50% (Wood et al., 2013). Production of milk-containing foods, modification of milk by food processing methods, and cross-contamination of non-milk containing foods by use of shared equipment and facilities contribute to the risk allergic consumers have to accidental ingestion and reaction. Commercially-available immunochemical methods are widely available and determine the presence, absence, or concentration of a target analyte present in a food. These methods represent one of the best available techniques at the current time for assessing the presence of target analyte in a food, especially at low concentrations. Processing can affect the allergenicity of foods, but the effect of processing on immunochemical detection of target analyte has not been established. This thesis seeks to identify the capabilities and limitations of commercially available milk detection methods and describe their efficacy especially in relation to fermented foods.

2. Food Allergens and Allergy

The prevalence of food allergy is increasing; recent reports indicate that between 3-6% of the US population has a food allergy (Sicherer, 2011a). Almost 90% of allergic reactions to foods are caused by eight groups of allergenic foods; milk, egg, peanut, tree nuts, soy, wheat, fish, and shellfish (1995; Bush and Hefle, 1996). The prevalence rates of food allergy according to geographic region as food consumption patterns are not globally consistent. Buckwheat and celery, for example, are unique to the priority allergen lists in Asia and Europe, respectively (Fernandez-Rivas and Ballmer-Weber, 2007). These allergenic foods are commonly consumed in these respective regions which increases overall exposure to the allergenic proteins. Increased exposure may contribute to increased sensitization compared to regions of the world where these foods are not commonly consumed.

The prevalence of food allergy is higher in infants and young children, averaging near 5% in westernized countries (Sicherer and Sampson, 2010). Food allergies are more common in children than adults, as many will outgrow their allergies within the first few years of life (Sampson, 1999). Allergies to milk, soy, wheat, and eggs are commonly outgrown, whereas peanut, tree nut, shellfish, and fish allergies are more likely to persist through adulthood (Hourihane, 1998). Although food allergy is most prevalent in the 0-9yr age group, most fatalities occur in the 10-19yr age group to milk, peanut, and tree nuts (Bock et al., 2001, 2007). The likelihood of outgrowing a food allergy may depend on the structure of the allergenic epitope(s) to which the individual is sensitized.

While it is not well known what renders certain proteins allergenic, many share similar biochemical characteristics. Typically, food allergens are relatively small and highly water-soluble proteins. They are often resistant to heat, proteolytic degradation, and pH extremes and are capable of reaching the small intestine largely intact and ready to provoke the immune system (Fu et al., 2002).

Most major food allergens are abundant within the food, accounting for typically greater than 1% of the total protein from the allergenic source (Metcalfe et al., 1996). Proteins present at higher concentrations have an increased chance of interacting with the immune system. This is thought to be important for sensitization and has been documented to be important for provocation of an allergic reaction based on clinical low dose food threshold challenges. Higher concentrations of allergenic protein present in the food increase the probability of exceeding the allergic individual's minimum eliciting dose. Allergenic proteins usually have several epitopes capable of binding IgE antibodies. The structure of the allergenic protein and epitopes, whether conformational or sequential, is largely responsible for the relationships among processing, reaction severity, remission, and other allergenic properties of the molecule (Poms and Anklam, 2004).

3. Adverse Reactions to Food

Adverse reactions to foods are classified into two main groups; food hypersensitivities and food intolerances (Sampson, 2004). Food intolerances are not immunological responses and can be a result of anaphylactoid reactions, metabolic food disorders, or idiosyncratic reactions. Food hypersensitivity and food allergy are immunological responses to food and can be IgE-mediated or non-IgE-mediated.

a. Food Intolerances

The best known metabolic food disorders include lactose intolerance and favism. Lactose intolerance is a condition in humans that occurs due to β-galactosidase enzyme deficiency, rendering the individuals incapable of completely digesting lactose from dairy products. Ingestion of lactose by affected individuals causes abdominal pain, flatulence, and diarrhea (Taylor et al., 2001). Favism is another intolerance characterized by deficiency of the glucose-6-phosphate dehydrogenase enzyme. Ingestion of fava beans by affected individuals results in hemolytic anemia. Lactose intolerance and favism have high prevalence among are

certain populations. While enzyme supplements are available for managing lactose intolerance, food intolerances are best treated and managed by avoidance diets (Taylor et al, 2001).

In anaphylactoid responses, ingestion of the offending food causes the release of histamine and other chemical mediators from basophils and mast cells without the degranulation signal from IgE binding and recognition (Taylor, 1997). Certain foods, including strawberries, egg white, chocolate, citrus fruits, and shellfish muscle are capable of triggering the release of histamine upon ingestion (Anderson, 1984; Baldwin, 1996). While ingestion of the food may cause anaphylaxis-like symptoms, the reaction lacks an immunological component (Taylor, 1997).

Idiosyncratic reactions are the third category of food intolerance, affect only certain individuals, and occur through unknown mechanisms. Some people have unexplainable sensitivities to products such as sulfites, aspartame, and monosodium glutamate (MSG) (Bush and Taylor, 1998) . These sensitivities have been attributed to induce symptoms of asthma, rash and hives, headaches, and muscle spasms. Although MSG naturally occurs in proteins, its individual addition to foods is claimed to be associated with development of adverse symptoms.

b. IgE-mediated Food Allergy

IgE-mediated reactions to food are referred to as immediate hypersensitivity reactions and "true" food allergies. Upon exposure to an allergenic protein, individuals may become sensitized and form antigen-specific IgE molecules. Little is known about the factors that predispose certain individuals to develop food allergies. Factors that influence sensitization of individuals may include genetic predisposition, an immature mucosal immune system, deficient antibody production, improper or late microbial colonization of the infant gastrointestinal tract, diet, vitamin D intake, and route of exposure (oral vs. cutaneous exposure) (Lack, 2008; Murphy,

2007). IgE antibodies are produced by plasma cells in the lymph nodes and at the site of allergic exposure, which, in the case of food allergens, is often the gastrointestinal tract.

The gastrointestinal tract has many physiologic barriers to prevent penetration by allergens (Murphy, 2007). The tight junctions between intestinal epithelial cells and the mucus layer covering the intestinal wall are especially important. Even with the physiologic barriers of the gastrointestinal tract, nearly 2% of food antigens are still capable of crossing the gut wall and entering the lymphoid tissue as intact proteins (Husby et al., 1986).

Once absorbed across the mucosal epithelium, antigens are bound by antigen-presenting cells (APCs) (Murphy, 2007). Professional APCs, such as dendritic cells and macrophages, bind to the foreign material, recognize it as "non-self", become activated, and transport the bound antigen further into the lymphoid tissue. In the lymphoid tissue, antigens are processed and broken down into small peptides and presented to a specific class of T cells, which then release chemical mediators. If the peptides are deemed a threat, chemokines trigger antigen-specific immunoglobulin production by B-cells. Non-professional APCs, such as intestinal epithelial cells, can also process and present antigen to T cells, but they are not capable of inducing tolerance to food antigens (Sampson, 2004). Once produced, IgE circulates through the bloodstream and attaches to high-affinity receptors on basophils and mast cells. These cells reside and travel near surfaces exposed to pathogens and allergens, including the skin and gut lymphoid tissue. After the sensitization phase, mature tissue-bound antigen-specific IgE molecules are primed and ready for exposure to antigen.

With repeat exposure to the antigen in sensitized individuals, IgE antibodies recognize the antigen and signal basophils and mast cells to elicit a response (Murphy, 2007). For a response to occur, crosslinking of two conserved epitopes on the antigen by IgE antibodies is required. While some IgE is free-floating, most is bound to immune cells. Crosslinking of

antigen by IgE initiates the release of inflammatory mediators and granules from mast cells and basophils. Cytokines, including histamine, interleukins, and tissue-destructive enzymes travel throughout the body and initiate immune responses. Histamine is a prevalent mediator in allergic responses and increases local blood flow to the reaction site and throughout the body, often causing swelling, redness, and itching and triggering additional antigen-specific IgE production by B cells. Additional symptoms of an allergic response to food include vomiting, diarrhea, hives, and uncommonly anaphylaxis (Murphy, 2007). Immunoregulation is the key to controlling allergic disease.

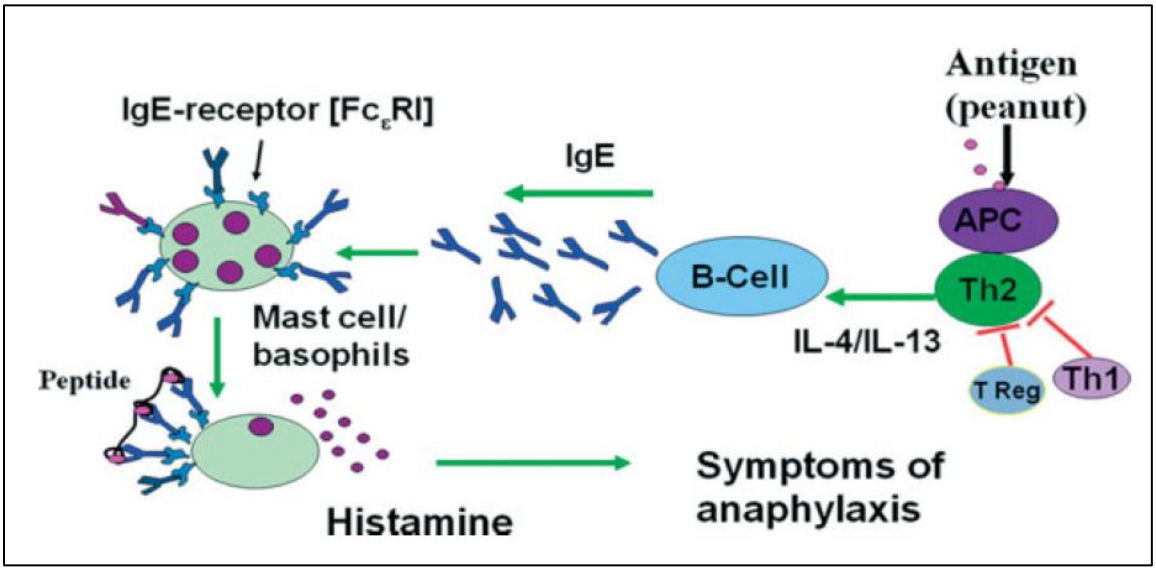


Figure 1.1. Sensitization and Degranulation of IgE- mediated food allergy. Taken from Li et al. (2001).

From the immediate response, cytokines initiate production of late-phase response immune mediators. The immunological development of an allergic response is depicted in Figure 1.1. Late-phase reactions occur in up to 20% of patients with immediate-hypersensitivity response (Brazil and MacNamara, 1998; Douglas et al., 1994; Kemp and Lockey, 2002; Lee and Greenes, 2000). These responses are caused by induced synthesis and release of mediators, including prostaglandins, leukotrienes, chemokines, and cytokines from activated mast cells and basophils. The late- phase response is associated with a second phase of T-cell mediated smooth

muscle contraction. Sustained edema and tissue remodeling are characteristic of late-phase responses (Murphy, 2007).

Certain food allergens, including peanuts, tree nuts, and shellfish are associated with a more severe IgE-mediated allergic response upon ingestion than other allergens (Sampson, 2004). A severe systemic response, termed anaphylaxis, can result in multiple organ failure and death. Unfortunately, allergic reactions are not an uncommon event, accounting for about 125,000 emergency room visits and 53,000 episodes of anaphylaxis per year in the US (Decker et al., 2008; Ross et al., 2008). Other allergens are capable of prompting anaphylactic responses, although typically less frequent and of less severity.

c. Non-IgE-mediated Food Allergy

In addition to IgE-mediated allergies, there are other types of hypersensitivity reactions. Some of these reactions are mediated by other antibodies (IgG) and others are mediated by T-cells. T-cell mediated responses include food protein-induced enterocolitis, food protein-induced proctocolitis, food protein-induced enteropathy, and celiac disease, among others. Cell-mediated reactions typically occur several hours after ingestion of the allergenic food and are grouped as delayed hypersensitivity reactions (Murphy, 2007).

The rate of celiac disease has increased in the recent decade, and is probably the most-reviewed example of cell-mediated food allergy (Rubio-Tapia et al., 2009). Celiac disease is an inappropriate T-cell mediated immune response to certain peptides formed during the catabolism of gluten. Specific subsets of T-cells are abnormally primed to recognize α-gliadin, a precursor of gluten. Individuals with cell-mediated food hypersensitivities like celiac disease have high rates of genetic predisposition and experience serious and potentially life-threatening consequences of their illness, including chronic intestinal inflammation, body wasting, diarrhea, and anemia. The ingestion of wheat, rye, and barley by celiac-individuals triggers the

inflammatory response that eventually destroys the absorptive capacity of the small intestine. The only known treatment for celiac disease is the avoidance of gluten-containing foods.

The remainder of this review will focus on IgE-mediated food allergies.

d. Diagnosis of Food Allergy

The "gold standard" of methods to diagnose food allergy is the double-blind, placebo-controlled food challenge (DBPCFC) (Binslev-Jensen et al., 2004). While more time-consuming and difficult to orchestrate than *in vitro* analyses, the DBPCFC allows clinicians and researchers to obtain definitive, quantitative, and unbiased data on allergy thresholds. Safety concerns when undergoing food challenges are common, especially for young children or those who have a clinical history of severe reactions. Even with medical staff present, there is risk of developing severe symptoms and possibly anaphylaxis. DBPCFC have additional drawbacks; false negative responses as a result of insufficient challenge doses, alteration of allergenicity during preparation of challenge material, and matrix effects. While the DBPCFC does not reliably identify all allergic patients, it is the favored method for diagnosing food allergy (Fernandez-Rivas and Ballmer-Weber, 2007). The most informational method for assessing processing effects on allergenicity is through DBPCFCs. However, these methods are expensive and time-consuming to properly orchestrate. Few DBPCFC have been performed using fermented milk proteins, but some data are available. More data are available regarding the allergenicity of enzymatically treated food proteins.

Other methods, such as skin prick tests (SPTs), rapidly provide useful information about levels of tissue-bound IgE (Hill et al., 2001). Because SPTs do not involve ingestion of the suspected allergen, the risk of severe reactions to food is largely reduced (Sicherer and Sampson, 2010). SPTs are often favored for infants and young children and as an initial screening for food sensitivities because of their reduced risk of severe reaction. Depending on the size of wheal

development upon pricking with an allergen or allergen-extract, SPT can indicate the presence and severity of clinical reactivity. A negative skin test often, but not always, indicates the absence of IgE-mediated food sensitivity to the tested antigen. It is also possible for a positive SPT reaction to occur in tolerant individuals (Sicherer and Sampson, 2010). Several studies have determined that reactions to SPTs for milk, egg, peanut, and fish were more than 95% predictive of clinical reactivity, although the predictive accuracy depends upon the wheal diameter size (Hill et al., 2001; Sampson, 2001). SPTs with fresh fruits and vegetables are often less accurate in predicting allergic reactivity due to the instability of these allergens during extraction and preparation. With allergens of this nature, prick-to-prick testing is used to preserve the protein's structure.

Radioallergosorbent tests (RASTs) and enzyme-allergosorbent tests (EASTs) are not currently relied on to predict clinical reactivity to food allergens although related methods such as ImmunoCAPs (see below) are popular. These types of assays provide only semi-quantitative information about patient sensitivity. RASTs and EASTs measure the amount of circulating antigen-specific IgE but can be less accurate than SPTs in predicting reactivity (Hill et al., 1993). More accurate tests for measuring severity of food sensitivities have been developed and are increasingly favored over the semiquantitative RAST and EAST methods.

ImmunoCAP methods also measure the amount of food-specific serum IgE, but have improved specificity and accuracy over other allergosorbent assays. Recent studies have established ImmunoCAP as the standard method for quantitative measurement of serum-specific IgE (Sampson, 2001; Wang et al., 2008). Differences in food allergy diagnosis among diagnostic methods are possibly due to the differences between detecting tissue-bound or circulating IgE. Some authors favor the theory that children with severe IgE food allergy have high levels of tissue-bound and circulating food-specific IgE. Studies have shown that children with prolonged

milk allergy have higher levels of milk-specific IgE than patients who will become tolerant (Ito et al., 2012).

e. *Treatment and Therapy for Food Allergy*

While the only proven therapy for food allergy is avoidance of the offending food, other therapies have been suggested to improve the quality of life of food-allergic individuals (Sicherer and Sampson, 2010). Certain drug treatments for allergy focus on the prevention of IgE production or the downstream pathways initiated by IgE-antigen crosslinking. After accidental exposure to food allergens, symptoms are commonly treated with antihistamine and/or epinephrine. Desensitization and specific allergen immunotherapy have gained much recent support (Burks et al., 2008). The goal of desensitization therapy is to decrease antigen-specific IgE response by increasing exposure to the antigen in a controlled manner.

Ingestion of certain plant-derived supplements has also gained support as a method of preventing allergic reactions. A Chinese herbal formula, FAHF-2, has undergone extensive testing in murine models with some success in decreasing allergenic phenotypes and circulation of allergy-associated interleukins and cytokines (Li et al., 2001).

4. Bovine Milk

Bovine milk and milk products have extremely widespread use in the food industry as it is capable of gelation, emulsification, and binding within food matrices (Damodaran and Parkin, 2008). Its nutritional aspects render it an ideal primary food for most children.

Bovine milk is a complex substance with an average protein content of 30-36g/L (Wal, 2002b). The nutritional, chemical, and sensory properties of milk change during lactation, environmental changes, and with geography.

All major milk proteins are allergenic. The four main allergenic proteins in bovine milk are the caseins (Bos d 8), BLG (Bos d 5), ALA (Bos d 4), and bovine serum albumin (Bos d 6)

(Poms et al., 2004b). Additional milk proteins, including immunoglobulins and lactoferrin also display allergenic activity. Roughly 80% of milk proteins are caseins, while the remaining 20% are whey proteins. The casein proteins are characterized by their precipitation and separation from the whey fraction of milk at pH 4.6 or during hydrolysis by chymosin. The caseins naturally cross-link and form ordered aggregates called micelles within a milk suspension (Wal, 2002b).

Table 1.1. Sizes of Major Allergenic Milk Proteins (Jost, 1988)

Protein Fraction	Size (kDa)	Length of AA sequence
α_{s1}-casein	23.6	199
α_{s2}-casein	25.2	207
β-casein	24.0	209
κ-casein	19.0	169
α-lactalbumin	14.2	123
β-lactoglobulin	36.6 (dimer); 18.3 (monomer)	162 (monomer)

Recent research has determined that great variability exists in affinity, specificity, and magnitude of IgE response to milk proteins. A study by Wal et al. (1995b) of 92 milk-allergic patient sera determined that most patients were sensitized to more than one milk protein, with no particular trend in protein combinations. According to the research, patients were sensitized to casein 66% of the time, BLG 68%, ALA 58%, BSA 50%, immunoglobulins 36%, and lactoferrin 45% (Wal et al., 1995a). Additionally, Bernard et al. (1999) determined that of 58 IgE sera from milk-allergic individuals, only one patient had a monospecific reaction (κ-casein).

a. Composition

Caseins

Caseins are a functional part of the milk transfer system (Horne, 2002). The casein fraction of bovine milk contains 80% of the total milk proteins. There are four casein proteins: α_{s1}, α_{s2}, β, and κ-casein. Although genes for the proteins are coded on the same chromosome, the caseins share little similarity in structural homology (Chatchatee, 2001a).

While each of the caseins is a unique chemical compound, the caseins aggregate in solution. In fluid milk, caseins clump together in ordered aggregates called micelles. Micelles are composed of 92% protein and 8% milk salts of calcium, magnesium, and citrate (Damodaran and Parkin, 2008). Caseins occur in fluid milk in generally stable proportions, with α_{s1}, α_{s2}, β, and κ-casein comprising 37%, 13%, 37%, 13% of total casein, respectively (Wal, 2001a). The distribution of caseins is not uniform within micelles; the center of the micelle is rich in calcium-sensitive α- and β- caseins, while the surface submicelles are dominated by κ-caseins. Assembly of micelles is facilitated by interactions with calcium ions and phosphoserine groups of α- and β-caseins. In micelles, polar fragments are exposed on the surface, especially in the case of k-casein. Polar regions of the other caseins also occur at the micelle periphery. A depiction of the casein micelle is shown in Figure 1.3.

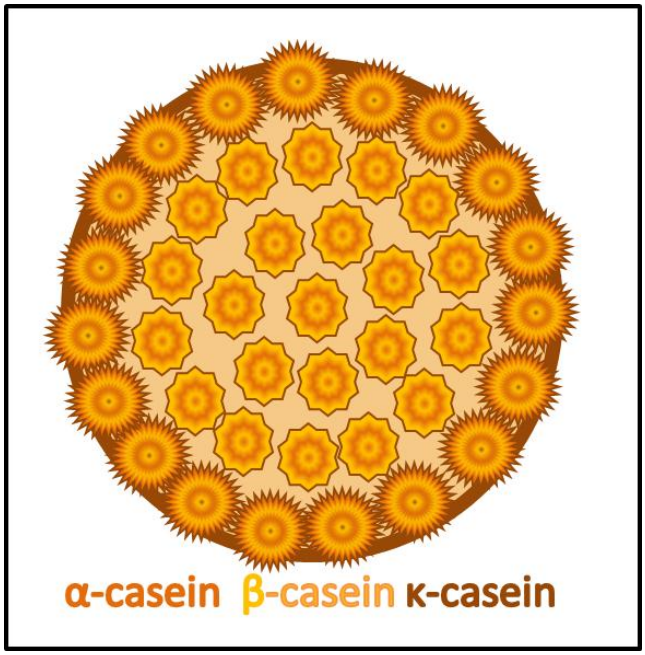


Figure 1.2. Casein micelle structure as occurs in bovine milk. Adapted from Schmidt (1982).

Homologs of bovine caseins are present in milk of other ruminant species. Some cow milk-allergic individuals may also exhibit IgE binding to milk of other ruminant species. Among

other ruminant species, especially ewe and goat, 80% to 90% homology exists among the protein sub-fractions that occur in bovine milk. Therefore, a high cross reactivity occurs in CMA patients between milk of different species (Wal, 2001b). Bernard et al. (1999) documented the cross reactivity of CMA patients to ewe and goat milk. According to the study, most CMA patients exhibit a reaction, albeit typically less intense, to ewe and goats milk.

Milk salts, especially calcium salts, affect not only the structure of casein micelles, but also the stability and function of individual milk proteins (Damodaran and Parkin, 2008). The α- and β-caseins are calcium sensitive, while κ-casein is insensitive to calcium. It is thought that calcium-sensitive caseins are derived from a common ancestral gene, while κ-casein evolved from a separate gene. Calcium-binding of caseins results in dehydration of the region that can alter the hydrophobic interactions and electrostatic forces and have a dramatic effect on structure.

Because of the sensitivity of the caseins to calcium, caseins cannot be crystallized, and thus their 3D structure has not been determined (Damodaran and Parkin, 2008). The chemical properties and proteolytic susceptibilities of individual caseins suggest that the tertiary structure is quite flexible. The large concentration of proline residues is thought to contribute to the flexibility, as these large residues interrupt the secondary structure of the proteins and prevent the formation of complex structures (Damodaran and Parkin, 2008). The lack of stable secondary and tertiary structures renders caseins relatively resistant to chemical or thermal denaturants, while the open structure exposes the caseins to proteolytic enzymes (Fox, 2001). Due to susceptibility to proteolysis, early researchers suspected caseins to be poorly immunogenic (Wal, 2002b; Wal, 1998b).

The presence of κ-casein is absolutely critical to maintain the micellar structure of caseins in fluid milk. The tail of κ-casein is a large polar sequence with several serine and threonine residues. These are often glycosylated during post-translational modifications. The

large hydrophobic domain of β-casein makes it more heat sensitive than the other caseins (Damodaran and Parkin, 2008).

Whey

The major protein constituents of whey are BLG and ALA. Both are globular proteins that have allergenic properties and are synthesized in the bovine mammary gland. Less prevalent proteins present in whey include bovine serum albumin, the immunoglobulins, and lactoferrin. These three proteins originate in the blood and are minor allergens (Restani et al., 1999). The whey fraction of milk may also contain some casein-derived fragments, particularly γ-caseins and casein macropeptide (derived from the polar portion of κ-casein), as their polarity renders them soluble in whey (Wal, 2002b). Function may be added to the whey proteins by modifications such as the addition of disulfide bonds and sulfhydryl groups.

At the typical pH of milk, 6.6-6.8, BLG forms dimers. Between pH 3.5 and pH 5.2, the dimers often aggregate and form octamers. BLG is highly sensitive to thermal treatments, but displays some resistance to acid hydrolysis and proteolysis (Astwood and Fuchs, 1996). Pasteurization or UHT processing causes irreversible unfolding of BLG (Wal, 2001b). Denaturation by heat treatment exposes a key sulfhydryl group which can interact with κ-casein and drastically alter milk structure (Reddy et al., 1988). According to Wal (2001b), the cleavage of the disulfide bonds of BLG has little effect on protein immunoreactivity. However, upon denaturation, BLG becomes much less soluble and is more likely to precipitate. Some whey proteins precipitate during cheese-making and can become incorporated in the cheese curd.

ALA is a 14.4 kDa protein with four disulfide bonds and a high affinity-calcium binding site (Wal, 2001b). It has a similar structure to lysozyme and plays an important role in regulating lactose synthesis. ALA is calcium sensitive. ALA unfolds at lower temperatures than BLG, but with intact disulfide bonds, the tertiary structure can unfold and refold reversibly. Under most

standard processing conditions, disulfide bonds remain intact, and ALA is not thermally denatured (Damodaran and Parkin, 2008). At temperatures above 70°C, whey proteins rapidly denature. In terms of irreversible heat damage, the sensitivity of whey proteins ranks as: IgG<BSA<BLG<ALA. Bovine-derived ALA exhibits a 74% homology to human ALA (Järvinen et al., 2001).

Ovine BLG shares sequence similarities with bovine BLG, but no counterpart exists in human milk (Järvinen, 2001). Traditionally, BLG had been largely attributed to CMA due to the uniqueness of the protein. In clinical studies, BLG showed the highest rate of positive oral challenges in children with milk allergy (Schutte and Paschke, 2007). Additionally, there is a high cross reactivity among ewe, goat, and cow milk in CMA patients, thought to be a result in homology of BLG molecules among the animals.

Other Potentially Allergenic Components of Milk

Research has shown that Maillard reaction products in milk have allergenic potential (Wal, 1998a). Additionally, other types of post-translational modification may induce allergenic potential of food proteins.

Other minor casein fractions exist in cow's milk. Hydrolysis of β-casein by naturally occurring milk enzymes produces a smaller sub-fraction called γ-casein (Wal, 2002). The γ-caseins are derived from the polar region of β-casein, and therefore are soluble in the whey fraction of milk. Additionally, casein macropeptide (derived from κ-casein) is also soluble in whey. It has been documented that casein macropeptide can retain allergenic activity even when separated from other caseins (Wal, 1998b).

Breakdown products of caseins by indigenous milk enzymes may play a role in allergic reactions. Plasmin, a naturally-occurring milk enzyme, catalyzes the breakdown of β-casein into γ-casein. Additionally, plasmin can produce λ-caseins from α_{s1} degradation. While it is known

that these compounds exist, the peptides have not been thoroughly identified, nor has their allergenic potential been assessed (Fox, 2001). Additional milk proteinases, such as the cathepsins, also produce peptides in milk, but these peptides have not been identified or evaluated.

b. Cow's Milk Allergy (CMA)

Nearly 80% of children diagnosed with CMA develop clinical tolerance within the first 3 years of life (Høst, 1997). Symptoms of CMA typically include urticaria, atopic dermatitis, and vomiting. It has been suggested that differences exist in the structure and sequence of epitopes recognized by individuals with transient or persistent CMA (Chatchatee et al., 2001a).

The resistance of BLG to acid hydrolysis and proteolysis allows relatively large derivative peptides to persist in the human body and transfer to human breast milk (Axelsson et al., 1986). The persistence of BLG-derivatives has been attributed to the development of colic in infants (Wal, 2001b).

In some sera, the IgE response is greatly reduced by modifying the major phosphorylation sites on cow's milk proteins (Otani et al., 1987). It is believed that post-translational modifications (phosphorylation, glycation, etc.) may play a critical role in the allergenicity of food proteins. A conserved region of phosphoserine residues are thought to be important in the allergenicity of calcium-sensitive caseins.

In a study evaluating allergenic and immunogenic responses of germ-free and conventional mice to adjuvant-free milk proteins, germ-free mice synthesized IgE and/or $IgG_1$to BLG, α_{S1}-, and κ-casein (Morin et al., 2011). No significant antibody response was observed in conventional mice. The research concluded that gut colonization by bacteria can have a significant impact on the susceptibility of mice to the allergenic and immunogenic effects of milk proteins. Additionally, the research described that with heat treatment, there was a decrease in

IgE response to BLG in germ free mice, but an increase in production of anti-BLG and anti-κ-casein IgG_1 in both germ free and conventional mice (Morin et al., 2011).

One of the major explanations for the change of immunoreactivity with processing is through the destruction of protein structure. Much research on egg and milk suggests that patients whose IgE antibodies recognize sequential (linear) epitopes on food proteins have an increased likelihood of lifelong, chronic allergy (Poms et al., 2004). On the other hand, patients whose sera bind to conformational epitopes on a food protein often have less severe and transient allergies (Figure 1.3). Allergy to foods other than egg and milk may not exhibit the same trends in allergenicity and structure. The destruction of conformational epitopes, however, can expose previously hidden sequential epitopes.

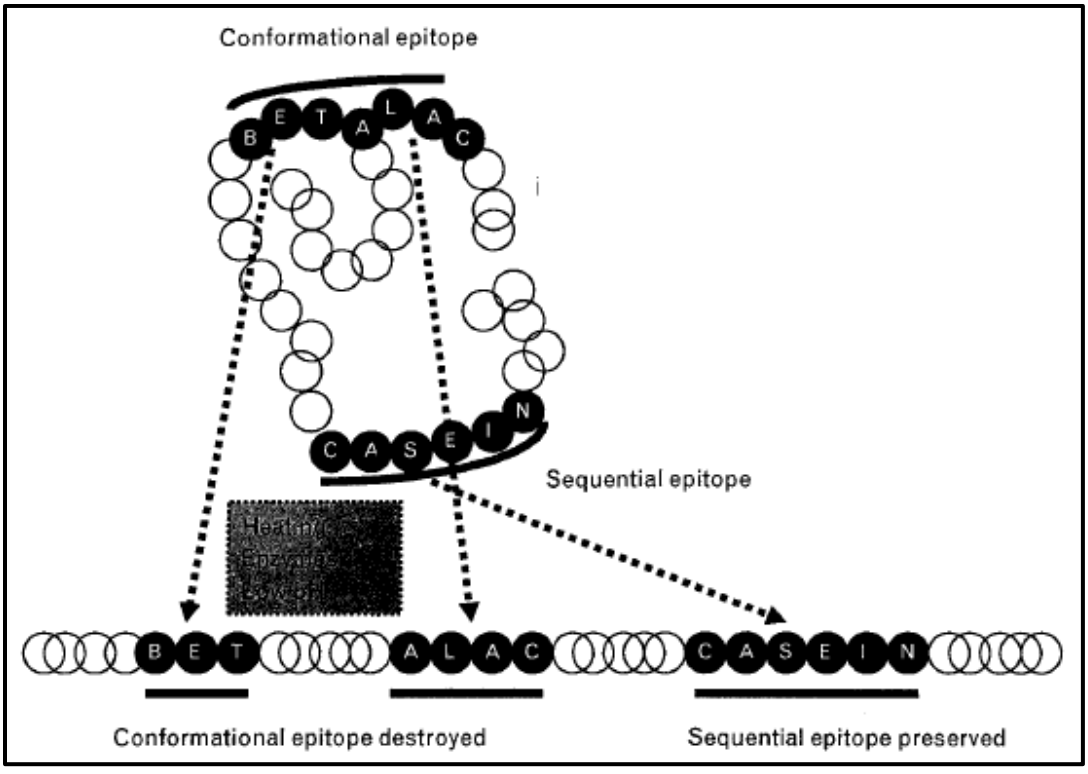


Figure 1.3. Potential effects of processing on protein structure and epitopes. Taken from (Nowak-Wegrzyn and Fiocchi, 2009). Illustration displays potential effects of processing on protein structure and conformational or sequential epitopes.

An increase in temperature is suggested to decrease allergenicity by destroying conformational epitopes (Nowak-Wegrzyn and Fiocchi, 2009). However, evidence suggests that some epitopes are stable and capable of retaining allergenicity in thermally processed foods. Thermostability is highly variable, even among food allergens belonging to the same protein family (Nowak-Wegrzyn and Fiocchi, 2009). Cow's milk caseins and BSA have increased thermostability over other milk proteins. After 2 hours of boiling at $100^{O}C$, caseins retained structural bands on electrophoretic gels whereas LF, BLG, and ALA exhibited extreme sensitivity to heat treatment. Matrix effects have been described as highly important for milk proteins, as the heating of BLG allows the formation of disulfide bonds and binding to other proteins. This binding can decrease allergenicity of the food product (Thomas et al., 2007). The ingestion of extensively heated milk products by milk-allergic patients gave no immediate symptoms for 70% of patients (Lemon-Mule et al., 2008; Nowak-Wegrzyn and Fiocchi, 2009).

c. Major Allergenic Epitopes of Bovine Milk

Caseins

As mentioned above, the casein subfragments share similar structural homology, but are encoded by different genes on the same chromosome. Evidence suggests that children with IgE that recognizes structural epitopes are more likely to have a transient food allergy than children who have IgE that recognizes sequential epitopes. This trend has been documented among patients with specific-IgE to each of the four casein fragments.

The most prevalent casein subunit in bovine milk is α_{s1}-casein (Wal et al., 2001). It is a 199 amino acid (AA) peptide with high proline content and lacks established secondary and tertiary structure. According to Chatchatee et al. (2000), there are 9 IgE binding regions and 6 IgG binding regions on α_{s1}-casein. Two IgE-binding regions have sequence homology; AA69-78 and AA109-120. Of 24 human sera tested for reactivity, each individual reacted to an average of

5 IgE binding regions on α_{s1}-casein (Chatchatee et al., 2001a). Patients were split among two groups, based on age, to further evaluate trends in IgE binding. Seven of nine regions were recognized by patients from both groups, while two regions, AA 69-78 and AA173-194 were only bound by individuals displaying clinical and immunological history of persistent CMA. Follow-up studies determined that individuals less than 3 years of age that had IgE that recognized the two epitopes dominated by older individuals were more likely to have persistent CMA. Alternately, individuals that did not recognize these two epitopes were likely to outgrow their CMA. AA69-78 on α_{s1}-casein is a unique IgE binding region that IgG from patient sera does not recognize. Some evidence suggests that IgE and IgG-production are not triggered by the same peptide sequences (Murphy, 2007; Chatchatee et al., 2001a). However, other evidence suggests that sometimes class-switching during antibody production may skip the IgG stage and directly switch from IgM production to IgE production, thus producing IgE antibodies with no IgG counterpart. The linear IgE and IgG binding epitopes of α_{s1}-casein are displayed in Figure 1.4.

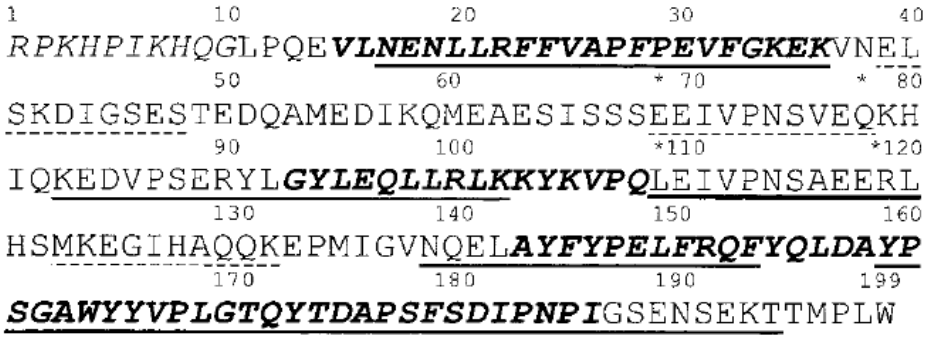


Figure 1.4. α_{s1}-casein sequence with IgE and IgG binding epitopes. Taken from (Chatchatee et al., 2001a). Thick or broken underlines correspond with major and minor IgE binding epitopes, respectively. Major IgG epitopes are shown in bold italics.

The next casein, α_{s2}-casein, is a 207-AA protein with a mass of 25.2kD. It has one disulfide bond and four genetic variants. According to Busse et al. (2002), α_{s2}-casein has 4 major

and 6 minor IgE binding epitopes. Two of the major epitopes, AA83-100 and AA165-188, share 50% sequence homology. There are two phosphorylation sites on α_{s2}-casein, one in the region of AA143-158, and another at AA 168. Phosphorylation at the former site has been shown to potentially increase allergenicity, while dephosphorylation at AA168 has been shown to decrease allergenicity of α_{s2}-casein. Treatment of α_{s2}-casein with strong denaturing agents, including urea, hydrochloric acid, sodium dodecyl sulfate, sodium hydroxide, or excessive heating, had little effect on the allergenicity of the protein (Busse et al., 2002). It is thought that sequential epitopes are most important for allergenicity of α_{s2}-casein; degradation of any 2^o or 3^ostructure has limited effects on the binding of sera from most milk-allergic patients. The AA sequence and IgE binding epitopes of α_{s2}-casein are shown in Figure 1.5.

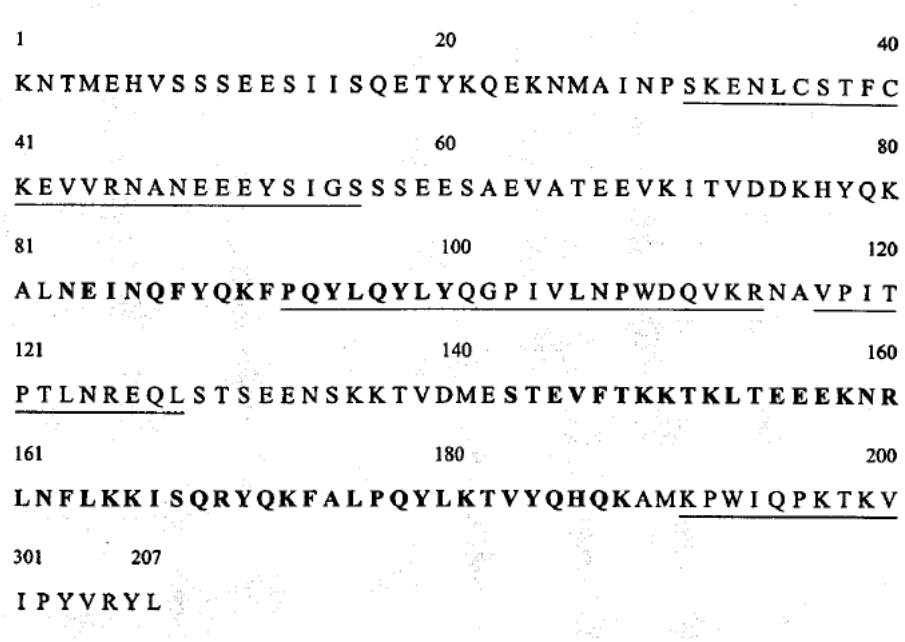


Figure 1.5. α_{s2}-casein amino acid sequence and sequential IgE binding epitopes. Taken from (Busse et al., 2002)

B-casein is a 209AA peptide that has 6 major and 3 minor IgE binding epitopes. Additionally, β-casein has 9 IgG binding epitopes. The sequence is pictured in Figure 1.6. Structurally, β-casein is similar to α_{s1}-casein (Chatchatee et al., 2001b). β-casein is a phosphoprotein with high proline content, no disulfide bonds, and minimal, if any, 2^o or

3^{o}structure. These characteristics point to linear epitopes on the molecule, due to lack of advanced peptide folding beyond the primary structure. AA1-16 is the primary epitope on β-casein recognized by sera from 15 CMA patients. However, AA83-92 and AA135-144 are recognized with the highest intensity of the patients examined (Chatchatee et al., 2001b). Two of the major IgE-binding epitopes on β-casein are not recognized by sera obtained from younger patients. AA149-164 and AA 167-184 are only recognized by older patients; those characterized by persistent CMA. The IgE and IgG binding epitopes of β-casein are depicted in Figure 1.6.

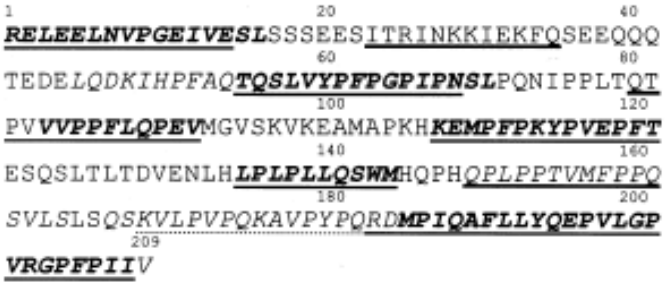


Figure 1.6. β-casein amino acid sequence and IgE and IgG binding epitopes. From (Chatchatee et al., 2001b)Major IgE binding epitopes shown in bold italic. Minor IgE epitopes shown in italic. Major and minor IgG epitopes shown as solid and broken underline, respectively.

κ-casein is the smallest of the casein fragments, at 169AA. It contains a bound carbohydrate moiety, and has disulfide bonds and a characteristic 3^{o} structure. κ-casein has 8 major IgE binding epitopes (Chatchatee et al., 2001b). There are 6 linear epitopes on the molecule that are recognized only by older patients; AA 9-26, AA67-78, AA95-116, AA111-126, AA137-148, and AA 149-166. Two epitopes on k-casein were recognized by young patients only. The IgE and IgG binding epitopes of κ-casein are depicted in Figure 1.7.

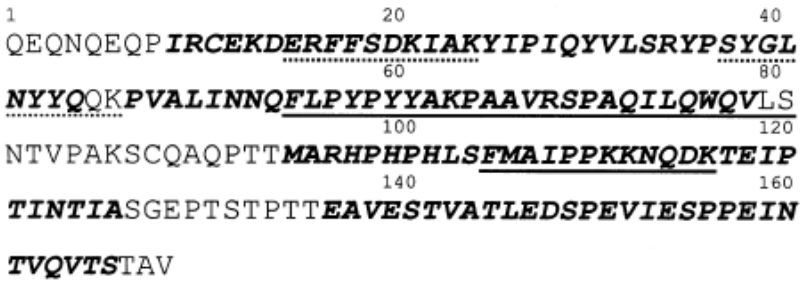


Figure 1.7. κ-casein IgE and IgG binding epitopes. From Chatchatee et al. (2001b). Major IgE epitopes shown in bold italic. Minor epitopes shown in italic only. Major and minor IgG epitopes shown as solid and broken underline, respectively.

Whey Proteins

ALA is a 123AA globular protein with a weight of 14.4kD. It has extensive secondary and tertiary structure, supported by its four disulfide bonds and calcium-dependence. Bovine ALA has a high level of homology (74%) to human ALA (Järvinen et al., 2001). There are four IgE-binding epitopes on ALA. The major antigenic site on ALA is AA5-18, which interestingly, is homologous to an antigenic site on BLG: AA124-134. Conformational epitopes appear to be particularly important to the allergenicity of ALA. While sera from eight of eleven CMA patients >3 years of age bound to one linear epitope, a high level of milk-specific IgE does not necessarily coordinate linear epitope recognition on ALA (Järvinen et al., 2001). Sera from young patients (<3 years of age) with CMA did not recognize any of the allergenic linear epitopes commonly recognized by older milk-allergic patients. Three IgG epitopes were identified on ALA, with all patients (including the five control subjects) exhibiting a high level of binding to the antigenic epitopes. Other studies have agreed that AA5-18 is the prominent IgE-binding epitope of ALA, but intense and frequent IgE recognition is also observed on tryptic digest fragments containing AA17-58 (Järvinen et al., 2001).

A study by Maynard et al. (1997) determined that more than 60% of patient sera evaluated responded to native ALA and had limited reaction after ALA was unfolded and/or

digested. It has been suggested that tryptic cleavage of ALA leads to longer residues and a potential for increased reactivity in CMA subjects. The most intense binding regions on bovine ALA share little sequence homology with human ALA. The IgE and IgG binding regions of ALA are depicted in Figure 1.8. Again, it is thought that most allergenic regions on ALA are conformational.

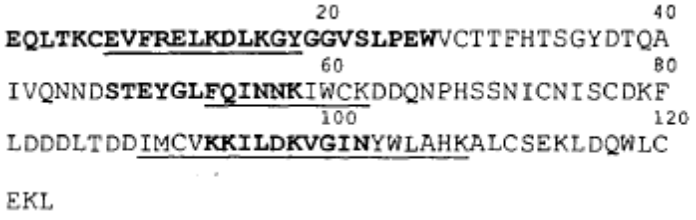


Figure 1.8. IgE and IgG binding epitopes on ALA. Taken from (Järvinen et al., 2001).IgE epitopes are shown in bold, and IgG epitopes are underlined.

BLG is a 36kD dimer of the lipocalin-family; each monomer comprises a 162AA protein. The two disulfide bonds and free cysteine residue give BLG unique structural and reactive properties. 7 IgE and 6 IgG binding regions have been identified on BLG by Järvinen et al. (2001), with general agreement among other researchers. Younger patients (< 3 years of age) recognized only 3 of 7 epitopes. AA119-128 exhibited intense binding for young patients, but weak binding by older patients. Of the identified IgG binding regions, studies with patient sera determined that these epitopes are frequently recognized by non-allergic control subjects. Tryptic cleavage of BLG molecules in other studies has been shown to largely reduce immunoreactivity for most patients (Järvinen et al., 2001). While the relationship between clinical reactivity and level of milk-specific IgG in patient sera has not been established, children who are likely to retain their allergy have been observed to have significantly higher serum milk-specific IgG than patients who will outgrow their milk allergy. IgE and IgG binding epitopes are displayed in Figure 1.8.

In ALA and BLG, many of the IgE and IgG epitopes align. However, three regions on ALA and one region on BLG are recognized only by IgE, supporting the theory that epitopes responsible for IgE synthesis are different than epitopes that promote production of IgG (Järvinen et al., 2001). These trends possibly contribute to the persistence of CMA.

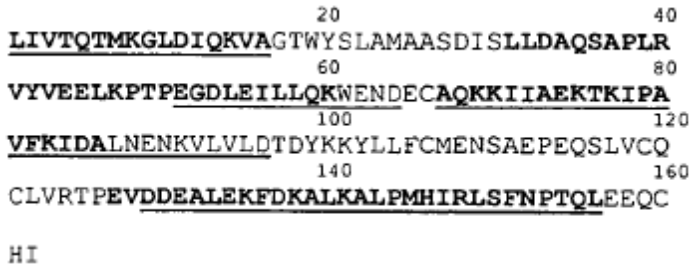


Figure 1.9. IgE and IgG binding epitopes on BLG. Taken from (Järvinen et al., 2001). IgE epitopes are shown in bold, and IgG epitopes are underlined.

5. Detection of Food Allergens

Because food-allergic patients often have extremely low sensitivities to the offending food, the food industry must be able to detect allergens at a sufficiently low level to mitigate the risk of reaction among consumers. Since the passage of the Food and Allergen Labeling and Consumer Protection Act of 2004 (FALCPA), an increasing number of companies make it a consistent practice to label products with the potential to contain allergens. Due to widespread use of allergen-containing products, industry compliance with a zero tolerance policy is almost impossible (Kerbach et al., 2009). The establishment of action levels for product labeling and is a topic of much recent debate. Consumer perspective of the establishment of these levels often provides a negative view of the food industry.

In addition to its use in diagnosis of food allergy, radioallergosorbent (RAST) assays have been used to detect allergens in foods. These methods have been phased out in recent years in favor of more accurate and sensitive procedures (Poms et al., 2004b). The RAST assay is

based on competitive inhibition of binding using a radiolabeled antibody for detection of antigen-specific IgE isolated from human sera. While sensitive and allergen-specific, the assay is labor-intensive, expensive, inconsistent, and only provides semi-quantitative results. Because most commercial ELISA kits are incapable or invalidated for detecting allergens in highly processed products, RAST may be one of the only ways to verify the presence or absence of allergenic proteins in specific foods.

PCR methods have been used to detect genetic sequences specific to the allergenic source using specialized DNA-primers (Poms et al., 2004a). PCR-based methods are not used in industry settings due to the cost of instrumentation and the high likelihood of assay contamination. The key disadvantage of this method is that the PCR test does not definitely prove or deny presence of an allergen. DNA may also be susceptible to the effects of processing. Milk, however, contains only low amounts of bovine DNA and does not make a good target for PCR-based detection methods (Koppelman and Hefle, 2006; Poms et al., 2004a).

a. ELISAs

Enzyme-Linked Immunosorbent Assay (ELISA)-based technologies are the most popular assays used to detect allergens in food (Koppelman and Helfle, 2006). By using a consistent source of animal antisera for assay development, kits are designed to maintain integrity between production lots. ELISA assays are developed using antibodies that have been obtained from animals sensitized to proteins within the food. Target proteins are not always allergens, but often are proteins that are selected for their stability to the effects of processing and digestion, and can indicate presumptive presence or absence of the allergenic food. Antibodies cross-link antigen sequences. During antigen processing and presentation, proteins are hydrolyzed and short sequences are presented to T and B cells for antibody maturation and class switching. Once activated, each B-cell has the capability of producing an antibody with a unique specificity. At

any time, there may be several antibodies capable of recognizing individual epitopes on a single protein (Murphy, 2007).

There are several types of quantitative ELISA, including sandwich and direct and indirect competitive. ELISA assays may use antibodies with a single specificity for detection (monoclonal), or may use two or more antibodies, each with individual specificities (polyclonal). Many researchers support the theory that a polyclonal ELISA is more robust than a monoclonal ELISA, especially if the antigen has undergone any type of processing or heat treatment. Some proteins are easily modified under processing conditions, and these are not ideal target proteins for detection. However, the more epitopes that are detectable by an ELISA, the more likely the assay is to detect the presence of target proteins.

In a sandwich ELISA, antigen-specific IgG antibodies are obtained from sensitized animals (often rabbit, sheep, goat, or mouse) and are used to coat the bottom of wells in a non-pyrogenic plastic microtiter plate. Extracted and centrifuged samples suspected of containing the target antigen are applied to the wells and incubated. Target antigen, if present, will be bound by the IgG antibodies, and equilibrium between bound and unbound antigen develops during the incubation period. Unbound antigen is rinsed away, and an enzyme-bound conjugate antibody is added. Binding will occur to the antigen-antibody complex. Typically, the enzyme-linked conjugate antibody is obtained from a different animal than used to generate the primary antibody to prevent false positive results. The bound enzyme is often horseradish peroxidase or alkaline phosphatase. Unbound conjugate is rinsed from the wells, and a substrate solution is added. The substrate is selected for its specificity to the enzyme label on the detector antibody. The substrate is allowed to incubate with the antibody-antigen-antibody-enzyme conjugate complex for a set amount of time and a colorimetric reaction occurs as a result of enzyme action on the substrate. The intensity of the color is directly proportional to the concentration of target antigen in the sample. Acidic stop solution is then added to deactivate the enzyme, and the solution maintains a

consistent color for spectrophotometric measurement. A standard curve is developed from the optical density of solutions containing a known concentration of target antigen. Quantification of the concentration of target analyte present in samples can be interpolated from the standard curve.

Competitive ELISAs also employ IgG antibodies. In this type of assay, antigen is bound directly to the microtiter plate. In a non-pyrogenic tube, sample is incubated with antigen-specific IgG antibodies. If the antigen is present, some of the antibodies will bind in solution. The sample-IgG solution is then applied to the microtiter plate. Any unbound IgG will bind to the antigen coated in the wells. Unbound material will be washed away. Enzyme-labeled conjugate antibody is added to the well plate and will bind to the primary antibody. The addition of substrate results in a colorimetric reaction which can be read at appropriate wavelengths. Unlike the sandwich ELISA, the intensity of the developed color in the competitive ELISA is inversely proportional to the concentration of the antigen present in the sample. A stronger color development in the microtiter plate indicates a lower concentration of antigen in the sample. Quantitation of unknown samples is done by interpolating values from a standard curve developed using standards of known concentration.

The two main factors affecting ELISA performance are extraction and detection efficiency. For many proteins, even low levels of heating can cause precipitation and aggregation. While some proteins are more sensitive than others to the various effects of processing, it is generally accepted that an increase in the severity of processing typically destroys conformational antibody binding epitopes on target proteins. However, the destruction of some epitopes that provide structure for the molecule may expose previously-hidden sequential epitopes (Dumont et al., 2010). In addition, processing may facilitate chemical modification of the food, including glycosylation and oxidation, which may also alter the allergenicity of food proteins. The effects of processing on allergen structure and function is discussed further in Section 6.

ELISA assays are sensitive, specific, rapid, and relatively consistent. They may be quantitative or qualitative, depending on stringency of analysis. For many commercial assay kits, quantitative results can be obtained from a sample in less than one hour. Some commercial quantitative ELISA assays can accurately quantify less than 1 part per million (ppm) of an allergen or protein present in a sample. Detection at this level is typically sufficiently sensitive to make informed decisions regarding the safety of a product.

Qualitative ELISAs are typically much faster than quantitative analyses, and can provide results in as few as five minutes. Lateral flow or dipstick ELISAs are used in semi-quantitative analyses.

Some ELISAs that show particular integrity in detection and sensitivity with consistent antisera production are adapted for use by external laboratories. Milk ELISAs were initially developed to detect the presence of bovine milk in ovine and caprine dairy products. Therefore, the development of these assays was specifically focused on the sequences and structural differences in proteins between cow and sheep and goat's milk. Anguita et al. (1996) developed an ELISA specific to bovine β-casein. Negroni et al. (1998) discussed the development of an ELISA using two monoclonal antibodies; one reactive to native BLG, and the other to denatured BLG. A publication by Mariager et al. (1994) documented the development of an ELISA assay that used polyclonal antibodies specific to both native and heat-treated BLG.

b. Commercially-available ELISAs: Interpretation of Results and Limitations

While being simple and relatively consistent, commercially-available ELISA assays are not without issues. Each kit manufacturer generates antigen-specific antibodies for ELISA kit development. While possible for manufacturers to build kits with similar target protein sensitivities, commercial kits often have different analytical targets. Knowing the specificity of ELISA antibodies is necessary to correctly interpret results obtained by each kit. Understanding

the type of antibodies used in assay development (whether monoclonal or polyclonal, or reactive to native or denatured protein) is crucial to correct kit selection and interpretation of results. In addition to choosing specific materials for sensitization of animals and antibodies with target specificities, each kit manufacturer may choose to develop a standard curve with unique calibration material; whether pure dilute target material, an extract of the target material, a specific group of proteins, or even one protein in particular. Based on these selections, many ELISA manufacturers apply quantitative conversion factors or other arbitrary values to calculations to obtain results with theoretically relevant units. For example, a kit manufacturer may select antibodies that target α-casein, but provide standards in some other unit, such as milk protein. Because of the diversity of allergen-derived food ingredients, it would be nearly impossible to develop a kit capable of accurately detecting and quantifying every derivative of a specific allergenic food.

There is little validation data available for commercial ELISAs. Due to large variation in kit performance as a result of differences in antibody preparation, extraction, and calibrants used in kit development, comparison among commercialized analytical kits is highly erroneous (Poms et al., 2005). In 2009, the European Union funded a working group, entitled Monitoring and Quality Assurance (MoniQA), to harmonize methods used worldwide for validation of safety and quality of foods. The group was funded with the goal of providing unified validation protocols and reference materials for calibration and development of ELISA methods (Poms et al., 2009). The MoniQA working group has identified four main challenges with harmonization of allergen detection tools. These challenges include: lack of certified reference material, matrix dependence of antibody recognition in processed foods, need for improved validation procedures, and lack of defined clinically-relevant thresholds to define limits of quantification for allergenic methods (Poms et al., 2009).

In concert with MoniQA, the AOAC Presidential Task Force on Food Allergens published a set of validation procedures for food allergen ELISA methods (Abbott et al., 2010). The authors suggest that the major challenges with ELISA development and consistency deal with the solubility and extractability of food antigens and the ability of ELISA antibodies to recognize them. Some of the main sources of variation within a kit deal with food matrix interactions, changes in solubility and reactivity of proteins as a result of processing, and different protein profile as a result of growth conditions and species variety (Abbott et al., 2010; Koppelman and Hefle, 2006). In the publication, the guidelines address required information to be included by the development laboratory and recommend interlaboratory validation procedures. The AOAC Task Force recommends use of NIST nonfat milk powder (NIST-RM-1549) as the reference material for calibration and quantification of milk ELISAs. If a different calibration material is used to build the standard curve, it is suggested that the manufacturer provide an experimentally validated conversion factor between units of calibration and the NIST milk powder.

Without use of a consistent certified reference material by kit manufacturers and kit users, false positive and negative samples, kit sensitivity, matrix effects, and recovery efficiency cannot be appropriately evaluated (Abbott et al., 2010). Adoption of a single reference material would allow kit manufacturers to develop consistent reporting units for assays, and provide conversion factors among detected antigen, antibody sensitivity, and reference material. There is critical and widespread need for the adaptation of a single reference material for food allergen testing. Without this, identical samples will give differing results when analyzed by kits of different manufacturers, comparisons are not possible, and observed differences in data cannot be appropriately attributed to experimental factors.

Additional collaboration among regulatory and advisory groups to validate current analytical test kits for various food allergens and set standards for the development of improved

methods is ongoing. The European Committee for Standardization, the European Commission's Joint Research Center, the German Institute for Standardization, and Health Canada have adopted initiatives to validate methods for allergen detection and quantitation.

Another topic of debate surrounding ELISA development regards sensitivity. At the present time, kit manufacturers often strive to develop kits that are highly sensitive to the presence of targeted material. Because, however, a zero tolerance action level on the presence of allergens in foods is unachievable, many current kits may be far more sensitive than actually necessary. While work is being done on threshold doses required for the elicitation of a reaction in allergic individuals, there is still much disagreement about how low detection limits should be set for analytical methods and how those levels should be applied to labeling requirements. Individuals may react at doses lower than one milligram or may not begin to react until they have ingested over one gram of allergenic protein (Taylor et al., 2002). There is agreement that analytical methods should be sufficiently sensitive to protect allergic consumers. Given the varying sensitivities of allergic individuals, it is difficult to determine how many consumers can feasibly be protected by analytical methods. While no official regulation has been established regarding the detection limits for application to labeling policies, the general assumption is that detection limits should be around 10 ppm of allergenic protein (Koppelman et al., 1996; Poms and Anklam, 2004b). Clinical data obtained in double-blind placebo-controlled food challenges is currently being critically assessed to determine suggested thresholds of detection, alert, and action for application in research, industry, and regulatory settings.

There are some types of samples that cannot be analyzed with ELISAs. Due to the aqueous nature of the assay, oils and oil-derived ingredients are not validated for ELISA evaluation. Some proteins may be extracted from oils using an aqueous buffer system, but the extraction efficiency is inconsistent and unpredictable. While allergenic proteins may be present in the sample, if they cannot be pulled from the matrix and into solution, an ELISA will not detect

their presence. In this way, a negative result does not necessarily indicate that there is no allergenic residue in the sample.

Hurst et al. (2002) performed an interlaboratory validation study of ELISA methods, in which the research suggested that because commercialized ELISAs were developed at different sites, using different reagents, and different antibody sources, individual kits will develop substantially different data. Additionally, it was determined that laboratories often choose kits based on geographical considerations, as opposed to technical considerations regarding the ideality of the kit for analysis of matrix, species variations, or processing effects on the target protein.

The major impairments during detection and quantitation by ELISA kits come as a result of three issues; matrix interference, extraction efficiency, and antibody affinity (Diaz-Amigo et al., 2010; Abbot et al., 2010). Processing of any kind has the potential to cause issues in all three areas. Additionally, the use of ELISA kits without the requirement of a standard reference material renders correlations among kits and among different varieties, species, and environmental conditions nearly impossible. As discussed further in Section 1.6, processing can have an enormous impact on solubility and reactivity of proteins as well as trigger reactions with other non-target compounds in the product matrix.

6. Effects of Processing on Allergens

The relationship between protein structure and function with regards to the effects of processing on allergenicity and immunoreactivity remains unclear. While certain trends can be established among allergenic food proteins of the same family, most do not hold true for all proteins.

Processing methods can be separated into two groups: thermal and non-thermal treatments. No definite relationship has been established between type of processing method and

effect on protein allergenicity. Thermal treatments include dry heat and wet heat methods. Methods are listed below in Table 1.2.

Table 1.2. Food Processing Methods and Classification

Thermal Processing		**Non-thermal Processing**
Dry Heat	Wet Heat	
Oven Roasting	Microwaving	Infrared Radiation
Oil Roasting	Pressure Cooking	Soaking
Infrared Heating	Extrusion	Milling
	Blanching	Germination
	Boiling	Fermentation
	Steaming	High Pressure Processing
		Dehulling
		Grinding

Food processing alters the structure of food proteins in two major ways; protein unfolding and aggregation and covalent modification by other food components. Processing methods can destroy or reduce allergenicity of proteins, such as the Bet v 1 homolog proteins-mostly contained within fresh fruits and vegetables. The Bet v 1 homologs unfold easily, especially with heat treatment, and become modified with plant polyphenols (Nicoletti et al., 2007). Proteins can also retain their allergenicity through the presence of stable protein scaffolds that prevent unfolding or allow refolding, typically occurring upon cooling. Proteins that have been characterized with stable scaffold structures include members of the prolamin superfamily, non-specific lipid transfer proteins (nsLTPs), and the 2S albumins (Barbosa-Cánovas, 2009). Other proteins, including caseins and some seed storage prolamins are thermostable due to their mobile structures. Some proteins are more susceptible to processing, and will partially unfold but retain their allergenic epitopes. The food matrix has dramatic effects on the stability of proteins and susceptibility to processing methods (Nicoletti et al., 2007). This will be discussed further in Section 6a.

Even at low levels, heating is capable of triggering protein precipitation and aggregation and affecting solubility. Processing can both destroy and reveal antibody-binding epitopes. Conformational epitopes, or those that depend on protein structure for allergenic activity, are often destroyed by heat treatment, as a result of protein unfolding. Linear epitopes are not easily destroyed by heat treatment, but are often hydrolyzed during fermentation and enzymatic modification (Dumont et al., 2010).

Processing may trigger chemical modification within food products such as oxidation, Maillard reactions, glycosylation, or protein-protein interactions. These modifications may create new epitopes, expose existing epitopes, or destroy conformational epitopes on the proteins required for IgE-binding (Sanchez and Frémont, 2003). The food matrix containing the protein has a large impact on the release and stability of allergens. For example, interaction with sugar or other moieties during processing may trigger Maillard reactions or other chemical modifications that affect the structure and functionality of allergenic or potentially allergenic proteins. Cross-reactive carbohydrate determinants (CCDs), also called haptens, are IgE binding epitopes that may occur as glycosylated regions on proteins and can represent thermally stable epitopes.

Detection assays that use antibodies measure immunoreactivity. A decrease in immunoreactivity does not necessarily indicate a decrease in allergenicity. Likewise, it is noteworthy to mention that lack of detection of an allergen does not necessarily correlate to absence of the allergen from the food product. The modification of proteins during fermentation could alter the immunoreactivity of proteins by changing their stability, conformation, or protein sequence. Literature regarding the effects of fermentation on protein structure and immunoreactivity is present, but there is a lack of sound evidence to support the theory that a decrease in immunoreactivity correlates to a decrease in allergenicity, or that a change in structure results in a change in protein function.

Where data are available, the effects of processing on milk proteins will be described and reviewed. Where data on milk proteins is limited, other allergen data will be provided as appropriate. Due to the focus of this research, a section specifically devoted to the documented effects of fermentation on various food proteins will be included.

a. Thermal Denaturation of Proteins

Proteins can be classified into several groups based on thermostability. Thermolabile proteins are not resistant to heat treatments and rapidly unfold upon exposure to heat. Conformational allergenic epitopes can be destroyed by thermal exposure, as marked by a decrease in IgE reactivity and lower potential to trigger an allergic reaction in a sensitized individual.

Some proteins are only slightly susceptible to effects of heat treatment. Limited unfolding and some aggregation among proteins occur, drastically altering textural properties in food matrices. Examples are the whey proteins BLG and ALA, and the 11S and 7S seed storage globulins. As BLG unfolds, the buried cysteine residue is exposed and becomes susceptible to disulfide bond rearrangement. After thermal treatments, IgE binding of BLG decreases, but trace IgE binding remains. At moderate temperatures or at low pH, ALA is partially unfolded and exists in a molten globule state. Heat treatment of the 11S and 7S seed storage globulins increases the likelihood that these proteins will aggregate.

Another class of protein is highly thermostable and proteins in this class resist unfolding. Even if unfolding occurs, the proteins are capable of refolding rapidly upon removal from heat. These proteins typically retain their allergenicity even after extensive heat treatment. The prolamin superfamily is included in this class. Proteins in this class are utilized in the food industry for their heat-induced gelation capabilities. Soy globulins, β-lactoglobulin, bovine

serum albumin, ovalbumin, and myosin are commonly used for these properties (Damodaran, 1994).

Yet another protein class is the thermostable rheomorphic proteins. These proteins have minimal secondary and tertiary structures, and their interactions are driven by their open and flexible primary structure. The caseins are a group of rheomorphic proteins with stable scaffold structures that withstand heat treatment without drastic effects on protein structure or reactivity. The proline-rich primary structure of the caseins prevents the β-strands of the secondary structure from forming stable tertiary structures. The proline residues force the structure of the caseins open, and is largely responsible for the stability of the protein.

b. Processing-induced changes in allergenicity and immunoreactivity

In the late 20th Century, the terms neoallergen and neoantigen were used in many publications to describe the activation or increased capability of proteins to elicit an allergic response upon a change in exposure or environment. Neoepitopes are defined as novel epitopes that are introduced as a consequence of processing (Mills et al., 2007). While the terms are not widely used, the activation or exposure of allergenic epitopes as a result of processing or other effects remains relevant. Pecans are well documented to have increased allergenicity for some patients after heating (Malanin et al., 1995). Some foods, such as shrimp, seem to exhibit little or no change in reactivity to processing (Leung et al., 1994). Reports of sensitivity only to processed foods are rare. A 1921 report described an allergy to cooked fish, but not to raw fish (Prausnitz and Küstner, 1921).

Heating has been documented to reduce the allergenicity of beef, purified bovine allergens, lupine, almond, and potato (Fiocchi et al., 1995). Heating of milk in the presence of other food proteins has been documented to decrease the allergenic reactivity of the products (Nowak-Wegrzyn and Fiocchi, 2009). Especially when interaction with wheat occurred, a baked

muffin or cupcake was less likely to induce an allergic reaction to the same amount of milk. The occurrence of a reaction with other molecules is dependent on the intensity of heating, water activity, pH, salt content, and concentration of other molecules in the matrix (Paschke and Besler, 2002). An early study described the lactosylation of milk proteins by the Maillard reaction and its reductive impact on allergenicity (Bleumink and Berrens, 1966).

Even harvest, transport, and storage of food can facilitate changes in protein stability. During transport and storage of soybeans, the heat generated by mold and microbes can raise the temperature and potentially generate additional allergens and expose previously hidden epitopes (Codina et al., 1998). Storage, especially of fruits and vegetables, can impact allergenicity. During storage, the allergenicity of apples increased, while no change was observed in allergenicity of mangoes (Paschke and Ulberth, 2009). During storage, enzymes have been documented to reduce the allergenicity of hazelnuts, rice, soybeans, and wheat, but have no effect on the allergenicity of peanut or peach. This has been assessed by skin prick testing and double-blind placebo-controlled food challenges.

The effects of pasteurization and homogenization on the allergenicity and antigenicity of milk proteins are well studied. Høst and Samuelsson (1988) reviewed studies on the allergenicity of raw, pasteurized, and pasteurized/homogenized milk. The authors suggested that processed milk products induce quicker and more severe reactions than raw milk. According to Høst and Samuelsson (1988), the trends observed indicate that milk-allergic patients have a lower threshold for processed milk products than raw milk products. However, whether raw, pasteurized, or pasteurized and homogenized, milk proteins remain able to induce similar adverse reactions among allergic children (Fiocchi et al., 2004). Although occurring at high temperature, pasteurization methods for most milk products consumed in developed countries (flash and HTST pasteurization) occur for such a short time that protein structure should not be significantly modified. Boiling milk for 10 minutes has been shown to decrease sensitivity in skin prick tests.

However, oral challenges with boiled milk failed to significantly reduce allergic reactions (Fiocchi et al., 2004).

A murine study by Poulsen et al. (1987) found that high fat content and homogenization increase the ability of milk to induce allergic reactions. Other studies, however, have found that heat treatment reduces the allergenicity of milk proteins (Gjesing et al., 1986). Kilshaw et al. (1982) discussed that severe heat treatment eliminates the capability of whey proteins to sensitize individuals and reduces the antigenicity of caseins. Regardless of heat treatment, however, allergic children retained reactivity to all types of milk, whether processed or not (Høst and Samuelsson, 1988).

Literature regarding the increased tolerability of baked or extensively heated milk products in milk-allergic children is available. Nowak-Wegrzyn et al. (2008) evaluated 100 milk allergic children for their reaction to extensively heated milk. Of the children, 68 tolerated extensively heated milk, 23 reacted to heated milk, and 9 reacted to both unheated and heated milk. The study concluded that the 70-80% of children with cow milk allergy can tolerate ingestion of heated milk. Additionally, a study by Bartnikas et al. (2012)determined that there is a strong correlation between SPT wheal diameter and ability to tolerate baked milk in open challenge. Smaller wheal diameter corresponded with increased ability to tolerate baked milk. Additional studies have supported the evidence that analysis of serum from children with persistent CMA shows an increased level of IgE to linear epitopes when compared to children who have achieved tolerance.

In a follow-up study, the researchers identified that there are significant differences between children who react to baked milk and those who do not. Baked milk-tolerant milk-allergic children have a less severe type of allergy and lower risk of anaphylaxis than CM allergic children who cannot tolerate baked milk (Caubet et al., 2013; Kim et al., 2011b).

Lara-Villoslada et al. (2005) described a study in which Balb/c mice were exposed to dairy preparations containing different casein: whey ratios. They determined that a higher amount of casein in the preparation leads to an increase in plasma histamine level and an increase in lymphocyte sensitization. Further, lower amounts of casein (increased whey protein) sensitized mice at a significantly lower level than the 80:20 ratio present in native cow milk. The authors suggest that the ratio of casein to whey is the reason why milk of different species has different sensitization capacities, even though the protein sources are similar.

c. Enzymatic Hydrolysis and Fermentation

Studies regarding allergenicity modification by cheese making have not found the process to affect allergenicity of bovine or ovine proteins. Lactic acid fermentation was reported to have a 99% decrease in antigenicity of ALA and BLG by ELISA, but allergenicity in skin testing was minimally affected. Because sensitization typically occurs at a very young age, particular attention is paid to the infant diet in terms of allergen exposure. Milk formulas are highly hydrolyzed with the goal of hypoallergenicity and ease of digestion for infants. In addition to prolonged exposure to proteolytic enzymes, infant formulas undergo ultrafiltration to separate remaining proteins from small peptides and amino acids. Ultrafiltration suppresses its allergenicity as a result (Thomas et al., 2007). Morisset et al. (2008) performed a study in which 115 infants were fed infant formula containing cow's milk proteins. Half of the infants were fed fermented infant formula and the remaining infants were fed standard (unfermented) infant formula. A significantly lower number of infants fed fermented milk formula became sensitized to cow's milk (prevalence of 1.7% compared to 12.5% in standard formula group). Björkstén et al. (2001) showed that statistically significant differences in allergic sensitization occur among infants with specific gut microflora.

During the production of milk-derived infant formulas, proteins are modified by heat denaturation, enzymatic hydrolysis, and sometimes ultrafiltration. Still, some individuals have

severely reacted to residual antigenic activity of milk-derived infant formulas in vitro and in vivo (Fiocchi et al., 2004). Clinical reactions have been reported with every type of available cow's milk hydrolysate, but 90% of milk-allergic children tolerate extensively hydrolyzed formulas (Fiocchi et al., 2004). It has been documented that the allergenic potency of casein-derived infant formulas is less than the allergenicity of whey-derived formulas for milk-allergic children.

Proteolysis of milk proteins during fermentation can affect immunoreactivity, but evidence regarding the effects on allergenicity is not as prevalent. Two studies have assessed the effects of fermentation on immunoreactivity and allergenicity of the major bovine whey proteins (Jedrychowski and Wróblewska, 1999; Paschke and Besler, 2002). The research showed that fermentation of sterilized milk reduced immunoreactivity by up to 99%, but allergenicity in skin prick tests (SPT) of milk-allergic patients was minimally affected. Kleber et al. (2006) also observed that allergenicity was retained when fermented skim milk and sweet whey (90% and 70% reduction in immunoreactivity, respectively) were analyzed in SPT with milk-allergic individuals. All three studies concluded that fermentations can reduce immunoreactivity, but does not necessarily correspond to a decrease in allergenicity.

Some microorganisms besides lactic acid bacteria show potential to reduce the immunoreactivity of milk proteins. Lakshman et al. (2011) evaluated the action of two fungal proteases isolated from *Monascus pilosus* on whey proteins. *M. pilosus* is used in Asia to produce fermented tofu and rice foods. After exposure to the proteinases, the antigenicity of whey proteins was measured by sandwich ELISA. The evaluated *M. pilosus* enzymes hydrolyzed α-lactalbumin and reduced its detection in their laboratory-developed ELISA. The enzymes, however, failed to reduce the detection of β-lactoglobulin by ELISA. No comment was made as to the degree of hydrolysis of BLG by *M. pilosus* proteases.

The microbial enzyme transglutaminase (MTG) has undergone much study as it possesses the capacity to decrease the allergenicity of casein and wheat. MTG catalyzes the formation of a cross-linking lysine-glutamine bond between two proteins. In milk, the enzyme

uses both casein and whey proteins as substrate. MTG is also capable of cross-linking soybean globulins, gluten, actin, myosins, and egg proteins (Watanabe et al., 2005). This newly formed bond is highly resistant to proteolytic degradation and has other dramatic effects on protein behavior.

The effects of enzymatic crosslinking by MTG have been evaluated for β-casein (Stanic et al., 2010). Highly polymerized caseins had an increased potential to inhibit IgE binding compared to untreated β-casein. Upon exposure to pepsin, cross-linked β-casein was digested slower than untreated β-casein. Stanic et al. (2010) also evaluated the effects of MTG cross-linking on allergenicity; in a study of non-atopic individuals, the reactivity of treated and control β-casein was equal, indicating that allergenicity was not enhanced in non-atopic individuals. However, Koppelman et al. (1999) described a case of anaphylaxis caused by ingestion of casein in MTG-treated salmon. The patient had previously reported allergic reactions to unintentionally ingested milk-containing products. Treatment by MTG has also been attributed to increasing serum IgA binding to gliadins and prolamins in wheat and maize in celiac patients over 8 years of age (Cabrera-Chavez et al., 2009).

Asian countries produce many fermented soy products. Soy sauce, miso, tempeh, and tofu have all been described to lose a large portion of their IgE binding through fermentation during production as assessed by a RAST IgE binding study (Herian et al., 1993). The research described that degradation of soy proteins did not occur consistently, providing evidence that some epitopes are favorably hydrolyzed by enzymes, while others are more resistant to hydrolysis.

7. Cheese Manufacture

In the United States, cheese and yogurt are among the most commonly consumed fermented dairy products. Cheeses made from bovine milk are more popular in the US than sheep or goat's milk cheeses. While cheese-making procedures differ slightly depending on

desired flavor and style characteristics, the production principles remain the same. Cheese manufacture is a lengthy dehydration process that serves to concentrate the fat and caseins in proportions roughly 6-12 times that of fluid milk (Fox et al., 2000). Although flavor and aroma development in cheese is a result of the combination of products of lipolysis, proteolysis, and carbohydrate utilization, the focus of this thesis is on the fermentation of bovine proteins with respect to allergenicity. Therefore, the biochemical mechanisms of cheese-making in this review will focus mainly on proteolysis and amino acid catabolism.

a. Milk Selection and Pre-treatment

The process of making cheese begins with proper milk selection. Even if obtained from the same species, the breed, climate, lactation stage, and diet of the animal has profound effects on the flavor and composition of the milk. Milk for the production of cheese must be free of antibiotics, which can otherwise inhibit starter bacteria and reduce product quality. Raw milk typically contains bacteria that are either naturally occurring or have been collected through contact with bovine skin and hair, milking equipment, transport vehicles, cheese vats, and other environmental sources.

To reduce the content of unwanted bacteria in milk, heat treatment is one of the first steps in the production of most cheeses. If milk is untreated, United States safety regulation dictates that any cheese made from unpasteurized milk must be aged for more than 60 days. Cheese produced from pasteurized milk has less intense flavor and ripens slower than raw milk cheeses, but reduces the numbers of potentially pathogenic and non-starter bacteria (Fox et al., 2004). Additionally, pasteurization, especially at high temperatures, denatures whey proteins and allows interaction with micellar κ-casein, which can have effects on texture, flavor, and moisture content of finished cheeses (Fox et al., 2004). While pasteurization has a noticeable effect on cheese flavor, many large US manufacturers use pasteurized milk for cheese production, regardless of intended age, due to concerns about food safety and product consistency.

b. Starter Culture Addition

Starter cultures are added to pasteurized milk to produce lactic acid and facilitate a decrease in pH. Cultures are selected on the basis of acid production, cook temperature, growth temperature, phage resistance, and development of flavor and texture. For cheeses like Cheddar, Edam, Gouda, and Camembert, the cook temperature is no more than 40^{o}C, and cultures such as *Lactococcus lactis* subspecies *lactis* or *Lactococcus lactis* subsp. *cremoris* are added. For cheeses with cook temperatures above 40^{o}C (often around 55^{o}C) , thermophilic LAB are added, such as *Streptococcus thermophilus*, *Lactobacillus delbrueckii* (subsp. *bulgaricus*, *casei*, or *lactis*), or *Lactobacillus helveticus* are added (Fox et al., 2004). High cook temperature cheeses include those of Swiss (Emmentaller and Gruyere) and Italian varieties (Mozzarella, Grana, Peccorino).

Acid production by starter LAB affects many aspects of cheese, especially concerning the activity of chymosin. Chymosin activity is pH-dependent; a large level of lactic acid in solution can increase the rate of casein coagulation. Additionally, acidification prior to draining due to lactic acid production influences the amount of chymosin trapped in the curd, gel strength, and moisture content. Residual coagulant influences the rate of proteolysis during ripening and affects cheese quality.

k-casein is hydrolyzed to para-kappa-casein and glycomacropetide, the micelle is destabilized, calcium bridges form, and coagulation of the curd occurs (Lucey, 2002). The presence of calcium during rennet coagulation is important; the positively charged calcium ions neutralize negatively charged casein residues, which increase the aggregation of casein micelles and reduce the total coagulation time (Lucey and Fox, 1993). A rapid pH decrease affects the dissolution of colloidal calcium phosphate, disrupts the casein micelle stability, and renders the caseins more susceptible to proteolysis.

c. Post-acidification

After coagulation, curd is cut with large wires to promote syneresis, and the milk-culture-chymosin mixture is cooked. The efficiency of syneresis is influenced by how small the curd is cut. Cooking temperature, as mentioned previously, affects the gel strength and moisture content of cheeses. High moisture cheeses are cooked at a temperature of less than 40^{o}C, while low moisture cheeses are cooked at higher temperatures than 40^{o}C. After cooking, the whey is drained from the coagulated curds. A high heat treatment of milk provides the potential for maximizing cheese yield by encouraging the inclusion of whey proteins in the curd. However, whey proteins have poor gel-formation characteristics, and their incorporation or remnants in curd can reduce cheese stability and structure (Damodaran and Parkin, 2008). With heat treatment, BLG denatures and often cross-links with intact κ-casein, reducing its susceptibility to chymosin (Fox et al., 2004).

The lactose in the cheese is fermented to lactic acid and cutting and salting of the curd enhances syneresis. Cheese is then milled, pressed, salted, and left for aging. Depending on variety, cheese can mature from two weeks to several years. As cheese is aged, it loses moisture and develops complex flavors. Typically, fresh cheeses (aged for short times) have higher moisture content and milder flavor than aged cheeses. Many primary biochemical changes occur during ripening, including metabolism of lactose, lactate, and citrate, lipolysis, and proteolysis. Secondary biochemical processes, including amino acid catabolism, fatty acid catabolism, and lactic acid catabolism, can also be responsible for flavor and texture development, whether desirable or undesirable.

8. Proteolysis during Cheese Manufacture and Ripening

In most cheeses, proteolysis is the major metabolic process attributed to flavor and texture development, although lipolysis is also partially responsible for specific flavors in some cheeses. Proteolysis is primarily responsible for changes in hardness, elasticity, cohesiveness,

brittleness, and other textural properties of cheese. The level of proteolysis can range from limited in some cheese varieties (e.g. Mozzarella) to quite extensive in others (Blue cheeses).

Two main types of proteases occur in most organisms; proteinases and peptidases. Proteinases are enzymes capable of hydrolyzing proteins and large oligopeptides, while peptidases hydrolyze smaller oligopeptides and di- and tri-peptides to free amino acids. The proteases present during cheese manufacture and ripening come from a variety of sources. Sources include enzymes naturally present in raw milk, added proteases (chymosin), enzymes from primary and secondary starter organisms, and enzymes from non-starter microorganisms (Fox, 1989). Levels of moisture, pH, salt, temperature, and microorganisms influence the biochemical changes that occur in cheese during ripening (Fox et al., 2004). Flavors and aromas are largely produced by both primary and secondary microflora and developed as a result of production and catabolism of low molecular weight peptides and free amino acids (Law and Tamime, 2011). Large casein-derived peptides often exhibit a bitter flavor. Beneficial and characteristic flavors and are developed through the catabolism of small oligopeptides and free amino acids (Law and Tamime, 2011).

a. Chymosin

Chymosin is absolutely critical in cheese manufacture and is arguably the most important enzyme involved in flavor and texture development during cheese ripening. Also called rennet, chymosin is a heat-labile aspartic proteinase, and its activity increases as the pH of the milk drops from lactic acid production by added starter culture. Activity of chymosin is highest at pH 6.3-6.6. Chymosin is a coagulant with limited proteolytic activity, hydrolyzing specifically one bond of κ-casein (Upadhyay et al., 2004). The Phe_{105}-Met_{106} bond of κ-casein, when cleaved, releases the hydrophilic casein macropeptide and para-κ-casein remains temporarily bound to the casein micelle. The cleavage of this bond and the acidification of the matrix initiate the reactivity of calcium present in the serum. Hydrolysis of other caseins by chymosin can also occur, but only

during aging. Chymosin is primarily responsible for casein catabolism in low-cooked cheeses and makes insignificant contributions to ripening of high-cooked cheeses (Upadhyay et al., 2004).

Traditionally, chymosin derived from calf stomachs was used to facilitate the coagulation of caseins and separation from the whey portion of milk. However, natural production of chymosin is dependent upon the veal market and has limited availability. Because calf rennet and other aspartic proteinases exhibit a high degree of structural homology, scientists have isolated effective alternate rennet enzymes.

Today, the majority of chymosin used in the cheese-making industry is produced in industrial scale fermenters. The most commonly used chymosin-producing cultures are clones of *E. coli*, *Kluyveromyces lactis*, and *Aspergillus niger*. Commercial production of chymosin substitutes is significantly less expensive than the traditional production of calf rennet and allows consistent availability. However, bacterial and fungal proteinases cause substantial non-specific hydrolysis of both κ-casein and para-κ-casein. Non-specific casein hydrolysis affects coagulation, flavor and texture development, and protein profiles in cheese. Microbial-derived chymosin is influenced by the host species used for cloning and typically exhibits varying caseinolytic specificity when obtained from different sources (Fox et al., 2000). Two commercially-produced recombinant chymosin enzymes, Chymax (Chr. Hansen, Denmark) and Maxiren (DSM Food Specialties, the Netherlands), are responsible for a large portion of world cheese production, cornering 35% of the cheese market as of the year 2000 (Fox et al., 2000).

Upon whey draining, chymosin can become trapped in the cheese matrix and proteolyze non-target caseins during aging. While the main role of chymosin is hydrolysis of the Phe_{105}-Met_{106} of κ-casein and coagulation of milk, hydrolysis of other proteins will occur during aging at a slower rate. Up to 30% of rennet can be retained in the curd, depending on cooking

temperature, pH at draining, and moisture content (Upadhyay et al., 2004). More chymosin will be retained in the curd as the pH is decreased (Creamer et al., 1985).

If chymosin is obtained from traditional calf sources, it can contain 10%-50% bovine pepsin, depending on quality (Fox et al., 2000). Pepsin is a digestive protease with broad specificity and can drive the differential hydrolysis of caseins. Electrophoresis gels of renneted cheese displays numerous additional casein-derived peptides not seen in cheese produced by microbial-derived chymosin. This is described to be a result of pepsin activity (Fox et al., 2000). Additionally, pepsins are more pH sensitive than chymosin, and the activity level in cheese is strongly dependent on the cheese pH at draining (Sousa et al., 2001).

b. Indigenous Milk Enzymes

Milk contains many indigenous proteases; the most important of these during cheese ripening is plasmin. Plasmin is a heat stable serine-proteinase that has similar specificity to the digestive enzyme trypsin (Fox et al., 2000). Plasmin hydrolyzes caseins with the following specificity: $\beta \approx \alpha_{s2} >> \alpha_{s1}$, while κ-casein is largely resistant to plasmin proteolysis (Bastian and Brown, 1996). Hydrolysis of β-casein and α_{s1}-casein produces two unique casein subclasses, the γ-caseins and λ-caseins, respectively.

The activity level of plasmin in cheese is largely dependent upon cooking temperature and pH during ripening (Farkye and Fox, 1990). Chymosin can be inactivated during the cooking step of high-cook cheeses (55^{o}C), and plasmin is majorly responsible for texture and flavor development in these varieties. Cheeses that are smear- or mold-ripened exhibit increased plasmin activity throughout aging due to increasing pH from metabolic byproducts (O'Farrell et al., 2002).

Another indigenous milk protein, cathepsin D, has been noted for proteolytic activity in milk. In studies employing model systems, cathepsin D has similar caseinolytic specificity to chymosin, but lacks the ability to cause coagulation (Fox et al., 2004; McSweeney et al., 1995).

Cathepsin D is a heat-labile acid proteinase, but its contribution to cheese ripening is unclear (Fox et al., 2000). Additionally, it has been suggested that because it is a serum protein, very little cathepsin D survives in the curd after draining during cheese manufacture (Upadhyay et al., 2004). However, Hurley et al. (2000) described that about 8% of cathepsin D survives in cheese after pasteurization and does play a contributing role in proteolysis of rennet-free cheeses. Other indigenous proteinases have been detected in ripened cheeses, although their contribution to flavor and texture has not been established as significant.

Cheeses made from pasteurized milk are markedly different in terms of proteolytic patterns than cheeses produced from raw milk. High temperature treatments during pasteurization inactivate many enzymes present in raw milk, including plasmin, can affect milk flavor, and can also affect the structure of some proteins.

c. Primary Starter Cultures

Lactic acid bacteria (LAB) starter cultures are added during their active growth stage and begin the production of lactic acid from lactose in cheese milk. The main LAB species used as starter cultures in cheese manufacture are *Lactococcus lactis, Leuconostoc* species, *Streptococcus thermophilus, Lactobacillus delbruecki* subsp. *lactis* and subsp. *bulgaricus*, and *Lb. helveticus.* High concentrations of active cells are added to cheese milk and acidification occurs quite rapidly, and some flavor and aroma compounds are also produced.

LAB employ complex proteolytic machinery to meet their nutritional requirements and grow to high concentrations in milk. Most LAB have similar proteolytic systems, including a cell wall bound proteinase, amino acid transport systems, and several intracellular peptidases and proteinases. The main LAB proteinase involved in cheese ripening is anchored to the cell wall and is known as the cell envelope proteinase (CEP). All CEPs are serine-proteases and target the degradation of caseins to oligopeptides (Juillard et al., 1995). The most well-defined CEP is PrtP of *Lactococcus lactis.* Amino acid substitutions in the binding cleft of PrtP are responsible for

varying specificity of α_{s1}-, β-, and κ-casein degradation. Other CEPs have been studied in both thermophilic and mesophilic LAB, with high homology in enzyme active sites. When used for cheese manufacture, it has been suggested that CEPs act on caseins, typically releasing 4-10 oligopeptide residues (Fox and McSweeney, 1996). All LAB peptidases are internal, and oligopeptides of 4-18 residues are transported into the cell via the non-specific oligopeptide transport system. After uptake into the cell, oligopeptides are hydrolyzed to shorter peptides and free amino acids by endopeptidases and exopeptidases, respectively (Christensen et al., 1999; Kunji et al., 1996; Law and Haandrikman, 1997). Many cheese-makers and scientists claim that the breakdown of κ-casein to peptides and free amino acids by a combination of chymosin and proteinases is the most important aspect of cheese-making.

Typically due to low pH, salt sensitivity, and limited fermentable carbohydrates, starter culture cells lyse after primary proteolysis, releasing intracellular enzymes into the surrounding matrix. Lysis is a critical step in cheese making as it releases classically intracellular enzymes into the surrounding matrix where they can continue proteolysis. Autolysis can also occur as a result of thermoinducible phage. Heating the cheese milk above a certain cook temperature triggers the lysis of many starters. The released peptidases continue to hydrolyze oligopeptides in the matrix during fermentation. The action of released peptidases during aging is termed secondary proteolysis (Rank et al., 1985).

d. Non-starter Lactic Acid Bacteria

Especially in traditionally manufactured cheeses, non-starter Lactic Acid Bacteria (NSLAB) play important roles in texture and flavor development. Lactobacilli are the most commonly occurring NSLAB, especially *Lactobacillus casei, L. paracasei, L. plantarum,* and *L. curvatus* (Fox et al., 2004). While they impart unique characteristics during aging, these bacteria are adventitiously obtained through environmental contact, and are usually unpredictable in concentration and species. Due to variability and minimal control over NSLAB growth and

metabolism, most large-scale cheese manufacturers employ pasteurization or additional processing methods to remove or minimize unwanted or unpredictable cultures. The proteolytic activity of NSLAB is similar to that of starter LAB. NSLAB occur in unripened cheese in low numbers, and their concentration increases through ripening. A review by Peterson and Marshall (1990) described the consensus of NSLAB, determining that they cause detrimental effects on cheese quality equally as often as they benefit the final product.

A study by Fitzsimons et al. (1999) assessed the genetic similarities among NSLAB isolated from Cheddar cheeses produced at three separate locations from the same starting materials. The research determined that NSLAB from cheeses produced at the same factory were genetically similar to each other, while distinctively different from the NSLAB produced at different factories. Further, NSLAB present in defective cheeses were distinctly different from NSLAB in premium-quality cheeses.

McSweeney et al. (1993) compared quality and sensory characteristics as well as count of NSLAB after three months of aging in cheeses produced from microfiltered, pasteurized, and raw milk. The research showed that at pressing, the cheeses contained $4.3x10^{1}$, $3.9x\ 10^{2}$, and $1.47x10^{5}$ cfu/g NSLAB, respectively. After 11 weeks of aging, the cheeses contained $9.3x10^{6}$, $1.12x10^{7}$, and $1.19x10^{8}$ cfu/g NSLAB, respectively. Sensory panels determined that the cheese produced from raw milk quickly developed off-flavors, while the filtered and pasteurized milks maintained equivalent taste and quality.

e. Secondary Cultures and Adjunct Microflora

Additional cultures are sometimes intentionally added to develop unique flavor and texture characteristics in ripening cheese. Smear-ripened, surface-ripened, and internal mold – ripened cheeses are among those which require further treatment prior to and/or during aging.

Secondary starter cultures are typically added at low levels at the same time as the primary starter LAB, but have no function in the acidification of cheese. Their role is to produce

biochemical and organoleptic changes in cheese during ripening. The main groups of secondary cultures are non-starter lactic acid bacteria, propionibacteria, coryneforms, staphylococci, yeasts, and molds. These cultures are present at low cell concentrations during early stages of cheese ripening, and flourish during aging after lysis of starter cultures. Secondary cultures are key for the development of unique flavors through the release of free amino acids during aging.

Molds are traditionally implemented for aging surface-ripened or blue-veined cheeses. While lipolysis is often considered the dominant biochemical process performed by molds, proteolytic activity is still considered significant. The most common species of molds used as adjuncts during cheese ripening are *Penicillium roqueforti* and *Penicillium camemberti* . *P. camemberti* is a white mold used to make Camembert and Brie- type cheeses, while *P. roqueforti* is a blue-green mold used in the production of Roquefort and other Blue cheeses. Both species have proteinase activity that typically begins on the surface. The extracellular proteinases specifically hydrolyze α_{s1}-, β-, and κ-casein and other chymosin-derived peptides (Law and Tamime, 2011). The free amino acids released by *Penicillium* peptidases are known for their debittering activity.

Yeasts are commonly used in conjunction in surface-ripened cheeses as they promote growth of molds and bacteria. They can be added directly to cheese milk with primary starters or can be applied to the surface of cheese after pressing in brine form. Yeasts contribute some proteolytic activity during ripening, as they employ caseinolytic and peptidolytic activity.

Coryneform bacteria and Staphylococci are used during the aging of bacterial-smear ripened cheeses, including Limburger, Munster, Tilsit, Raclette, Brick, and Monterrey. These bacteria are grouped together based on their characteristic production of red-orange pigments on cheese rinds. While both Staphylococci and Coryneform are present in the adjunct cultures for these cheeses, Staphylococci are outnumbered by Coryneforms early in the ripening process (Fox et al., 2004). The most popular Coryneform for cheese production is *Brevibacterium linens*, and is used in the ripening of Munster cheese. *B. linens* has targeted specificity for α_{s1}- and β-casein.

Cultures used in the production of smear- or bacterial-surface ripened cheese have two functions; enzyme production and deacidification. These bacteria are capable of metabolizing lactic acid to carbon dioxide and water, causing a pH increase during ripening. While these bacteria have a minor contribution to casein proteolysis, they catabolize many small casein-derived oligopeptides and amino acids and contribute to the production of characteristic aromas and flavors.

Propionic acid bacteria (PAB) are known for their ability to metabolize many carbon sources, especially alcohols and lactic acid, to produce carbon dioxide, propionic acid, and acetate. These bacteria are responsible for eye development in Swiss-type cheeses and are especially active on proline-containing peptides (Fox et al., 2000). This cheese family includes Emmental cheese. They exhibit minor caseinolytic activity, but possess extensive peptidolytic activity that contributes to flavor production. The most commonly used PAB in cheese making is *Propionibacterium freudenreichii* subsp. *shermanii*.

9. Monitoring Proteolysis in Dairy Products

Comparing extent and patterns of proteolysis in cheese is the basis for classification and measurement of cheese quality and maturity. Because of the vast differences in milk, treatments, enzymes, microflora, and environmental conditions during production and aging, proteolytic patterns of ripening are distinctly different among cheeses, especially those of different varieties. Several methods of analysis have been described for monitoring cheese proteolysis, and include classification by nitrogen content, gel and capillary electrophoresis, chromatography, and free amino acid content. Extent of proteolysis correlates well with age.

a. Non-specific Methods

Non-specific methods of monitoring proteolysis include evaluation of cheese solubility and protein extraction in various buffers. Commonly used buffers for extracting proteins from cheese include pH 4.6 citrate buffers, water, sodium chloride, ethanol, trichloroacetic acid, phosphotungstic acid, and sulfosalicylic acid (Christensen et al., 1991; Fox and McSweeney,

1996; McSweeney and Fox, 1997; Sousa et al., 2001). Extracts can be evaluated in several ways: by various nitrogen determination methods including Kjeldahl, Lowry, Hull, absorbance at 280nm, or by electrophoretic methods. The selectivity of various extraction buffers provides different protein concentrations and profiles when analyzed, even for identical samples. Depending on the cheese conditions during manufacture and aging, different buffers will provide improved or less efficient protein extraction from the matrix. Buffer solubility is a rapid, inexpensive, and valuable technique for routine assessment of cheese maturity and quality. As cheese ages, the profiles obtained through fractionation will appear more concentrated as a result of the increased level of released peptides and amino acids.

If analysis of particular proteins is desired, fractionation is a useful technique to discriminate among the proteins and peptides of specific size and solubility (Christensen et al., 1991). The soluble and insoluble fractions obtained from extraction with these chemicals display distinctly different electrophoretic profiles.

Many studies use Kjeldahl analysis to determine the ratio of soluble nitrogen to total nitrogen in cheeses. As cheese ages and proteolysis continues the amount of soluble nitrogen increases. The Kjeldahl method, while useful, is a very laborious and time-consuming procedure for evaluation of cheese proteolysis. More rapid techniques have been suggested for measuring free amino groups in cheese based on spectrometry and titration. Most of the rapid methods require a fractionation step prior to analysis, while only two methods can be performed on whole cheese.

b. Specific Methods

Polyacrylamide gel electrophoresis (PAGE) is one of the most widely used techniques for assessing proteolysis in cheese. As cheese ages, proteins are broken down into larger peptides, from larger peptides to smaller peptides, and then into free amino acids. PAGE allows visual observation of changing proportions of milk proteins during fermentation to be observed (Chin

and Rosenberg, 1998). The efficiency of various electrophoretic protocols, buffers, and staining procedures for cheese analysis has been extensively reviewed (Kuchroo and Fox, 1982; Ledford et al., 1966; Morr, 1971; Shalabi and Fox, 1987; Veloso et al., 2004).

Much research conducted in the late 20th century relied upon urea-PAGE to monitor proteolysis. Urea, however, is a strong denaturant, and can cause changes in protein structure, function, or response in downstream analyses. Difficulties in resolving and staining milk proteins derived from cheese samples was common when using 1-D sodium dodecyl sulfate (SDS)-PAGE for milk proteins due to limited construction of sufficient separation matrices within acrylamide gels, but using urea-PAGE was documented to provide improved separation of caseins (Creamer, 1991; O'Sullivan and Fox, 1990; Shalabi and Fox, 1987). Casein fractions, especially α_{s1}- and α_{s2}-, migrate closely within SDS-polyacrylamide and native gels of low and broad concentrations, as they share similar molecular weights and isoelectric points(see Table 1.1) (Marshall and Williams, 1988). Two-dimensional electrophoresis, using isoelectric focusing (IEF) as the first dimension and PAGE as the second, has been suggested as an superior method of separating protein fractions with increased specificity (Chin and Rosenberg, 1998; Marshall and Williams, 1988). Recent advances, however, have improved the capabilities of SDS-gels and buffers for electrophoresis. These gels are commercially available and useful for 1-D separation of milk proteins at specific concentrations.

10. Conclusions

The relationship between protein structure and function is unclear. This thesis seeks to determine the effects of fermentation-driven proteolysis on the detection of allergen-specific residues in cheese. Different levels of proteolysis, whether limited or extensive, will be evaluated using imaging and immunoassay methods. The relationship between structure and allergenicity is also unclear. Theoretically, the detection of protein fragments derived from allergenic proteins will be decreased as a result of structural degradation. However, research suggests that new

allergenic epitopes are commonly exposed upon destruction of conformational protein structures, but this relationship, again, is unclear. This thesis seeks to add supporting data and insight to these scientific uncertainties.

11. References

1995. in: Food and Agriculture Organization of the United Nations (Ed.), Report of the FAO Technical Consultation on Food Allergies. FAO, Rome.

Abbott, M., Hayward, S., Ross, W., Godefroy, S.B., Ulberth, F., van Hengel, A.J., Roberts, J., Akiyama, H., Popping, B., Yeung, J.M., Wehling, P., Taylor, S.L., Poms, R.E., Delahaut, P., 2010. Validation procedures for quantitative food allergen ELISA methods: community guidance and best practices. Journal of AOAC International 93, 442-450.

Anderson, J.A., 1984. Non-immunologically-mediated food sensitivity. Nutr Rev 42, 109-116.

Anguita, G., Martin, R., Garcia, T., Morales, P., Haza, A.I., Gonzalez, I., Sanz, B., Hernández, P.E., 1996. Immunostick ELISA for detection of cow's milk in ewe's milk and cheese using a monoclonal antibody against bovine ß-casein. J Food Prot 59, 436-437.

Astwood, J.D., Fuchs, R.L., 1996. Food allergens are stable to digestion in a simple model of the gastrointestinal tract. J Allergy Clin Immunol 97, 241.

Baldwin, J.L., 1996. Pharmacologic food reactions, 2nd ed. Blackwell Science Publications, Oxford.

Barbosa-Cánovas, G.V., 2009. Global issues in food science and technology. Elsevier ; Academic Press, Amsterdam; Boston; Burlington, MA.

Bartnikas, L.M., Sheehan, W.J., Hoffman, E.B., Permaul, P., Dioun, A.F., Friedlander, J., Baxi, S.N., Schneider, L.C., Phipatanakul, W., 2012. Predicting food challenge outcomes for baked milk: role of specific IgE and skin prick testing. Annals of Allergy, Asthma & Immunology.

Bastian, E.D., Brown, R.J., 1996. Plasmin in milk and dairy products: an update. International Dairy Journal 6, 435-457.

Bernard, H., Créminon, C., Negroni, L., Peltre, G., Wal, J.-M., 1999. IgE cross-reactivity with caseins from different species in humans allergic to cow's milk. Food Agric Immunol 11, 101-111.

Bindslev-Jensen, C., Ballmer-Weber, B.K., Bengtsson, U., Blanco, C., Ebner, C., Hourihane, J., Knulst, A.C., Moneret-Vautrin, D.A., Nekam, K., Niggemann, B., Osterballe, M., Ortolani, C., Ring, J., Schnopp, C., Werfel, T., 2004. Standardization of food challenges in patients with immediate reactions to foods - position paper from the European Academy of Allergology and Clinical Immunology. Allergy 59, 690-697.

Björkstén, B., Sepp, E., Julge, K., Voor, T., Mikelsaar, M., 2001. Allergy development and the intestinal microflora during the first year of life. Journal of Allergy and Clinical Immunology 108, 516-520.

Bleumink, E., Berrens, L., 1966. Synthetic approaches to the biological activity of b-lactoglobulin in human allergy to cows' milk. Nature 212, 541-543.

Bock, S.A., Muñoz-Furlong, A., Sampson, H.A., 2001. Fatalities due to anaphylactic reactions to foods. Journal of Allergy and Clinical Immunology 107, 191-193.

Bock, S.A., Muñoz-Furlong, A., Sampson, H.A., 2007. Further fatalities caused by anaphylactic reactions to food, 2001-2006. Journal of Allergy and Clinical Immunology 119, 1016-1018.

Brazil, E., MacNamara, A.F., 1998. 'Not so immediate' hypersensitivity - the danger of biphasic anaphylactic reactions. J Accid Emerg Med 15, 252-253.

Burks, A.W., Laubach, S., Jones, S.M., 2008. Oral tolerance, food allergy, and immunotherapy: implications for future treatment. J Allergy Clin Immunol 121, 1344-1350.

Bush, R.K., Hefle, S.L., 1996. Food allergens. Crit Rev Food Sci Nutr 36, S119-S163.

Bush, R.K., Taylor, S.L., 1998. Adverse reactions to food and drug additives, in: E. Middleton, Jr., C.E. Reed, E.F. Ellis, N.F. Adkinson, Jr., J.W. Yunginger, W.W. Busse (Eds.), Allergy: Principles & Practice, 5th ed. Mosby, St. Louis, pp. 1183-1198.

Busse, P.J., Järvinen, K.-M., Vila, L., Beyer, K., Sampson, H.A., 2002. Identification of sequential IgE-binding epitopes on bovine a_{s2}-casein in cow's milk allergic patients. International Archives of Allergy and Immunology 129, 93-96.

Cabrera-Chavez, F., Rouzaud-Sandez, O., Sotelo-Cruz, N., Calderon de la Barca, A.M., 2009. Bovine milk caseins and transglutaminase-treated cereal prolamins are differentially recognized by IgA of celiac disease patients according to their age. J Agric Food Chem 57, 3754-3759.

Caubet, J.-C., Nowak-Węgrzyn, A., Moshier, E., Godbold, J., Wang, J., Sampson, H.A., 2013. Utility of casein-specific IgE levels in predicting reactivity to baked milk. Journal of Allergy and Clinical Immunology 131, 222-224. e224.

Chatchatee, P., Bardina, L., Sampson, H.A., 2000. Identification of IgE and IgG binding epitopes of a-casein in cow milk allergic patients. Journal of Allergy and Clinical Immunology 105, S185.

Chatchatee, P., Järvinen, K.-M., Bardina, L., Beyer, K., Sampson, H.A., 2001a. Identification of IgE- and IgG-binding epitopes on as1-casein: differences in patients with persistent and transient cow's milk allergy. Journal of Allergy and Clinical Immunology 107, 379-383.

Chatchatee, P., Järvinen, K.-M., Bardina, L., Vila, L., Beyer, K., Sampson, H.A., 2001b. Identification of IgE and IgG binding epitopes on b- and k-casein in cow's milk allergic patients. Clinical and Experimental Allergy 31, 1256-1262.

Chin, H.W., Rosenberg, M., 1998. Monitoring Proteolysis During Cheddar Cheese Ripening Using Two-Dimensional Gel Electrophoresis. Journal of Food Science 63, 423-428.

Christensen, J.E., Dudley, E.G., Pederson, J.A., Steele, J.L., 1999. Peptidases and amino acid catabolism in lactic acid bacteria. Antonie van Leeuwenhoek 76, 217-246.

Christensen, T., Bech, A.M., Werner, H., 1991. Methods for crude fractionation (extraction and precipitation) of nitrogen components in cheese. Bulletin-International Dairy Federation, 4-9.

Codina, R., Lockey, R.F., Ardusso, L., 1998. Soybean hull allergens induced by heating and storage. J Allergy Clin Immunol 101, S229.

Creamer, L., 1991. Electrophoresis of Cheese. Bulletin of the International Dairy Federation, 14-28.

Creamer, L., Lawrence, R., Gilles, J., 1985. Effect of acidification of cheese milk on the resultant Cheddar cheese. New Zealand Journal of Dairy Science and Technology 20, 185-203.

Damodaran, S., 1994. Structure-function relationship of food proteins. Protein functionality in food systems, 1-37.

Damodaran, S., Parkin, K., 2008. Fennema's food chemistry. CRC Press/Taylor & Francis.

Decker, W.W., Campbell, R.L., Manivannan, V., Luke, A., St. Sauver, J.L., Weaver, A., Bellolio, M.F., Bergstralh, E.J., Stead, L.G., Li, J.T.C., 2008. The etiology and incidence of anaphylaxis in Rochester, Minnesota: a report from the Rochester Epidemiology Project. J Allergy Clin Immunol 122, 1161-1165.

Douglas, D.M., Sukenick, E., Andrade, P., Brown, J.S., 1994. Biphasis systemic anaphylaxis: an inpatient and outpatient study. J Allergy Clin Immunol 93, 977-985.

Dumont, V., Kerbach, S., Poms, R., Johnson, P., Mills, C., Popping, B., Tömösközi, S., Delahaut, P., 2010. Development of milk and egg incurred reference materials for the validation of food allergen detection methods. Quality Assurance and Safety of Crops & Foods 2, 208-215.

Farkye, N.Y., Fox, P.F., 1990. Observations on plasmin activity in cheese. Journal of Dairy Research 57, 413-418.

Fernandez-Rivas, M., Ballmer-Weber, B., 2007. Food allergy: current diagnosis and management, in: C. Mills, H. Wichers, K. Hoffmann-Sommergruber (Eds.), Managing Allergens in Food. CRC Press, LLC, Boca Raton, FL, p. 315.

Fiocchi, A., Bouygue, G.R., Sarratud, T., Terracciano, L., Martelli, A., Restani, P., 2004. Clinical tolerance of processed foods. Annals of Allergy, Asthma and Immunology 93, S38-S46.

Fiocchi, A., Restani, P., Riva, E., Restelli, A.R., Biasucci, G., Galli, C.L., Giovannini, M., 1995. Meat allergy: II - Effects of food processing and enzymatic digestion on the allergenicity of bovine and ovine meats. J Am Coll Nutr 14, 245-250.

Fitzsimons, N., Cogan, T., Condon, S., Beresford, T., 1999. Phenotypic and genotypic characterization of non-starter lactic acid bacteria in mature cheddar cheese. Applied and environmental microbiology 65, 3418-3426.

Fox, P.F., 1989. Proteolysis During Cheese Manufacture and Ripening. Journal of Dairy Science 72, 1379-1400.

Fox, P.F., 2001. Milk proteins as food ingredients. International Journal of Dairy Technology 54, 41-55.

Fox, P.F., McSweeney, P.L., Cogan, T.M., Guinee, T.P., 2000. Fundamentals of cheese science. Springer.

Fox, P.F., McSweeney, P.L.H., 1996. Proteolysis in cheese during ripening. Food Reviews International 12, 457-509.

Fox, P.F., McSweeney, P.L.H., Cogan, T.M., Guinee, T.P., 2004. Cheese: Chemistry, Physics and Microbiology: General Aspects. Elsevier Science.

Fu, T.-F., Abbott, U.R., Hatzos, C., 2002. Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid - a comparative study. J Agric Food Chem 50, 7154-7160.

Gjesing, B., Osterballe, O., Schwartz, B., Wahn, U., Lowenstein, H., 1986. Allergen-specific IgE antibodies against antigentic components in cow milk and milk substitutes. Allergy 41, 51-56.

Herian, A.M., Taylor, S.L., Bush, R.K., 1993. Allergenic reactivity of various soybean products as determined by RAST inhibition. Journal of Food Science 58, 385-388.

Hill, D.J., Firer, M.A., Ball, G., Hosking, C.S., 1993. Natural history of cows' milk allergy in children: immunological outcome over 2 years. Clinical and Experimental Allergy 23, 124-131.

Hill, D.J., Hosking, C.S., Reyes-Benito, L.V., 2001. Reducing the need for food allergen challenges in young children: a comparison of in vitro with in vivo tests. Clinical and Experimental Allergy 31, 1031-1035.

Horne, D.S., 2002. Casein structure, self-assembly and gelation. Current opinion in colloid & interface science 7, 456-461.

Høst, A., 1997. Cow's milk allergy. J R Soc Med 90, 34-39.

Høst, A., Samuelsson, E.-G., 1988. Allergic reactions to raw, pasteurized, and homogenized/ pasteurized cow milk: a comparison. Allergy 43, 113-118.

Hourihane, J.O.B., 1998. Prevalence and severity of food allergy - need for control. Allergy 53, 84-88.

Hurley, M., Larsen, L., Kelly, A., McSweeney, P., 2000. The milk acid proteinase cathepsin D: a review. International Dairy Journal 10, 673-681.

Hurst, W.J., Krout, E.R., Burks, W.R., 2002. A comparison of commercially available peanut ELISA test kits on the analysis of samples of dark and milk chocolate. Journal of Immunoassay and Immunochemistry 23, 451-459.

Husby, S., Jensenius, J.C., Svehag, S.-E., 1986. Passage of undegraded dietary antigen into the blood of healthy adults. Further characterization of the kinetics of uptake and the size distribution of the antigen. Scandinavian Journal of Immunology 24, 447-455.

Ito, K., Futamura, M., Moverare, R., Tanaka, A., Kawabe, T., Sakamoto, T., Borres, M.P., 2012. The usefulness of casein-specific IgE and IgG4 antibodies in cow's milk allergic children. Clin Mol Allergy 10, 1.

Järvinen, K.-M., Chatchatee, P., Bardina, L., Beyer, K., Sampson, H.A., 2001. IgE and IgG binding epitopes on a-lactalbumin and b-lactoglobulin in cow's milk allergy. International Archives of Allergy and Immunology 126, 111-118.

Jedrychowski, L., Wróblewska, B., 1999. Reduction of the antigenicity of whey proteins by lactic acid fermentation. Food Agric Immunol 11, 91-99.

Jost, R., 1988. Physicochemical treatment of food allergens: application to cow's milk proteins. Vevey/ Raven Press, Ltd., New York.

Juillard, V., Le Bars, D., Kunji, E.R., Konings, W.N., Gripon, J.C., Richard, J., 1995. Oligopeptides are the main source of nitrogen for Lactococcus lactis during growth in milk. Applied and environmental microbiology 61, 3024-3030.

Kemp, S.F., Lockey, R.F., 2002. Anaphylaxis: a review of causes and mechanisms. J Allergy Clin Immunol 110, 341-348.

Kerbach, S., Alldrick, A.J., Crevel, R.W., Dömötör, L., DunnGalvin, A., Clare Mills, E., Pfaff, S., Poms, R.E., Popping, B., Tömösközi, S., 2009. Managing food allergens in the food supply chain–viewed from different stakeholder perspectives. Quality Assurance and Safety of Crops & Foods 1, 50-60.

Kilshaw, P.J., Heppell, L.M.J., Ford, J.E., 1982. Effects of heat treatment of cows' milk and whey on the nutritional quality and antigenic properties. Archives of Disease In Childhood 57, 842-847.

Kim, J.S., Nowak-Węgrzyn, A., Sicherer, S.H., Noone, S., Moshier, E.L., Sampson, H.A., 2011. Dietary baked milk accelerates the resolution of cow’s milk allergy in children. Journal of Allergy and Clinical Immunology 128, 125-131. e122.

Kleber, N., Weyrich, U., Hinrichs, J., 2006. Screening for lactic acid bacteria with potential to reduce antigenic response of b-lactoglobulin in bovine skim milk and sweet whey. Innovative Food Science and Emerging Technologies 7, 233-238.

Koppelman, S.J., Bleeker-Marcelis, H., van Duijn, G., Hessing, M., 1996. Detecting peanut allergens: the development of an immunochemical assay for peanut proteins. The World of Food Ingredients, 35-38.

Koppelman, S.J., Hefle, S.L., 2006. Detecting allergens in food. Woodhead Publishing Ltd.

Koppelman, S.J., Wensing, M., De Jong, G.A.H., Knulst, A.C., 1999. Anaphylaxis caused by the unexpected presence of casein in salmon. Lancet 354, 2136.

Kuchroo, C., Fox, P., 1982. Soluble nitrogen in Cheddar cheese: Comparison of extraction procedures. Anglais 37, 331-335.

Kunji, E.R.S., Mierau, I., Hagting, A., Poolman, B., Konings, W.N., 1996. The proteotytic systems of lactic acid bacteria. Antonie van Leeuwenhoek 70, 187-221.

Lack, G., 2008. Epidemiologic risks for food allergy. Journal of Allergy and Clinical Immunology 121, 1331-1336.

Lakshman, P., Toyokawa, Y., Tachibana, S., Toyama, H., Taira, T., Yasuda, M., 2011. Reducing the antigenicity of milk whey protein using acid proteinases from< i> Monascus pilosus</i>. Process Biochemistry 46, 806-810.

Lara-Villoslada, F., Olivares, M., Xaus, J., 2005. The balance between caseins and whey proteins in cow's milk determines its allergenicity. J Dairy Sci 88, 1654-1660.

Law, B.A., Tamime, A., 2011. Technology of cheesemaking. Wiley-Blackwell.

Law, J., Haandrikman, A., 1997. Proteolytic enzymes of lactic acid bacteria. International Dairy Journal 7, 1-11.

Ledford, R.A., O'Sullivan, A.C., Nath, K.R., 1966. Residual Casein Fractions in Ripened Cheese Determined by Polyacrylamide-Gel Electrophoresis. Journal of Dairy Science 49, 1098-1101.

Lee, J.M., Greenes, D.S., 2000. Biphasic anaphylactic reactions in pediatrics. Pediatrics 106, 762-766.

Lemon-Mule, H., Sampson, H.A., Sicherer, S.H., Shreffler, W.G., Noone, S., Nowak-Wegrzyn, A., 2008. Immunologic changes in children with egg allergy ingesting extensively heated egg. Journal of Allergy and Clinical Immunology 122, 977-983.

Leung, P.S.C., Chu, K.H., Chow, W.K., Ansari, A., Bandea, C.I., Kwan, H.S., Nagy, S.M., Gershwin, M.E., 1994. Cloning, expression, and primary structure of *Metapenaeus ensis* tropomyosin, the major heat-stable shrimp allergen. J Allergy Clin Immunol 94, 882-890.

Li, X.-M., Zhang, T.-F., Huang, C.-K., Srivastava, K., Teper, A.A., Zhang, L., Schofield, B.H., Sampson, H.A., 2001. Food allergy herbal formula-1 (FAHF-1) blocks peanut-induced anaphylaxis in a murine model. J Allergy Clin Immunol 108, 639-646.

Lucey, J., 2002. Formation and physical properties of milk protein gels. Journal of Dairy Science 85, 281-294.

Lucey, J., Fox, P., 1993. Importance of calcium and phosphate in cheese manufacture: a review. Journal of Dairy Science 76, 1714-1724.

Malanin, K., Lundberg, M., Johansson, S.G.O., 1995. Anaphylactic reaction caused by neoallergens in heated pecan nut. Allergy 50, 988-991.

Mariager, B., Solve, M., Eriksen, H., Brogren, C.-H., 1994. Bovine ß-lactoglobulin in hypoallergenic and ordinary infant formulas measured by an indirect competitive ELISA using monoclonal and polyclonal antibodies. Food Agric Immunol 6, 73-83.

Marshall, T., Williams, K.M., 1988. High resolution two-dimensional electrophoresis and sodium dodecyl sulphate-polyacrylamide gel electrophoresis of cheese proteins after rapid solubilisation. ELECTROPHORESIS 9, 143-147.

Maynard, F., Jost, R., Wal, J.-M., 1997. Human IgE binding capacity of tryptic peptides from bovine a-lactalbumin. Int Arch Allergy Immunol 113, 478-488.

McSweeney, P., Fox, P., Lucey, J., Jordan, K., Cogan, T., 1993. Contribution of the indigenous microflora to the maturation of Cheddar cheese. International Dairy Journal 3, 613-634.

McSweeney, P., Fox, P., Olson, N., 1995. Proteolysis of bovine caseins by cathepsin D: preliminary observations and comparison with chymosin. International Dairy Journal 5, 321-336.

McSweeney, P.L.H., Fox, P.F., 1997. Chemical methods for the characterization of proteolysis in cheese during ripening. Lait 77, 41-76.

Metcalfe, D.D., Astwood, J.D., Townsend, R., Sampson, H.A., Taylor, S.L., Fuchs, R.L., 1996. Assessment of the allergenic potential of foods derived from genetically engineered crop plants. Critical Reviews in Food Science and Nutrition 36, S165-S186.

Mills, C., Sancho, A., Moreno, J., Kostyra, H., 2007. The effects of food processing on allergens, in: C. Mills, H. Wichers, K. Hoffmann-Sommergruber (Eds.), Managing allergens in food. CRC Press, LLC, Boca Raton, FL, p. 315.

Morin, S., Bernard, H., Przybylski-Nicaise, L., Corthier, G., Rabot, S., Wal, J.-M., Hazebrouck, S., 2011. Allergenic and immunogenic potential of cow's milk β-lactoglobulin and caseins evidenced without adjuvant in germ-free mice. Molecular Nutrition & Food Research 55, 1700-1707.

Morisset, M., Soulaines, P., Aubert-Jacquin, C., Codreanu, F., Maamri, N., Hatahet, R., Kalindjian, A., Cuny, J., Babchia, H., Guenard, L., Frentz, P., Bouillot, F., Florac, L., Cordebar, V., Beaudouin, E., Kanny, G., Moneret-Vautrin, D., Dupont, C., 2008. Double blind trial of a fermented infantile formula in cow's milk allergy prevention. Journal of Allergy and Clinical Immunology 121, S244.

Morr, C.V., 1971. Comparison of Protein Preparation Procedures and Starch versus Polyacrylamide Gel Electrophoresis for Examining Casein Degradation Products in Cheese. Journal of Dairy Science 54, 339-342.

Murphy, K.M., 2007. Janeway's Immunobiology (Immunobiology: The Immune System (Janeway)) Author: Kenneth M. Murphy, Paul Travers, Mark Walport.

Negroni, L., Bernard, H., Clement, G., Chatel, J.M., Brune, P., Frobert, Y., Wal, J.M., Grassi, J., 1998. Two-site enzyme immunometric assays for determination of native and denatured b-lactoglobulin. J Immunol Methods 220, 25-37.

Nicoletti, C., Temblay, J., Ivory, K., 2007. The immune system and allergic responses to food, in: C. Mills, H. Wichers, K. Hoffmann-Sommergruber (Eds.), Managing allergens in food. CRC Press, LLC, Boca Raton, FL, p. 315.

Nowak-Wegrzyn, A., Bloom, K.A., Sicherer, S.H., Shreffler, W.G., Noone, S., Wanich, N., Sampson, H.A., 2008. Tolerance to extensively heated milk in children with cow's milk allergy. Journal of Allergy and Clinical Immunology 122, 342-347.

Nowak-Wegrzyn, A., Fiocchi, A., 2009. Rare, medium, or well done? The effect of heating and food matrix on food protein allergenicity. Current Opinion in Allergy and Clinical Immunology 9, 234-237.

O'Farrell, I.P., Jeremiah, J.S., Martin, G.W., Dermot, H., Alan, L.K., 2002. Influence of addition of plasmin or mastitic milk to cheesemilk on quality of smear-ripened cheese. Lait 82, 305-316.

O'Sullivan, M., Fox, P.F., 1990. A scheme for the partial fractionation of cheese peptides. Journal of Dairy Research 57, 135-139.

Otani, H., Hori, H., Hosono, A., 1987. Antigenic reactivity of dephosphorylated alpha(S1)-casein, phosphopeptide from beta-casein and O-phospho-L-serine towards the antibody to native alpha(S1)-casein. Agric Biol Chem 51, 2049-2054.

Paschke, A., Besler, M., 2002. Stability of bovine allergens during food processing. Annals of Allergy, Asthma and Immunology 89, 16-20.

Paschke, A., Ulberth, F., 2009. Allergens in foods. Anal Bioanal Chem 395, 15-16.

Peterson, S., Marshall, R., 1990. Nonstarter lactobacilli in Cheddar cheese: a review. Journal of Dairy Science 73, 1395-1410.

Poms, R., Thomas, M., Finglas, P., Astley, S., Spichtinger, D., Rose, M., Popping, B., Alldrick, A., Van Egmond, H., Solfrizzo, M., 2009. MoniQA (Monitoring and Quality Assurance): an EU-funded Network of Excellence working towards the harmonization of worldwide food quality and safety monitoring and control strategies–status report 2008. Quality Assurance and Safety of Crops & Foods 1, 9-22.

Poms, R.E., Agazzi, M.-E., Bau, A., Brohee, M., Capelletti, C., Norgaard, J.V., Anklam, E., 2005. Inter-laboratory validation study of five commercial ELISA test kits for the determination of peanut proteins in biscuits and dark chocolate. Food Additives and Contaminants 22, 104-112.

Poms, R.E., Anklam, E., 2004. Trends in detecting food allergens. G. I. T. Laboratory Journal 1, 43-46.

Poms, R.E., Anklam, E., 2004. Effects of chemical, physical, and technological processes on the nature of food allergens. Journal of AOAC International 87, 1466-1474.

Poms, R.E., Anklam, E., Kuhn, M., 2004a. Polymerase chain reaction techniques for food allergen detection. J AOAC Int 87, 1391-1397.

Poms, R.E., Klein, C.L., Anklam, E., 2004b. Methods for allergen analysis in food: a review. Food Additives and Contaminants 21, 1-31.

Poulsen, O.M., Hau, J., Kollerup, J., 1987. Effect of homogenization and pasteurization on the allergenicity of bovine milk analysed by a murine anaphylactic shock model. Clinical Allergy 17, 449-458.

Prausnitz, C., Küstner, H., 1921. Studien über die Ueberempfindlichkeit. Centralbl. fur Bakteriol Abt Orig 86, 160-169.

Rank, T.C., Grappin, R., Olson, N.F., 1985. Secondary Proteolysis of Cheese During Ripening: A Review. Journal of Dairy Science 68, 801-805.

Reddy, I.M., Kella, N.K., Kinsella, J.E., 1988. Structural and conformational basis of the resistance of. beta.-lactoglobulin to peptic and chymotryptic digestion. Journal of Agricultural and Food Chemistry 36, 737-741.

Restani, P., Gaiaschi, A., Plebani, A., Beretta, B., Cavagni, G., Fiocchi, A., Poiesi, C., Velona, T., Ugazio, A.G., Galli, C.L., 1999. Cross-reactivity between milk proteins from different animal species. Clinical and Experimental Allergy 29, 997-1004.

Ross, M.P., Ferguson, M., Street, D., Klontz, K., Schroeder, T., Luccioli, S., 2008. Analysis of food-allergic and anaphylactic events in the National Electronic Injury Surveillance System. J Allergy Clin Immunol 121.

Rubio–Tapia, A., Kyle, R.A., Kaplan, E.L., Johnson, D.R., Page, W., Erdtmann, F., Brantner, T.L., Kim, W., Phelps, T.K., Lahr, B.D., 2009. Increased prevalence and mortality in undiagnosed celiac disease. Gastroenterology 137, 88-93.

Sampson, H.A., 1999. Food allergy. Part 1: Immunopathogenesis and clinical disorders. J Allergy Clin Immunol 103, 717-728.

Sampson, H.A., 2001. Utility of food-specific IgE concentrations in predicting symptomatic food allergy. J Allergy Clin Immunol 107, 891-896.

Sampson, H.A., 2004. Update on food allergy. J Allergy Clin Immunol 113, 805-819.

Sanchez, C., Frémont, S., 2003. [Consequences des traitements thermiques et de la formulation sur la structure et l' allergenicite des proteines alimentaires]//Consequences of heat treatment and processing of food on the structure and allergenicity of component proteins. Revue Francaise d'Allergologie et d'Immunologie Clinique 43, 13-20.

Schmidt, D., 1982. Association of caseins and casein micelle structure. Developments in dairy chemistry 1, 61-86.

Schutte, L., Paschke, A., 2007. Reducing allergens in milk and milk products, in: C. Mills, H. Wichers, K. Hoffmann-Sommergruber (Eds.), Managing allergens in food. CRC Press LLC, Boca Raton, FL, p. 315.

Shalabi, S., Fox, P., 1987. Electrophoretic analysis of cheese: comparison of methods. Irish Journal of Food Science and Technology 11, 135-151.

Sicherer, S.H., 2011a. Epidemiology of food allergy. Journal of Allergy and Clinical Immunology 127, 594-602.

Sicherer, S.H., 2011b. Epidemiology of food allergy. J Allergy Clin Immunol 127, 594-602.

Sicherer, S.H., Sampson, H.A., 2010. Food allergy. Journal of Allergy and Clinical Immunology 125, S116-S125.

Sousa, M.J., Ardö, Y., McSweeney, P.L.H., 2001. Advances in the study of proteolysis during cheese ripening. International Dairy Journal 11, 327-345.

Stanic, D., Monogioudi, E., Dilek, E., Radosavljevic, J., Atanaskovic-Markovic, M., Vuckovic, O., Raija, L., Mattinen, M., Buchert, J., Cirkovic Velickovic, T., 2010. Digestibility and allergenicity assessment of enzymatically crosslinked b-casein. Mol Nutr Food Res 54, 1273-1284.

Taylor, S.L., 1997. Food Allergies and Intolerances, 2nd ed. Academic Press, San Diego.

Taylor, S.L., Hefle, S.L., 2001. Food allergies and other food sensitivities. Food Technology 55, 68-83.

Taylor, S.L., Hefle, S.L., Bindslev-Jensen, C., Bock, S.A., Burks, A.W., Jr., Christie, L., Hill, D.J., Høst, A., Hourihane, J.O.B., Lack, G., Metcalfe, D.D., Moneret-Vautrin, D.A., Vadas, P.A., Rancé, F., Skrypec, D.J., Trautman, T.A., Yman, I.M., Zeiger, R.S., 2002. Factors affecting the determination of threshold doses for allergenic foods: how much is too much? J Allergy Clin Immunol 109, 24-30.

Thomas, K., Herouet-Guicheney, C., Ladics, G., Bannon, G., Cockburn, A., Crevel, R., Fitzpatrick, J., Mills, C., Privalle, L., Vieths, S., 2007. Evaluating the effect of food processing on the potential human allergenicity of novel proteins: International workshop report. Food and Chemical Toxicology 45, 1116-1122.

Upadhyay, V.K., McSweeney, P.L.H., Magboul, A.A.A., Fox, P.F., 2004. Proteolysis in cheese during ripening, in: P.L.H.M.T.M.C. Patrick F. Fox, P.G. Timothy (Eds.), Cheese: Chemistry, Physics and Microbiology. Academic Press, pp. 391-VIII.

Veloso, A.C.A., Teixeira, N., Peres, A.M., Mendonça, Á., Ferreira, I.M.P.L.V.O., 2004. Evaluation of cheese authenticity and proteolysis by HPLC and urea–polyacrylamide gel electrophoresis. Food Chemistry 87, 289-295.

Wal, J.-M., 1998a. Cow's milk allergens. Allergy 53, 1013-1022.

Wal, J.-M., 2001a. Structure and function of milk allergens. Allergy 56, 35-38.

Wal, J.-M., 2002. Cow's milk proteins/ allergens. Annals of Allergy, Asthma and Immunology 89, 3-10.

Wal, J.-M., Bernard, H., Créminon, C., Hamberger, C., David, B., Peltre, G., 1995a. Cow's milk allergy: the humoral immune response to eight purified allergens, Part B ed. Plenum Press, New York.

Wal, J., 2001b. Structure and function of milk allergens. Allergy 56, 35-38.

Wal, J.M., 1998b. Immunochemical and molecular characterization of milk allergens. Allergy 53, 114-117.

Wal, J.M., Bernard, H., Yvon, M., Peltre, G., David, B., Créminon, C., Frobert, Y., Grassi, J., 1995b. Enzyme immunoassay of specific human IgE to purified cows' milk allergens. Food Agric Immunol 7, 175-187.

Wang, J., Godbold, J.H., Sampson, H.A., 2008. Correlation of serum allergy (IgE) tests performed by different assay systems. Journal of Allergy and Clinical Immunology 121, 1219-1224.

Watanabe, H., Kai, S., Mitani, T., Yokoyama, H., Kishi, M., 2005. Study on detection of allergenic substances (egg and milk) in processed meat products and frozen foods. Journal of the Food Hygienic Society of Japan 46, 139-147.

Wood, R.A., Sicherer, S.H., Vickery, B.P., Jones, S.M., Liu, A.H., Fleischer, D.M., Henning, A.K., Mayer, L., Burks, A.W., Grishin, A., Stablein, D., Sampson, H.A., 2013. The natural history of milk allergy in an observational cohort. The Journal of allergy and clinical immunology 131, 805-812.e804.

CHAPTER 2. EVALUATION AND VALIDATION OF COMMERCIAL MILK ELISA KITS

1. Abstract

Reliable and sensitive methods of allergen quantitation are needed to ensure protection of allergic consumers and to allow food manufacturers to assess the effectiveness of their preventive allergen control practices. The consistency and accuracy of twelve commercial milk ELISA kits were evaluated based on the performance of calibrator solutions. Calibration standards were run in triplicate wells on duplicate ELISA plates. The suitability of using certificates of analysis, %CV, and r^2 to assess and describe ELISA kit performance was evaluated. Recommended benchmarks for acceptability of ELISA kit performance are suggested. Standard curves were constructed from the manufacturer provided calibration standards, and the ELISA data for all commercial kits should be expected to meet or exceed an $r^2 \geq 0.98$ and %CV $\leq 20\%$ when replicates are evaluated. Three commercial kits did not meet the suggested performance criteria: Romer Labs® AgraQuant® Casein, Morinaga® Casein, and Morinaga® BLG. Unless the manufacturers of these kits improve the stability and performance of calibrator solutions during transport, storage, and proper kit use, these kits should not be used to quantify trace amounts of milk residues in food samples. The remaining 9 kits performed in accordance with expectations. Establishing harmonized guidelines for kit performance will improve the accuracy and reliability of results. When selecting ELISA kits, efforts should be made to assure that the kit offers consistent and reliable performance.

2. Introduction

Enzyme-linked immunosorbent assays (ELISAs) exist to quantitatively detect allergenic residues in foods. ELISAs are one of the most commonly used immunochemical methods for the detection of residues of allergenic foods. ELISAs are specific, sensitive, and quantitative. In

recent years, ELISA methods for the detection of many food allergens have been commercialized and improved to quantify residues in less than an hour. While commercial ELISA kits are excellent tools for allergen detection, they still have limitations. ELISA kits are developed with antibodies of limited sensitivity. Either monoclonal or polyclonal antibodies can be used for ELISA kits, with many manufacturers favoring polyclonal antibodies that detect several epitopes on one or more proteins from an allergenic food. If an epitope is identified that is stable to a variety of processing methods, monoclonal antibodies may be developed for its specific detection.

None of the commercially-available or published ELISA methods have been validated. An approach to validate ELISA kits has been recommended, but the criteria have not been widely adopted. Some collaborative inter-laboratory validation data is available for detection of trace amounts of milk in foods or spiked food matrices, but these kits still lack certification by standardization organizations. Validation would ensure that available ELISA methods are suitable for their intended use in detecting allergen residues.

Many sources of variation exist within ELISA kits. It is well known that evaluation of an identical sample with different ELISA test kits can provide vastly differing results. Antibody sensitivity, calibration materials, extraction reagents and procedures, and reporting units all contribute to differences in results when identical samples are analyzed with different ELISA kits. Variation observed in kit performance can be a result of stress from shipping, storage, improper handling, or contamination.

Because commercial ELISA kits do not typically use antibodies with reactivity specifically toward all proteins or epitopes within an allergenic food, calibration materials are selected and used to normalize data. Solutions of different concentrations of calibration materials are used to build a standard curve for quantitation of allergenic residues within a food. Often, regardless of antibody specificities or calibration materials, kits will use mathematical conversion

factors to report results in yet another unit. Taylor et al. (2009) discussed that kit antibodies may be calibrated against the whole food, total or soluble protein, or a specific protein. Additionally, manufacturers may use calibrator solutions derived from purified, partially purified, the entire spectrum of soluble proteins, or even from the whole food. For example, antibodies of milk ELISA kits may be sensitive to one or several epitopes on an individual milk protein, while whole casein is used as the calibration material and data are quantified and reported in units of non-fat dry milk (NFDM).

The quantitative nature of ELISA kits relies on the use of a standard curve composed of several solutions of known analyte concentrations. Each assay requires running the standard solutions in parallel to samples of unknown concentration. The results obtained from the analysis of the known solutions are used to build a standard curve. Analyte concentration in a test sample can be interpolated from the standard curve. AOAC guidelines for ELISA detection suggest that acceptable recovery of spiked samples should be between 50%-150%. The guidelines, however, do not recommend limits for maximum levels of variation in an acceptable assay (Abbott et al., 2010).

Because ELISA kits do not express results in the same units, comparison between kits is difficult. Not only do commercial milk kit antibodies target different milk fractions, but they also use different calibration materials and different units of reporting. Diaz-Amigo and Popping (2010) suggest that conversion factors between detected and reporting units should be based on soluble protein in a commodity food, not total protein contained in a whole food. Conversion factors likely introduce additional uncertainty into ELISA results because they are not standardized between different kits. Public health and clinical concerns may necessitate that commercial ELISAs express results in clinically relevant units. Because food challenges to determine threshold doses for allergic individuals are performed using the whole food and not individual proteins, ELISA manufacturers may present results in converted units. Kits that are

developed using calibration materials other than the whole food may need to provide mathematical conversion factors for data analysis and interpretation.

The development and adoption of reference materials for kit validation must account for natural differences in protein composition among varieties of the same allergenic food. Ideally, reference materials will also account for the effects of processing. No widely accepted standard materials exist for allergen test kits, although some reference materials are widely available.

Abbott et al. (2010) suggest the adoption of NIST RM 1549 (non-fat dry milk) as the standard reference material for milk allergen test kits. Additionally, if kit manufacturers decide to use a different reference material, then a conversion factor must be provided with the test kit for proper data quantitation.

Certain assumptions regarding ELISA kit performance are often implied; users expect that when protocols are followed, data obtained with test kits will be free from significant bias, kit response will be proportional to the concentration of antigen present in the sample, and errors are randomly and consistently distributed throughout the data (Thompson et al., 2002). Ideal recovery for spiked samples is between 80%-120%, although acceptable recoveries should fall within the 50%-150% range (Abbott et al., 2010; Thompson et al., 2002).

The coefficient of variation (CV), also known as the relative standard deviation (RSD), is a dimensionless unit and is typically expressed as a percentage. During analyses where sample replicates are evaluated, the coefficient of variation provides a useful measure of consistency among replicate samples. In statistics, the standard deviation also measures data consistency, but %CV has the advantage of expressing consistency with relation to the mean and is a standardization of the SD (Reed et al., 2002). Regardless of analyte concentration, the CV estimates the magnitude of variation. A low %CV indicates that sample points are distributed closely around the mean. Consistent data suggests acceptable and homogenous kit performance.

Likewise, a high %CV may indicate biased or inappropriate kit performance. Although standard solutions are not typically evaluated, they should be expected to meet stringent requirements for consistency.

Depending on the concentrations of standards, kit calibration curves display relatively clear limits of antigen detection and quantitation. Kits always provide or recommend the use of a zero standard. The zero ppm calibrator gives an estimation of the background interference and is often affected by contamination, incomplete washing, and user proficiency and experience. Because ELISA kits typically provide a certificate of analysis (COA) for calibrators, the acceptability of kit performance can be inferred from the optical density of standard solutions. Evaluated samples, either in absence of detectable analyte or at low concentrations can give readings at or below the zero calibrator and the lowest positive solution. Because quantitation of unknown samples is performed using the linear part of the standard curve, enzyme kinetics reveal that the response observed between the zero standard and the lowest positive calibrator does not always give a linear response. OD values that fall in this region cannot be accurately quantified, and are given a 'below the level of quantitation' (BLQ) designation.

The coefficient of determination, often referred to as the r^2, is another commonly used statistic for data evaluation. When a calibration curve is fit to the data, the r^2 measures how closely the data fits the curve. Values that are closer to 1 indicate the curve fits the data well, while lower values suggest that the results are not accurately predicted by the curve. The r^2 value obtained from kit calibration curves are recommended to exceed 0.98 (Cordle, 2006).

The calibrator solutions provided with each kit provide quantitative estimates of allergen concentration in a tested food product. The reporting units of a kit are expected to reflect its detection specificity. Adoption of proper reference materials and unification of reporting units will help minimize differences observed in kit results.

An evaluation of peanut kits by Pomés et al. (2003) claimed that because calibrators and reporting units in different ELISA kits do not agree, results cannot be correlated. Some research suggests that because ELISA results are expressed differently, the exact mathematical factors and calculations used to correlate the reporting units of different kits are specific to each test. Others, however, suggest that correlation factors between peanut protein and other units are relatively consistent and correlation can be performed between kits (Zeleny and Schimmel, 2010). Regardless of position on correlation, research agrees that protocol provided with a kit must be closely followed for results to be accurate. Variation in environmental conditions and incubation time during assay performance should be ≤ 5% for obtaining acceptable results (Immer, 2006). Because ELISA kits rely heavily on the principles of chemical equilibrium and enzyme kinetics, disruption of the assay prior to the recommended incubation time can dramatically alter assay performance.

Published ELISA methods often list the variation in performance of standard calibrator solutions, while commercial kits typically do not. A peanut ELISA developed by Holzhauser and Vieths (1999) listed the range of optical densities for replicates of standard solutions between 1.4% and 3.1% CV. Typical accuracies in sample detection fall within ± 5%-20% (Cordle, 2006). An assessment of commercial milk ELISA kits determined that experimental %CVs for standard solutions were in the range of 1-5% (Monaci et al., 2011). The accuracy of standards is used to evaluate the precision and stability of the test. It has been suggested that a high %CV among standards may indicate poor washing technique or improper pipetting by the user. If replicates of a standard solution vary by more than 20%CV, the assay must be repeated (Immer, 2006).

The Food Allergy Research and Resource Program (FARRP) at the University of Nebraska-Lincoln uses internal control points to assess the performance of each kit tested. These control points include a ± 20% confidence interval for calibrator solutions and sample replicates

in ELISA analyses. Additionally, FARRP requires an r^2 value of ≥ 0.98 for the standards of all kits. The applicability of using these guidelines for the quality control of commercial ELISA kits will be assessed.

The objective of this research was to assess the validity of twelve commercial milk ELISA kits using manufacturer-provided calibration solutions. The OD values obtained will be compared to the expected values on the Certificate of Analysis (COA) provided with each kit. R^2 values, %CV, and % difference from expected values will also be calculated for each data set. These analyses will investigate the consistency of each kit within a lot code and will contribute to establishing criteria for acceptable performance of commercial ELISA kits.

3. Materials and Methods

Kit Selection and Procurement

To assess the validity of commercial ELISA milk kits, twelve kits were selected for analysis. Neogen Veratox® Total Milk, Casein, and BioKits™ BLG (β-lactoglobulin) were obtained from Neogen® Corporation (Lansing, MI, USA). ELISA Systems™ Casein and β-lactoglobulin kits were obtained from ELISA Systems (Windsor, Queensland, Australia). R-Biopharm RIDASCREEN® Fast Casein, Fast Milk, and Fast BLG (Darmstadt, Germany) and Romer Labs® AgraQuant® Casein and BLG (Union, MO, USA) were also used and obtained from their respective distributors. Morinaga® BLG and Morinaga® Casein were obtained from Morinaga Institute of Biological Science (Yokohama, Japan). Each commercial kit provides its own set of standard solutions at given concentrations. Lot numbers of each kit evaluated are listed in Table 2.1.

Table 2.1 Commercial milk ELISA kits and lot numbers used

KIT	LOT #
Neogen Veratox® Casein	31105
ELISA Systems™ Casein	CAS11-283
R-Biopharm RIDASCREEN® Fast Casein	14350
Romer Labs® AgraQuant® Casein	1005-1106
Morinaga® Casein	1201SACA93SA
Neogen Veratox® Total Milk	12158
R-Biopharm RIDASCREEN® Fast Milk	11421
Neogen BioKits™ BLG	146,009
ELISA Systems™ BLG	11-104
R-Biopharm RIDASCREEN® Fast BLG	14121
Romer Labs® AgraQuant® BLG	BL1005-1111
Morinaga® BLG	1110SABL30A

ELISA Analysis

Each of the standard solutions provided by kit manufacturers were evaluated using respective kits. For each commercial kit, standards were evaluated in triplicate wells on two independent plates. While each kit recommends a specific extraction procedure for the analysis of unknown samples, no extraction is required for standard solutions; they are applied directly to antibody-coated wells. A few sets of standards require additional dilution prior to ELISA analysis; this was performed according to manufacturer's instructions.

All kits used in the analysis are sandwich ELISAs, with the exception of Neogen BioKits™ BLG, which uses an indirect competitive format. The sandwich ELISA kits provide antibody-coated wells, standard solutions, conjugate antibody solution, substrate solution, stop solution, wash buffer, and extraction buffer. The indirect competitive-ELISA kit (Biokits BLG) provides protein-coated wells and avidin peroxidase, an enzyme that allows the detection of antigen in wells. Differences between the mechanisms of sandwich and competitive ELISA formats are discussed in detail in Chapter 1.

Each kit, with the exception of Morinaga kits, is accompanied by a Certificate of Analysis (COA) for each solution and standard present in the kit, verifying its purity and performance by a quality assurance laboratory. The COA of each kit lot contains optical density (OD) values for the standards as obtained in quality analysis and deemed acceptable by the manufacturer. In data analysis, the values on the COA were regarded as the expected results for the OD of the standards.

Protocol for each ELISA kit was followed as outlined in kit inserts. Briefly, for each of the sandwich-ELISA assays, 100 µL-150 µL of each standard was added to milk-specific antibody coated wells in triplicate and was allowed to incubate at room temperature for a recommended period of time as stipulated by the manufacturer's instructions. For r-Biopharm® RIDASCREEN Fast Casein, Fast Milk, Fast BLG, and Neogen Veratox® Total Milk and Casein, the incubation period was 10 minutes. For ELISA Systems™ BLG and Casein kits, the incubation period was 15 minutes. For Romer Labs® AgraQuant® Casein and BLG, the incubation period was 20 minutes. The incubation time for Morinaga® BLG and Casein was 60 minutes. After incubation, the wells were thoroughly washed with wash buffer solution provided by the manufacturer a determined number of times. In most kits, the wash buffer solution was a dilute solution of PBS-Tween. In the R-Biopharm RIDASCREEN® Fast Casein kit, wells were washed 3 times; R-Biopharm RIDASCREEN® Fast Milk and Fast BLG, 4 times; ELISA Systems, Romer, and Neogen BioKits™ BLG kits, 5 times; Morinaga Kits, 6 times; and Neogen Veratox® kits, 10 times. Well plates were inverted and tapped repeatedly on paper towel after washing to remove excess wash solution. Remaining bubbles in the wells were popped with a clean pipette tip to prevent assay interference.

Next, 100 µL of enzyme-conjugated antibody solution was added to each well using a multichannel pipette and incubated for the prescribed time period. Wells were washed and tapped dry using the protocol described above. Substrate solution (100 µL) was added to each

well with a multichannel pipette and the well plate was allowed to incubate as a colorimetric reaction occurred. After the incubation, acidic stop solution was added to each well to deactivate the enzymatic reaction and prevent additional color formation.

The optical density of each well was then read using a plate reader at the absorbance wavelength recommended by each kit manufacturer. Two replicate plates were run, each with calibration standards analyzed in triplicate. Expected values as provided on the COA were compared to actual values. Data was analyzed in Microsoft Excel, GraphPad Prism, and the manufacturer-provided software program, if supplied. Standard curves were created using the curve fits recommended by kit manufacturers or using a four-parameter sigmoidal dose-response curve if no curve fit was suggested. Curves were created in GraphPad Prism version 4.03.

4. Results

All kits were accompanied by a Certificate of Analysis (COA) with the exceptions of the Morinaga® Casein and Morinaga® BLG kits. Values listed on COAs by kit quality assurance labs were considered expected values. Kit manufacturers often recommend that experimental optical density values meet certain criteria to confirm kit stability and analyst proficiency. When the optical density of standard solutions falls outside of a certain suggested range, error is indicated, sample data must be discarded, and the assay must be performed again to alleviate or confirm error.

The percent coefficient of variation obtained during data analysis was quite high for several kits. The Romer Labs® AgraQuant® Casein and Morinaga® Casein kits display the highest levels of variation among replicates of the same standards, both within and between replicates (see Table 2.2). These two kits also display the lowest r^2 values. ELISA kits are characterized by a high correlation coefficient, with acceptable r^2 values typically falling between 0.97-1.0. For kits that provided a COA, recommended values for standard solutions were used as

expected values. The means of experimentally obtained data were compared to the expected values and the difference from expected values, represented as a percentage (%DFE), was calculated (Equation 2.1). The significance of %DFE measurements represents the variation observed in standard solutions as a potential result of kit transport and/or storage. Larger %DFE represents the conditions that the standard solutions exhibited greater differences from values listed on the COA. %DFE was calculated using the following equation:

Equation 2.1. Calculation of % Difference from expected values (%DFE). OD represents optical density. Please note the equation absolute value of the difference between the mean experimental and expected optical density is taken.

$$\%DFE = \frac{\left|OD_{expected} - \overline{OD}_{actual}\right|}{OD_{expected}} \times 100\%$$

Table 2.2. Results from standard solution analysis of commercial ELISA kits.

Key	KIT	%CV[a]	r^2	%DFE[b]
1	Neogen Veratox® Casein	5.6%	0.997	16.3%
2	ELISA Systems™ Casein	9.1%	0.991	49.7%
3	R-Biopharm RIDASCREEN® Fast Casein	5.0%	0.999	49.6%
4	Romer Labs® AgraQuant® Casein	**28.1%**	**0.973**	44.1%
5	Morinaga® Casein	**22.6%**	**0.920**	n/a
6	Neogen Veratox® Total Milk	3.8%	0.999	13.1%
7	R-Biopharm RIDASCREEN® Fast Milk	8.5%	0.998	27.2%
8	Neogen BioKits™ BLG	3.8%	0.988	17.8%
9	ELISA Systems™ BLG	11.2%	0.998	40.4%
10	R-Biopharm RIDASCREEN® Fast BLG	9.3%	0.997	37.4%
11	Romer Labs® AgraQuant® BLG	7.4%	0.992	19.4%
12	Morinaga® BLG	8.1%	0.998	n/a

[a] %CV represents percent coefficient of variation

[b] %DFE represents the percentage difference from expected values

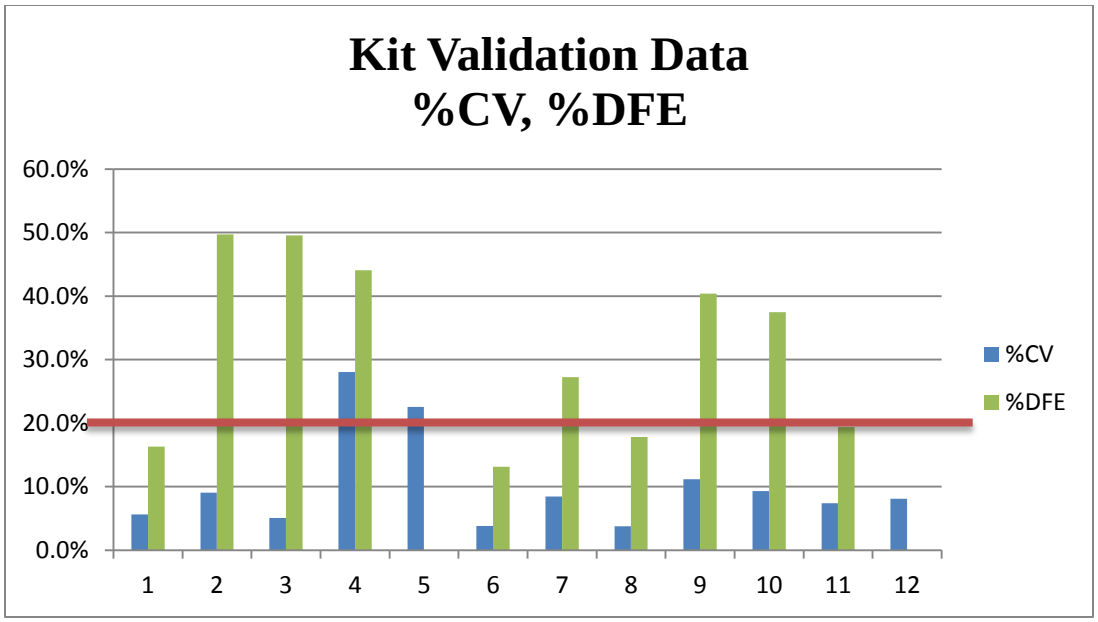


Figure 2.1. Analysis of manufacturer-provided standard solutions. Kits are listed on the x-axis according to numerical assignment in Table 2.1. %CV represents percent coefficient of variation. %DFE represents the mathematical difference from expected values expressed as a percentage. Because the Morinaga® Casein and BLG kits lacked COAs, no expected values were available, and %DFE was not calculated.

Because the Morinaga® Casein and BLG kits (kits 5 and 12, respectively) lacked a COA, no expected values were available for calculations. Therefore, %DFE could not be determined for these kits, and this data is not shown in Table 2.2 or Figure 2.1. The Morinaga® assay insert claims ≤ 10% intra- and inter-assay percent coefficient of variation, according to the product inserts for both the Casein and BLG kits although these values could not be confirmed.

Because ELISA methods are recommended to display a %CV among sample replicates of ≤20%, the standard solutions should be expected to meet, if not exceed, the constraints (Immer, 2006). Two kits, Romer Labs® AgraQuant® Casein and Morinaga® Casein exhibit high %CVs, at 28.1% and 22.6%, respectively. The kits do not meet the 20%CV cutoff mark (kits 4 and 5, Table 2.2, Figure 2.1). The Neogen Veratox® Total Milk and Neogen BioKits™ BLG ELISA kits display the lowest %CV, at 3.8% and 3.0%, respectively. All other evaluated kits fall within the acceptable range for observed %CV data. With the exception of the ELISA Systems™ BLG

kit, Romer Labs® AgraQuant® Casein, and Morinaga® Casein kits, the other assay standards fall below 10% CV. A more stringent requirement for %CV observed among replicates of kit standards may be appropriate.

%DFE is a valuable measure of kit stability and differences in performance observed between the manufacturer testing facility and user laboratories. Kit instability during shipping and storage may suggest that the standards or other reagents in the kit are unstable and fail to provide consistent results. While the differences from expected values do not have suggested points for validity, it might be reasonable to recommend that experimental values to fall within 20% of the values observed during post-production kit testing by the manufacturer. ELISA protocol dictates that a new standard curve must be prepared and analyzed with each evaluation. Day-to-day variability in observed optical densities for standard solutions is not uncommon, but large variation is unusual and cause for concern.

According to the observed data, only four of the kits meet the 20% DFE criteria: Neogen Veratox® Casein, Neogen Veratox® Total Milk, Neogen BioKits™ BLG, and Romer Labs® AgraQuant® BLG. The Morinaga® Casein and BLG kits do not provide expected values for standard solutions on a COA and therefore, %DFE cannot be calculated for these two kits. The ELISA Systems™ Casein, R-Biopharm RIDASCREEN® Fast Casein, and the Romer Labs® AgraQuant® Casein kits display the largest differences from the COA values, at 49.7%, 49.6%, and 44.1%DFE, respectively (Table 2.2).

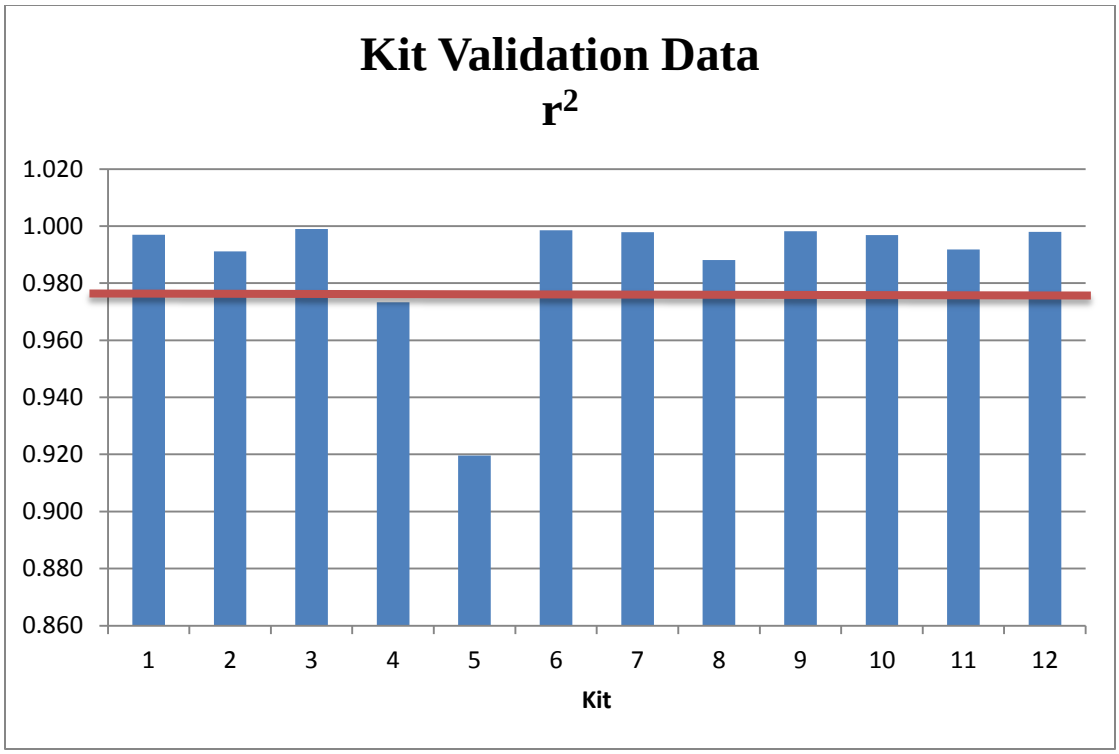


Figure 2.2. Coefficient of Determination data for Commercial ELISA kits. The red bar at 0.98 displays the minimum r^2 value for acceptable results as recommended by the Food Allergy Research and Resource Program at the University of Nebraska-Lincoln and Cordle (2006).

Two kits fail to meet the recommended r^2 values for standard analysis; Romer Casein (kit 4) and Morinaga® Casein (kit 5) (Figure 2.2). The Morinaga® Casein kit exhibits especially poor values, averaging near 0.92. Additional replicates were performed of these kits, but improvements were not observed.

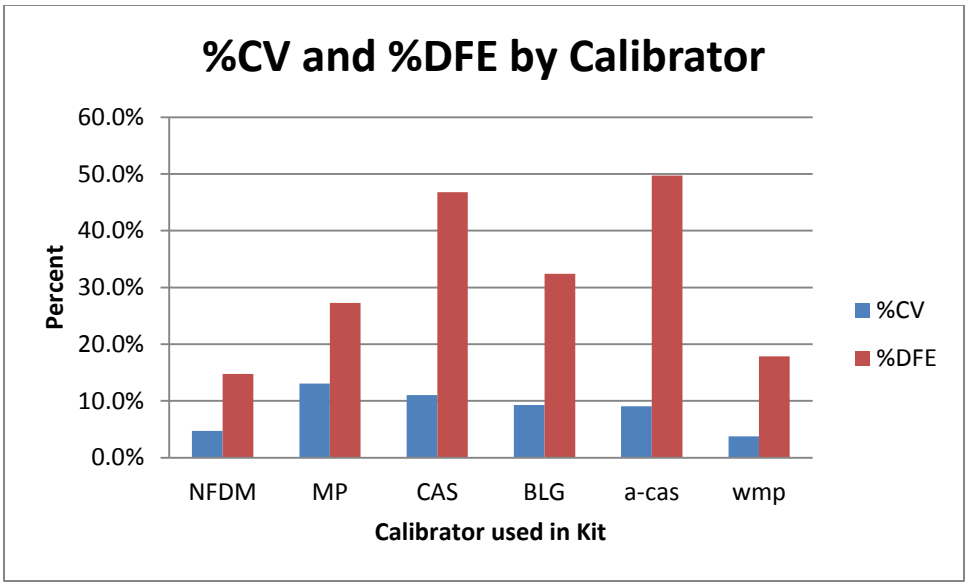


Figure 2.3. Performance of kit standards by common calibrator. WMP: whole milk powder, a-cas: α-casein. Note, there are an unequal number of replicates for each calibration material.

Table 2.3. Kit performance sorted by common calibrator.

		averages	
	n	CV	DFE
NFDM	2	4.7%	14.7%
MP	3, 1	13.0%	27.2%[a]
CAS	2	11.0%	46.8%
BLG	3	9.3%	32.4%
a-cas	1	9.1%	49.7%
wmp	1	3.8%	17.8%

[a] Because Morinaga® Casein and BLG Kits lacked COA, no DFE could be calculated. CV for MP has n=3, DFE for MP has n=1.

Considering the results on the basis of calibration materials, some trends are apparent. On the basis of %CV, it appears that whole milk powder (WMP) and non-fat dry milk (NFDM) are the highest performing calibrators, displayed on Figure 2.3 and Table 2.3. Each displays a low %CV (3.8% and 4.8%CV, respectively). Considering the %DFE measurement, NFDM and WMP are again the highest performing calibration standards, at 14.7% and 17.8% DFE, respectively. These inferences, however, may be inappropriate, as there are an unequal number of kits that employ each calibrator (see Tables 2.3 and 2.4). Further, normalization of the data

prior to performing statistics may contribute to statistical error, as the true distribution of data is not represented.

Table 2.4. Information provided in kit inserts regarding calibrators and reporting units.

KIT	Standards	Reporting Units
Neogen Veratox® Casein	NFDM	NFDM
ELISA Systems™ Casein	a-casein	skim milk powder
R-Biopharm RIDASCREEN® Fast Casein	casein	casein
Romer Labs® AgraQuant® Casein	casein	casein
Morinaga® Casein	milk protein	milk protein
Neogen Veratox® Total Milk	NFDM	NFDM
R-Biopharm RIDASCREEN® Fast Milk	milk protein	milk protein
Neogen BioKits™ BLG	whole milk powder	BLG
ELISA Systems™ BLG	BLG	BLG
R-Biopharm RIDASCREEN® Fast BLG	BLG	BLG
Romer Labs® AgraQuant® BLG	BLG	BLG
Morinaga® BLG	milk protein	milk protein

Given the data, NFDM appears to be the most favorable calibration material for milk ELISA kits. NFDM standards exhibit the lowest average %DFE and second-lowest %CV on the kits where it is employed. The characteristics of high consistency and low variability exhibited by kits that use NFDM are indicators of kit stability. Whole milk powder also performs with high consistency in the Neogen BioKits™ BLG kit. However, the NIST Standard Reference Material for whole milk powder used in this kit is no longer available and production has been discontinued indefinitely as of February 2011 (NIST-SRM-8435). In concert with ending production of NIST-SRM-8435, the use of whole milk powder for the calibration of current assays and the development of new milk-specific immunoassays should be reconsidered.

The three kits with the highest %DFE are all based on casein calibrants (Figures 2.2 and 2.3, Tables 2.2 and 2.3). After the caseins (whole or α-casein), BLG has the next highest %DFE at 32.4%. Composite milk powders, whether NFDM or whole milk powder, have less variability in performance than individual proteins or fractions (casein or BLG) and are often more consistent with the values recommended by manufacturers or quality assurance laboratories.

5. Discussion

According to the results observed when manufacturer-provided standard solutions are analyzed, two kits fail to meet criteria for acceptance. The Romer Casein kit exceeds acceptable values for %CV and does not meet requirements for r^2 of standard curves. It is possible that the data is better suited to a curve-fit other than the four-parameter sigmoidal dose-response curve used and applied in GraphPad Prism. Without manufacturer direction, the user is left to make assumptions regarding data manipulation and interpretation. For calculation of results from raw data using the Romer Labs® AgraQuant® Casein kit, Romer Labs provides a Microsoft Excel spreadsheet with built-in formulas. However, the r^2 is not provided, nor is an appropriate curve fit

recommended in the product insert. Morinaga Institute of Biological Science suggests that either of two curve fits be performed with the standard curve of each assay: a linear curve fit or a 4-parameter curve fit (cubic regression). No software is provided for performing these calculations, therefore certain assumptions must be made by the user. The two fits suggested by Morinaga can provide drastically different results, especially if the standards are unstable. Variation during data analysis compounds the error of kit stability.

Although day-to-day assay variation is not unexpected, the application of calibrator solutions and the development of a standard curve within each assay attempt to limit the differences in quantitation between assays. Some kit developers argue that regardless of deterioration in standards or other sources of variability, the slope of the standard curve should remain the same. However, even though the slope may remain relatively consistent, the y-intercept will likely change and affect the results of sample quantitation. The stability of standard solutions should be improved to minimize errors in sample quantitation.

Applying a maximum of 20% DFE seems reasonable; however, only four of the kits evaluated meet the criteria. The applicability of using %DFE to evaluate kit performance should be evaluated with ELISA kits for additional allergen residues. The variability of calibration standards during shipping and storage and their performance during assay protocol should be minimized. Ideally, variation in assay results will be a result of deliberate experimental alterations, as opposed to external and uncontrollable circumstances. However, this does not necessarily translate to kit design and manufacturing practices.

In addition to establishing a widely adopted and certified reference material, some literature suggests the importance of internal quality control points to indicate how well methods perform over time (Thompson et al., 2002). Many suggest that the internal quality control standard also be developed from the same reference material used as a kit calibrator. A reference

material should be established to allow more accurate data comparisons and validation of ELISA methods.

The %CV is an acceptable and effective measure of variability in ELISA kits. Conservative criteria for acceptable %CV are between 15%-20%, while stringent levels are recommended to be set near 5%-10% (Cordle, 2006; Immer, 2006; Monaci et al., 2011; Reed et al., 2002). Holzhauser and Vieths (1999) recommend that the precision of triplicate samples should be ≤ 15%. In this analysis, 75% of kits evaluated have %CVs less than 10%. The two kits with unacceptable %CVs (≥20%) also displayed r^2 values below 0.98; both measures suggest kit inconsistencies and poor performance. The stringency of acceptable limits for the %CV of standard curves could be increased to 10-15% with minimal negative consequences.

Accurate detection and quantitation of milk residues with ELISA kits is challenging. Comparing results among assay kits that use antibodies of different specificities, different calibration materials, and different reporting units adds to the difficulty of appropriate comparisons and interpretations. This research evaluated twelve commercialized milk ELISA kits for their consistent and precise detection of analyte in calibration materials. NFDM exhibits a low %CV, DFE, and a high r^2 value when used to construct standard curves. In this research, NFDM is shown to be the most consistent, robust, and stable calibrator of those evaluated. Individual proteins and protein fractions, such as casein and BLG, display higher levels of instability as assessed by %DFE than do powders containing multiple unfiltered milk proteins. Commercial ELISA kits used to analyze milk residues in food should meet the criteria of $r^2 \geq 0.98$ and %CV ≤ 15%. Use of kits that fail to meet these conditions cannot be recommended for quantitative analysis.

6. References

1995. in: Food and Agriculture Organization of the United Nations (Ed.), Report of the FAO Technical Consultation on Food Allergies. FAO, Rome.

Abbott, M., Hayward, S., Ross, W., Godefroy, S.B., Ulberth, F., van Hengel, A.J., Roberts, J., Akiyama, H., Popping, B., Yeung, J.M., Wehling, P., Taylor, S.L., Poms, R.E., Delahaut, P., 2010. Validation procedures for quantitative food allergen ELISA methods: community guidance and best practices. J AOAC Int 93, 442-450.

Addeo, F., Garro, G., Intorcia, N., Pellegrino, L., Resmini, P., Chianese, L., 1995. Gel electrophoresis and immunoblotting for the detection of casein proteolysis in cheese. Journal of Dairy Research 62, 297-309.

Alessandri, C., Sforza, S., Palazzo, P., Lambertini, F., Paolella, S., Zennaro, D., Rafaiani, C., Ferrara, R., Bernardi, M.L., Santoro, M., Zuzzi, S., Giangrieco, I., Dossena, A., Mari, A., 2012. Tolerability of a Fully Maturated Cheese in Cow's Milk Allergic Children: Biochemical, Immunochemical, and Clinical Aspects. PLoS ONE 7, e40945.

Anderson, J.A., 1984. Non-immunologically-mediated food sensitivity. Nutr Rev 42, 109-116.

Anguita, G., Martin, R., Garcia, T., Morales, P., Haza, A.I., Gonzalez, I., Sanz, B., Hernández, P.E., 1996. Immunostick ELISA for detection of cow's milk in ewe's milk and cheese using a monoclonal antibody against bovine ß-casein. J Food Prot 59, 436-437.

Astwood, J.D., Fuchs, R.L., 1996. Food allergens are stable to digestion in a simple model of the gastrointestinal tract. J Allergy Clin Immunol 97, 241.

Baldwin, J.L., 1996. Pharmacologic food reactions, 2nd ed. Blackwell Science Publications, Oxford.

Bansal, N., Piraino, P., McSweeney, P.L.H., 2009. Determination of Proteolysis in Cheese. Handbook of Dairy Foods Analysis, 405.

Barbosa-Cánovas, G.V., 2009. Global issues in food science and technology. Elsevier ; Academic Press, Amsterdam; Boston; Burlington, MA.

Bartnikas, L.M., Sheehan, W.J., Hoffman, E.B., Permaul, P., Dioun, A.F., Friedlander, J., Baxi, S.N., Schneider, L.C., Phipatanakul, W., 2012. Predicting food challenge outcomes for baked milk: role of specific IgE and skin prick testing. Annals of Allergy, Asthma & Immunology.

Bastian, E.D., Brown, R.J., 1996. Plasmin in milk and dairy products: an update. International Dairy Journal 6, 435-457.

Bernard, H., Créminon, C., Negroni, L., Peltre, G., Wal, J.-M., 1999. IgE cross-reactivity with caseins from different species in humans allergic to cow's milk. Food Agric Immunol 11, 101-111.

Besler, M., Steinhart, H., Paschke, A., 2001. Stability of food allergens and allergenicity of processed foods. Journal of Chromatography B 756, 207-228.

Björkstén, B., Sepp, E., Julge, K., Voor, T., Mikelsaar, M., 2001. Allergy development and the intestinal microflora during the first year of life. Journal of Allergy and Clinical Immunology 108, 516-520.

Bleumink, E., Berrens, L., 1966. Synthetic approaches to the biological activity of b-lactoglobulin in human allergy to cows' milk. Nature 212, 541-543.

Bock, S.A., Muñoz-Furlong, A., Sampson, H.A., 2001. Fatalities due to anaphylactic reactions to foods. Journal of Allergy and Clinical Immunology 107, 191-193.

Bock, S.A., Muñoz-Furlong, A., Sampson, H.A., 2007. Further fatalities caused by anaphylactic reactions to food, 2001-2006. Journal of Allergy and Clinical Immunology 119, 1016-1018.

Bock, S.A., Sampson, H.A., Atkins, F.M., Zeiger, R.S., Lehrer, S., Sachs, M., Bush, R.K., Metcalfe, D.D., 1988. Double-blind, placebo-controlled food challenge (DBPCFC) as an office procedure: a manual. J Allergy Clin Immunol 82, 986-997.

Brazil, E., MacNamara, A.F., 1998. 'Not so immediate' hypersensitivity - the danger of biphasic anaphylactic reactions. J Accid Emerg Med 15, 252-253.

Bush, R.K., Hefle, S.L., 1996. Food allergens. Crit Rev Food Sci Nutr 36, S119-S163.

Bush, R.K., Taylor, S.L., 1998. Adverse reactions to food and drug additives, in: E. Middleton, Jr., C.E. Reed, E.F. Ellis, N.F. Adkinson, Jr., J.W. Yunginger, W.W. Busse (Eds.), Allergy: Principles & Practice, 5th ed. Mosby, St. Louis, pp. 1183-1198.

Busse, P.J., Järvinen, K.-M., Vila, L., Beyer, K., Sampson, H.A., 2002. Identification of sequential IgE-binding epitopes on bovine α_{s2}-casein in cow's milk allergic patients. International Archives of Allergy and Immunology 129, 93-96.

Cabrera-Chavez, F., Rouzaud-Sandez, O., Sotelo-Cruz, N., Calderon de la Barca, A.M., 2009. Bovine milk caseins and transglutaminase-treated cereal prolamins are differentially recognized by IgA of celiac disease patients according to their age. J Agric Food Chem 57, 3754-3759.

Caubet, J.-C., Nowak-Węgrzyn, A., Moshier, E., Godbold, J., Wang, J., Sampson, H.A., 2013. Utility of casein-specific IgE levels in predicting reactivity to baked milk. Journal of Allergy and Clinical Immunology 131, 222-224. e224.

Chapman, J.A., Bernstein, I., Lee, R.E., Oppenheimer, J., Nicklas, R.A., Portnoy, J.M., Sicherer, S.H., Schuller, D.E., Spector, S.L., Khan, D., 2006. Food allergy: a practice parameter. Annals of Allergy, Asthma & Immunology 96, S1-S68.

Chatchatee, P., Bardina, L., Sampson, H.A., 2000. Identification of IgE and IgG binding epitopes of a-casein in cow milk allergic patients. Journal of Allergy and Clinical Immunology 105, S185.

Chatchatee, P., Järvinen, K.-M., Bardina, L., Beyer, K., Sampson, H.A., 2001a. Identification of IgE- and IgG-binding epitopes on as1-casein: differences in patients with persistent and transient cow's milk allergy. Journal of Allergy and Clinical Immunology 107, 379-383.

Chatchatee, P., Järvinen, K.-M., Bardina, L., Vila, L., Beyer, K., Sampson, H.A., 2001b. Identification of IgE and IgG binding epitopes on b- and k-casein in cow's milk allergic patients. Clinical and Experimental Allergy 31, 1256-1262.

Chin, H.W., Rosenberg, M., 1998. Monitoring Proteolysis During Cheddar Cheese Ripening Using Two-Dimensional Gel Electrophoresis. Journal of Food Science 63, 423-428.

Christensen, J.E., Dudley, E.G., Pederson, J.A., Steele, J.L., 1999. Peptidases and amino acid catabolism in lactic acid bacteria. Antonie van Leeuwenhoek 76, 217-246.

Christensen, T., Bech, A.M., Werner, H., 1991. Methods for crude fractionation (extraction and precipitation) of nitrogen components in cheese. Bulletin-International Dairy Federation, 4-9.

Codina, R., Lockey, R.F., Ardusso, L., 1998. Soybean hull allergens induced by heating and storage. J Allergy Clin Immunol 101, S229.

Cordle, C., 2006. Allergen quality assurance for hypoallergenic formula, in: S.J. Koppelman, S.L. Hefle (Eds.), Detecting allergens in food. Woodhead Publishing Ltd, Cambridge, England, pp. 294-314.

Creamer, L., 1991. Electrophoresis of Cheese. Bulletin of the International Dairy Federation, 14-28.

Creamer, L., Lawrence, R., Gilles, J., 1985. Effect of acidification of cheese milk on the resultant Cheddar cheese. New Zealand Journal of Dairy Science and Technology 20, 185-203.

Cucu, T., Platteau, C., Taverniers, I., Devreese, B., De Loose, M., De Meulenaer, B., 2013. Effect of partial hydrolysis on the hazelnut and soybean protein detectability by ELISA. Food Control 30, 497-503.

Damodaran, S., 1994. Structure-function relationship of food proteins. Protein functionality in food systems, 1-37.

Damodaran, S., Parkin, K., 2008. Fennema's food chemistry. CRC Press/Taylor & Francis.

Decker, W.W., Campbell, R.L., Manivannan, V., Luke, A., St. Sauver, J.L., Weaver, A., Bellolio, M.F., Bergstralh, E.J., Stead, L.G., Li, J.T.C., 2008. The etiology and incidence of anaphylaxis in Rochester, Minnesota: a report from the Rochester Epidemiology Project. J Allergy Clin Immunol 122, 1161-1165.

Deegan, K.C., McSweeney, P.L., 2013. Effects of low-pressure homogenisation pre-treatment of cheesemilk on the ripening of Cheddar cheese. Dairy science & technology, 1-15.

Di Luccia, A., Faccia, M., Incoronato, C., Inglese, F., Lamacchia, C., Lamparelli, S., Occidente, M., Matassino, D., 2013. Cheese Processing, Proteomics in Foods. Springer, pp. 247-259.

Diaz-Amigo, C., Popping, B., 2010. Analytical testing as a tool for the enforcement of future regulatory thresholds for food allergens. J AOAC Int 93, 434-441.

Douglas, D.M., Sukenick, E., Andrade, P., Brown, J.S., 1994. Biphasis systemic anaphylaxis: an inpatient and outpatient study. J Allergy Clin Immunol 93, 977-985.

Downs, M.L., Taylor, S.L., 2010. Effects of thermal processing on the enzyme-linked immunosorbent assay (ELISA) detection of milk residues in a model food matrix. Journal of Agricultural and Food Chemistry 58, 10085-10091.

Dumont, V., Kerbach, S., Poms, R., Johnson, P., Mills, C., Popping, B., Tömösközi, S., Delahaut, P., 2010. Development of milk and egg incurred reference materials for the validation of food allergen detection methods. Quality Assurance and Safety of Crops & Foods 2, 208-215.

Farkye, N.Y., Fox, P.F., 1990. Observations on plasmin activity in cheese. Journal of Dairy Research 57, 413-418.

Fernandez-Rivas, M., Ballmer-Weber, B., 2007. Food allergy: current diagnosis and management, in: C. Mills, H. Wichers, K. Hoffmann-Sommergruber (Eds.), Managing Allergens in Food. CRC Press, LLC, Boca Raton, FL, p. 315.

Fiocchi, A., Bouygue, G.R., Sarratud, T., Terracciano, L., Martelli, A., Restani, P., 2004. Clinical tolerance of processed foods. Annals of Allergy, Asthma and Immunology 93, S38-S46.

Fiocchi, A., Restani, P., Riva, E., Restelli, A.R., Biasucci, G., Galli, C.L., Giovannini, M., 1995. Meat allergy: II - Effects of food processing and enzymatic digestion on the allergenicity of bovine and ovine meats. J Am Coll Nutr 14, 245-250.

Fitzsimons, N., Cogan, T., Condon, S., Beresford, T., 1999. Phenotypic and genotypic characterization of non-starter lactic acid bacteria in mature cheddar cheese. Applied and environmental microbiology 65, 3418-3426.

Fox, P., Guiney, J., 1973. Casein micelle structure: susceptibility of various casein systems to proteolysis. J. Dairy Res 40, 229.

Fox, P.F., 1989. Proteolysis During Cheese Manufacture and Ripening. Journal of Dairy Science 72, 1379-1400.

Fox, P.F., 2001. Milk proteins as food ingredients. International Journal of Dairy Technology 54, 41-55.

Fox, P.F., McSweeney, P.L., Cogan, T.M., Guinee, T.P., 2000. Fundamentals of cheese science. Springer.

Fox, P.F., McSweeney, P.L.H., 1996. Proteolysis in cheese during ripening. Food Reviews International 12, 457-509.

Fox, P.F., McSweeney, P.L.H., Cogan, T.M., Guinee, T.P., 2004. Cheese: Chemistry, Physics and Microbiology: General Aspects. Elsevier Science.

Gaiaschi, A., Beretta, B., Poiesi, C., Conti, A., Giuffrida, M.G., Galli, C.L., Restani, P., 2001. Proteolysis of β-Casein as a Marker of Grana Padano Cheese Ripening. Journal of Dairy Science 84, 60-65.

Gjesing, B., Osterballe, O., Schwartz, B., Wahn, U., Lowenstein, H., 1986. Allergen-specific IgE antibodies against antigentic components in cow milk and milk substitutes. Allergy 41, 51-56.

Grappin, R., Beuvier, É., Bouton, Y., Pochet, S., 1999. Advances in the biochemistry and microbiology of Swiss-typecheeses. Le Lait 79, 3-22.

Grappin, R., Rank, T.C., Olson, N.F., 1985. Primary Proteolysis of Cheese Proteins During Ripening. A Review. Journal of Dairy Science 68, 531-540.

Green, M.L., Foster, P., 1974. Comparison of the rates of proteolysis during ripening of Cheddar cheeses made with calf rennet and swine pepsin as coagulants. J. Dairy Res 41, 269-282.

Guinee, T., Kilcawley, K., 2004. Cheese as an ingredient. Cheese: Chemistry, physics and microbiology 2, 395-428.

Herian, A.M., Taylor, S.L., Bush, R.K., 1993. Allergenic reactivity of various soybean products as determined by RAST inhibition. Journal of Food Science 58, 385-388.

Hildebrandt, S., Garber, E.A.E., 2010. Effects of processing on detection and quantification of the parvalbumin gene in Atlantic salmon (*Salmo salar*). Food Chem 119, 75-80.

Hill, D.J., Firer, M.A., Ball, G., Hosking, C.S., 1993. Natural history of cows' milk allergy in children: immunological outcome over 2 years. Clinical and Experimental Allergy 23, 124-131.

Hill, D.J., Hosking, C.S., Reyes-Benito, L.V., 2001. Reducing the need for food allergen challenges in young children: a comparison of in vitro with in vivo tests. Clinical and Experimental Allergy 31, 1031-1035.

Holzhauser, T., Vieths, S., 1999. Indirect competitive ELISA for determination of traces of peanut (Arachis hypogaea L.) protein in complex food matrices. J Agric Food Chem 47, 603-611.

Horne, D.S., 2002. Casein structure, self-assembly and gelation. Current opinion in colloid & interface science 7, 456-461.

Høst, A., 1997. Cow's milk allergy. J R Soc Med 90, 34-39.

Høst, A., Halken, S., 1990. A prospective study of cow milk allergy in Danish infants during the first 3 years of life. Allergy 45, 587-596.

Høst, A., Samuelsson, E.-G., 1988. Allergic reactions to raw, pasteurized, and homogenized/ pasteurized cow milk: a comparison. Allergy 43, 113-118.

Hourihane, J.O.B., 1998. Prevalence and severity of food allergy - need for control. Allergy 53, 84-88.

Hurley, M., Larsen, L., Kelly, A., McSweeney, P., 2000. The milk acid proteinase cathepsin D: a review. International Dairy Journal 10, 673-681.

Hurst, W.J., Krout, E.R., Burks, W.R., 2002. A comparison of commercially available peanut ELISA test kits on the analysis of samples of dark and milk chocolate. Journal of Immunoassay and Immunochemistry 23, 451-459.

Husby, S., Jensenius, J.C., Svehag, S.-E., 1986. Passage of undegraded dietary antigen into the blood of healthy adults. Further characterization of the kinetics of uptake and the size distribution of the antigen. Scandinavian Journal of Immunology 24, 447-455.

Hutkins, R.W., 2008. Microbiology and technology of fermented foods. Wiley. com.

Immer, U., 2006. Factors affecting the effectiveness of allergen detection, in: S.J. Koppelman, S.L. Hefle (Eds.), Detecting allergens in food. Woodhead Publishing Ltd, Cambridge, England, pp. 330-347.

Ito, K., Futamura, M., Moverare, R., Tanaka, A., Kawabe, T., Sakamoto, T., Borres, M.P., 2012. The usefulness of casein-specific IgE and IgG4 antibodies in cow's milk allergic children. Clin Mol Allergy 10, 1.

Järvinen, K.-M., Chatchatee, P., Bardina, L., Beyer, K., Sampson, H.A., 2001. IgE and IgG binding epitopes on a-lactalbumin and b-lactoglobulin in cow's milk allergy. International Archives of Allergy and Immunology 126, 111-118.

Jedrychowski, L., Wróblewska, B., 1999. Reduction of the antigenicity of whey proteins by lactic acid fermentation. Food Agric Immunol 11, 91-99.

Jost, R., 1988. Physicochemical treatment of food allergens: application to cow's milk proteins. Vevey/ Raven Press, Ltd., New York.

Juillard, V., Le Bars, D., Kunji, E.R., Konings, W.N., Gripon, J.C., Richard, J., 1995. Oligopeptides are the main source of nitrogen for Lactococcus lactis during growth in milk. Applied and environmental microbiology 61, 3024-3030.

Kemp, S.F., Lockey, R.F., 2002. Anaphylaxis: a review of causes and mechanisms. J Allergy Clin Immunol 110, 341-348.

Kerbach, S., Alldrick, A.J., Crevel, R.W., Dömötör, L., DunnGalvin, A., Clare Mills, E., Pfaff, S., Poms, R.E., Popping, B., Tömösközi, S., 2009. Managing food allergens in the food supply chain–viewed from different stakeholder perspectives. Quality Assurance and Safety of Crops & Foods 1, 50-60.

Khuda, S., Slate, A., Pereira, M., Al-Taher, F., Jackson, L., Diaz-Amigo, C., Bigley, E.C., Whitaker, T., Williams, K., 2012a. Effect of Processing on Recovery and Variability Associated with Immunochemical Analytical Methods for Multiple Allergens in a Single Matrix: Dark Chocolate. J Agric Food Chem 60, 4204-4211.

Khuda, S., Slate, A., Pereira, M., Al-Taher, F., Jackson, L., Diaz-Amigo, C., Bigley, E.C., Whitaker, T., Williams, K.M., 2012b. Effect of Processing on Recovery and Variability Associated with Immunochemical Analytical Methods for Multiple Allergens in a Single Matrix: Sugar Cookies. J Agric Food Chem 60, 4195-4203.

Kilcawley, K., Wilkinson, M., Fox, P., 2000. A survey of the composition and proteolytic indices of commercial enzyme-modified Cheddar cheese. International Dairy Journal 10, 181-190.

Kilcawley, K., Wilkinson, M., Fox, P., 2002. Determination of key enzyme activities in commercial peptidase and lipase preparations from microbial or animal sources. Enzyme and microbial technology 31, 310-320.

Kilcawley, K.N., Wilkinson, M.G., Fox, P.F., 1998. Enzyme-modified cheese. International Dairy Journal 8, 1-10.

Kilshaw, P.J., Heppell, L.M.J., Ford, J.E., 1982. Effects of heat treatment of cows' milk and whey on the nutritional quality and antigenic properties. Archives of Disease In Childhood 57, 842-847.

Kim, J.S., Nowak-Wegrzyn, A., Sicherer, S.H., Noone, S., Moshier, E.L., Sampson, H.A., 2011a. Dietary baked milk accelerates the resolution of cow's milk allergy in children. J Allergy Clin Immunol 128, 125-131.

Kim, J.S., Nowak-Węgrzyn, A., Sicherer, S.H., Noone, S., Moshier, E.L., Sampson, H.A., 2011b. Dietary baked milk accelerates the resolution of cow's milk allergy in children. Journal of Allergy and Clinical Immunology 128, 125-131. e122.

Kleber, N., Weyrich, U., Hinrichs, J., 2006. Screening for lactic acid bacteria with potential to reduce antigenic response of β-lactoglobulin in bovine skim milk and sweet whey. Innovative Food Science and Emerging Technologies 7, 233-238.

Koppelman, S.J., Bleeker-Marcelis, H., van Duijn, G., Hessing, M., 1996. Detecting peanut allergens: the development of an immunochemical assay for peanut proteins. The World of Food Ingredients, 35-38.

Koppelman, S.J., Hefle, S.L., 2006. Detecting allergens in food. Woodhead Publishing Ltd.

Koppelman, S.J., Wensing, M., De Jong, G.A.H., Knulst, A.C., 1999. Anaphylaxis caused by the unexpected presence of casein in salmon. Lancet 354, 2136.

Kuchroo, C., Fox, P., 1982. Soluble nitrogen in Cheddar cheese: Comparison of extraction procedures. Anglais 37, 331-335.

Kunji, E.R.S., Mierau, I., Hagting, A., Poolman, B., Konings, W.N., 1996. The proteotytic systems of lactic acid bacteria. Antonie van Leeuwenhoek 70, 187-221.

Lack, G., 2008. Epidemiologic risks for food allergy. Journal of Allergy and Clinical Immunology 121, 1331-1336.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lakshman, P., Toyokawa, Y., Tachibana, S., Toyama, H., Taira, T., Yasuda, M., 2011. Reducing the antigenicity of milk whey protein using acid proteinases from< i> Monascus pilosus</i>. Process Biochemistry 46, 806-810.

Lara-Villoslada, F., Olivares, M., Xaus, J., 2005. The balance between caseins and whey proteins in cow's milk determines its allergenicity. J Dairy Sci 88, 1654-1660.

Larsen, L.B., Wedholm-Pallas, A., Lindmark-Månsson, H., Andrén, A., 2010. Different proteomic profiles of sweet whey and rennet casein obtained after preparation from raw versus heat-treated skimmed milk. Dairy science & technology 90, 641-656.

Law, B.A., Tamime, A., 2011. Technology of cheesemaking. Wiley-Blackwell.

Law, J., Haandrikman, A., 1997. Proteolytic enzymes of lactic acid bacteria. International Dairy Journal 7, 1-11.

Ledford, R.A., O'Sullivan, A.C., Nath, K.R., 1966. Residual Casein Fractions in Ripened Cheese Determined by Polyacrylamide-Gel Electrophoresis. Journal of Dairy Science 49, 1098-1101.

Lee, J.M., Greenes, D.S., 2000. Biphasic anaphylactic reactions in pediatrics. Pediatrics 106, 762-766.

Lemon-Mule, H., Sampson, H.A., Sicherer, S.H., Shreffler, W.G., Noone, S., Nowak-Wegrzyn, A., 2008. Immunologic changes in children with egg allergy ingesting extensively heated egg. Journal of Allergy and Clinical Immunology 122, 977-983.

Leonard, S.A., Sampson, H.A., Sicherer, S.H., Noone, S., Moshier, E.L., Godbold, J., Nowak-Wegrzyn, A., 2012. Dietary baked egg accelerates resolution of egg allergy in children. J Allergy Clin Immunol 130, 473-480.

Leung, P.S.C., Chu, K.H., Chow, W.K., Ansari, A., Bandea, C.I., Kwan, H.S., Nagy, S.M., Gershwin, M.E., 1994. Cloning, expression, and primary structure of *Metapenaeus ensis* tropomyosin, the major heat-stable shrimp allergen. J Allergy Clin Immunol 94, 882-890.

Li, X.-M., Zhang, T.-F., Huang, C.-K., Srivastava, K., Teper, A.A., Zhang, L., Schofield, B.H., Sampson, H.A., 2001. Food allergy herbal formula-1 (FAHF-1) blocks peanut-induced anaphylaxis in a murine model. J Allergy Clin Immunol 108, 639-646.

Lipton, C., Dautlick, J., Grothaus, G., Hunst, P., Magin, K., Mihaliak, C., Rubio, F., Stave, J., 2000. Guidelines for the validation and use of immunoassays for determination of introduced proteins in biotechnology enhanced crops and derived food ingredients. Food and Agricultural Immunology 12, 153-164.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J biol chem 193, 265-275.

Lucey, J., 2002. Formation and physical properties of milk protein gels. Journal of Dairy Science 85, 281-294.

Lucey, J., Fox, P., 1993. Importance of calcium and phosphate in cheese manufacture: a review. Journal of Dairy Science 76, 1714-1724.

Malanin, K., Lundberg, M., Johansson, S.G.O., 1995. Anaphylactic reaction caused by neoallergens in heated pecan nut. Allergy 50, 988-991.

Marcos, A., Esteban, M., 1993. Iberian cheeses, Cheese: Chemistry, physics and microbiology. Springer, pp. 173-219.

Marcos, A., Esteban, M.A., León, F., Fernández-Salguero, J., 1979. Electrophoretic Patterns of European Cheeses: Comparison and Quantitation. Journal of Dairy Science 62, 892-900.

Mariager, B., Solve, M., Eriksen, H., Brogren, C.-H., 1994. Bovine ß-lactoglobulin in hypoallergenic and ordinary infant formulas measured by an indirect competitive ELISA using monoclonal and polyclonal antibodies. Food Agric Immunol 6, 73-83.

Marshall, T., Williams, K.M., 1988. High resolution two-dimensional electrophoresis and sodium dodecyl sulphate-polyacrylamide gel electrophoresis of cheese proteins after rapid solubilisation. ELECTROPHORESIS 9, 143-147.

Maynard, F., Jost, R., Wal, J.-M., 1997. Human IgE binding capacity of tryptic peptides from bovine a-lactalbumin. Int Arch Allergy Immunol 113, 478-488.

McSweeney, P., Fox, P., Lucey, J., Jordan, K., Cogan, T., 1993. Contribution of the indigenous microflora to the maturation of Cheddar cheese. International Dairy Journal 3, 613-634.

McSweeney, P., Fox, P., Olson, N., 1995. Proteolysis of bovine caseins by cathepsin D: preliminary observations and comparison with chymosin. International Dairy Journal 5, 321-336.

McSweeney, P.L.H., Fox, P.F., 1997. Chemical methods for the characterization of proteolysis in cheese during ripening. Lait 77, 41-76.

Mejbaum-Katzenellenbogen, W., Dobryszycka, W.M., 1959. New method for quantitative determination of serum proteins separated by paper electrophoresis. Clinica Chimica Acta 4, 515-522.

Metcalfe, D.D., Astwood, J.D., Townsend, R., Sampson, H.A., Taylor, S.L., Fuchs, R.L., 1996. Assessment of the allergenic potential of foods derived from genetically engineered crop plants. Critical Reviews in Food Science and Nutrition 36, S165-S186.

Mills, C., Sancho, A., Moreno, J., Kostyra, H., 2007. The effects of food processing on allergens, in: C. Mills, H. Wichers, K. Hoffmann-Sommergruber (Eds.), Managing allergens in food. CRC Press, LLC, Boca Raton, FL, p. 315.

Monaci, L., Brohée, M., Tregoat, V., van Hengel, A., 2011. Influence of baking time and matrix effects on the detection of milk allergens in cookie model food system by ELISA. Food Chemistry 127, 669-675.

Monaci, L., Tregoat, V., van Hengel, A.J., Anklam, E., 2006. Milk allergens, their characteristics and their detection in food: a review. European Food Research and Technology 223, 149-179.

Mooney, J., Fox, P., Healy, A., Leaver, J., 1998. Identification of the principal water-insoluble peptides in Cheddar cheese. International Dairy Journal 8, 813-818.

Morin, S., Bernard, H., Przybylski-Nicaise, L., Corthier, G., Rabot, S., Wal, J.-M., Hazebrouck, S., 2011. Allergenic and immunogenic potential of cow's milk β-lactoglobulin and caseins evidenced without adjuvant in germ-free mice. Molecular Nutrition & Food Research 55, 1700-1707.

Morisset, M., Soulaines, P., Aubert-Jacquin, C., Codreanu, F., Maamri, N., Hatahet, R., Kalindjian, A., Cuny, J., Babchia, H., Guenard, L., Frentz, P., Bouillot, F., Florac, L., Cordebar, V., Beaudouin, E., Kanny, G., Moneret-Vautrin, D., Dupont, C., 2008. Double blind trial of a fermented infantile formula in cow's milk allergy prevention. Journal of Allergy and Clinical Immunology 121, S244.

Morr, C.V., 1971. Comparison of Protein Preparation Procedures and Starch versus Polyacrylamide Gel Electrophoresis for Examining Casein Degradation Products in Cheese. Journal of Dairy Science 54, 339-342.

Moskowitz, G.J., Noelck, S.S., 1987. Enzyme-modified cheese technology. Journal of Dairy Science 70, 1761-1769.

Mulvihill, D., Fox, P., 1980. Proteolysis of-Casein by Chymosin in Dilute Nac1 Solutions and in Cheddar Cheese. Irish Journal of Food Science and Technology, 13-23.

Murphy, K.M., 2007. Janeway's Immunobiology (Immunobiology: The Immune System (Janeway)) Author: Kenneth M. Murphy, Paul Travers, Mark Walport.

Negroni, L., Bernard, H., Clement, G., Chatel, J.M., Brune, P., Frobert, Y., Wal, J.M., Grassi, J., 1998. Two-site enzyme immunometric assays for determination of native and denatured b-lactoglobulin. J Immunol Methods 220, 25-37.

Nicoletti, C., Temblay, J., Ivory, K., 2007. The immune system and allergic responses to food, in: C. Mills, H. Wichers, K. Hoffmann-Sommergruber (Eds.), Managing allergens in food. CRC Press, LLC, Boca Raton, FL, p. 315.

Nowak-Wegrzyn, A., Bloom, K.A., Sicherer, S.H., Shreffler, W.G., Noone, S., Wanich, N., Sampson, H.A., 2008. Tolerance to extensively heated milk in children with cow's milk allergy. Journal of Allergy and Clinical Immunology 122, 342-347.

Nowak-Wegrzyn, A., Fiocchi, A., 2009. Rare, medium, or well done? The effect of heating and food matrix on food protein allergenicity. Current Opinion in Allergy and Clinical Immunology 9, 234-237.

O'Farrell, I.P., Jeremiah, J.S., Martin, G.W., Dermot, H., Alan, L.K., 2002. Influence of addition of plasmin or mastitic milk to cheesemilk on quality of smear-ripened cheese. Lait 82, 305-316.

O'Keeffe, R.B., Fox, P.F., Daly, C., 1976. Contribution of rennet and starter proteases to proteolysis in Cheddar cheese. Journal of Dairy Research 43, 97-107.

O'Sullivan, M., Fox, P.F., 1990. A scheme for the partial fractionation of cheese peptides. Journal of Dairy Research 57, 135-139.

Otani, H., Hori, H., Hosono, A., 1987. Antigenic reactivity of dephosphorylated alpha(S1)-casein, phosphopeptide from beta-casein and O-phospho-L-serine towards the antibody to native alpha(S1)-casein. Agric Biol Chem 51, 2049-2054.

Paschke, A., Besler, M., 2002. Stability of bovine allergens during food processing. Annals of Allergy, Asthma and Immunology 89, 16-20.

Paschke, A., Ulberth, F., 2009. Allergens in foods. Anal Bioanal Chem 395, 15-16.

Peterson, S., Marshall, R., 1990. Nonstarter lactobacilli in Cheddar cheese: a review. Journal of Dairy Science 73, 1395-1410.

Polenta, G., Weber, D., Godefroy-Benrejeb, S., Abbott, M., 2012. Effect of Processing on the Detectability of Pecan Proteins Assessed by Immunological and Proteomic Tools. Food Anal Methods 5, 216-225.

Pomés, A., Helm, R.M., Bannon, G.A., Burks, A.W., Tsay, A., Chapman, M.D., 2003. Monitoring peanut allergen in food products by measuring Ara h 1. J Allergy Clin Immunol 111, 640-645.

Poms, R., Thomas, M., Finglas, P., Astley, S., Spichtinger, D., Rose, M., Popping, B., Alldrick, A., Van Egmond, H., Solfrizzo, M., 2009. MoniQA (Monitoring and Quality Assurance): an EU-funded Network of Excellence working towards the harmonization of worldwide food quality and safety monitoring and control strategies–status report 2008. Quality Assurance and Safety of Crops & Foods 1, 9-22.

Poms, R.E., Agazzi, M.-E., Bau, A., Brohee, M., Capelletti, C., Norgaard, J.V., Anklam, E., 2005. Inter-laboratory validation study of five commercial ELISA test kits for the determination of peanut proteins in biscuits and dark chocolate. Food Additives and Contaminants 22, 104-112.

Poms, R.E., Anklam, E., 2004a. Effects of chemical, physical, and technological processes on the nature of food allergens. Journal of AOAC International 87, 1466-1474.

Poms, R.E., Anklam, E., 2004b. Trends in detecting food allergens. G. I. T. Laboratory Journal 1, 43-46.

Poms, R.E., Anklam, E., Kuhn, M., 2004a. Polymerase chain reaction techniques for food allergen detection. J AOAC Int 87, 1391-1397.

Poms, R.E., Klein, C.L., Anklam, E., 2004b. Methods for allergen analysis in food: a review. Food Additives and Contaminants 21, 1-31.

Poms, R.E., Schäppi, G., Agazzi, M.-E., Koch, P., Bau, A., Anklam, E., 2004c. Allergenicity vs detectability of processed peanut residues in cookies, in: E. Anklam, F. Morlin (Eds.), 9th International Symposium on Immunological, Chemical and Clinical Problems of Food Allergy, Budapest, Hungary.

Poulsen, O.M., Hau, J., Kollerup, J., 1987. Effect of homogenization and pasteurization on the allergenicity of bovine milk analysed by a murine anaphylactic shock model. Clinical Allergy 17, 449-458.

Prausnitz, C., Küstner, H., 1921. Studien über die Ueberempfindlichkeit. Centralbl. fur Bakteriol Abt Orig 86, 160-169.

Quesnel, V.C., 1968. Fractionation and properties of the polymeric leucocyanidin of the seeds of Theobroma cacao. Phytochemistry 7, 1583-1592.

Rank, T.C., Grappin, R., Olson, N.F., 1985. Secondary Proteolysis of Cheese During Ripening: A Review. Journal of Dairy Science 68, 801-805.

Reddy, I.M., Kella, N.K., Kinsella, J.E., 1988. Structural and conformational basis of the resistance of. beta.-lactoglobulin to peptic and chymotryptic digestion. Journal of Agricultural and Food Chemistry 36, 737-741.

Reed, G.F., Lynn, F., Meade, B.D., 2002. Use of coefficient of variation in assessing variability of quantitative assays. Clinical and Diagnostic Laboratory Immunology 9, 1235-1239.

Restani, P., Gaiaschi, A., Plebani, A., Beretta, B., Cavagni, G., Fiocchi, A., Poiesi, C., Velona, T., Ugazio, A.G., Galli, C.L., 1999. Cross-reactivity between milk proteins from different animal species. Clinical and Experimental Allergy 29, 997-1004.

Ross, M.P., Ferguson, M., Street, D., Klontz, K., Schroeder, T., Luccioli, S., 2008. Analysis of food-allergic and anaphylactic events in the National Electronic Injury Surveillance System. J Allergy Clin Immunol 121.

Sampson, H.A., 1999. Food allergy. Part 1: Immunopathogenesis and clinical disorders. J Allergy Clin Immunol 103, 717-728.

Sampson, H.A., 2001. Utility of food-specific IgE concentrations in predicting symptomatic food allergy. J Allergy Clin Immunol 107, 891-896.

Sampson, H.A., 2004. Update on food allergy. Journal of Allergy and Clinical Immunology 113, 805-819.

Sampson, H.A., Konstantinou, G.N., Kattan, J.D., Masilamani, M., Strong, B.D., Paynter, E., Bahnson, T., Nowak-Wegrzyn, A.H., 2013. Tolerance to Extensively Heated (Baked) Milk-Clinical and Immunologic Phenotype. J Allergy Clin Immunol 131, AB83.

Sanchez, C., Frémont, S., 2003. [Consequences des traitements thermiques et de la formulation sur la structure et l' allergenicite des proteines alimentaires]//Consequences of heat treatment and processing of food on the structure and allergenicity of component proteins. Revue Francaise d'Allergologie et d'Immunologie Clinique 43, 13-20.

Sathe, S.K., Teuber, S.S., Roux, K.H., 2005. Effects of food processing on the stability of food allergens. Biotechnology Advances 23, 423-429.

Schmidt, D., 1982. Association of caseins and casein micelle structure. Developments in dairy chemistry 1, 61-86.

Schutte, L., Paschke, A., 2007. Reducing allergens in milk and milk products, in: C. Mills, H. Wichers, K. Hoffmann-Sommergruber (Eds.), Managing allergens in food. CRC Press LLC, Boca Raton, FL, p. 315.

Shalabi, S., Fox, P., 1987. Electrophoretic analysis of cheese: comparison of methods. Irish Journal of Food Science and Technology 11, 135-151.

Sicherer, S.H., 2011a. Epidemiology of food allergy. Journal of Allergy and Clinical Immunology 127, 594-602.

Sicherer, S.H., 2011b. Epidemiology of food allergy. J Allergy Clin Immunol 127, 594-602.

Sicherer, S.H., Sampson, H.A., 2010. Food allergy. Journal of Allergy and Clinical Immunology 125, S116-S125.

Singh, T., Fox, P., Højrup, P., Healy, A., 1994. A scheme for the fractionation of cheese nitrogen and identification of principal peptides. International Dairy Journal 4, 111-122.

Singh, T.K., Fox, P.F., Healy, A., 1995. Water-soluble peptides in Cheddar cheese: isolation and identification of peptides in the diafiltration retentate of the water-soluble fraction. Journal of Dairy Research 62, 629-629.

Singh, T.K., Fox, P.F., Healy, Á., 1997. Isolation and identification of further peptides in the diafiltration retentate of the water-soluble fraction of Cheddar cheese. Journal of Dairy Research 64, 433-443.

Sousa, M.J., Ardö, Y., McSweeney, P.L.H., 2001. Advances in the study of proteolysis during cheese ripening. International Dairy Journal 11, 327-345.

Spinnler, H.-E., Gripon, J.-C., 2004. Surface mould-ripened cheeses. Cheese: Chemistry, physics and microbiology 2, 157-174.

Stanic, D., Monogioudi, E., Dilek, E., Radosavljevic, J., Atanaskovic-Markovic, M., Vuckovic, O., Raija, L., Mattinen, M., Buchert, J., Cirkovic Velickovic, T., 2010. Digestibility and allergenicity assessment of enzymatically crosslinked β-casein. Mol Nutr Food Res 54, 1273-1284.

Taylor, S.L., 1997. Food Allergies and Intolerances, 2nd ed. Academic Press, San Diego.

Taylor, S.L., Hefle, S.L., Bindslev-Jensen, C., Bock, S.A., Burks, A.W., Jr., Christie, L., Hill, D.J., Høst, A., Hourihane, J.O.B., Lack, G., Metcalfe, D.D., Moneret-Vautrin, D.A., Vadas, P.A., Rancé, F., Skrypec, D.J., Trautman, T.A., Yman, I.M., Zeiger, R.S., 2002. Factors affecting the determination of threshold doses for allergenic foods: how much is too much? J Allergy Clin Immunol 109, 24-30.

Taylor, S.L., Nordlee, J.A., Niemann, L.M., Lambrecht, D.M., 2009. Allergen immunoassays - considerations for use of naturally incurred standards. Anal Bioanal Chem 395, 83-92.

Thomas, K., Herouet-Guicheney, C., Ladics, G., Bannon, G., Cockburn, A., Crevel, R., Fitzpatrick, J., Mills, C., Privalle, L., Vieths, S., 2007. Evaluating the effect of food processing on the potential human allergenicity of novel proteins: International workshop report. Food and Chemical Toxicology 45, 1116-1122.

Thompson, M., Ellison, S.L., Wood, R., 2002. Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report). Pure and Applied Chemistry 74, 835-855.

Trayer, H.R., Trayer, I.P., 1988. Fluorescence resonance energy transfer within the complex formed by actin and myosin subfragment 1. Comparison between weakly and strongly attached states. Biochemistry 27, 5718-5727.

Upadhyay, V.K., McSweeney, P.L.H., Magboul, A.A.A., Fox, P.F., 2004. Proteolysis in cheese during ripening, in: P.L.H.M.T.M.C. Patrick F. Fox, P.G. Timothy (Eds.), Cheese: Chemistry, Physics and Microbiology. Academic Press, pp. 391-VIII.

Veloso, A.C.A., Teixeira, N., Peres, A.M., Mendonça, Á., Ferreira, I.M.P.L.V.O., 2004. Evaluation of cheese authenticity and proteolysis by HPLC and urea–polyacrylamide gel electrophoresis. Food Chemistry 87, 289-295.

Wal, J.-M., 1998a. Cow's milk allergens. Allergy 53, 1013-1022.

Wal, J.-M., 2001a. Structure and function of milk allergens. Allergy 56, 35-38.

Wal, J.-M., 2002a. Cow's milk proteins/ allergens. Ann Allergy Asthma Immunol 89, 3-10.

Wal, J.-M., 2002b. Cow's milk proteins/ allergens. Annals of Allergy, Asthma and Immunology 89, 3-10.

Wal, J.-M., Bernard, H., Clement, G., Chatel, J.-M., Adel-Patient, K., 2001. Characterization of allergens in milk. Current Allergy and Clinical Immunology 14, 4-6, 8-10.

Wal, J.-M., Bernard, H., Créminon, C., Hamberger, C., David, B., Peltre, G., 1995a. Cow's milk allergy: the humoral immune response to eight purified allergens, Part B ed. Plenum Press, New York.

Wal, J., 2001b. Structure and function of milk allergens. Allergy 56, 35-38.

Wal, J.M., 1998b. Immunochemical and molecular characterization of milk allergens. Allergy 53, 114-117.

Wal, J.M., 2003. Thermal processing and allergenicity of foods. Allergy 58, 727-729.

Wal, J.M., Bernard, H., Yvon, M., Peltre, G., David, B., Créminon, C., Frobert, Y., Grassi, J., 1995b. Enzyme immunoassay of specific human IgE to purified cows' milk allergens. Food Agric Immunol 7, 175-187.

Wallace, J., Fox, P., 1998. Rapid spectrophotometric and fluorimetric methods for monitoring nitrogenous (proteinaceous) compounds in cheese and cheese fractions: a review. Food Chemistry 62, 217-224.

Wang, J., Godbold, J.H., Sampson, H.A., 2008. Correlation of serum allergy (IgE) tests performed by different assay systems. Journal of Allergy and Clinical Immunology 121, 1219-1224.

Warburg, O., Christian, W., 1942. Insulation and Crystalisation of the Fermenting Process of Enolase. Biochem Z 310, 384-421.

Watanabe, H., Kai, S., Mitani, T., Yokoyama, H., Kishi, M., 2005. Study on detection of allergenic substances (egg and milk) in processed meat products and frozen foods. Journal of the Food Hygienic Society of Japan 46, 139-147.

Wood, R.A., Sicherer, S.H., Vickery, B.P., Jones, S.M., Liu, A.H., Fleischer, D.M., Henning, A.K., Mayer, L., Burks, A.W., Grishin, A., Stablein, D., Sampson, H.A., 2013. The natural history of milk allergy in an observational cohort. The Journal of allergy and clinical immunology 131, 805-812.e804.

Wróblewska, B., Jedrychowski, L., Mierzejewska, D., 2000. Reduction of protein allergenicity by lactic acid fermentation. Polish Journal of Food and Nutrition Sciences 9, 37-40.

Zarmpoutis, I., McSweeney, P., Fox, P., 1997. Proteolysis in blue-veined cheese: an intervarietal study. Irish journal of agricultural and food research, 219-229.

Zeleny, R., Schimmel, H., 2010. Towards comparability of ELISA results for peanut proteins in food: a feasibility study. Food Chem 123, 1343-1351.

CHAPTER 3: COMMERCIAL MILK ELISA KIT SENSITIVITES TO PURIFIED MILK PROTEINS AND MILK-DERIVED INGREDIENTS

1. Abstract

Commercial enzyme-linked immunosorbent assay (ELISA) kits exist to quantitatively detect bovine milk residues. Milk contains many proteins that can serve as ELISA targets including caseins (α-, β-, or κ-casein) and whey proteins (α-lactalbumin or β-lactoglobulin). However, because not all ELISAs target the same protein fractions, comparison among assay kits is challenging. To correlate the detection levels and specificity of ELISAs, nine milk-specific commercial kits were selected for evaluation. Samples of five purified milk proteins (α-,β-, and κ-casein, α-lactalbumin (ALA) , and β-lactoglobulin (BLG)), and three milk-derived ingredients (non-fat dry milk (NFDM), whey protein concentrate (WPC), and sodium caseinate) were evaluated with commercial ELISA kits that target casein, BLG, or total milk. All milk kits tested are capable of quantifying NFDM, but do not necessarily detect all of its protein components. No kits are capable of detecting α-lactalbumin, even though it is considered an important milk allergen. One total milk kit detects only the caseins, while the common whey allergens (BLG and ALA) are virtually undetectable. Another total milk kit detects BLG, but is incapable of detecting α- and κ-casein. While the milk-derived ingredients (NFDM, WPC, and sodium caseinate) are detected by the kits, their quantitation is inaccurate due to the use of different calibrators, reference materials, and antibodies. The evaluated ELISA kits rely on mathematical conversion factors for quantitation of target antigen in a sample. Mathematical assumptions may affect the quantitative accuracy of ELISA kits, especially when allergenic foods have been modified or processed. Milk ELISA kits target specific proteins as opposed to broad spectrum detection of protein fractions and sub-fractions. The establishment of a standard reference material for calibration of milk ELISA kits is increasingly important. Appropriate selection and

understanding of milk ELISA kits for food analysis is critical to accurate quantification of milk residues and informed risk management decisions.

2. Introduction

The prevalence of food allergies is increasing around the world (Sicherer, 2011a). While the "Big Eight" food allergens are responsible for 90% of allergic reactions to foods, nearly 80% of reactions among infants are to milk, egg, and peanut (Bock et al., 1988). Allergy to cow's milk is especially common among children, affecting between 2-3% of young children in the United States (Monaci et al., 2006; Sampson, 2004). While nearly 80% of children diagnosed with milk allergy will outgrow their sensitivity prior to adolescence, milk still presents a risk for many who ingest it (Chapman et al., 2006; Høst and Halken, 1990). The importance of rapid, sensitive, robust, and specific methods for the detection of allergenic residues in packaged and processed foods, along with ensuring allergenic protein residue is effectively removed from shared processing equipment, is critical for the protection of food-allergic consumers and compliance with labeling and food processing regulations.

Bovine milk contains several major allergens. Milk can be separated into two fractions by acid precipitation at pH 4.6; casein and whey (Damodaran and Parkin, 2008). The casein fraction of milk contains nearly 80% of the total milk protein, while the whey fraction contains the remaining 20%. There are three major casein groups, α-, β-, and κ-casein. While the genes for casein production are coded on the same chromosome, there are major differences among the caseins in their sequences, functionality, reactivity, and allergenicity (Chatchatee et al., 2000; Chatchatee et al., 2001b). The whey fraction of bovine milk contains two major allergens, α-lactalbumin (ALA) and β-lactoglobulin (BLG). No counterpart of bovine BLG exists in human breast milk. Research now indicates that milk-allergic patients are typically allergic to more than one milk protein, and BLG is not the predominant milk allergen (Wal et al., 2001; Wal et al., 1995a).

The proportions of proteins within fluid milk remain relatively consistent across breeds and geographical changes. For the casein fractions, the proportions of α-,β-,and κ-casein comprise roughly 50%, 37%, and 13% of total casein protein (Wal, 2002b). BLG and ALA comprise roughly 50% and 25% of the total protein present in the whey fraction of fluid milk. Other milk proteins are documented to exhibit some allergenic activity, but their low concentration in milk renders ELISA analysis based on these fragments challenging and largely irrelevant, especially in processed foods (Wal, 2002b).

Due to widespread use of milk and derivative ingredients in the food industry and the allergenic importance of milk to consumers, accurate and reliable methods of detecting small amounts of milk in products, equipment, and facilities must be available. One of the most commonly used methods to quantitatively analyze target proteins in food is the enzyme-linked immunosorbent assay (ELISA). Many commercialized ELISAs are available for use in industry laboratories and by regulatory agencies, as are contract services for evaluating samples and analyzing data. Commercial ELISA kits are typically based on detection of a specific protein or group of proteins that are generally always present in the allergenic food, stable to matrix interactions and processing effects, and are easily extractable. The target proteins are not always allergens, but rather are proteins that fit the above criteria, are found in conjunction with the allergen and do not occur outside of the allergenic food (Abbott et al., 2010). ELISA kits must be appropriately sensitive to detect trace amounts of allergenic food residue. Abbott et al. (2010) suggested guidelines that all ELISA kits should meet before the kit can be considered for AOAC method validation. In addition to requiring a standard reference material, spiking methods and matrices must be established.

Fluid milk is modified in numerous ways to maximize desirable characteristics for use in the food industry. While many milk ingredients contain whey and casein proteins at their conception, the differential susceptibility of these proteins to denaturation by various methods of

processing (heating, drying, high pressure, etc.) can alter the prevalence and specificity of antibody binding epitopes in foods (Poms and Anklam, 2004a). Some milk-derived ingredients undergo processes to remove certain milk components.

Among the most commonly used milk-derived ingredients in the food industry are caseinate and whey protein concentrate. The concentration of protein and other materials in these products is modified by manufacturers to suit customer needs. Caseinates undergo acid precipitation to facilitate the removal of whey proteins. Through various processes, caseinates can reach a level of greater than 90% protein, of which, nearly all is casein and very little is whey-derived. Caseinates are derivatives of various salts, commonly sodium, potassium, or calcium. The minerals used in precipitation can affect the function and reactivity of the caseinate in a food. Using an ELISA kit that relies on detection of whey protein for quantitation of milk in a food product containing caseinate residues will be inaccurate and may yield a false negative result. Additionally, use of a kit that detects milk proteins in equivalent presence of nonfat dry milk (NFDM) or milk powder would incorrectly indicate the presence of whey proteins in the sample. One of the most important principles of ELISA kits is that lack of detection does not indicate absence of the protein or allergenic food—it does, however, indicate that fewer targets are present in the sample.

Whey protein concentrates contain only traces of casein proteins. These concentrates can range from 35% to upwards of 85% protein, chosen depending on desired applications. A milk powder manufactured with similar procedures and containing more than 85% protein is typically referred to as a whey protein isolate. The detection of caseinate and whey derivatives by commercial ELISA kits has not been evaluated.

One of the most commonly used milk-derived ingredients used by the food industry is NFDM. Like other milk ingredients, NFDM can be manufactured under various conditions.

These conditions affect the structure and function of milk proteins. One of the most common variables in the production of NFDM is the drying temperature. NFDM can be dried under high, medium, or low heat conditions. Unlike caseinates and whey protein-derived ingredients, the proportions of milk proteins in NFDM remain relatively consistent with those observed in fluid milk (Wal, 2002b).

Various processing methods, especially thermal treatments, have been documented to affect allergenicity of food products (Fiocchi et al., 2004; Lemon-Mule et al., 2008; Nowak-Wegrzyn et al., 2008; Sampson et al., 2013; Wal, 2003; Wróblewska et al., 2000). Additionally, antigen detection by ELISA is altered by processing (Cucu et al., 2013; Downs and Taylor, 2010; Hildebrandt and Garber, 2010; Khuda et al., 2012a; Khuda et al., 2012b; Polenta et al., 2012; Poms et al., 2004c). Both of these trends occur due to the effects of processing on protein structure and function. Due to the occurrence of conformational and sequential epitopes on native and denatured proteins, no definite relationship can be established between protein structure, function, and allergenicity. Some allergens are more resistant to the effects of processing than others. Susceptibility of allergens to effects of processing does not always indicate that processing reduces protein allergenicity. While protein denaturation can destroy conformational allergenic epitopes, it can also expose new sequential epitopes. Additionally, some unfolded proteins will aggregate with other proteins or moieties within the food and create novel molecules with allergenic potential (Sathe et al., 2005; Thomas et al., 2007).

The specificity of antibodies used in commercial ELISA kits varies. It has been suggested that the main sources of variation within a kit are food matrix interactions, changes in solubility and reactivity of proteins as a result of processing, and different protein composition as a result of environmental conditions and species variety (Abbott et al., 2010; Koppelman and Hefle, 2006). The effects of common food processing methods on the detection of milk residues have been studied with three commercially-available milk ELISA kits in a model food matrix

(Downs and Taylor, 2010). However, the detection specificities of the kits themselves have not been reviewed. Without understanding the antibody sensitivities used in each kit, comparing results and kits is highly challenging.

In addition to selection of antibodies with specific sensitivities, kit manufacturers use various materials to serve as kit calibrants, standards, and reporting units. Kit information is listed in Table 3.1. Even within a single kit, these materials are not always consistent. These units are rarely comparable among kits. The lack of an established standard reference material for the development of ELISA kits increases the complexity of kit comparison. The AOAC Task Force recommends use of National Institute of Standards and Technology (NIST) nonfat milk powder (NIST-RM-1549) as the standard reference material for calibration and quantification of commercial milk ELISAs (Abbott et al., 2010). If a calibration material other than NIST NFDM is used to build the standard curve, it is suggested that the manufacturer provide an experimentally validated conversion factor between the calibration material and NIST milk powder.

Many ELISA kit users are unaware of the importance of kit specificities in accurate detection and quantification of antigens. In this research, the sensitivity of nine milk-specific ELISA kits to five purified milk proteins and four milk-derived ingredients were be determined. Kit selection and usage by food industry as well as regulators must be driven by an appropriate understanding of the specificities, assumptions, and limitations of commercial milk ELISAs.

3. Materials and Methods

Materials, Equipment, and Kits

Milk protein fractions were purchased from Sigma-Aldrich (St. Louis, MO). Fractions evaluated include α_s-casein (≥ 70% purity), β-casein (≥ 98% purity), κ-casein (≥ 70% purity), β-lactoglobulin (≥ 90% purity), and α-lactalbumin (≥ 85% purity). Samples of whey protein

concentrate at two concentrations, 34% protein and 80% protein, and sodium caseinate were donated by Erie Foods International (Erie, IL). Whey protein concentrate at 34% and 80% protein will be referred to as WPC34 and WPC80, and sodium caseinate will be referred to as NaCas. Low-heat processed nonfat dry milk was obtained from Darigold (Seattle, WA). C & H Pure Granulated White Cane Sugar was purchased from a local retail store in Lincoln, NE (distributed by Domino Foods, Yonkers, NY, USA).

Neogen Veratox® Total Milk, Casein, and BioKits™ BLG ELISA kits were obtained from Neogen® Corporation (Lansing, MI, USA). ELISA Systems™ Casein and β-lactoglobulin ELISA kits were obtained from ELISA Systems (Windsor, Queensland, Australia). R-Biopharm RIDASCREEN® Fast Casein, Fast Milk, and Fast BLG (Darmstadt, Germany) and Romer Labs® AgraQuant® BLG (Union, MO, USA) ELISA kits were also used and obtained from their respective distributors. Kit information is listed below in Table 3.1.

Table 3.1. Sensitivity, range, calibration, and reporting units of commercial milk ELISA kits

Kit	LOD	LOQ	Dynamic Range	Calibrant	Reporting units (ppm)
Casein Kits					
ELISA Systems™ Casein	0.5 ppm	1.0 ppm	1.0-10 ppm	a-casein	skim milk powder
Neogen Veratox® Casein	1 ppm	2.5 ppm	2.5-15 ppm	NFDM	NFDM
R-Biopharm RIDASCREEN® Fast Casein	0.12 ppm	0.5 ppm	0.5-13.5 ppm	casein	casein
BLG Kits					
ELISA Systems™ BLG	0.05 ppm	0.1 ppm	0.1-1 ppm	BLG	BLG
Neogen BioKits™ BLG	2 ppm	2.5 ppm	2.5-40 ppm	whole milk powder	BLG
R-Biopharm RIDASCREEN® Fast BLG	0.19 ppm	0.5 ppm	0.5-13.5 ppm	BLG	BLG
Romer Labs® AgraQuant® BLG	1.5 ppb	10 ppb	10-400 ppb	BLG	BLG
Total Milk Kits					
Neogen Veratox® Total Milk	1 ppm	2.5 ppm	2.5-25 ppm	NFDM	NFDM
R-Biopharm RIDASCREEN® Fast Milk	0.7 ppm	2.5 ppm	2.5-67.5 ppm	milk protein	milk protein

Equipment used in this experiment included a Mr. Coffee® IDS77 Coffee Grinder (Jarden Consumer Solutions, Boca Raton, FL), DYNEX Spectra MR™ Plate Reader (Dynex Technologies, Chantilly, VA), Thermo Scientific Legend Micro 17 Centrifuge (Dubuque, IA), Thermo Scientific Titer Plate Shaker (Dubuque, IA), and a Julabo™ SW22 shaking waterbath (Julabo USA, Allentown, VA).

Spike Preparation

Homogenous spikes were created in a neutral matrix of granulated cane sugar at levels that correlate with the levels detectable by milk ELISA kits. First, a 250 ppm concentrated spike of each purified protein or milk ingredient was developed. Briefly, 0.025 g of each purified protein and milk ingredient was weighed and added to 99.975 g of granulated sugar. The mixture was ground for one minute in a coffee grinder. The sides of the bowl were scraped down with a rubber spatula, and grinding was continued for another minute. The finely ground sample was removed from the grinder and poured into a plastic zip-top bag. Nine subsamples of each concentrated spike were evaluated for homogeneity using the Neogen Veratox® Total Milk kit.

The concentrated 250 ppm spike was used to create spikes of lower concentration in granulated sugar. The concentrations were selected based on the standard solutions provided by each ELISA kit manufacturer. For example, a kit with standard solutions at 0 ppm, 2.5 ppm, 5 ppm, and 10 ppm NFDM would have spikes of 0, 2.5, 5, and 10 ppm of each purified protein. Spikes were produced using the same method of weighing and grinding, as described above. A total of 100 g of each concentration of spike was produced.

Sample Evaluation: Protein Concentration and Electrophoresis

The protein content of each milk-derivative was determined by LECO Dumas Protein analysis. Proportions were applied to ELISA results to evaluate the capability of kits to quantify milk proteins in samples where bovine milk micelle proportionality is not preserved.

Additionally, each purified protein and milk ingredient was evaluated using SDS-PAGE. Each analyte was evaluated using the protocol of Laemmli (1970). Samples were solubilized in 0.01M phosphate-buffered saline (PBS) and protein concentrations were estimated using the Lowry Protein Assay (Lowry et al., 1951). Proteins were prepared using reducing conditions. Briefly, 50 µg of each sample was boiled in Laemmli sample buffer containing 5.4% dithithreitol (w/v) for 5 minutes. Protein separation was performed using a Bio-Rad Mini-Protean® Tetracell electrophoresis unit (Bio-Rad Laboratories, Hercules, CA). 5 µL of Precision Plus Protein™ Dual X-tra Standard was loaded in the first lane of each gel (#161-0377; Bio-Rad Laboratories, Hercules, CA). Multiple protein levels of each reduced sample were loaded on 12% Mini-Protean® TGX™ Tris-HCl precast gel, 10 wells, with 30 µL maximum volume per well (Bio-Rad Laboratories, Hercules, CA). Gels were run at a constant voltage of 200V for approximately 30 minutes, or until the dye front reached the bottom of the gel.

Gels were fixed in a solution of 60% trichloroacetic acid (w/v) and 17.5% 5-sulfosalicylic acid (w/v) diluted 1:5 with deionized water (Sigma-Aldrich, St. Louis, MO). Fixed gels were stained overnight using Coomassie Brilliant-Blue R-250 Staining Solution (Bio-Rad Laboratories, Hercules, CA). After a minimum of 8 hours of staining, gels were destained using the Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad Laboratories, Hercules, CA). Gels images were captured using a Kodak Gel Logic 440 Imaging System (Eastman Kodak, Rochester, NY) and evaluated using Carestream Molecular Imaging software (v5.02.30, Carestream Health, Rochester, NY).

Kit Selection

The ELISA kits selected for analysis were the Neogen Veratox® Casein, Neogen Veratox® Total Milk, and Neogen BioKits™ BLG (Lansing, Michigan, USA), ELISA Systems™ Casein and β-lactoglobulin (Windsor, Queensland, Australia), r-Biopharm RIDASCREEN® Fast Casein, Fast Milk, and Fast BLG (Darmstadt, Germany), and Romer

Labs® BLG (Union, Missouri, USA). These kits were selected based on prior history of acceptable performance. Other commercial ELISA kits are available, but did not meet our internal minimum criteria for accuracy and precision. For kit results to be considered valid, standard curves must exhibit an $r^2 \geq 0.98$ and a %CV $\leq$ 20% among sample replicates. Spikes were weighed, extracted, and analyzed according to kit manufacturer's instructions.

Each purified protein was analyzed using appropriate kits. Sodium caseinate and whey protein concentrates were analyzed using only the total milk kits (Neogen Veratox® Total Milk and r-Biopharm RIDASCREEN® Fast Milk). NFDM was analyzed with all kits.

Sample Preparation and Extraction for ELISA ktis

Briefly, 1g of each sample was weighed into a 50 mL polypropylene Falcon™ tube (Fisher Scientific, Pittsburgh, PA) and the appropriate amount of pre-warmed extraction buffer (60^{o}C) was added to the tube according to the manufacturers' instructions in each kit. The samples were vortexed and placed in a 60^{o}C shaking waterbath for the allotted extraction period. Tubes were removed from the waterbath after the appropriate extraction period and were cooled to room temperature. 1mL of each extracted sample was pipetted into a 1.5 mL Eppendorf™ microcentrifuge tube (Fisher Scientific, Pittsburgh, PA). Samples were centrifuged in a Thermo Scientific Legend Micro 17 centrifuge for the recommended time and speed (ThermoScientific, Rockford, IL). Three independent extractions were performed of each sample.

The r-Biopharm RIDASCREEN® Fast Milk and Fast BLG kits utilize an extraction buffer containing β-mercaptoethanol (BME) and sodium dodecyl sulfate (SDS) to improve protein solubility. Extraction buffers containing BME or SDS were disposed according to guidelines for hazardous material collection procedures as indicated by Environmental Health and Safety at the University of Nebraska-Lincoln.

ELISA Analysis

Extracted samples were analyzed with respective ELISA kits. Of the kits selected, eight were sandwich-ELISAs and the Neogen® BioKits BLG was an indirect-competitive format. For the eight sandwich ELISAs, antibody-coated wells, conjugate antibody, substrate, stop solution, wash buffer, and solutions of standard concentration were provided with each kit. For the indirect-competitive ELISA kit, antibody-coated wells, avidin peroxidase, conjugate antibody, substrate, and stop solution were provided. Prepared samples were applied to respective kits according to manufacturer's instructions and allowed to incubate. Wells were washed using wash buffer in 500 mL wash buffer bottles. Each well on the plate was filled with wash buffer. The plate was inverted to remove the wash buffer, and filling was repeated. Washing was repeated for the appropriate number of times as directed by the kit manufacturers. After washing was completed, microplates were inverted and excess wash buffer was repeatedly tapped out onto an absorbent paper towel.

Enzyme-labeled conjugate antibody was added to each well using a Ranin® multichannel pipette. The plate was allowed to incubate for the appropriate amount of time, and washing was repeated, as described above. Substrate solution was added to each well, producing a colorimetric reaction throughout the incubation period. Acidic stop solution was added to each well to stop the enzyme reaction after incubation. The optical density of each well was read at the appropriate wavelength recommended for each kit with a DYNEX Spectra MR™ plate reader.

The optical density readings of each manufacturer-provided standard solution was used to build a standard curve. The optical density of each sample was then applied to the curve to establish the analyte concentration in the recommended reporting units.

Criteria for acceptable ELISA results

The Food Allergy Research and Resource Program at the University of Nebraska-Lincoln utilizes certain criteria for acceptability of ELISA results. Manufacturer-provided standard curves must have an r^2 value of ≥ 0.98 and a coefficient of variation (CV) less than 20% among sample replicates for acceptance. These criteria have been adapted from Lipton et al. (2000). In the present study, these guidelines were adjusted and followed; any sample with a CV greater than 15% among replicates or a standard curve r^2 value of less than 0.97 was rerun.

Data Analysis

Data was quantified and analyzed in Microsoft Excel, GraphPad Prism Version 4.03, and any manufacturer-provided software (Neogen Veratox® Version 3 and r-Biopharm RIDASCREEN® Ridasoft Win Version 1.42). Curve fits were applied with software as recommended per manufacturer instructions. If no curve was recommended, a four-parameter sigmoidal dose response curve was used for quantitation. Statistical analyses were performed in Microsoft Excel and GraphPad Prism Version 4.03.

Kit Comparison Approaches

Upon literature review, it is apparent that casein sub-fractions occur in relatively stable proportions in bovine milk micelles. As suggested by Wal (2001b), the proportions for α-,β-, and κ-casein compose roughly 50%, 37%, and 13% of total casein in the bovine milk micelle. These proportions remain fairly consistent across breeds of cattle, climate, and geographical differences (Wal et al., 2001). ELISA kits that rely on casein detection for quantitation of milk residues use conversion factors based on the proportions of specific casein in the bovine milk micelle.

To compare results observed with different kits, several equations were used. To compare kits that use different reporting units, the Equations 3.1- 3.4 were used. Detailed equations are listed in Appendix A. Estimates for the proportion of individual protein fractions to

the overall milk protein concentration were obtained from (Wal et al., 2001). Estimates for the concentration of milk protein in NFDM were obtained from the USDA Nutrient Database and through LECO Dumas Analysis; NFDM contains approximately 35% milk protein.

Equation 3.1. Converting ppm casein to ppm milk protein

$$ppm\ casein\ \times \frac{1\ ppm\ milk\ protein}{0.8\ ppm\ casein} = 1.25\ ppm\ milk\ protein$$

Equation 3.2. Converting ppm BLG to ppm milk protein

$$ppm\ BLG \times \frac{1\ ppm\ milk\ protein}{0.1\ ppm\ BLG}\ = 10\ ppm\ milk\ protein$$

Equation 3.3. Converting ppm milk protein to ppm NFDM

$$ppm\ milk\ protein\ \times \frac{1\ ppm\ NFDM}{0.35\ ppm\ milk\ protein} = 2.86\ ppm\ NFDM$$

Equation 3.4. Converting ppm NFDM to ppm milk protein

$$ppm\ NFDM\ \times \frac{0.35\ ppm\ milk\ protein}{1\ ppm\ NFDM} = 0.35\ ppm\ milk\ protein$$

Equation 3.5. Applying conversion factors to spike concentrations for equivalent casein estimate

Protein	Conversion Factor
α-casein	**2**
β-casein	**2.7**
κ-casein	**7.7**

$$ppm\ spike\ (\alpha-, \beta-, \kappa-casein) \times conversion\ factor\ = equivalent\ ppm\ casein$$

Equation 3.6. Applying conversion factors to kit results for whey protein spikes

Protein	Conversion Factor
BLG	**10**
ALA	**20**

$$ppm\ spike\ (BLG\ or\ ALA) \times conversion\ factor = equivalent\ ppm\ milk\ protein$$

Equation 3.7. Calculating ratios of quantitation for ELISA kits using milk proteins

	Proportions		
Spike Material	**Proportion of casein**	**Proportion of MP**	**Proportion of NFDM**
α-casein	**0.5**	**0.4**	**0.14**
β-casein	**0.37**	**0.3**	**0.100**
κ-casein	**0.13**	**0.1**	**0.036**
BLG		**0.1**	**0.035**
ALA		**0.05**	**0.018**

$$\frac{\sum\left[\left(\frac{detected\ ppm\ with\ kit}{ppm\ spike\ level}\right)\times(proportion)\right]}{total\ number\ of\ spike\ levels^{a}} = average\ ratio\ of\ detection$$

[a]Applicable only for spike levels > 0 ppm.

4. Results

SDS-PAGE

All purified proteins (α-, β-, and κ-casein, BLG, and ALA) and milk ingredients (WPC34, WPC80, NaCas) were analyzed with SDS-PAGE. The primary purpose of electrophoresis was to evaluate the purity of individual proteins and the milk-derived ingredients to be examined with the commercial milk ELISA kits. Results obtained were used to estimate appropriate concentrations of milk proteins within each sample and account for the presence and quantity of unforeseen protein remnants (β-casein in purified α-caseins, casein in whey protein concentrates, etc.). NFDM was not evaluated with SDS-PAGE because it is documented to maintain the proportionality observed in bovine milk micelles (Wal, 2002b). Gel images are displayed in Figures 3.1-3.5. Purified protein samples are denoted with Greek letters, with the exception of the molecular weight marker, which is denoted above each lane as "X". Subscripts indicate μg of protein loaded in each well.

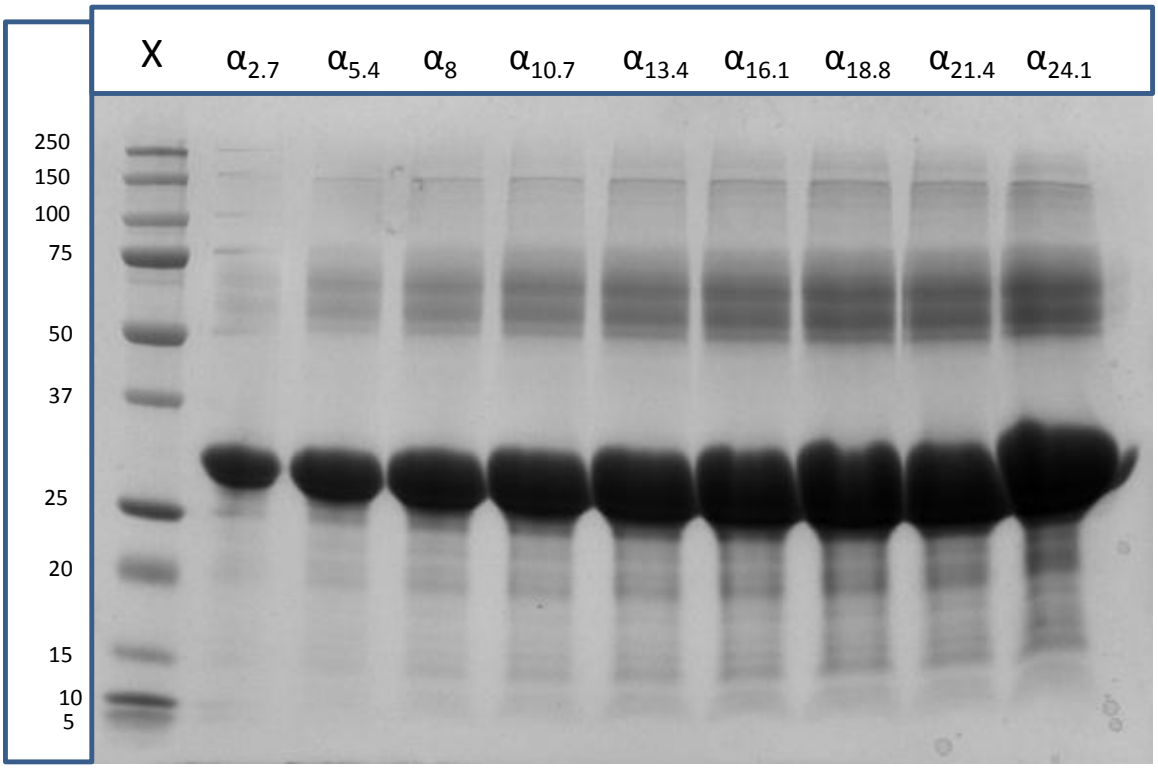


Figure 3.1. SDS-PAGE image of α-casein; ≥70% purity (Sigma-Aldrich). The purified protein sample was prepared under reducing conditions and the gel was run at 200V, fixed, stained, and imaged. Subscripts indicate μg of protein loaded in each well. The molecular weight marker (kDa) is denoted as lane "X".

Figure 3.1 displays the protein profile of purified α-casein. The sample was marketed as ≥70% purity. The gel image displays an intense band near the documented molecular masses of α_{s1}- and α_{s2}-casein (23.6 kDa and 25.2 kDa, respectively) (Jost, 1988). The band near 150 kDa corresponds with traces of immunoglobulins, which typically compromise about 3% of total protein in cow's milk (Wal, 2002b). The stained proteins between 50-75 kDa may correspond with bovine serum albumin (66 kDa) and lactoferrin (76 kDa). These proteins are responsible for about 1% of the proteins in fluid bovine milk. Low molecular weight protein bands that have migrated further than the α_s-caseins may be breakdown products. Some α_s-casein derived proteins are identified as members of the λ-casein subclass, but have yet to be thoroughly described. While the lowest concentration of protein loaded on the gel (2.7 μg) does not clearly

separate several bands within the casein region, it is known that caseins often migrate closely together in an SDS-PAGE format (Creamer, 1991).

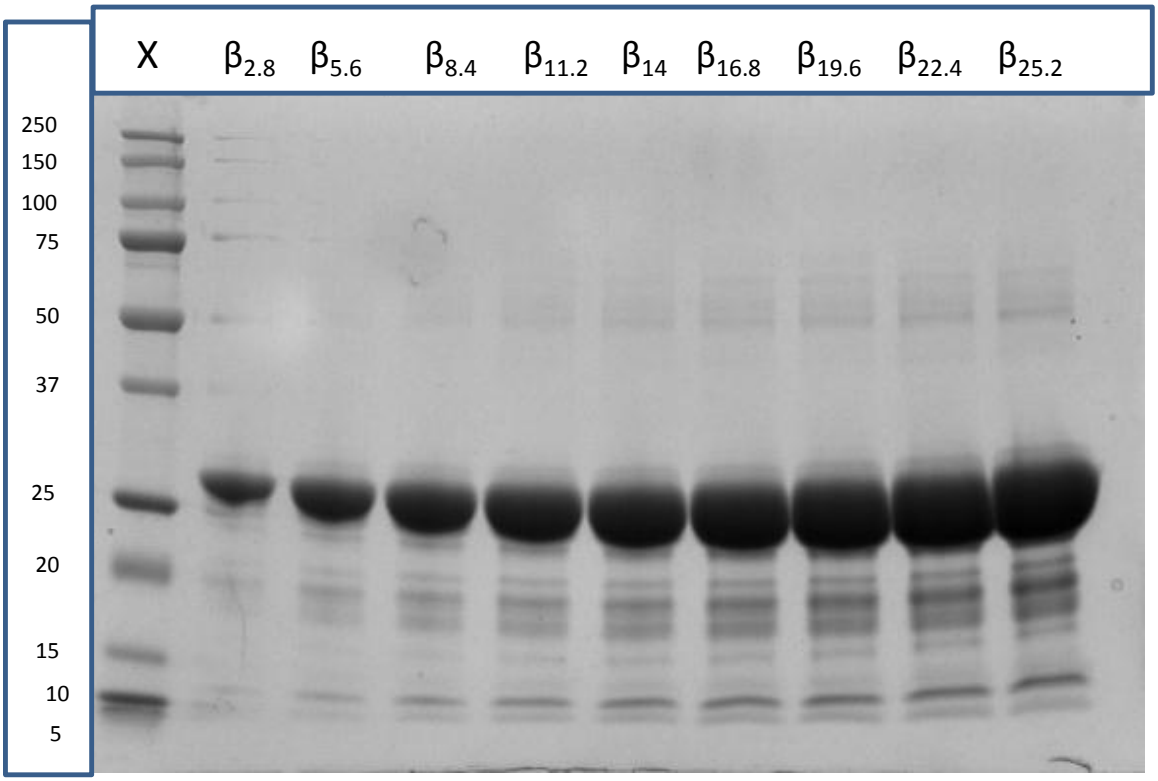


Figure 3.2. SDS-PAGE image of β-casein; ≥98% purity (Sigma-Aldrich). The purified protein sample was prepared under reducing conditions and the gel was run at 200V, fixed, stained, and imaged. Subscripts indicate μg of protein loaded in each well. The molecular weight marker (kDa) is denoted as lane "X".

The β-casein purchased for this experiment is listed as ≥98% purity. The gel displays an intensely stained band near 25 kDa (Figure 3.2). β-casein comprises nearly 30% of total milk protein and has a size molecular mass of 24 kDa (Wal, 2002b). Low intensity bands appear between 50-65 kDa, and may correspond to bovine serum albumin (66 kDa). Breakdown products in this gel (shown between 5-20 kDa) display different profiles from those bands observed in the separation of purified α-casein (see Figure 3.1). These bands may be identified as γ-caseins, the subclass of protein derived from β-casein breakdown.

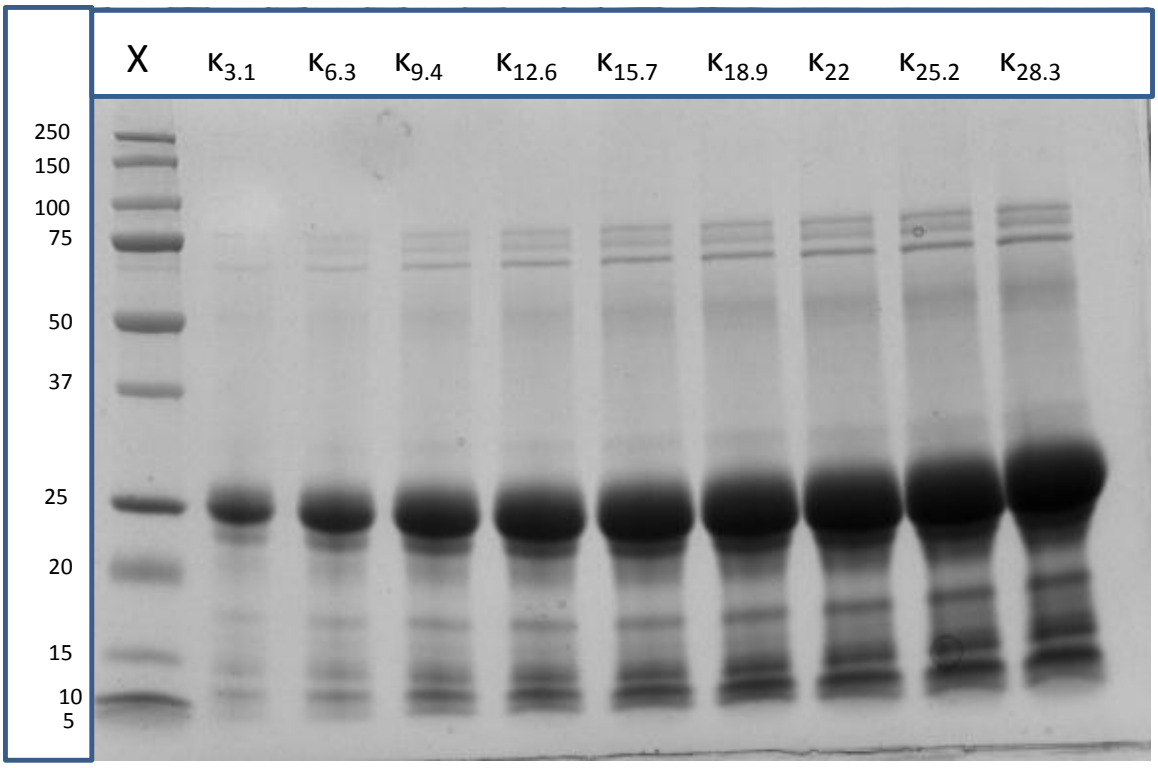


Figure 3.3. SDS-PAGE Image of κ-casein; ≥70% purity (Sigma-Aldrich). The purified protein sample was prepared under reducing conditions and the gel was run at 200V, fixed, stained, and imaged. Subscripts indicate μg of protein loaded in each well. The molecular weight marker (kDa) is denoted as lane "X".

In the κ-casein gel shown in Figure 3.3, a large band is observed around 25 kDa. While κ-casein is documented to have a molecular weight of 19 kDa, the caseins are known to migrate differently in SDS gel systems than their documented molecular weights would suggest (Veloso et al., 2004). The purity of the κ-casein purchased from Sigma-Aldrich is ≥70% purity. The band observed near 17-18 kDa may be intact κ-casein or another derivative; para-κ-casein is suggested to migrate between 10-15 kDa (Fox, 1989). However, the Phe_{105}-Met_{106} bond in κ-casein is typically only susceptible to proteolysis during cheesemaking (Fox, 1989). Other derivatives of κ-casein can be produced as well. The band observed near 70-75 kDa is possibly lactoferrin (76 kDa).

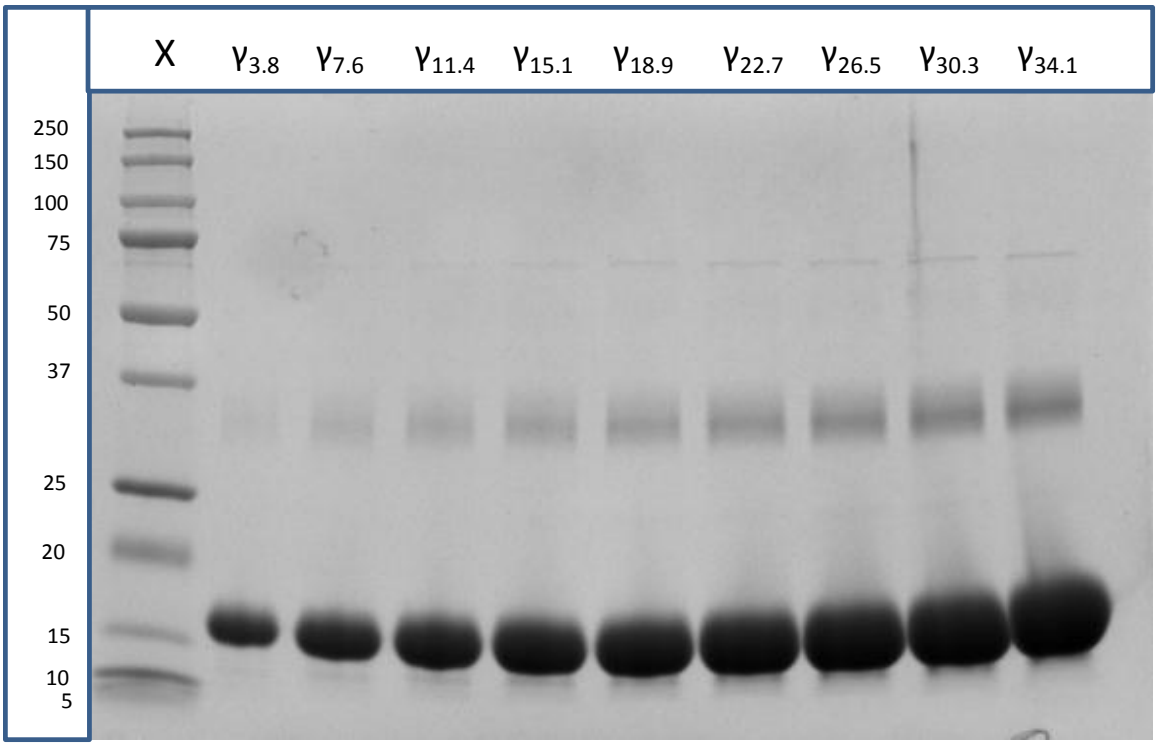


Figure 3.4. SDS-PAGE image of β-lactoglobulin: ≥90% purity (Sigma-Aldrich). The purified protein sample was prepared under reducing conditions and the gel was run at 200V, fixed, stained, and imaged. Subscripts indicate μg of protein loaded in each well. The molecular weight marker (kDa) is denoted as lane "X".

Figure 3.4 displays the gel image of βLG at ≥90% purity. The most intense band observed on the gel migrates near 17 kDa. BLG is documented to have a molecular weight of about 18 kDa (Wal, 2002b). A medium-intensity band is observed near 32-35 kDa and may correspond to BLG in dimer form. BLG commonly forms dimers when present at high concentrations, and the bands may indicate inefficient reduction by SDS and dithiothreitol during sample preparation. However, it is also possible for α- and β-caseins to migrate in this region. A low-intensity band is observed near 70 kDa and may correspond to bovine serum albumin (66 kDa) or lactoferrin (76 kDa). The level of contaminants in this sample is quite low in comparison to the samples evaluated in Figures 3.1 and 3.3 (α- and κ-casein, respectively).

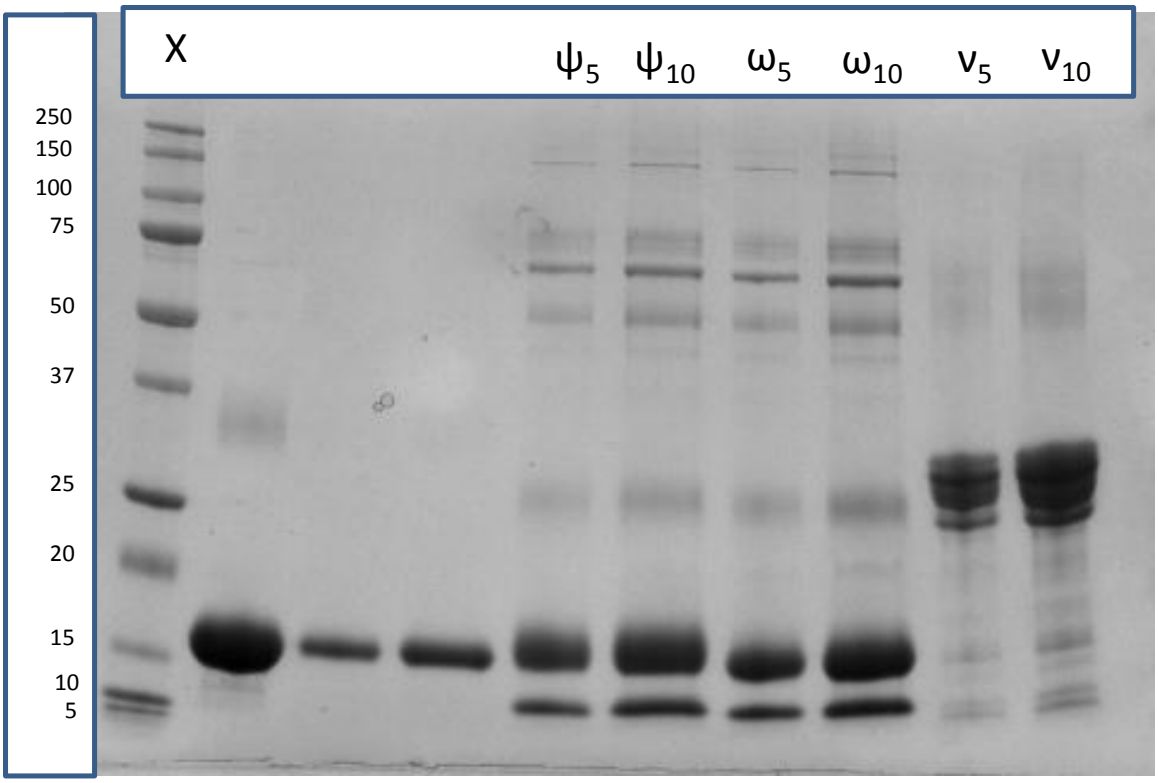


Figure 3.5. SDS-PAGE of Milk-Derived ingredients. Samples were as follows: ψ: whey protein (WPC34); ω: whey protein (WPC80); ν: sodium caseinate. Subscripts indicate μg protein loaded in each well. The molecular weight marker (kDa) is denoted as lane "X". Samples were run under reducing conditions at 200V, fixed, stained, and imaged.

The analysis of whey protein concentrates revealed that when compared at equivalent levels of protein, the sample purity was similar (see ψ and ω lanes, Figure 3.5). Whey protein concentrates at 34% and 80% protein exhibit similar separation patterns with intense bands migrating near the appropriate locations for BLG and ALA (18 and 14 kDa, respectively). In WPC80, the band corresponding to BLG (near 17 kDa on the gel) appears slightly smaller, yet of similar intensity, than the same band observed in WPC34. Breakdown products of the whey proteins are not observed in Figure 3.5, as the bands have migrated beyond the bottom of the gel. As observed in Figures 3.3 and 3.4, the band near 70-75 kDa may parallel lactoferrin (76 kDa) or bovine serum albumin (66 kDa). The band displayed near 150 kDa in Figure 3.5 corresponds with the molecular weight of immunoglobulins (150 kDa) and is also observable in Figure 3.1. In

the casein migration region (20-25 kDa), a wide low-intensity band is displayed and corresponds to trace levels of caseins.

In the sodium caseinate lane where 5 μg of protein is loaded, at least two distinct bands are observable in the region where casein typically migrates (lane ν_5, Figure 3.5). Some breakdown products are observed in sodium caseinate samples, migrating between 5-20 kDa (ν lanes, Figure 3.5). Bands migrating in the low molecular weight region may also be derivatives of BLG and ALA. Non-specific shadowing seen in Figure 3.5 between 50 kDa is also shown in the casein gels (Figures 3.1-3.3). While the shadowing may be a result of traces of bovine serum albumin or lactoferrin, it could also be derived from residues of small milk enzymes.

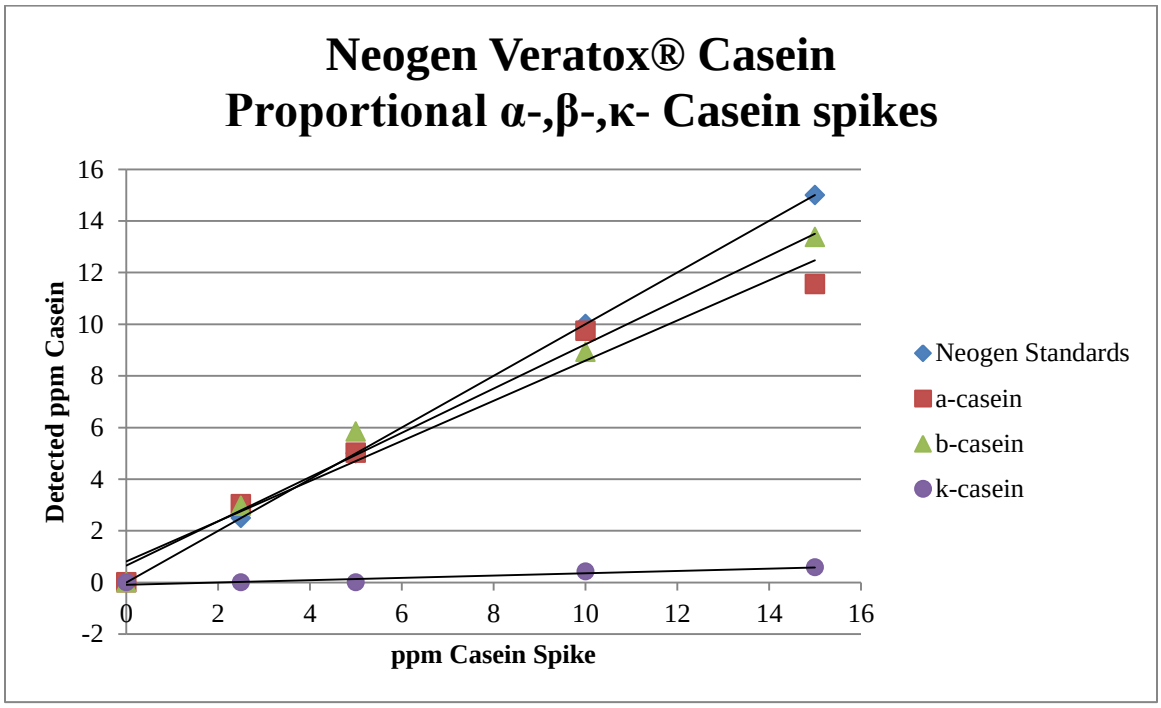
Electrophoresis results indicate that purified protein samples are sufficiently pure for ELISA analysis. Purity levels for purchased proteins are estimated to meet or exceed the values listed on the container packaging. Specifically, purity levels listed on the packaging for α-, β-, and κ-casein and BLG are at or above 80%, 98%, 85%, and 90%, respectively.

Casein Kits and Detection of α-, β-, and κ-casein

Spiked samples of α-,β-,and κ-casein were analyzed with Neogen Veratox® Casein, ELISA Systems™ Casein, and r-Biopharm RIDASCREEN® Fast Casein ELISA kits. Figures 3.6-3.8 display the differential detection of casein fragments by the various casein ELISA kits.

For the Neogen Veratox® Casein kit, several equations and conversions were used to prepare data for comparison. Spike concentrations were created by weight. To convert the spike concentration from units of α-, β-, and κ-casein to the units of the calibrant used in the kit (ppm NFDM), Equations 3.5, 3.1, and 3.3 were applied. The ratios of quantitation to kit standards supplied with the kit were calculated using Equation 3.7. For more detailed information and equations regarding conversions, see Appendix A. Spikes of α-and β-casein are detected at similar levels to the kit standards and exhibits ratios of quantitation of 1.0 for both materials.

However, κ-casein is not detected at any of the spike levels evaluated with the Neogen Veratox® Casein kit and exhibits a ratio of 0.04.

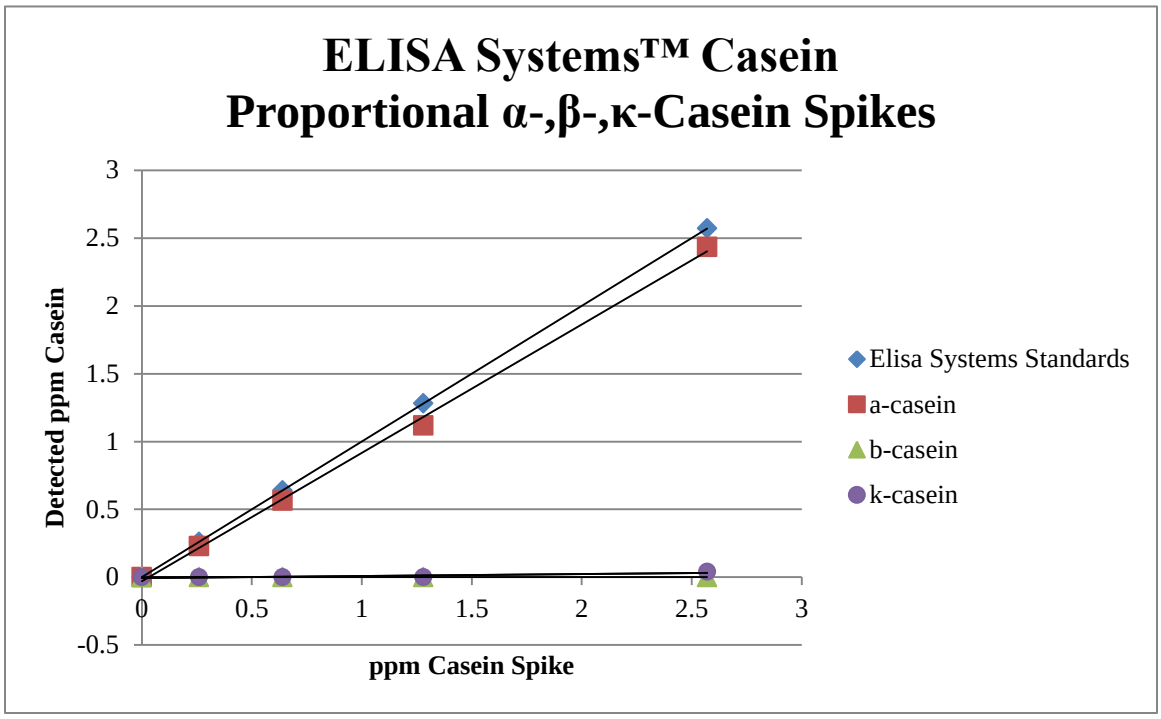


	α-casein	β-casein	κ-casein
%CV	5.82%	5.91%	8.76%
Correlation	0.9849	0.9941	0.9502
Ratio to kit Stds	1.0	1.0	0.04

Figure 3.6. Neogen Veratox® Casein Proportional ELISA Results

In the ELISA Systems™ Casein kit, the spike concentrations were converted to equivalent values in ppm casein using Equation 3.4. Ratios of quantitation for each spike were calculated using Equation 3.7. With the ELISA Systems™ Casein kit, α-casein is primarily detected. Spikes of β- and κ-casein are virtually undetectable (Figure 3.7). α-casein spikes are

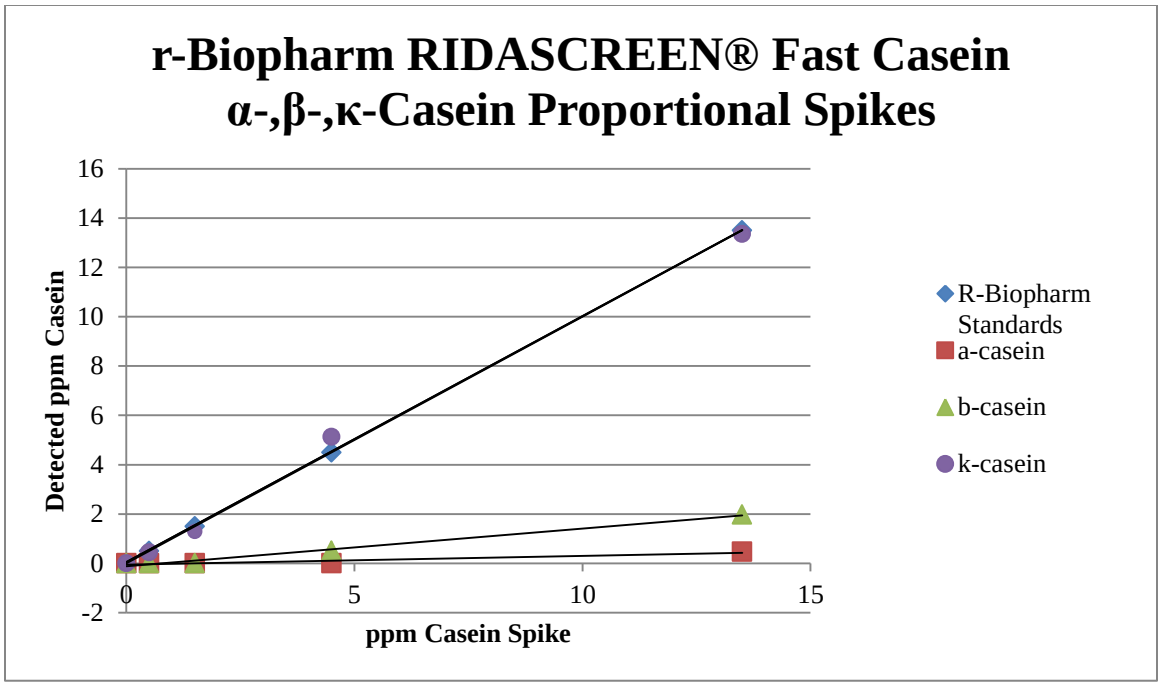
detected at a ratio of 0.9, while ratios are non-calculable for β- and κ-casein.



	α-casein	β-casein	κ-casein
%CV	11.59%	N/A	N/A
Correlation	0.9992	N/A	N/A
Ratio to kit standards	0.9	0.0	0.0

Figure 3.7. ELISA Systems™ Casein Proportional ELISA Analysis

The r-Biopharm RIDASCREEN® Fast Casein kit supplies standards in units of ppm casein. Spikes concentrations were converted to the units of the kit calibrant using Equation 3.5. Ratios of quantitation for each spiking material were calculated using Equation 3.7. According to analysis performed with the r-Biopharm RIDASCREEN® Fast Casein kit, the antibodies in the kit are highly sensitive to the presence of κ-casein (ratio of 1.0) (Figure 3.8). α-casein is minimally detected (ratio of 0.01), while detection of β-casein occurs at a ratio of 0.1 to the kit standards.



	α-casein	β-casein	κ-casein
%CV	4.07%	6.18%	7.47%
Correlation	0.9500	0.9945	0.9982
Ratio to kit Stds	0.01	0.1	1.0

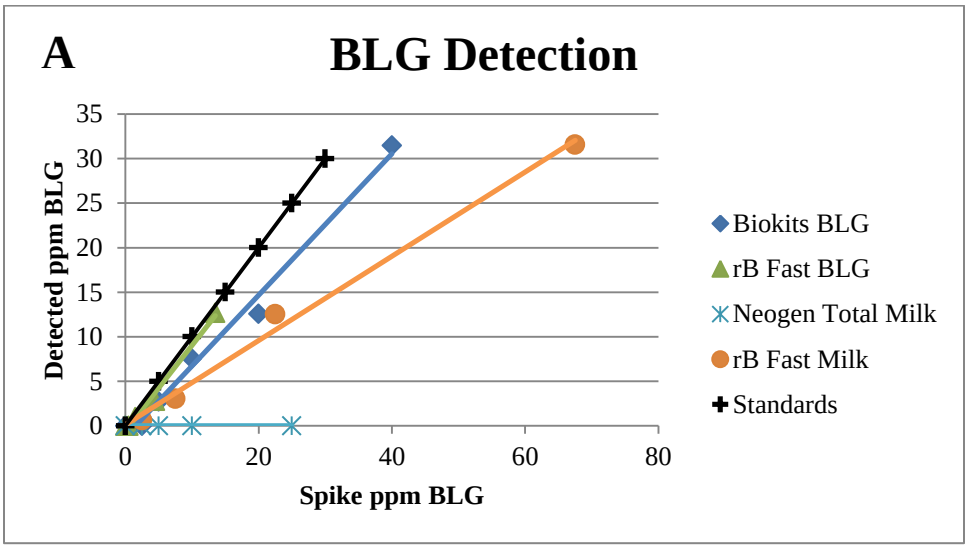
Figure 3.8. r-Biopharm RIDASCREEN® Fast Casein Proportional ELISA Analysis

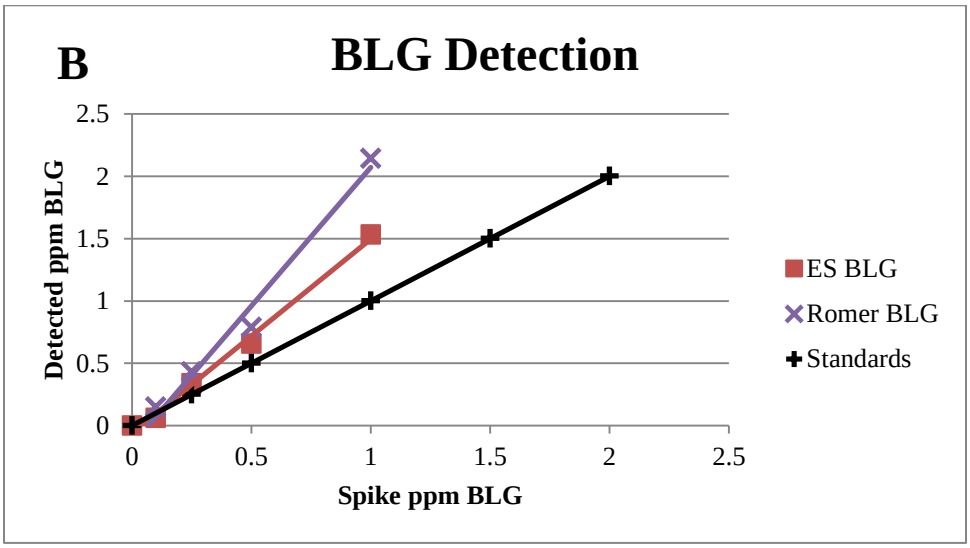
When the bovine milk micelle proportions are applied to the data, results indicate that the r-Biopharm RIDASCREEN® Fast Casein kit detects κ-casein at the same capacity as observed with the standard solutions (Figure 3.8). The β- casein spikes are minimally detected at the highest spike concentration evaluated, while the α-casein spikes are not significantly detected at any level capable of influencing kit quantitation.

BLG Kits and Detection of Whey Proteins

BLG kits evaluated include Neogen BioKits™ BLG, ELISA Systems™ BLG, r-Biopharm RIDASCREEN® Fast BLG, and Romer Labs® BLG. Spikes were created of ALA and BLG at concentrations equivalent to those provided by the kit manufacturers. Total milk kits were also used to evaluate BLG and ALA spikes. Equations 3.2, 3.3, and 3.6 were used to

correlate concentrations of BLG and ALA spikes to units of milk protein and NFDM. Ratios of quantitation were calculated using Equation 3.7. Results are displayed in Figure 3.9.





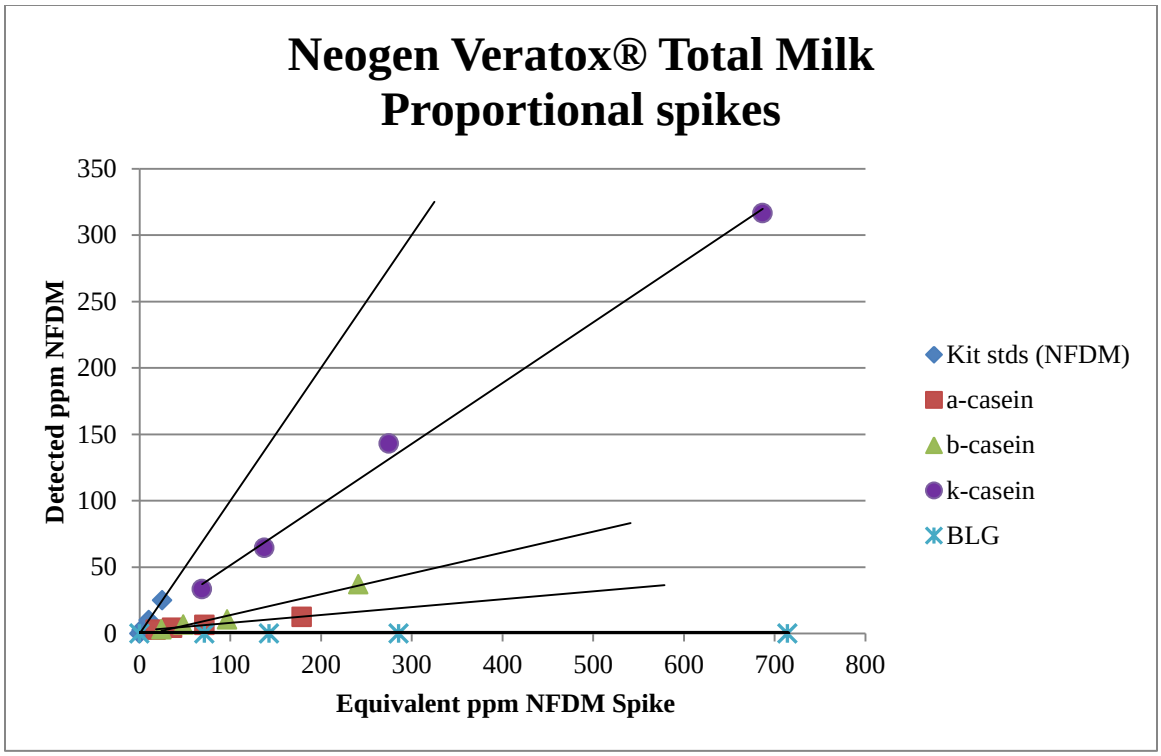
	BIOKITS BLG	ES BLG	rB Fast BLG	Romer BLG	Neogen TM	rB Fast Milk
%CV	10.9%	14.0%	10.0%	8.2%	N/A	3.5%
Correlation	0.9944	0.9983	0.9954	0.9912	N/A	0.9970
Ratio to Stds	0.68	1.20	0.73	1.73	0.00	0.42

Figure 3.9. Results of BLG ELISA Kits. Kit standards are manufacturer-provided solutions of BLG. In the total milk kits, results are converted to ppm BLG using Equations 3.4 and 3.2, where appropriate. A. Kits with dynamic ranges at 0-60 ppm BLG. B. Kits with dynamic ranges at 0-2.5 ppm BLG.

Less variation is observed in detection among BLG kits than with casein kits (Figure 3.9). All BLG-specific kits are capable of detecting BLG. The sensitivity of these kits to BLG spikes varies (Figure 3.9). Interestingly, none of the kits evaluated detect BLG at the same sensitivity as calibration solutions provided with the kits (Figure 3.9). The ELISA Systems™ BLG and r-Biopharm RIDASCREEN® Fast BLG kit have the closest ratios of quantitation to the kit standards (1.20 and 0.73, respectively). The Neogen Veratox® Total Milk kit is unable to detect BLG at any of the levels tested; this is discussed further in the following section.

Total Milk Kits and Detection of Milk Proteins

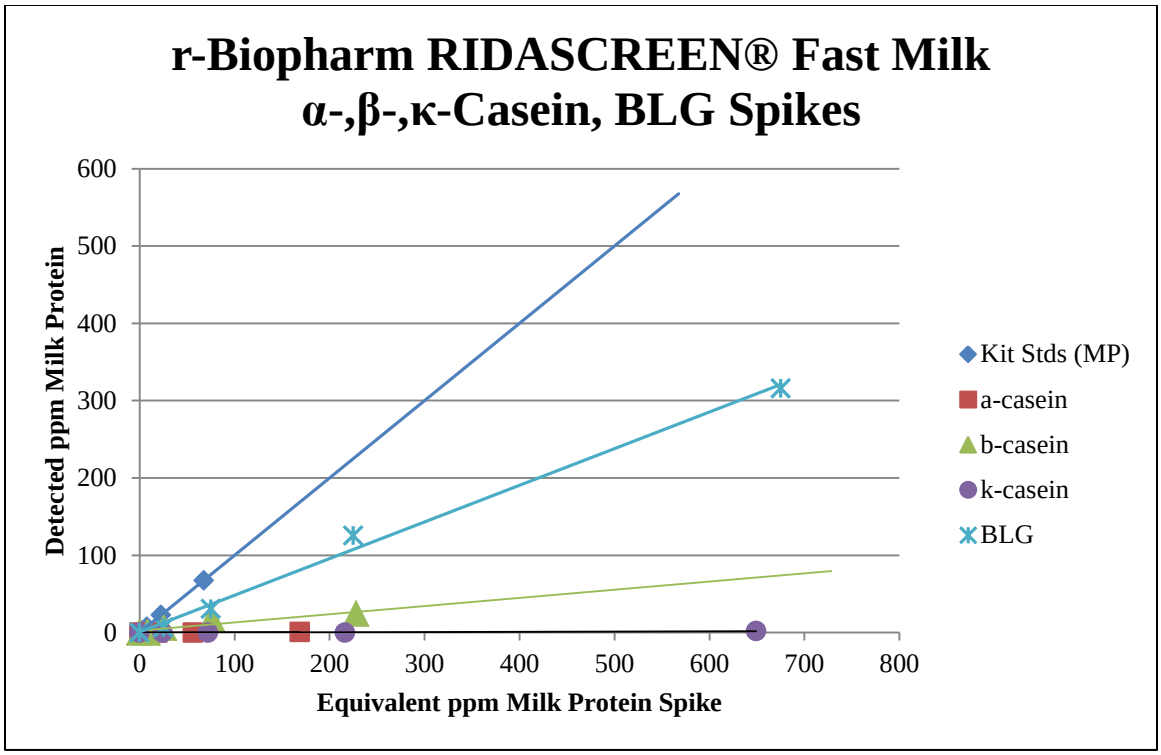
Two total milk kits were analyzed in this study; Neogen Veratox® Total Milk and r-Biopharm RIDASCREEN® Fast Milk. The Neogen Veratox® Total Milk kit reports results in ppm NFDM, while the r-Biopharm RIDASCREEN® Fast Milk kit reports results in ppm milk protein. Equations 3.1-3.7 were applied to the data to compare results across kits and analytes. Neither kit was capable of detecting ALA at any concentration evaluated, and these points are not displayed in Figures 3.10 or 3.11. Data obtained with the Neogen Veratox® Total Milk kit is displayed in Figure 3.10.



	α-casein	β-casein	κ-casein	BLG
Proportion of NFDM	14%	10%	4%	4%
Ratio to kit standards	0.11	0.13	0.48	N/A

Figure 3.10. Neogen Veratox® Total Milk Proportional Spikes

While the Neogen Veratox® Total Milk is capable of detecting α-, β-, and κ-casein, the kit fails to detect BLG or ALA at any level evaluated (Figures 3.10). As shown, the ratios of detection of individual milk proteins are quite low compared to ratios observed in other evaluated kits (ratios of 0.1, 0.1, and 0.5, for α-, β-, and κ-casein, respectively). Detection and quantification with the Neogen Veratox® Total Milk kit primarily depends on sensitivity to κ-casein and less on sensitivity to β- and α-casein (Figure 3.10).



	α-casein	β-casein	κ-casein	BLG
Proportion of MP	40%	30%	10%	10%
Ratio to kit standards	0.00	0.19	0.00	0.42

Figure 3.11. Proportional detection with r-Biopharm RIDASCREEN® Fast Milk ELISA. Kit standards are manufacturer-provided solutions of milk protein.

According to results, the r-Biopharm Fast Milk kit also has limitations. α-casein and κ-casein are undetectable using the kit (Figure 3.11). The r-Biopharm Fast Milk kit fails to detect ALA. However, the kit is able to detect one protein from each fraction (casein and whey) of bovine milk; β-casein and βLG. The r-Biopharm RIDASCREEN® Fast Milk kit relies primarily on BLG detection and to a lesser extent on β-casein for quantitation (ratios of 0.4 and 0.2, respectively) (Figure 3.11).

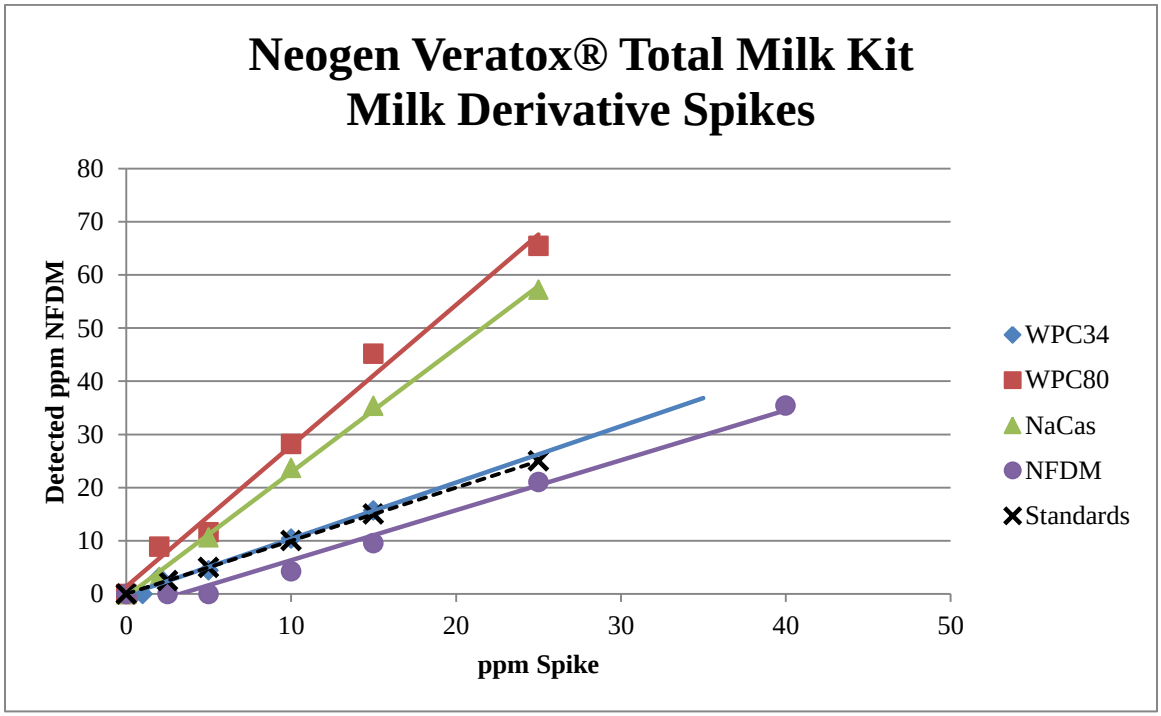
Detection of Milk-Derived Ingredients with ELISA kits

Spikes of milk-derived ingredients were evaluated with Neogen Veratox® Total Milk and r-Biopharm RIDASCREEN® Fast Milk kits. In the Neogen Veratox® Total Milk kit, results were reported in ppm NFDM. The results of the r-Biopharm RIDASCREEN® Fast Milk kit

were reported in ppm milk protein. The protein concentration of each milk-derived ingredient was determined using LECO Dumas Analysis (Table 3.2). The protein estimates displayed in Table 3.2 were applied to Equation 3.8 to determine the equivalent or expected concentration of spikes of derivative ingredients. Results without conversions for the Neogen Veratox® Total Milk are presented in Figure 3.12.

Table 3.2. LECO Dumas Protein Analysis of Milk-Derived Ingredients

Derivative	% Protein (g/100g)
NFDM	36%
WPC34	32.55%
WPC80	78.47%
NaCas	89.88%



Neogen Veratox® Total Milk kit				
Ingredient	NFDM	WPC34	WPC80	NaCas
Ratio to Stds	0.70	1.13	3.04	2.14

Figure 3.12. Neogen Veratox® Total Milk Analysis of Milk Ingredient Spikes. Standards are manufacturer-provided solutions of NFDM.

The results in Figure 3.12 display the detection of milk residues in milk-derived ingredients. The Neogen Veratox® Total Milk kit detects a higher concentration of milk residues in WPC80 and sodium caseinate than in the calibrator solutions. These results are expected, given the higher concentration of protein present in these materials (Table 3.2). WPC 80 contains approximately 78.5% protein, while sodium caseinate contains 90% protein. WPC34 is detected at almost the same level as the standard solutions provided by Neogen®. NFDM spikes display the lowest level of detection with this kit although the detection ratio does not vary substantially from the standard. Ratios of quantitation are also displayed; WPC80 and sodium caseinate exhibit high ratios (3.04 and 2.14, respectively). The advantage of viewing the milk ingredient data in a non-proportional format (Figure 3.12) is that it reflects the conditions of incidental contamination during manufacturing. The spikes were created on a w/w basis, all other conditions were normalized, and ELISA detection is based on protein content and kit sensitivity. While the trends observed in Figure 3.12 agree with the percentage of protein in the milk derived ingredients, the spike concentration and results are not presented in the same units, resulting in skewed depiction and comparison of residue detection. Conversion factors for converting spike ingredients to reporting units were calculated and results are listed in Table 3.3. Detailed equations for the calculation of conversion factors are shown in Equations A.11-A.16. To correct the skewed results, Equation 3.8 was applied to convert spike concentrations to ppm milk protein for the r-Biopharm RIDASCREEN® Fast Milk kit. Equation 3.9 was applied to milk ingredient spike concentrations to calculate the equivalent ppm NFDM.

Table 3.3. Conversion Factors for ppm spike to ppm reporting unit

Spike Ingredient	CF_1 - equivalent MP	CF_2- equivalent NFDM
WPC34	1.62	4.63
WPC80	3.92	11.21
NaCas	1.12	3.21

Equation 3.8. Converting spike concentration to ppm milk protein

$$ppm\ spike\ \times CF_1^a = equivalent\ ppm\ MP$$

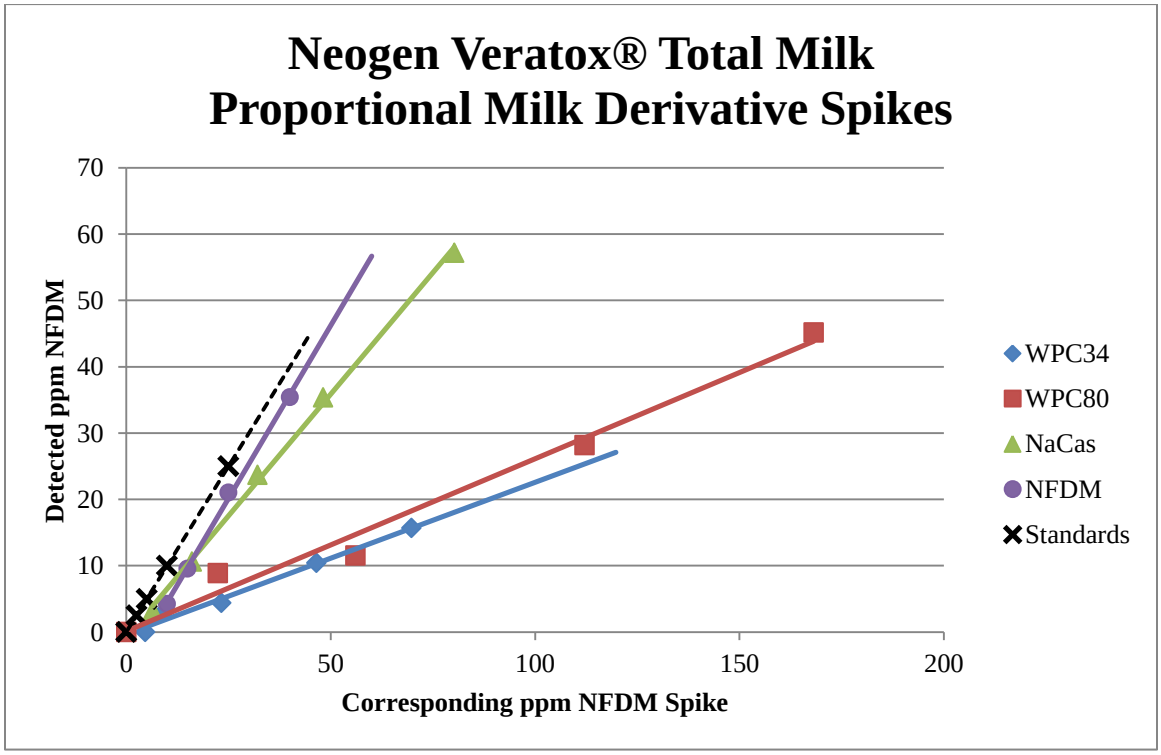
[a]Conversion factors taken from Table 3.3. Detailed equations shown in Equations A.11, A.13, amd A.15.

Equation 3.9. Converting spike concentrations to ppm NFDM

$$ppm\ spike\ \times CF_2^a = equivalent\ ppm\ NFDM$$

[a]Conversion factors listed in Table 3.3. Detailed equations for the calculation of conversion factors are shown in Equations A.12, A.14, and A.16.

Applying Equation 3.9 to the spike concentrations used depicts a more accurate representation of kit ability to detect milk residues in various milk-derived ingredients. Figure 3.13 displays corrected data for the Neogen Veratox® Total Milk kit. Milk residues in NFDM and sodium caseinate spikes are detected most accurately with the Neogen Veratox® Total Milk kit (0.70 and 0.71, respectively). Milk residues in spikes of WPC are detected with similar affinity, exhibiting ratios of 0.27 and 0.24 for WPC34 and WPC80, respectively. The detection of WPC with the Neogen Veratox® Total Milk kit is somewhat surprising considering the lack of detection of spikes of BLG and ALA with this kit.

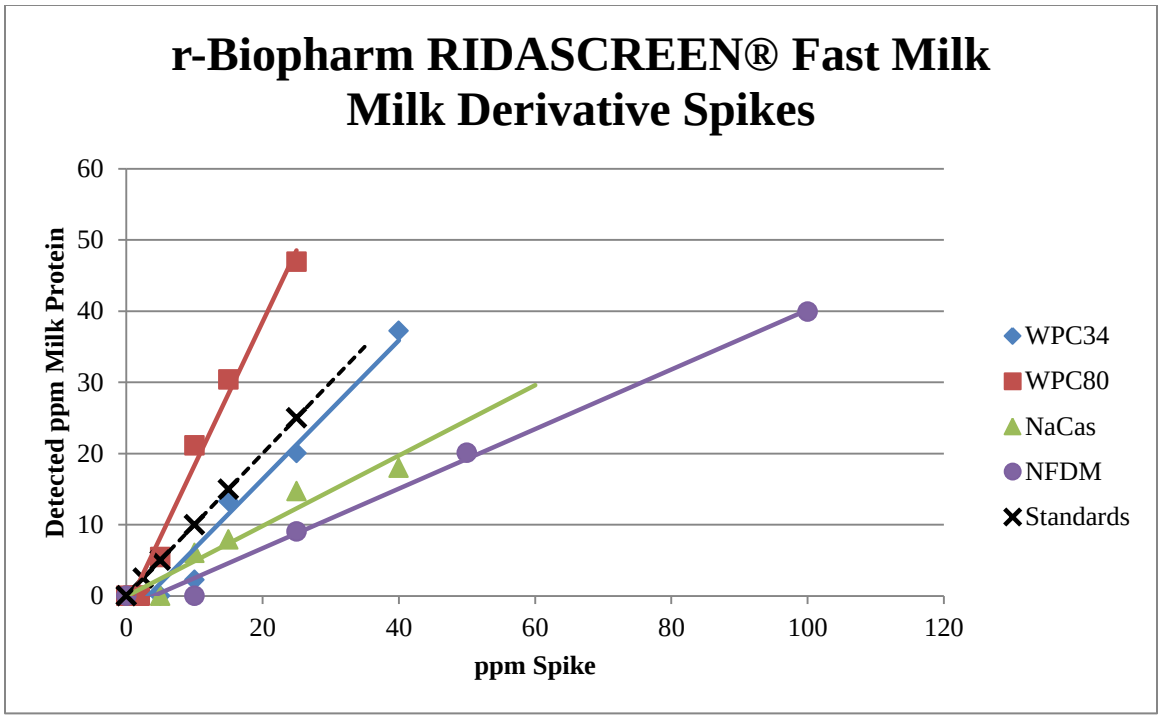


Neogen Veratox Total Milk				
Ingredient	NFDM	WPC34	WPC80	NaCas
%CV	8.11%	8.7%	9.78%	11.1%
Correlation	0.9991	0.9923	0.9924	0.9987
Ratio of Detection	0.70	0.24	0.27	0.71

Figure 3.13. Proportional Neogen Veratox® Total Milk Analysis of Milk Ingredients Spikes. Standards are manufacturer-provided solutions of NFDM.

With the r-Biopharm RIDASCREEN® Fast Milk kit, initial observations suggest that WPC80 is the most strongly detected with the kit (Figure 3.14, ratio of 1.78). Milk residues of WPC34 appear to be present at similar levels to those observed in the kit standards (ratio of 0.71). NFDM spikes are the least detected by the kit (ratio of 0.4, Figure 3.14). Based on antibody specificity, the kit is primarily sensitive to BLG and β-casein (ratios of 0.4 and 0.2, respectively) (Figures 3.8 and 3.9). Given the results, it is apparent that the kit has a higher sensitivity to whey proteins than caseins. The data depicted in Figure 3.13 give indications of potential results observed when using this kit to analyze traces of residue of different milk ingredients in manufacturing settings.

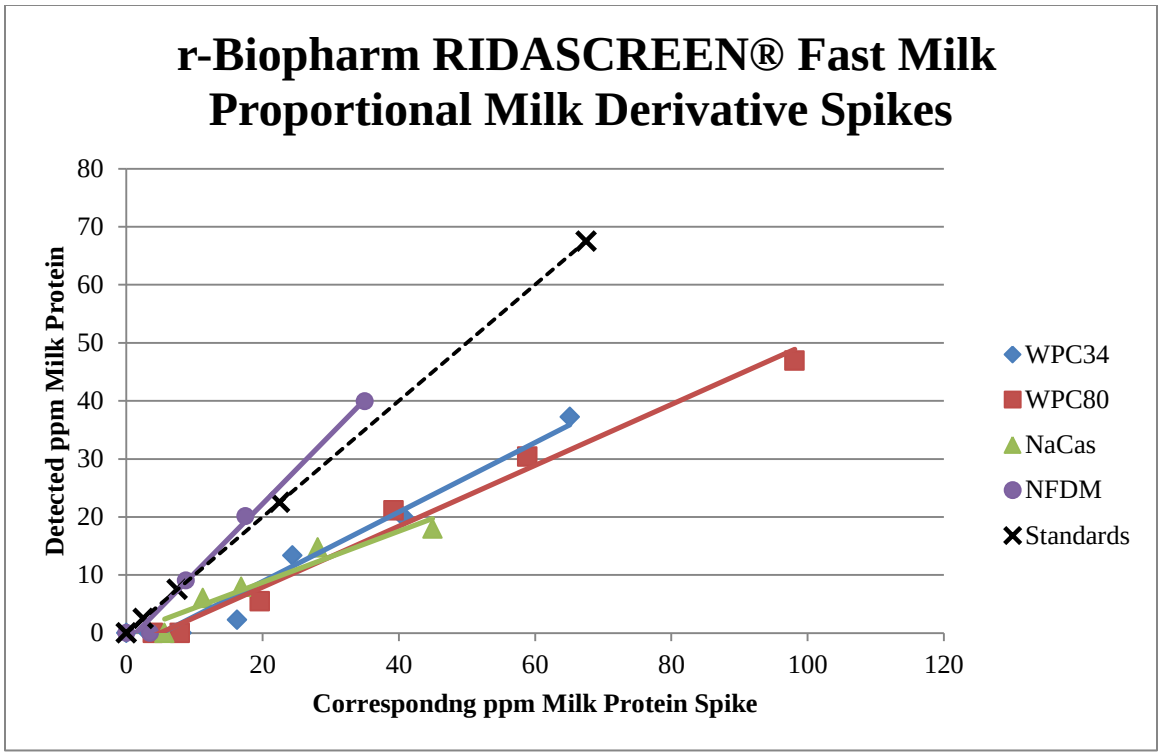
However, the data observed in Figure 3.14 are skewed; spike concentrations depicted on the X axis of Figure 3.14 and used to calculate ratios of detection are not represented in the same units as the kit calibrant and are not normalized to the protein content. To convert spike concentrations to equivalent ppm milk protein, Equation 3.8 was used with conversion factors represented in Table 3.3. For more detailed information regarding the calculation of conversion factors for the r-Biopharm RIDASCREEN® Fast Milk kit, see Equations A.11, A.13, and A.15.



r-Biopharm RIDASCREEN® Fast Milk				
Ingredient	NFDM	WPC34	WPC80	NaCas
Ratio to stds	0.40	0.71	1.78	0.54

Figure 3.14. r-Biopharm RIDASCREEN® Fast Milk Analysis of Milk Ingredient Spikes. Standards are manufacturer-provided solutions of milk protein.

Once the data has been corrected using Equation 3.8, more accurate assessments of kit results can be performed. With the r-Biopharm RIDASCREEN® Fast Milk kit, NFDM is detected with the highest sensitivity at a ratio of 1.14 of the actual milk residues present (Figure 3.15). Additionally, WPC34, WPC80, and NaCas are all detected near the same level (ratios of 0.53, 0.45, and 0.48, respectively) (Figure 3.15). According to previous work, the r-Biopharm Fast Milk kit is primarily sensitive to BLG (ratio of 0.4) and exhibits some sensitivity to β-casein (ratio of 0.2). Because of the accuracy and sensitivity exhibited with the detection of NFDM, it is possible that NFDM or a closely related milk derivative was used for kit calibration.



r-Biopharm RIDASCREEN Fast Milk				
Ingredient	NFDM	WPC34	WPC80	NaCas
%CV	5.52%	8.8%	7.76%	12.9%
Correlation	0.9996	0.9912	0.9875	0.9707
Ratio of Detection	1.14	0.54	0.45	0.48

Figure 3.15. Proportional r-Biopharm RIDASCREEN® Fast Milk Analysis of Milk Ingredient Spikes. Standards are manufacturer provided solutions of milk protein.

The r-Biopharm RIDASCREEN® Fast Milk kit detected WPC and NFDM at higher levels than the Neogen Veratox® Total Milk kit. However, sodium caseinate was detected more efficiently using the Neogen Veratox® Total Milk kit than when the r-Biopharm RIDASCREEN® Fast Milk kit was used. Given the increased sensitivity of the Neogen Veratox® Total Milk kit to κ-casein and the primary sensitivity of the r-Biopharm RIDASCREEN® Fast Milk kit to BLG, these results are expected (Figures 3.16 and 3.18). NFDM exhibited the highest level of reactivity for both total milk kits among milk-derived ingredients (Figures 3.13 and 3.15).

Detection of NFDM using milk ELISA kits

NFDM was evaluated using all nine commercial milk ELISA mentioned throughout this study (Figure 3.16). Results indicate that the Neogen BioKits™ BLG kit detects NFDM at the highest ratio of all kits evaluated (ratio of 2.0, Figure 3.16 and Table 3.4). However, this kit is not capable of detecting trace residues of NFDM. The kit with the highest level of sensitivity to NFDM is the Romer Labs™ BLG kit, which is capable of detecting milk residues in samples containing 1ppm NFDM.

Of the two total milk kits evaluated, the Neogen Veratox® Total Milk kit is more sensitive to milk residues in NFDM than the r-Biopharm RIDASCREEN® Fast Milk kit. The Neogen Veratox® Total Milk kit detects milk residues in the 10 ppm NFDM spike, while detection of milk residues with the R-Biopharm RIDASCREEN® Fast Milk kit does not occur until spikes reach 25 ppm NFDM. In terms of quantitative accuracy, the Neogen Veratox® Total Milk kit more accurately represents the concentration of milk residues present in the sample than the r-Biopharm® Fast Milk kit (ratios of 1.1 and 0.7, respectively) (Table 3.4).

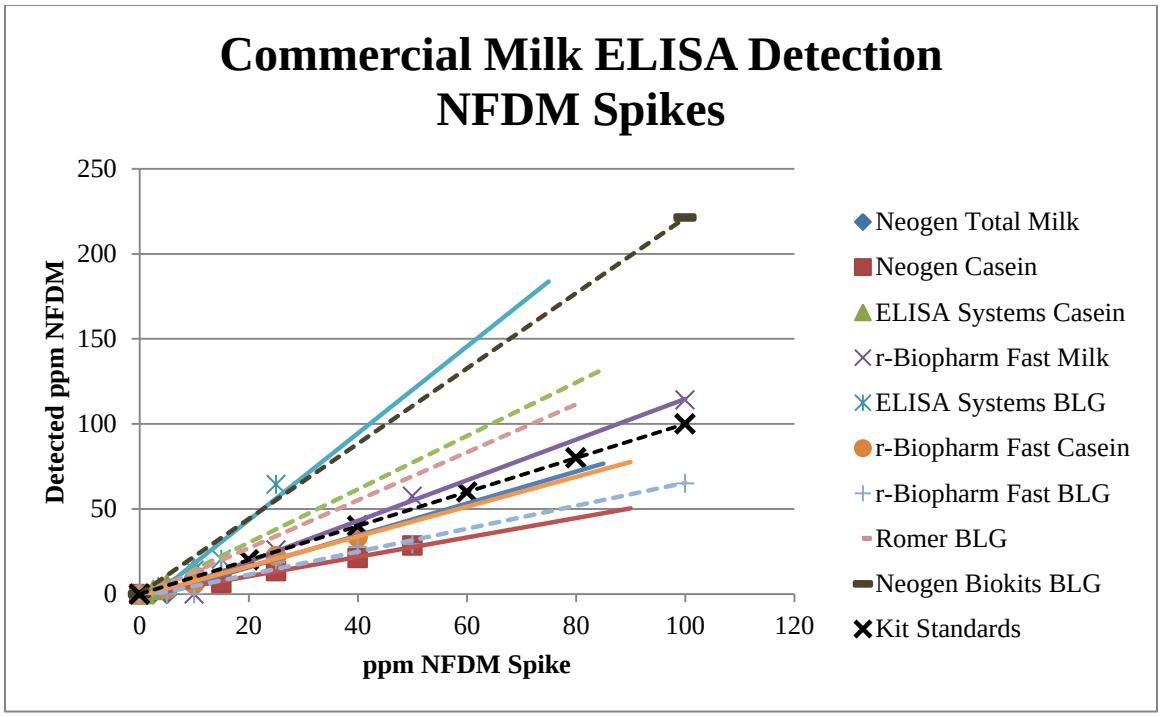


Figure 3.16. Detection of NFDM spikes by commercial milk ELISA kits. The Neogen BioKits™ BLG data point only displayed at lowest spike value (approximately 100 ppm NFDM spike, 220 ppm NFDM detected). Standards are manufacturer-provided solutions of milk residues converted to equivalent ppm NFDM using Equations 3.1, 3.2, and 3.3.

Table 3.4. Sensitivities of Milk ELISA Kits to Purified Milk Proteins and Milk Ingredients

Analyte	α-casein	β-casein	κ-casein	BLG	ALA	WPC34	WPC80	NaCas	NFDM
Proportion (Wal, 2001)	40%	30%	10%	10%	5%				
Ratio to kit standards									
Neogen Veratox® Casein	1	1	0						0.5
ELISA Systems Casein	0.9	0	0						1.2
r-Biopharm Fast Casein	0	0.1	1						0.7
Neogen Veratox® Total Milk	0.1	0.1	0.5	0	0	0.2	0.3	0.7	0.7
r-Biopharm Fast Milk	0	0.2	0	0.4	0	0.5	0.5	0.5	1.1
Neogen BioKits BLG				0.7	0				2.0
ELISA Systems BLG				1.2	0				1.5
r-Biopharm Fast BLG				0.7	0				0.7
Romer Labs BLG				1.7	0				0.9

5. Discussion

Detecting milk residues in foods is a challenge due to the variety of milk-based ingredients and the availability of ELISA kits with varying targets. The selection of the appropriate kit for any given application is an additional challenge. This research highlights that each kit manufacturer uses antibodies of varying specificity and affinity in the development of commercial ELISAs. While most ELISA manufacturers do not reveal the nature of the antibodies used in detection of milk residues, some information can be inferred from this data. Casein ELISA kits detect various casein fractions, BLG kits detect BLG, and total milk kits detect various milk proteins. Although a major whey protein and an important allergen, ALA is not detected by any milk kit tested. In addition to varying antibody specificities, kits recommend or provide extraction buffers comprised of various chemical components that affect the extraction of proteins from a food matrix and solubility in the buffer system, in turn affecting results.

All kits evaluated are capable of detecting milk residues in NFDM. However, variation in detection observed with NFDM spikes among kits highlights the lack of standardization among commercial ELISA kits. Because kits are not developed using the same materials, antibody sensitivities, or calibrated to a standard reference material, even testing the sample with multiple kits will not provide consistent results. Users are often required to select kits with little to no information about kit applicability for specific samples or processing methods.

Purified protein analysis reveals that r-Biopharm RIDASCREEN® Fast Milk kit detects one protein from both casein and whey fractions: β-casein and BLG. However, the Neogen Veratox® Total Milk kit only detects proteins from the casein fraction. Sole reliance of this kit on detection of κ-casein for accurate estimation of whole casein in a kit is cause for concern. In addition to being the least prevalent of the casein sub-fragments (approximately 13% of total casein), it is known that κ-casein is more sensitive to proteolysis than other caseins and will interact with other milk proteins upon heat treatment (Wal, 2002a). Using κ-casein as the

primary target for detecting milk residues with ELISA kits may only be suitable for specific food products and applications. Both total milk kits are capable of detecting milk residues in all milk-derived ingredients evaluated. Results suggest that even in samples that contain primarily whey protein, the Neogen Veratox® Total Milk kit is capable of detecting and quantifying substantial milk residues. In the analysis of NFDM spikes, Neogen Veratox® Total Milk kit is more quantitatively accurate and more sensitive to the presence of milk residues than the r-Biopharm RIDASCREEN® Fast Milk kit. While dependent on application, the Neogen Veratox® Total Milk kit appears to be the most suitable and broad-spectrum ELISA kit for the detection of trace levels of milk residues in foods.

In the absence of adopting a standard reference material for the calibration of milk ELISA kits, the quantitative accuracy of the kits is questionable. Without industry and government agreement on established threshold doses and action levels for allergen labeling and recall, unified positions on allergen levels that pose a threat to human health are unlikely. While numerous barriers to accurate quantitation exist, the semi-quantitative abilities of the kits may be sufficient for responding to contamination at or near arbitrary action levels set within each company. In order for industry to appropriately respond to the impending establishment of threshold values by regulatory agencies, the semi-quantitative nature of ELISA kits must be reevaluated. This is not to say that proprietary differences in kit development must be discarded, but rather that calibration of each kit to a standard reference material and conversion factors from kit reporting units to relevant reference units must be provided by kit manufacturers. As a mildly processed material with high consistency and accuracy of detection across all milk ELISA kits evaluated, NFDM appears to be a good choice for a standard reference material.

This research has especially highlighted the differences observed among kits in the ability to detect both purified milk proteins and commonly used milk-derived ingredients. While conversion factors can be applied to ELISA results to obtain relevant units and facilitate

comparisons among kits, this type of data extrapolation may lead to inappropriate regulatory and industry recommendations. Because individual proteins or protein groups do not directly represent the materials from which they are derived in concentration or proportion, the application of conversion factors to achieve relevant results is prone to error. Food processing methods have the capability to reduce the solubility and detection of allergenic foods by ELISA analyses. Therefore, even with the assumptions of conserved protein proportionality across all milk-containing foods and derivative ingredients, the effects of processing on antibody recognition, epitope reactivity, and protein solubility add high levels of variability and inconsistency in detection among ELISA kits. Use of ELISA kits that do not use standard reference materials for regulatory analysis in the food industry can lead to false positive, false negative, and other inaccurate results. The need to establish specific criteria for kit validation and adopt a standard reference material among ELISA kits is also discussed in this research. Current commercial ELISA kits should be considered semi-quantitative. The extreme variability observed in detection of purified milk proteins suggests critical limitations of ELISA kits. The importance of proper calibration and understanding of ELISA kits is crucial for the protection of milk-allergic consumers.

Kits that inaccurately quantify target antigens represent a risk for food industry laboratories and regulatory agencies. While the scientific community debates establishing allergen detection thresholds for labeling requirements of packaged foods, the available analytical methods fail to accurately detect and quantify trace amounts of purified and unprocessed allergens. In order for industry and regulatory agencies to make appropriate recommendations, the validation and unification of commercial ELISA methods is paramount.

6. References

Abbott, M., Hayward, S., Ross, W., Godefroy, S.B., Ulberth, F., van Hengel, A.J., Roberts, J., Akiyama, H., Popping, B., Yeung, J.M., Wehling, P., Taylor, S.L., Poms, R.E., Delahaut, P., 2010. Validation procedures for quantitative food allergen ELISA methods: community guidance and best practices. Journal of AOAC International 93, 442-450.

Bock, S.A., Sampson, H.A., Atkins, F.M., Zeiger, R.S., Lehrer, S., Sachs, M., Bush, R.K., Metcalfe, D.D., 1988. Double-blind, placebo-controlled food challenge (DBPCFC) as an office procedure: a manual. J Allergy Clin Immunol 82, 986-997.

Chapman, J.A., Bernstein, I., Lee, R.E., Oppenheimer, J., Nicklas, R.A., Portnoy, J.M., Sicherer, S.H., Schuller, D.E., Spector, S.L., Khan, D., 2006. Food allergy: a practice parameter. Annals of Allergy, Asthma & Immunology 96, S1-S68.

Chatchatee, P., Bardina, L., Sampson, H.A., 2000. Identification of IgE and IgG binding epitopes of a-casein in cow milk allergic patients. Journal of Allergy and Clinical Immunology 105, S185.

Chatchatee, P., Järvinen, K.-M., Bardina, L., Vila, L., Beyer, K., Sampson, H.A., 2001. Identification of IgE and IgG binding epitopes on b- and k-casein in cow's milk allergic patients. Clinical and Experimental Allergy 31, 1256-1262.

Creamer, L., 1991. Electrophoresis of Cheese. Bulletin of the International Dairy Federation, 14-28.

Cucu, T., Platteau, C., Taverniers, I., Devreese, B., De Loose, M., De Meulenaer, B., 2013. Effect of partial hydrolysis on the hazelnut and soybean protein detectability by ELISA. Food Control 30, 497-503.

Damodaran, S., Parkin, K., 2008. Fennema's food chemistry. CRC Press/Taylor & Francis.

Downs, M.L., Taylor, S.L., 2010. Effects of thermal processing on the enzyme-linked immunosorbent assay (ELISA) detection of milk residues in a model food matrix. Journal of Agricultural and Food Chemistry 58, 10085-10091.

Fiocchi, A., Bouygue, G.R., Sarratud, T., Terracciano, L., Martelli, A., Restani, P., 2004. Clinical tolerance of processed foods. Annals of Allergy, Asthma and Immunology 93, S38-S46.

Fox, P.F., 1989. Proteolysis During Cheese Manufacture and Ripening. Journal of Dairy Science 72, 1379-1400.

Hildebrandt, S., Garber, E.A.E., 2010. Effects of processing on detection and quantification of the parvalbumin gene in Atlantic salmon (*Salmo salar*). Food Chem 119, 75-80.

Høst, A., Halken, S., 1990. A prospective study of cow milk allergy in Danish infants during the first 3 years of life. Allergy 45, 587-596.

Jost, R., 1988. Physicochemical treatment of food allergens: application to cow's milk proteins. Vevey/ Raven Press, Ltd., New York.

Khuda, S., Slate, A., Pereira, M., Al-Taher, F., Jackson, L., Diaz-Amigo, C., Bigley, E.C., Whitaker, T., Williams, K., 2012a. Effect of Processing on Recovery and Variability Associated with Immunochemical Analytical Methods for Multiple Allergens in a Single Matrix: Dark Chocolate. J Agric Food Chem 60, 4204-4211.

Khuda, S., Slate, A., Pereira, M., Al-Taher, F., Jackson, L., Diaz-Amigo, C., Bigley, E.C., Whitaker, T., Williams, K.M., 2012b. Effect of Processing on Recovery and Variability Associated with Immunochemical Analytical Methods for Multiple Allergens in a Single Matrix: Sugar Cookies. J Agric Food Chem 60, 4195-4203.

Koppelman, S.J., Hefle, S.L., 2006. Detecting allergens in food. Woodhead Publishing Ltd.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lemon-Mule, H., Sampson, H.A., Sicherer, S.H., Shreffler, W.G., Noone, S., Nowak-Wegrzyn, A., 2008. Immunologic changes in children with egg allergy ingesting extensively heated egg. Journal of Allergy and Clinical Immunology 122, 977-983.

Lipton, C., Dautlick, J., Grothaus, G., Hunst, P., Magin, K., Mihaliak, C., Rubio, F., Stave, J., 2000. Guidelines for the validation and use of immunoassays for determination of introduced proteins in biotechnology enhanced crops and derived food ingredients. Food and Agricultural Immunology 12, 153-164.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J biol chem 193, 265-275.

Monaci, L., Tregoat, V., van Hengel, A.J., Anklam, E., 2006. Milk allergens, their characteristics and their detection in food: a review. European Food Research and Technology 223, 149-179.

Nowak-Wegrzyn, A., Bloom, K.A., Sicherer, S.H., Shreffler, W.G., Noone, S., Wanich, N., Sampson, H.A., 2008. Tolerance to extensively heated milk in children with cow's milk allergy. Journal of Allergy and Clinical Immunology 122, 342-347.

Polenta, G., Weber, D., Godefroy-Benrejeb, S., Abbott, M., 2012. Effect of Processing on the Detectability of Pecan Proteins Assessed by Immunological and Proteomic Tools. Food Anal Methods 5, 216-225.

Poms, R.E., Anklam, E., 2004. Effects of chemical, physical, and technological processes on the nature of food allergens. Journal of AOAC International 87, 1466-1474.

Poms, R.E., Schäppi, G., Agazzi, M.-E., Koch, P., Bau, A., Anklam, E., 2004. Allergenicity vs detectability of processed peanut residues in cookies, in: E. Anklam, F. Morlin (Eds.), 9th International Symposium on Immunological, Chemical and Clinical Problems of Food Allergy, Budapest, Hungary.

Sampson, H.A., 2004. Update on food allergy. Journal of Allergy and Clinical Immunology 113, 805-819.

Sampson, H.A., Konstantinou, G.N., Kattan, J.D., Masilamani, M., Strong, B.D., Paynter, E., Bahnson, T., Nowak-Wegrzyn, A.H., 2013. Tolerance to Extensively Heated (Baked) Milk-Clinical and Immunologic Phenotype. J Allergy Clin Immunol 131, AB83.

Sathe, S.K., Teuber, S.S., Roux, K.H., 2005. Effects of food processing on the stability of food allergens. Biotechnology Advances 23, 423-429.

Sicherer, S.H., 2011. Epidemiology of food allergy. Journal of Allergy and Clinical Immunology 127, 594-602.

Thomas, K., Herouet-Guicheney, C., Ladics, G., Bannon, G., Cockburn, A., Crevel, R., Fitzpatrick, J., Mills, C., Privalle, L., Vieths, S., 2007. Evaluating the effect of food processing on the potential human allergenicity of novel proteins: International workshop report. Food and Chemical Toxicology 45, 1116-1122.

Veloso, A.C.A., Teixeira, N., Peres, A.M., Mendonça, Á., Ferreira, I.M.P.L.V.O., 2004. Evaluation of cheese authenticity and proteolysis by HPLC and urea–polyacrylamide gel electrophoresis. Food Chemistry 87, 289-295.

Wal, J.-M., 2002a. Cow's milk proteins/ allergens. Annals of Allergy, Asthma and Immunology 89, 3-10.

Wal, J.-M., 2002b. Cow's milk proteins/ allergens. Ann Allergy Asthma Immunol 89, 3-10.

Wal, J.-M., Bernard, H., Clement, G., Chatel, J.-M., Adel-Patient, K., 2001. Characterization of allergens in milk. Current Allergy and Clinical Immunology 14, 4-6, 8-10.

Wal, J.-M., Bernard, H., Créminon, C., Hamberger, C., David, B., Peltre, G., 1995. Cow's milk allergy: the humoral immune response to eight purified allergens, Part B ed. Plenum Press, New York.

Wal, J., 2001. Structure and function of milk allergens. Allergy 56, 35-38.

Wal, J.M., 2003. Thermal processing and allergenicity of foods. Allergy 58, 727-729.

Wróblewska, B., Jedrychowski, L., Mierzejewska, D., 2000. Reduction of protein allergenicity by lactic acid fermentation. Polish Journal of Food and Nutrition Sciences 9, 37-40.

CHAPTER 4. EFFECT OF PROTEOLYSIS DURING CHEDDAR CHEESE AGING ON THE DETECTION OF MILK PROTEIN RESIDUES BY ENZYME-LINKED IMMUNOSORBENT ASSAY

1. Abstract

During cheese ripening, milk proteins are degraded by proteases from enzymes and bacterial sources. Commercialized allergen detection methods are not validated for detection of residues in fermented or hydrolyzed products. The objective of this research was to evaluate commercially-available milk enzyme-linked immunosorbent assay (ELISA) kits for their capability to detect milk residues in aged Cheddar cheese. Cheddar cheese was manufactured in Brainard, Nebraska and was aged at 40^{o}C for 24 months. Samples were removed at ten points throughout aging and transferred to a freezer until the time of analysis. Milk residues and protein profiles were measured using commercial ELISA kits and SDS-PAGE. The protein content of a 5% sodium chloride extract of each cheese sample was evaluated using the Lowry protein assay, absorbance at 280 nm, and the microtannin protein assay. Protein assay results were compared to residue detection by ELISA kits. Several commercial milk ELISA kits were evaluated including Neogen Veratox® Total Milk, Neogen Veratox® Casein, R-Biopharm RIDASCREEN® Fast Milk, and ELISA Systems™ Casein. ELISA data revealed a 90% loss of milk residue signal between the youngest and oldest Cheddar cheese samples (0.5 months and 24 months, respectively). SDS-PAGE analysis showed protein degradation throughout aging, with the highest levels of proteolysis observed at 24 months. Results suggest that current commercial ELISA methods can detect milk residues in young Cheddar cheese, but the detection signal dramatically decreases during aging. The four evaluated ELISA kits are not capable of detecting trace levels of milk residues in aged cheese. Reliable detection of allergen residues in fermented food products is critical for upholding allergen control programs, maintaining product safety, and protecting allergic consumers.

2. Introduction

Cow's milk represents one of the most commonly allergenic foods around the world (Sampson, 2004). Cow's milk contains several different allergenic proteins including casein, β-lactoglobulin, and α-lactalbumin (Wal et al., 2001). Milk-allergic individuals are advised to avoid all milk-derived food products and ingredients (Sicherer and Sampson, 2010). The level of risk of an allergic reaction is related to the dose of exposure to milk proteins. However, milk-allergic individuals vary widely with respect to their threshold doses for milk protein (Skripak et al., 2008). Many milk-allergic individuals outgrow their sensitivity to milk over a period of months to years (Skripak et al., 2007) and presumably their individual threshold doses increase during that period until they become fully tolerant. Oral immunotherapy (OIT) with milk can be used to accelerate the development of oral tolerance to milk (Skripak et al., 2008). Furthermore, studies have found that milk-allergic individuals can tolerate heat-treated (baked) foods containing milk before they can tolerate other heat-treated (pasteurized) foods (Kim et al., 2011a; Leonard et al., 2012). Recent studies evaluating milk oral immunotherapy have exposed patients to aged cheeses, either as a component of the therapy regimen or as an evaluation of the achieved level of tolerance. Approximately 58% of milk-allergic patients tolerated fully—matured Parmigiano-Reggiano (Alessandri et al., 2012). In general, milk-allergic individuals are advised to avoid cheeses but the degree of tolerance to various cheeses among milk-allergic individuals has not been examined.

Two protein classes comprise the majority of milk proteins; caseins, representing nearly 80% of total milk protein, and the whey proteins, representing the remaining 20%. The caseins are composed of four subfractions: α_{s1}-, β-,α_{s2}-,and κ-casein. These proteins comprise 37:37:13:13 of the casein proportions (Wal, 2002b). During cheese manufacture, several biochemical processes occur. Perhaps the most important process for developing structure, texture, and flavor characteristics of cheese is proteolysis. Proteolysis during manufacture and

aging of cheese has been reviewed for many cheese varieties (Fox and McSweeney, 1996; Ledford et al., 1966; Marcos et al., 1979; Mooney et al., 1998; O'Keeffe et al., 1976; Singh et al., 1997). Proteolytic activity is attributed to chymosin, indigenous milk proteases, and the proteases of starter and non-starter microorganisms. The most obvious function of proteases in cheese is to hydrolyze κ-casein and destabilize the milk micelle, coagulate the curd, and form the cheese matrix. Proteases also play a significant role in developing characteristic flavors and texture during ripening.

Primary proteolysis during cheese ripening is dominated by the actions of chymosin and the indigenous milk proteinase plasmin. The primary role of chymosin during cheese production is the hydrolysis of κ-casein and subsequent destabilization of the milk micelle. However, during the early stages of ripening, chymosin also hydrolyzes α_{s1}-casein at several sites. In Cheddar cheese, a_{s1}-casein is almost completely degraded by chymosin after ripening for 20 weeks(Mooney et al., 1998). Due to its low water activity, β-casein is highly resistant to hydrolysis by chymosin, but about 50% of it is degraded during Cheddar cheese ripening (Ledford et al., 1966; O'Keeffe et al., 1976). Chymosin has not been documented to degrade para-κ-casein during ripening (Green and Foster, 1974).

Plasmin preferentially hydrolyzes β-casein during primary proteolysis (Upadhyay et al., 2004). In addition, plasmin has minor activity on α_{s1}-casein and is responsible for producing caseins of the λ-subclass (Upadhyay et al., 2004). The fraction of κ-casein remaining in the cheese after coagulation is largely resistant to proteolysis during the early stages of ripening. Even though these similarities exist in primary proteolysis among many cheeses, dramatic differences in proteolysis still occur as a result of myriad environmental factors, including pH, water activity, cook temperature, storage content, fat content, and homogenization (Deegan and McSweeney, 2013; Di Luccia et al., 2013; Larsen et al., 2010).

While the concentration of α_{s2}-casein decreases during ripening, no large peptides have been isolated from its hydrolysis(Mooney et al., 1998). However, Singh et al. (1997) described the isolation of four α_{s2}-casein derived peptides from the water soluble fraction of Cheddar cheese. These peptides were produced through the action of plasmin and a starter-culture associated aminopeptidase (O'Keeffe et al., 1976; Singh et al., 1997).

Several extraction and fractionation schemes have been developed to evaluate the extent of ripening and proteolysis in cheese and are based on liberation of free amino acids and protein fragments (Christensen et al., 1991; Kuchroo and Fox, 1982; O'Sullivan and Fox, 1990; Quesnel, 1968; Singh et al., 1994). As aging continues, the number of large peptides isolated from cheese dramatically decreases (Addeo et al., 1995; Singh et al., 1994). Larger pH 4.6-soluble peptides are typically derived from the action of chymosin, while pH 4.6-insoluble fragments are typically γ-caseins derived from the action of plasmin on β-casein(O'Keeffe et al., 1976). Small peptides and free amino acids, however, are a result of peptidases derived from starter and non-starter cultures(O'Keeffe et al., 1976). The small peptides produced during ripening, especially those produced during secondary proteolysis, are particularly difficult to visualize with electrophoretic methods. These peptides are often only distinguishable using chromatography or mass spectrometry (Mooney et al., 1998; Singh et al., 1997).

In solution, α_{s1}-casein has 20 chymosin-susceptible bonds. In the cheese matrix, however, only six peptides are produced by the action of rennet on α_{s1}-casein (Mulvihill and Fox, 1980). One of the most consistently produced fragments is α_{s1}-I casein; it is characteristically present in all cheeses during the early stages of ripening (Grappin et al., 1985). α_{s1}-casein is completely degraded to α_{s1}-I casein (f24-199) and α_{s1}-casein (f1-23) in mature cheeses(Fox and Guiney, 1973). In many cheeses, α_{s1}-I casein is resistant to proteolysis (Marcos et al., 1979).

Plasmin activity is responsible for producing γ-caseins from β-casein. Three major subclasses of γ-caseins exist. γ_1: (f29-209), γ_2: (f106-209), and γ_3: (f108-209). The γ-caseins have been used in several studies as an indication of ripening, particularly with Parmigiano-Reggiano and Grana Padano cheeses (Addeo et al., 1995; Gaiaschi et al., 2001).

Secondary proteolysis is the term used to describe the hydrolysis of peptides generated during primary proteolysis. Secondary proteolysis is performed by chymosin, plasmin, and peptidases provided by the microflora in cheese (Rank et al., 1985; Sousa et al., 2001). Chymosin and lacotococcal cell envelope proteinases (CEP) continue to break down α_{s1}-I casein and α_{s1}-casein f(1-23) in Cheddar cheese during secondary proteolysis. Small peptides are produced from the α_{s1}-casein derived fragments and have been isolated from the water soluble fraction of Cheddar cheese(Singh et al., 1997). The breakdown of γ-caseins by lactococcal CEP also occurs during secondary proteolysis. Due to their size, many products of secondary proteolysis are only isolated using advanced methods of chromatography, mass spectrometry, and amino acid sequencing(Fox and McSweeney, 1996).

Literature indicates that throughout aging of Cheddar cheese, several epitopes capable of binding IgE and eliciting an allergic response remain intact on the most abundant caseins, α_{s1}- and β-casein (Mooney et al., 1998; Singh et al., 1997; Wal, 2002b). However, some allergenic epitopes can lose their immunoreactive potential as a result of proteolysis during cheese manufacture and aging. The comparative allergenic potency of various types of cheeses has not been carefully assessed.

For the food industry, labeling regulations in most countries require the labeling of milk and all ingredients derived from milk. According, milk is identified as a priority allergenic food in Allergen Control Plans by the food industry. Potential for allergen cross contact exists in manufacturing facilities where various food ingredients including milk-derived ingredients are

processed on shared equipment. Allergen Control Plans require the development and validation of sanitation procedures capable of removing allergen residues from equipment surfaces so that such residues do not appear in the following formulations. Detecting cross contact of food products with allergens is critical for the validation of Allergen Control Plans and the sanitation procedures incorporated into those plans. Detection of cross contact is also critical to maintain product and consumer safety. However, current immunochemical methods are not validated for evaluation of all forms of the allergenic food. Fermented or hydrolyzed food products are particularly difficult to assess for residues of allergenic foods because the immunoassay methods used for allergen detection are oriented to detect intact protein from those foods. While receiving a false positive result when analyzing food products can result in unnecessary regulatory action and product recall, a false negative analysis may not support a response sufficient to protect allergic consumers. The consequences of a false negative result may be more severe than those of a false positive result for food companies. Current commercial ELISA methods are not validated for the detection or accurate quantification of allergen residues in foods subjected to protein hydrolysis. The relationship between the extent of proteolysis and detection of milk proteins using commercial ELISA kits has not been evaluated.

This research seeks to measure the proteolytic effects in aging Cheddar cheese by evaluating the antibody detection of commercial ELISA kits and the polyacrylamide profiles of the cheeses. This research seeks to determine if proteolysis during cheese ripening affects the detection and quantitation of milk residues by commercial total milk and casein ELISA kits. Because whey proteins have limited and inconsistent presence in Cheddar cheese, evaluation of cheese for whey protein residues was not included. The commercial ELISA kits selected for analysis included three casein kits and one total milk kit. The specificities of the selected ELISA kits to milk proteins and milk ingredients were determined previously. Established scientific knowledge indicates that proteolytic enzymes can destroy or reveal allergenic epitopes on

proteins (Besler et al., 2001). The cumulative effects of proteolysis during long-term cheese aging on the capability for detection of milk protein residues by ELISA has not been previously assessed.

3. Materials and Methods

Cheese manufacture

Cheese was manufactured at Jisa's Farmstead Cheese in Brainard, Nebraska, under the supervision of David Jisa and according to standard procedure for New York Cheddar developed by the University of Nebraska-Lincoln Dairy Plant. Briefly, 9,000 pounds of raw milk (3.4% butterfat) from Holstein cows was pasteurized by HTST on location. The milk was piped to a 10,000 gallon steam-jacketed stainless steel cheese vat in the production facility. Once the vat was filled with 4,500 gallons of milk, freeze-dried Vivolac Vivopel MSM 960 starter culture was added, the vat was filled, and the mixture was agitated for one hour at 90^{o}F (Greenfield, Indiana). Next, 14oz. of double-strength rennet was added to the vat. The mixture was stirred until rennet was evenly distributed. After the curd had set (approximately 25 minutes after rennet addition), the curd was cut with ½ inch-spaced wire mesh and was allowed to rest for 15 minutes). Curds were then cooked at 100^{o}C with slow stirring. Once the whey acidity had reached the appropriate level after cutting, whey draining began. Curd was piled along each side of the vat while whey was drained. Upon completion of draining, matted curd was cut into wide slabs for Cheddaring. Slabs were rotated every 15 minutes until the appropriate acidity was reached, at which time slabs were milled and salted. Milled curds were pressed into 20 lb cheese hoops and held under pressure at 40psi overnight at 8-10^{o}C. A 20 lb. hoop of Cheddar cheese was transported from the production facility in Brainard, Nebraska to the University of Nebraska-Lincoln Dairy Plant for packaging and aging. The cheese was cut and vacuum packaged into eighty ¼ lb. blocks, and placed in the cheese cooler for ripening (40-44^{o}C).

Sampling during manufacture and aging

Ten samples of aged Cheddar cheese were collected at the following time points: 2 weeks, 1 month, 2 months, 4 months, 6 months, 8 months, 10 months, 13 months, 19 months, and 24 months of aging. At each sampling point, four ¼ lb. blocks of cheese were randomly selected and removed from the cheese cooler at the University of Nebraska-Lincoln Dairy Plant. Each of the four blocks was cut in half and one half was shredded using a fine-texture grater attachment with a 13-cup KitchenAid™ Food Processor. Shredded cheese was mixed and portioned into 10 g aliquots in plastic zip-top bags. The remaining portions of the blocks were resealed and placed into plastic zip-top bags. All samples were transferred to a -20^{o}C freezer for storage until time of analysis.

Sample Preparation for SDS-PAGE and ELISA Analyses

Approximately 30 grams of each finely-grated and aged cheese sample was removed from the -20^{O}C freezer and pulverized to a fine powder using a Spex 6850 CentriPrep Freezer/Mill (Metuchen, NJ).

Samples to be analyzed using SDS-PAGE were prepared as follows. 5 g of freezer-milled cheese were weighed into a 50mL Falcon™ tube (Fisher Scientific, Rockford, IL). 20 g of 5% sodium chloride solution was added to the cheese, and the sample was mixed using a pulse vortex technique for approximately 5 seconds. Samples were extracted with horizontal shaking for 60 minutes at room temperature (22^{o}C) on a Barnstead Thermolyne LabQuake Shaker (Thermo Fisher Scientific, Rockford, IL). Extracts were centrifuged at 4,200 RPM for 30 minutes at 10^{o}C in an IEC Centra MP4R Centrifuge (International Equipment Company, Needham Heights, MA). The aqueous layer was removed to a new 50 mL Falcon™ tube. Pellets and fat layers were discarded and extracts were frozen at -20^{o}C until time of analysis.

Samples to be analyzed with ELISA were defatted by hexane extraction prior to solubilization. Previous work with cheese highlighted that solubilized cheese provides a more homogenous solution and consistent results than analyses with solid cheese. Briefly, 20 g of freezer-milled cheese was weighed into a 500 mL Erlenmeyer flask. Approximately 180mL of cold hexane at -20^{o}C was added to the flask. Flasks were placed in an ice bath on a horizontal shaker. Flasks were shaken for 30 minutes at 50% max speed. The aqueous layer was decanted and the remaining sample was retained. Two additional hexane washes were performed, for a total of three -20^{o}C hexane washes. The final wash was gravity filtered through a fluted Whatman Size 1 Filter paper in a glass funnel. The filter and retained cheese were removed from the glass funnel and were allowed to dry overnight in a fume hood. Used hexane was disposed using standard operating procedure for hazardous material disposal dictated by the Office of Environmental Health and Safety at the University of Nebraska-Lincoln. Dried defatted cheese was stored in plastic zip-top bags at -20^{o}C until time of analysis.

Prior to ELISA analysis, 1 g of each aged defatted cheese was suspended in 5% sodium chloride solution at a 1:10 (w/v) ratio in a 50 mL Falcon™ tube. Tubes were shaken horizontally on a Barnstead Thermolyne LabQuake Shaker for 3 hours at room temperature. Extracts of defatted cheese were centrifuged in an IEC Centra MP4R Centrifuge (International Equipment Company, Needham Heights, MA). The aqueous layer was removed and reserved in a clean 50 mL Falcon™ tube and pellets were discarded. Extracts were frozen at -20^{o}C until further use.

Protein Concentration of Extracts

The concentration of 5% NaCl soluble protein in extracts was determined using the Lowry Protein Assay (27) as adapted for application in a 96-well microtiter plate The assay measures the intact protein concentration in a sample by allowing it to complex with cupric sulfate in alkaline conditions. Folin-Ciocalteu Phenol reagent is added to the solution and is

reduced in proportion to the chelated copper-protein complexes, producing a colorimetric reaction. Cheese samples were solubilized as described above for analysis with SDS-PAGE. Standard solutions at concentrations between 0-200 μg/mL were generated by dilution of a 2 mg/mL Bovine Serum Albumin Standard (Pierce Scientific, a division of Thermo Scientific, Rockford, IL). The absorbance of standards and samples were read at 490 nm with a BioTek EC808x Microplate Reader and evaluated with KC Junior Software (Winooski, VT).

Additionally, the UV-absorbance of extracts was measured at 280 nm using a NanoDrop 2000c Spectrophotometer (ThermoScientific, Rockford, IL). Proteins that contain tyrosine, tryptophan, or disulfide bonds will absorb UV radiation at 280 nm. Protein concentration was calculated using the Warburg-Christian equation (Warburg and Christian, 1942). Because both amino acids and nucleic acids absorb ultraviolet light at 280 nm, and ribonucleotides also absorb strongly at 260 nm, the 260/280 ratio can be used to correct the measurement for the contribution of nucleic acids to the sample absorbance. The Warburg-Christian Equation is applied to the 260/280 nm ratio to provide a more accurate estimate of protein concentration as compared to estimation based on absorbance readings at A_{280} alone. Using UV absorbance has been suggested to be a potentially problematic method for analyzing ripening cheeses (Wallace and Fox, 1998). Caseins are differentially sensitive to proteolysis. As cheese is fermented, α-casein is among the first to be hydrolyzed. The N-terminal residues of α_{s1}-casein that are released early in the aging process do not contain tyrosine or tryptophan residues, and the peptides do not absorb UV light. Even though proteolysis has occurred, a corresponding change in fluorescence at A_{280} is not observed (Marcos and Esteban, 1993). Because the Lowry Assay is a dye-binding assay that relies on intact protein fragments for quantitation, and the A_{280} assay can be affected by interference from several sources, the Microtannin Protein Assay was also used to measure protein concentration. The Microtannin Protein Assay is a rapid, reliable, and robust assay that relies on protein precipitation for protein content determination.

The Microtannin protein assay was performed according to the procedure developed by Mejbaum-Katzenellenbogen and Dobryszycka (1959) and modified by Trayer and Trayer (1988). Briefly, 1 mL of each defatted cheese extract was suspended 1:10 (w/v) in 5% NaCl. 1 mL of the diluted cheese solution was added to a 10mL disposable glass culture tube. A standard curve was created by diluting a standardized solution of Bovine Serum Albumin at 2 mg/mL to concentrations ranging from 0-75 μg/mL (Pierce Scientific, a division of Thermo Scientific, Rockford, IL). 1 mL of each standard was also added to 10 mL disposable glass culture tubes. Next, 1 mL of 10% (w/v) tannic acid in 2% phenol solution (v/v) in 1N HCl was added to each tube. For the phenol-HCl solution, 41.75mL of 12N HCl was mixed with 458.25 mL of d_2H_2O. After the solution was homogenous, 10 mL of phenol was added to the solution. The solution was heated to 80^{o}C and 50 g of tannic acid was added and mixed until completely dissolved. The solution was cooled to room temperature and gravity filtered using a fluted Whatman size 1 filter paper. A 0.2% solution of gum Arabic was created by dissolving 1 g of gum Arabic in 500mL d_2H_2O at 40^{o}C. After cooling, 0.1g of sodium azide was added to the solution to inhibit microbial growth. Solutions were stored at room temperature (≈22^{O}C) until use.

Sample tubes were briefly mixed in a vortex-type mixer and incubated at room temperature (approximately 22^{o}C) for 10 minutes. Next, 1 mL of 0.2% gum Arabic solution was added to each standard and sample tube to suspend the precipitated protein. Tubes were promptly mixed in a vortex-type mixer and the absorbance was read at 520 nm in disposable cuvettes using a Thermo Scientific NanoDrop 2000c Spectrophotometer. All samples were analyzed in duplicate, and the standard solutions were prepared and analyzed in triplicate. The standard curve was constructed in GraphPad Prism (Version 4.03) and the concentration of unknown samples was interpolated from the curve. Used reagents were disposed to the sewer after pH adjustment to pH 6.5-9.0 according to the Office of Environmental Health and Safety at the University of Nebraska-Lincoln.

Electrophoresis

Proteins were prepared under reducing conditions according to the procedure of Laemmli (1970). Briefly, 50 uL of each sample was boiled in a 100^{o}C waterbath in Laemmli sample buffer containing 5.4% dithiothreitol (w/v) for 5 minutes. Protein separation was performed using a Bio-Rad Mini-Protean® Tetracell electrophoresis unit (Bio-Rad Laboratories, Hercules, CA). 5 µL of Precision Plus Protein™ Dual X-tra Standard was loaded in the first lane of each gel (Bio-Rad Laboratories, Hercules, CA).

Briefly, 18% Tris-HCl precast polyacrylamide Ready Gels® for Mini-Protean® Systems, 10 wells, 30 µL maximum volume per well were used to separate proteins and evaluate banded regions (Bio-Rad Laboratories, Hercules, CA). Three sets of gels were run; each set was loaded with 10, 15, or 20 µg of protein per lane. Approximate loading volume was determined by the averaged protein concentrations as determined by the Lowry, A_{280}, and microtannin assays. Empty lanes were filled with 5 µL of sample buffer to improve profile quality. Gels were run at a constant voltage of 200V for approximately 30 minutes, or until the dye front reached the bottom of the gel.

Gels were fixed for 30 minutes in a solution of 60% trichloroacetic acid (w/v) and 17.5% 5-sulfosalicylic acid (w/v) diluted 1:5 with deionized water (DI) (Sigma-Aldrich, St. Louis, MO). Fixed gels were rinsed in DI water and stained overnight using Coomassie Brilliant-Blue R-250 Staining Solution (Bio-Rad Laboratories, Hercules, CA). After approximately 8 hours of staining, gels were destained using the Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad Laboratories, Hercules, CA). Gels images were captured using Kodak Gel Logic 440 Imaging System (Eastman Kodak, Rochester, NY) and evaluated using Carestream Molecular Imaging software (v5.02.30, Carestream Health, Rochester, NY).

ELISA Analysis

Four commercial ELISA kits were used. Neogen Veratox® Total Milk and Casein ELISA kits were obtained from Neogen ® Corporation (Lansing, MI, USA). ELISA Systems™ Casein kits were obtained from ELISA Systems (Windsor, Queensland, Australia). R-Biopharm RIDASCREEN® Fast Casein (Darmstadt, Germany) kits were obtained from a distributor, Pi Bioscientific (Seattle, WA, USA).

Prior to ELISA analysis, 1mL of each defatted cheese extract was diluted 1:100 (v/v) in 5% sodium chloride solution. Sample preparation and ELISA analysis were performed according to manufacturer's instructions for 1 mL of homogenous liquid sample. Briefly, 1 mL of diluted sample was added to the appropriate amount of prepared extraction solution heated to 60^{o}C. Extractions were performed in a 60^{o}C shaking water bath for the recommended time. Each sample was extracted in triplicate. Samples were cooled to room temperature and 1 mL of each extraction was centrifuged in a ThermoScientific Legend Micro 17 centrifuge for the recommended time and speed from the manufacturer-provided insert. Additional dilutions were performed in the extraction buffer provided by corresponding kits to fall within the manufacturer-provided standard curves.

For each ELISA, 100 µL of extracted and diluted sample was added to three wells of the antibody-coated microtiter wells provided by each kit manufacturer. Samples were incubated for 10 minutes and were washed the appropriate number of times with prepared manufacturer-provided wash buffer solution. Excess buffer was removed from wells by tapping the inverted plate on a paper towel. 100 µL of conjugate antibody solution was added to wells and the plate was allowed to incubate for an additional 10 minutes. Washing was repeated, and 100 µL of substrate was added to each well, followed by a final 10 minute incubation period to develop a colorimetric reaction. 100 µL of acidic stop solution was added to the wells, and the plates were

read in a Dynex Spectra MR Plate Reader at the manufacturer-recommended wavelength. The concentration of analyte in each sample was read from the standard curve.

Samples that contained concentrations of detectable milk proteins that fell outside the dynamic range of the kits were diluted with the manufacturer-provided extraction buffer until their concentration was quantifiable. Dilution factors were applied to the detected value of milk residue to determine a "final" concentration for each sample. The ranges of quantitation and other kit information for the kits used in the study are listed in Appendix B.

Data analysis was performed in Microsoft Excel, GraphPad Prism (Version 4.03), and manufacturer-provided software, if any. The Neogen Veratox® Software (Version 3.02) was used to analyze Neogen data, while the r-Biopharm RIDAWIN® Software (Version 1.42) was used to evaluate r-Biopharm data. Criteria for acceptable ELISA kit performance included an R^2 of ≥ 0.98 for the standard curve and a coefficient of variation (%CV) of $\leq 20\%$.

4. Results/Discussion

Protein Concentration of Extracts

Three assays, Lowry, Absorbance at 280nm, and the Microtannin Protein Assay were used to determine the concentration of protein in 5% NaCl-soluble extracts of cheese samples.

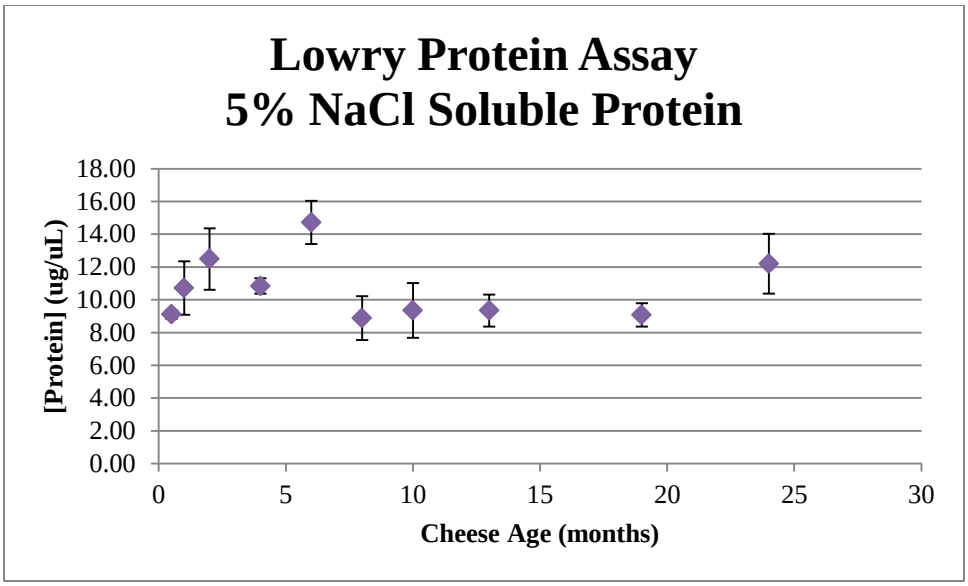


Figure 4.1. Protein Concentration of Cheddar cheese aged 0.5-24 months as determined by the Lowry Protein Assay. Vertical error bars represent standard deviation.

Results of the Lowry Protein assay display a nondescript and variable trend in the concentration of 5% NaCl soluble protein, with peaks observed at 2, 6, and 24 months of aging, corresponding to 12.5, 14.7, and 12.2 μg/mL (Figure 4.1 and Table 4.1). The lowest levels of soluble protein are observed at 0.5, 8, and 19 months of aging in the Lowry Assay, falling at 9.1, 8.9, and 9.1 μg protein/mL, respectively. The soluble protein concentration remains relatively stable in the samples taken between 8 and 19 months of aging; among these samples; the variation was only 5%. Because the observed response displayed variable trends, additional methods were used to evaluate and quantify protein concentration. The UV-absorbance of each 5% NaCl-soluble cheese extract at 520 nm and the microtannin protein assay were used as alternative methods to measure protein concentration.

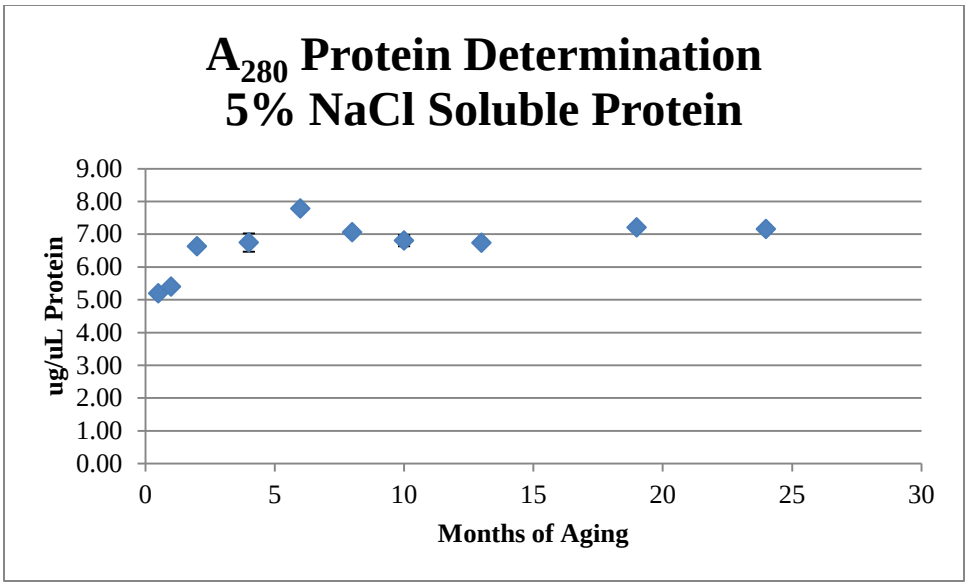


Figure 4.2. Protein concentration of Cheddar cheese aged 0.5-24 months as measured by absorbance at 280nm. Error bars represent standard deviation.

According to UV-absorbance data, the concentration of soluble protein is greater at the end of aging (24 months) than at the onset (0.5 months) (See Figure 4.2 and Table 4.1). At 24 months, approximately 7.2 µg/mL of protein is observed, whereas only 5.2 µg/mL soluble protein is measured at 0.5 months. A peak in soluble protein concentration is observed at 6 months of aging, corresponding to 7.8 µg/mL (Figure 4.2). The Warburg-Christian Method was used to correct for interference from ribonucleic acids at A_{280} and is described in greater detail in Chapter 4.3. The trends in concentration of soluble protein as determined by the A_{280} assay are less variable than the data observed using the Lowry Protein Assay.

A third method, the Microtannin Protein Assay, was used to confirm results obtained through the Lowry and A_{280} methods. Results are listed in Figure 4.3 and Table 4.1.

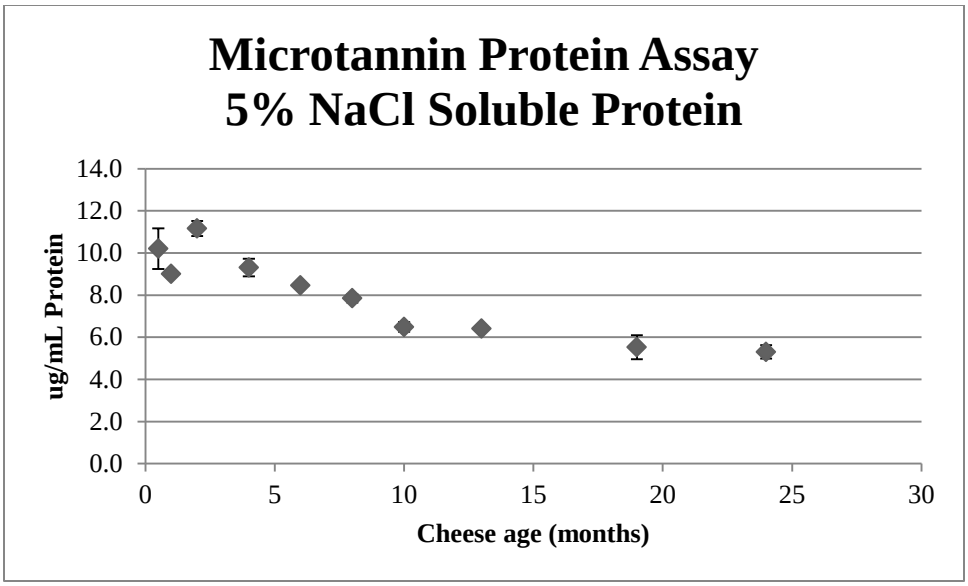


Figure 4.3. Protein concentration of Cheddar cheese aged 0.5-24 months, as measured using the microtannin protein assay. Vertical error bars represent standard deviation.

According to the Microtannin Protein assay, as aging continues, there is a decrease in 5% sodium chloride-soluble protein (Figure 4.3 and Table 4.1). A peak in soluble protein content is observed at 2 months of aging; in agreement with the Lowry protein assay (Figures 4.1 and 4.3).

Different results among the assays are expected. The Lowry and A_{280} assays rely on intact proteins to determine concentration, while the Microtannin assay can detect both intact proteins and free amino acids. In cheese, proteolytic enzymes release small peptides and free amino acids during the degradation of larger proteins throughout aging. Increasing levels of proteolysis should correspond to an increased concentration of free amino acids and a decrease in intact protein in sample extracts, although this is not observed (Kuchroo and Fox, 1982). Literature does not indicate any of the assays as superior to the others for accurately determining protein concentration in cheeses. To provide an estimate of actual concentration in extracts for visualization purposes only, the results of the three assays were averaged for each sample.

Table 4.1. Cumulative Protein Concentration Assay data for Cheddar cheese aged 0.5-24 months

Age (mo.)	Lowry (µg/µL)	A_{280} (µg/µL)	Microtannin (µg/µL)	Average (µg/µL)
0.5	9.1	5.2	10.2	8.2
1	10.7	5.4	9.0	8.4
2	12.5	6.6	11.2	10.1
4	10.8	6.7	9.3	9.0
6	14.7	7.8	8.5	10.3
8	8.9	7.1	7.8	7.9
10	9.4	6.8	6.5	7.5
13	9.3	6.7	6.4	7.5
19	9.1	7.2	5.5	7.3
24	12.2	7.2	5.3	8.2

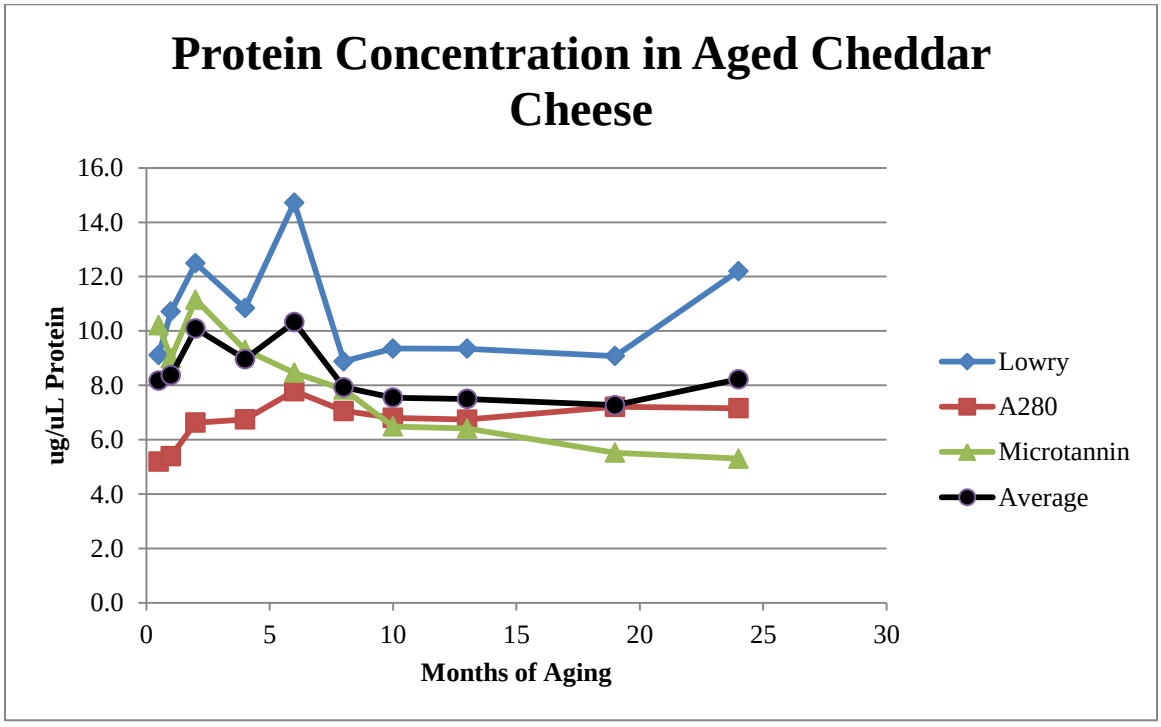


Figure 4.4. Protein concentration of Cheddar cheese aged 0.5-24 months as determined by the Lowry Protein Assay, Absorbance at 280nm, and the Microtannin Protein Assay.

According to the averaged data (Figure 4.4), 5% sodium chloride soluble protein remains relatively consistent over time, varying only 3 µg/µL throughout aging. The microtannin protein assay is the most robust method of the three used for protein analysis in this study. The assay

displays limited interference from sample buffers or matrix components and is highly reproducible. The other protein assays performed are generally consistent with the microtannin assay.

The observed increases and decreases in protein concentration during aging are possibly a result of the liberation and subsequent degradation of small peptides released during casein proteolysis. Peaks are observed in protein concentration at 2 and 6 months of aging in the Lowry and A_{280} methods. No peak is observed in the microtannin assay at the 6 month time point, but a peak is observed at 2 months.

Electrophoresis

Gels were run at conditions described above. Only the gels loaded with 10μg of protein per lane are displayed; see Figures 4.5 and 4.6.

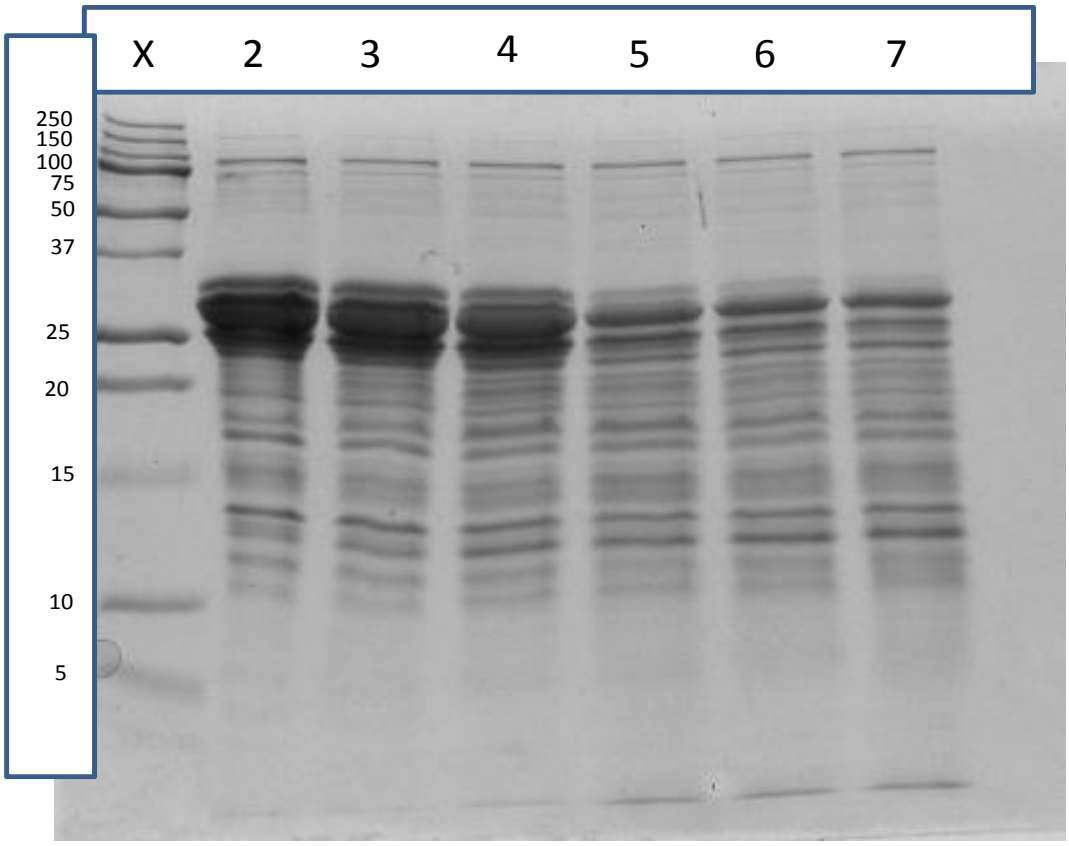


Figure 4.5. Profiles of aged Cheddar cheese. Lane X: molecular weight marker; lane 2: 0.5 months, lane 3: 1 month; lane 4: 2 months; lane 5: 4 months; lane 6: 6 months; lane 7: 8 months. Gels were run at 200V for 35 minutes. Each well contains approximately 10 μg of protein.

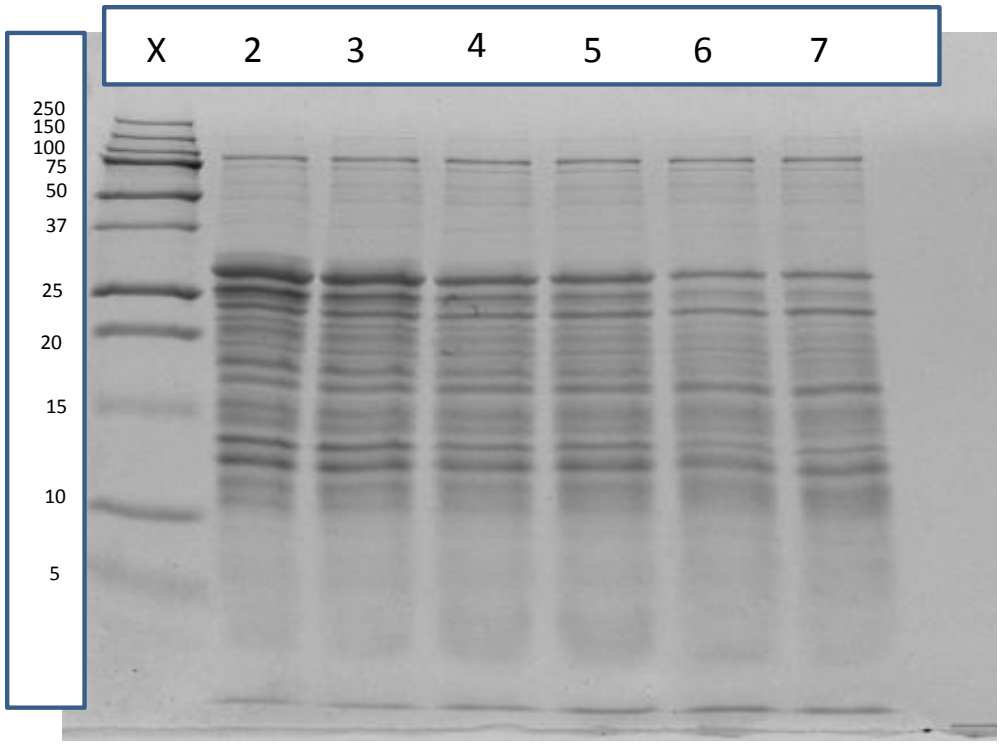


Figure 4.6. Profiles of aged Cheddar cheese. Lane X: BioRad Precision Plus Protein Dual Xtra molecular weight marker; Lane 2: 6 months; lane 3: 8 months; lane 4: 10 months; lane 5: 13 months; lane 6: 19 months; lane 7: 24 months. Gels were run at 200V for 35 minutes. Each well was loaded with approximately 10 µg of protein.

Samples were split between two gels. Protein degradation is easily observed throughout aging as displayed with the changes in intensity of bands as aging continues (Figures 4.5 and 4.6). 10 µg of each protein extract was loaded in each lane of the gels. The bands displayed in the 24-month Cheddar sample are distinctly less prominent than the protein bands observed in samples taken during the early stages of aging. The casein bands, observable between 20-30kDa, show a dramatic decrease in intensity over time. Low molecular weight peptides increase throughout aging, as shown by an increase in intensity of residues below the 10 kDa marker.

In concurrence with the data obtained in the assays to measure protein concentration, there is an increase in band intensity at the two month time point, displayed as a band migrating in the 12-13 kDa range. Where a peak was also observed in the Lowry and A_{280} assays at 6 months of aging, only a minimal increase in band intensity is observed in the 12-13 kDa range.

However, a large decrease in intensity is observed around 15 kDa and within the intact casein region (27-30 kDa), when the 6 month sample is compared to the 4 month time point.

According to Grappin et al. (1985), para-κ-casein is not degraded during cheese ripening, only during cheese manufacture. Electrophoretic analyses reveal a band near 16 kDa thought to be para-κ-casein. The intensity of this band remains visually consistent throughout aging.

ELISA Analysis

ELISA analysis was performed on defatted cheese samples using three commercial casein ELISA kits (Neogen Veratox® , ELISA Systems™ Casein, and R-Biopharm Ridascreen®) and one commercial Total Milk kit (Neogen Veratox®). Kit specificities to purified casein fractions and milk ingredients were determined in earlier work (see Chapter 3). Kit sensitivities are displayed in Table 4.2. The Neogen Veratox® Casein kit is specifically sensitive to α- and β-casein. Analyses with purified protein fractions revealed that this kit detects both protein fractions at approximately 100% of the present level. The ELISA Systems™ Casein kit is specifically sensitive to α-casein, at approximately 90% sensitivity to the actual quantity present in the sample. The kit fails to detect β- and κ-casein, regardless of the present concentration. The R-Biopharm RIDASCREEN® Fast Casein kit primarily detects κ-casein, at 100% of the ratio present, but also detects some β-casein, at approximately 10% of the level actually present. The Neogen Veratox® Total Milk kit is primarily sensitive to κ-casein (at 50% of the present concentration), but also detects some α- and β-casein (approximately 10% of the present levels).

Table 4.2. Sensitivities of selected milk ELISA kits as described in Chapter 3.

Analyte	α-casein	β-casein	κ-casein
Proportion (Wal, 2001)	50%	37%	13%
Ratio to kit standards			
Neogen Veratox® Casein	1.0	1.0	0.0
ELISA Systems™ Casein™	0.9	0.0	0.0
r-Biopharm Fast Casein	0.0	0.1	1.0
Neogen Veratox® Total Milk	0.1	0.1	0.5

The oldest cheese sample evaluated was collected 24 months from the date of production. The youngest sample evaluated was procured 0.5 months post-production. Observable differences in milk residue quantitation with ELISA kits were expected between the two extremes of aging, even if significant differences were not observed between sequential time points.

Table 4.3. Milk residues in aged Cheddar cheese as detected by the Neogen Veratox® Total Milk

Neogen Total Milk Analysis of Aged Cheddar Cheese				
Age (mo)	Sample	avg ppm NFDM	St. Dev.	%CV
0.5	116	12000	720	6%
1	117	14000	630	5%
2	118	15000	2700	19%
4	119	14000	550	4%
6	120	8700	740	9%
8	121	5100	620	12%
10	122	4400	960	22%
13	123	3600	490	14%
19	124	970	200	21%
24	136	1000	50	5%

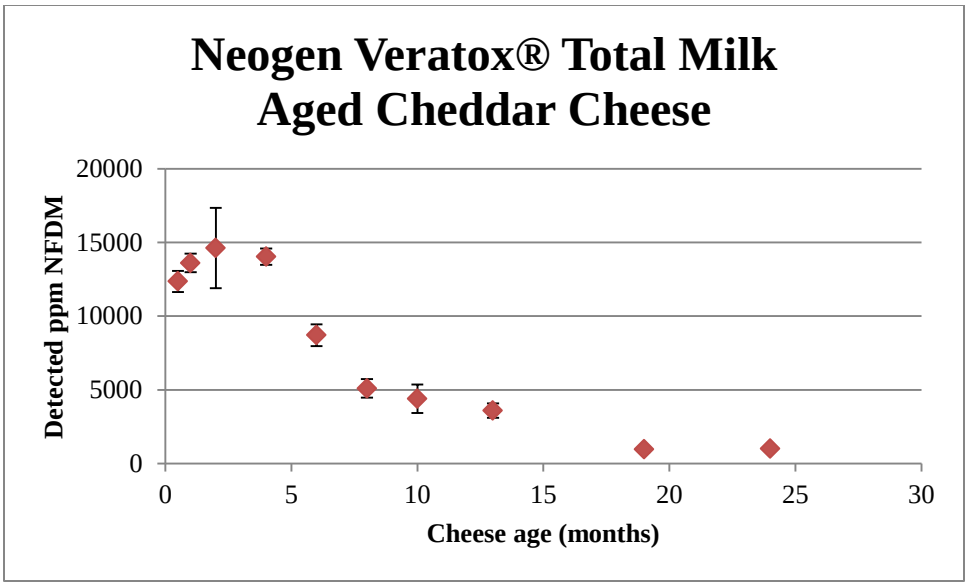


Figure 4.7. Detection of milk residues in aged Cheddar cheese with the Neogen Veratox® Total Milk kit. Error bars represent standard deviation (n=3).

Using the Neogen Veratox® Total Milk kit, a substantial decrease in detection of milk protein residues expressed as NFDM was observed with increasing cheese age. However, milk protein residues remained detectable even after fermentation and 24 months of aging using the Neogen Total Milk ELISA kit. Over the course of aging, milk protein detection was reduced by more than 90% with 93% of signal lost between the highest (2 months) to the lowest (19 months) detection points.

During the aging of Cheddar cheese, an increase in the detection of milk protein residues with the Neogen Veratox® Total Milk ELISA was observed after two and four months of aging. The increase in milk protein detection peaks after 2 months of aging is followed by decreases in detection throughout the remaining aging process. When comparing ELISA results to levels of soluble protein throughout cheese aging, there is a compatible peak in detectable protein content at 2 months of aging. However, compatible results between residual milk protein detection by ELISA and soluble protein were not similarly observed after 6 months of aging.

In previous analyses, it was determined that the Neogen Total Milk kit is primarily sensitive to κ-casein, detecting and quantifying at a ratio of about 50% of the concentration present in the sample. The kit displays only about 10% sensitivity to α- and β-casein. Although κ-casein residues remain in the sample, their prevalence as a ratio of the other present casein fractions is unknown. The peak in detection after 2 months of aging suggests the increased exposure of κ-casein epitopes or protein solubility in this sample. While the kit is capable of detecting some α- and β-casein (10%), the dramatic liberation of solely the soluble epitopes of α- and β-casein is unlikely to cause the observed peak in results observed with the Neogen Veratox® Total Milk kit.

The Neogen Veratox® Casein kit was also used to evaluate detectable milk residues throughout Cheddar cheese ripening.

Table 4.4. Detection of milk residues in aged Cheddar cheese with the Neogen Veratox® Casein kit.

Age (mo)	avg ppm NFDM	Standard Deviation	%CV
0.5	36000	4600	13%
1	49000	2900	6%
2	47000	5800	12%
4	37000	4300	12%
6	22000	5100	23%
8	8400	1500	18%
10	6700	870	13%
13	5800	430	7%
19	1200	70	6%
24	970	90	9%

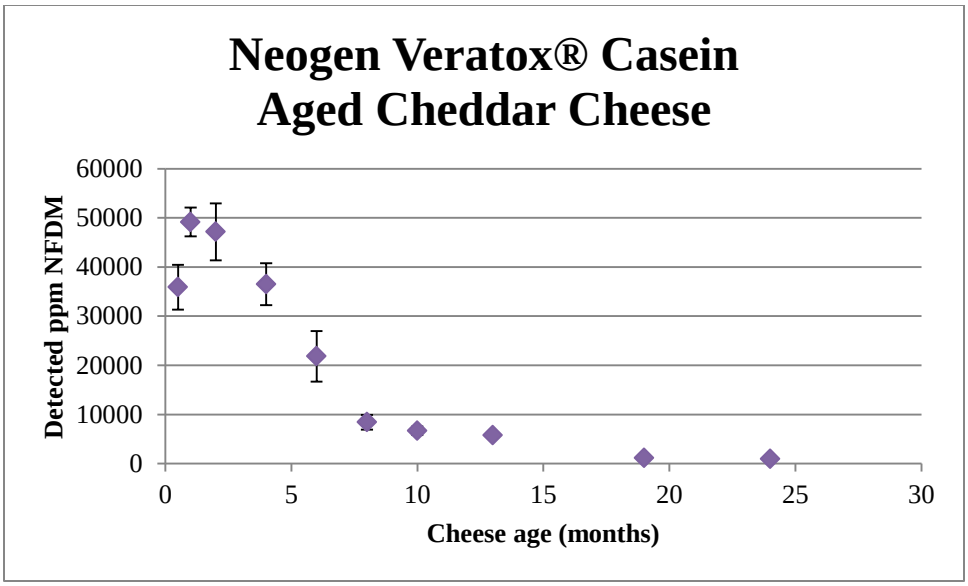


Figure 4.8. Detection of ppm NFDM in aged Cheddar cheese using the Neogen Veratox® Casein kit. Vertical error bars represent standard deviation.

A similar trend in curve shape was observed with the Neogen Veratox® Casein kit as was observed with the Neogen Veratox® Total Milk kit (Figures 4.7 and 4.8). A peak in detectable milk residues occurred in the cheese aged for two months. Over the entire time course of aging, the detection of casein residues using the Neogen Veratox casein ELISA decreased by 97% with a decrease of 98% from the peak level of casein detection. The increase in detectable milk residues observed in the cheese between 2 weeks and 1 month of aging may be a result of exposure of previously hidden linear epitopes upon changes resulting from proteolysis.

Using the Neogen Veratox® Casein kit, a peak in detection of milk residues occurs after 1-2 months of cheese aging. While the detection level is higher after 1 month of aging, there is no observable difference between detection after 1 and 2 months. Similarly to the results obtained using the Neogen Veratox® Total Milk kit, the increase in detection of casein residues after 2 months of cheese aging corresponds with an increase in soluble protein content but no such correlation was observed after 6 months of aging.

According to previous work, the Neogen Veratox® Casein kit quantifies milk residues at a level of 100% of α- and β-casein fragments detected. The peak in detection of casein residues observed after 2 months of cheese aging would suggest an increase in detectable α and/or β-casein residues. Even though the Neogen Veratox® Casein and Total Milk kits detect different fragments of casein in samples, similar trends are displayed in detection of residual milk proteins in Cheddar cheese over time with both kits.

The r-Biopharm Fast Casein kit was also used to evaluate Cheddar cheese. Results are displayed below.

Table 4.5. Detection of casein by the r-Biopharm® Fast Casein kit in aged Cheddar cheese

Age (mo)	avg ppm Casein	Standard Deviation	%CV
0.5	22000	3000	13%
1	24000	2100	8%
2	33000	2200	7%
4	22000	3400	16%
6	16000	150	1%
8	8700	250	3%
10	4500	520	12%
13	3700	160	4%
19	2400	100	4%
24	2200	140	7%

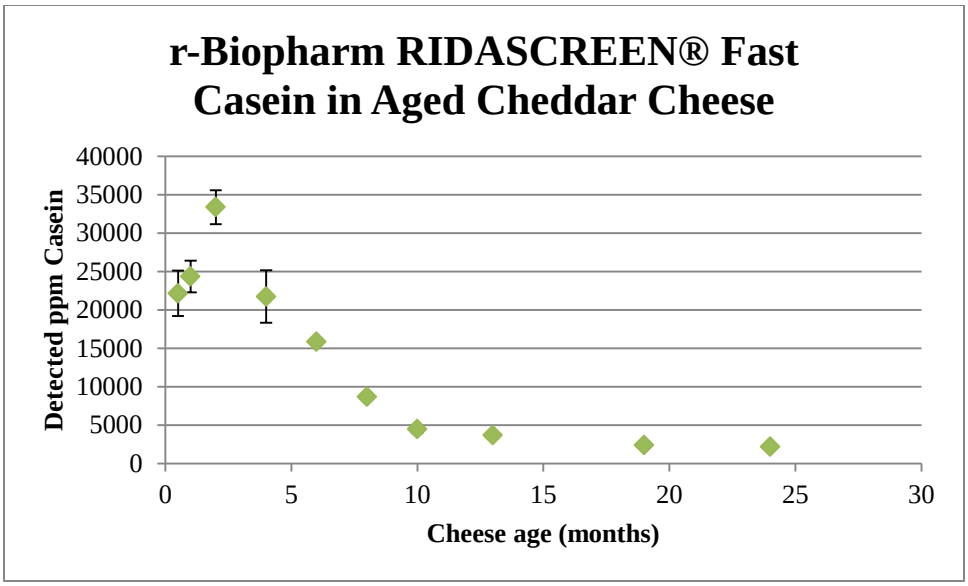


Figure 4.9. Detection of casein residues in aged Cheddar cheese using the r-Biopharm RIDASCREEN® Fast Casein kit. Vertical error bars represent standard deviation.

Using the R-Biopharm RIDASCREEN® Fast Casein kit, a similar trend is observed in detection of milk protein residues as with the two Neogen Kits. A peak in detection is observed after 2 months of cheese aging. The detectable casein content then decreases throughout the remaining period of cheese aging. The lowest level of detectable milk protein is observed after 24 months of aging. With this kit, the detection of casein residues decreased by approximately 93% over the time course of cheese aging between 2 months and 24 months. Over the entire time course of aging, the decrease in detection of casein residues with this kit is approximately 90%. Again, the increase in casein detection after 2 months of cheese aging corresponds to an increase in soluble protein detection but, as with the other ELISAs, a similar correlation was not observed after 6 months of cheese aging.

According to previous data (see Chapter 3 for more information), the r-Biopharm RIDASCREEN® Fast Casein kit relies on detection of κ-casein for quantification. The kit also has a low sensitivity to β-casein, at approximately 10%. Because the Neogen Veratox® Total Milk kit and the r-Biopharm® Fast Casein kit both detect primarily κ-casein, the similar results

obtained between these two kits is not surprising. The increase in detectable milk protein residues observed after 2 months of cheese aging can be explained by the liberation of κ- or β-casein residues due to proteolysis.

ELISA Systems™ Casein kit data is displayed below, in Table 4.6 and Figure 4.10.

Table 4.6. Detection of casein residues in aged Cheddar cheese using the ELISA Systems™ Casein kit

Age (mo)	avg ppm SMP	Standard Deviation	%CV
0.5	40400	2300	6%
1	33700	1500	4%
2	33400	1400	4%
4	23700	870	4%
6	12300	600	5%
8	6600	270	4%
10	2700	180	6%
13	2200	150	7%
19	310	20	7%
24	270	10	5%

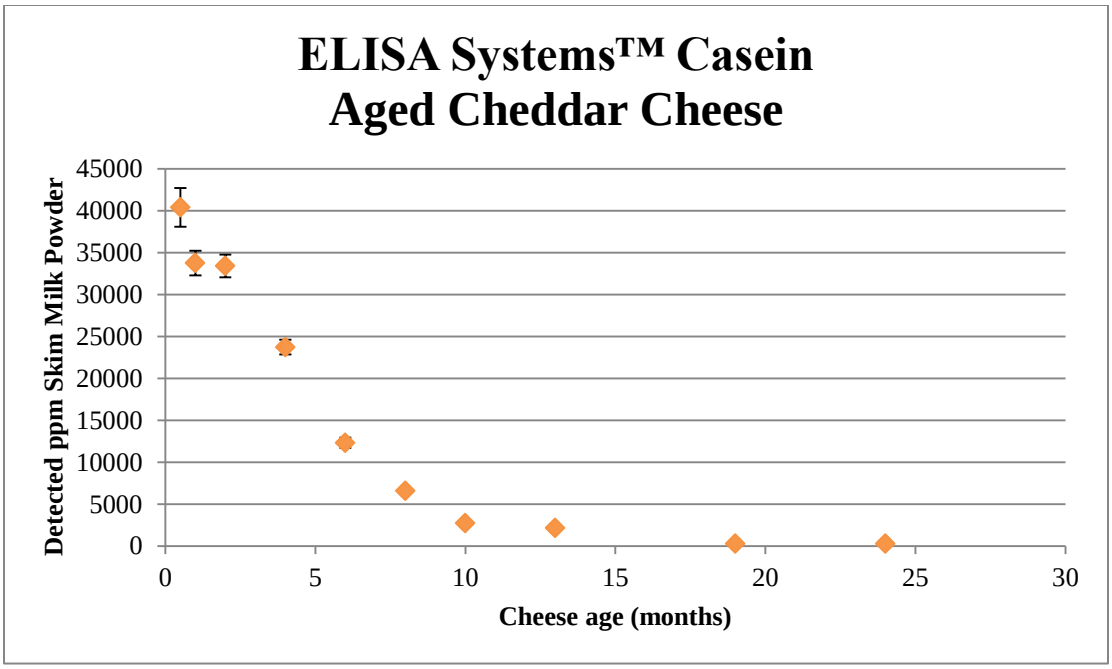


Figure 4.10. Detection of casein residues in aged Cheddar cheese using the ELISA Systems™ Casein kit. Vertical error bars represent standard deviation.

The detection of casein residues in aging cheese displayed a different trend with the ELISA Systems™ Casein kit. Unlike the other three kits tested, no peak in detectable milk protein residues was observed after 2 months of cheese aging. However, the decrease in detection over the entire time period of cheese aging was similar to the trend observed with the other evaluated kits. From production to two years of aging, a 99% decrease in detectability of milk protein residues in cheese by this kit was observed. From previous research, the ELISA Systems™ Casein kit relies strictly on detection of α_s-casein for quantitation (ratio of 0.9).

Because no peak was observed in the detection of milk residues after 2 months of cheese aging with the ELISA Systems™ Casein kit, the evidence initially suggests that the peaks observed with other kits may be a result of increased solubility or revealed epitopes of β or κ-casein. In contrast, the ELISA Systems™ Casein kit result seems to indicate continuous degradation of the α_s-casein without exposure of additional epitopes detected by the kit. Additionally, it is possible that the proteolysis that occurs during cheese aging and fermentation

decreases the solubility of proteins. Caseins are primarily linear proteins and proteolysis may or may not have an effect on their solubility. However, the results may also suggest that the ELISA Systems™ Casein kit was developed using antibodies that recognize only conformational epitopes of α-casein which are progressively lost during cheese aging.

Table 4.7. Cumulative ELISA analysis of milk residues in aged Cheddar cheese

	ppm NFDM			ppm SM
Age (m)	Neogen Total Milk	Neogen Casein	r-Biopharm Fast Casein	ELISA Systems™ Casein
0.5	12000	36000	22000	40400
1	14000	49000	24000	33700
2	15000	47000	33000	33400
4	14000	37000	22000	23700
6	8700	22000	16000	12300
8	5100	8400	8700	6600
10	4400	6700	4500	2700
13	3600	5800	3700	2200
19	970	1200	2400	310
24	1000	970	2200	270

Contrary to the results observed with the Lowry and A_{280} protein assays but consistent with the Microtannin Protein Assay, no peak in detection of milk protein residues was observed after 6 months of aging with any of the selected ELISA kits. However, in the Neogen Veratox® Casein and Total Milk kits and r-Biopharm RIDASCREEN® Fast Casein kit, a peak in milk protein residue detection was observed after 2 months of cheese aging, as also indicated by all protein assays. With all evaluated ELISA kits, the detection of milk protein residues dramatically decreases over the entire time course of cheese aging. A 90-99% reduction in the detection of milk protein residues is observed in all kits from production to 24 months of aging. However, even with this decrease, milk protein residues were still detected, even at the most advanced levels of proteolysis.

Literature suggests that α_{s1}-casein is degraded substantially more than other caseins during Cheddar cheese ripening (Singh et al., 1995). According to ELISA results, the kit that relies exclusively on detection of α-casein epitopes, ELISA Systems™ Casein, exhibits the most dramatic decrease in detection during ripening (99%). Additionally, this kit is the only one that displayed a progressive decrease in the detection of milk protein residues during the entire time course of cheese aging. Thus, this kit might uniquely offer an excellent approach to the monitoring of cheese proteolysis and especially the loss of α_s-casein structure.

While both the Neogen Veratox® Total Milk kit and the r-Biopharm RIDASCREEN® Fast Casein kit are capable of detecting and quantifying κ-casein, the r-Biopharm kit detects a significantly higher concentration of milk residues in aged Cheddar cheese (Figure 4.11). Previous work indicates that the r-Biopharm kit has a 1.0 ratio of detection for κ-casein, while the Neogen Total Milk kit has a 0.5 ratio. This observation may indicate that the kit targets an epitope stable to proteolysis in Cheddar cheese manufacture and ripening. Literature indicates that para-κ-casein is not broken down during cheese ripening, so the degradation observed by the kits must be a result of casein glycomacropeptide f(1-105) (Rank et al., 1985).

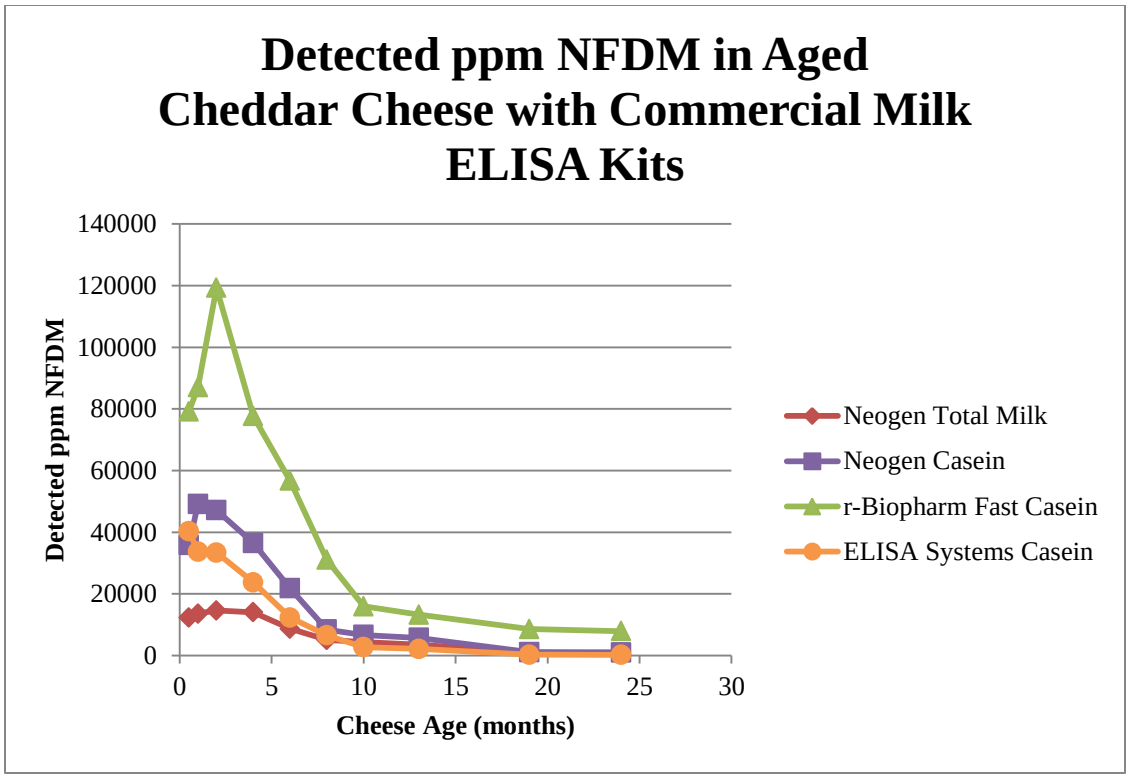


Figure 4.11. Detected ppm NFDM in aged Cheddar cheese as measured by commercial milk ELISA kits

This research highlights the importance of understanding the limitations of ELISA kits for the detection of allergen residues in food products. In this case, fermentation is shown to have a major effect on the detection of milk protein residues in aged Cheddar cheese. This finding is not especially surprising because the antibodies in commercial ELISA kits are oriented toward the detection of intact milk proteins while the milk proteins in aged Cheddar cheese have been subjected to considerable proteolysis. None of the current commercially-available milk ELISA methods are validated specifically for milk allergen detection in fermented foods. The dramatic loss in the ability of the antibodies in these kits to detect milk proteins between young and aged cheese may be a cause for some concern. While high concentrations of Cheddar cheese are detected with current commercially-available ELISA kits, low concentrations of aged or highly proteolyzed cheese will be a challenge to reliably detect and quantify. The recovery of trace amounts of aged cheese in various common food matrices has not yet been assessed by ELISA

methods. The importance of cross contact between cheese with other processed food products has not been fully assessed but might be difficult to assess with the current commercial milk ELISA kits. The construction of milk ELISA kits capable of the detection of milk allergen epitopes stable during fermentation and aging would seem to offer a path toward development of improved methods.

5. Acknowledgements

Special thanks to David Jisa of Jisa's Farmstead Cheese of Brainard, Nebraska, for donating the 20 lb. block of cheese used in this research.

6. References

Addeo, F., Garro, G., Intorcia, N., Pellegrino, L., Resmini, P., Chianese, L., 1995. Gel electrophoresis and immunoblotting for the detection of casein proteolysis in cheese. Journal of Dairy Research 62, 297-309.

Alessandri, C., Sforza, S., Palazzo, P., Lambertini, F., Paolella, S., Zennaro, D., Rafaiani, C., Ferrara, R., Bernardi, M.L., Santoro, M., Zuzzi, S., Giangrieco, I., Dossena, A., Mari, A., 2012. Tolerability of a Fully Maturated Cheese in Cow's Milk Allergic Children: Biochemical, Immunochemical, and Clinical Aspects. PLoS ONE 7, e40945.

Besler, M., Steinhart, H., Paschke, A., 2001. Stability of food allergens and allergenicity of processed foods. Journal of Chromatography B 756, 207-228.

Christensen, T., Bech, A.M., Werner, H., 1991. Methods for crude fractionation (extraction and precipitation) of nitrogen components in cheese. Bulletin-International Dairy Federation, 4-9.

Deegan, K.C., McSweeney, P.L., 2013. Effects of low-pressure homogenisation pre-treatment of cheesemilk on the ripening of Cheddar cheese. Dairy science & technology, 1-15.

Di Luccia, A., Faccia, M., Incoronato, C., Inglese, F., Lamacchia, C., Lamparelli, S., Occidente, M., Matassino, D., 2013. Cheese Processing, Proteomics in Foods. Springer, pp. 247-259.

Fox, P., Guiney, J., 1973. Casein micelle structure: susceptibility of various casein systems to proteolysis. J. Dairy Res 40, 229.

Fox, P.F., McSweeney, P.L.H., 1996. Proteolysis in cheese during ripening. Food Reviews International 12, 457-509.

Gaiaschi, A., Beretta, B., Poiesi, C., Conti, A., Giuffrida, M.G., Galli, C.L., Restani, P., 2001. Proteolysis of β-Casein as a Marker of Grana Padano Cheese Ripening. Journal of Dairy Science 84, 60-65.

Grappin, R., Rank, T.C., Olson, N.F., 1985. Primary Proteolysis of Cheese Proteins During Ripening. A Review. Journal of Dairy Science 68, 531-540.

Green, M.L., Foster, P., 1974. Comparison of the rates of proteolysis during ripening of Cheddar cheeses made with calf rennet and swine pepsin as coagulants. J. Dairy Res 41, 269-282.

Kim, J.S., Nowak-Wegrzyn, A., Sicherer, S.H., Noone, S., Moshier, E.L., Sampson, H.A., 2011. Dietary baked milk accelerates the resolution of cow's milk allergy in children. J Allergy Clin Immunol 128, 125-131.

Kuchroo, C., Fox, P., 1982. Soluble nitrogen in Cheddar cheese: Comparison of extraction procedures. Anglais 37, 331-335.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Larsen, L.B., Wedholm-Pallas, A., Lindmark-Månsson, H., Andrén, A., 2010. Different proteomic profiles of sweet whey and rennet casein obtained after preparation from raw versus heat-treated skimmed milk. Dairy science & technology 90, 641-656.

Ledford, R.A., O'Sullivan, A.C., Nath, K.R., 1966. Residual Casein Fractions in Ripened Cheese Determined by Polyacrylamide-Gel Electrophoresis. Journal of Dairy Science 49, 1098-1101.

Leonard, S.A., Sampson, H.A., Sicherer, S.H., Noone, S., Moshier, E.L., Godbold, J., Nowak-Wegrzyn, A., 2012. Dietary baked egg accelerates resolution of egg allergy in children. J Allergy Clin Immunol 130, 473-480.

Marcos, A., Esteban, M., 1993. Iberian cheeses, Cheese: Chemistry, physics and microbiology. Springer, pp. 173-219.

Marcos, A., Esteban, M.A., León, F., Fernández-Salguero, J., 1979. Electrophoretic Patterns of European Cheeses: Comparison and Quantitation. Journal of Dairy Science 62, 892-900.

Mejbaum-Katzenellenbogen, W., Dobryszycka, W.M., 1959. New method for quantitative determination of serum proteins separated by paper electrophoresis. Clinica Chimica Acta 4, 515-522.

Mooney, J., Fox, P., Healy, A., Leaver, J., 1998. Identification of the principal water-insoluble peptides in Cheddar cheese. International Dairy Journal 8, 813-818.

Mulvihill, D., Fox, P., 1980. Proteolysis of-Casein by Chymosin in Dilute Nac1 Solutions and in Cheddar Cheese. Irish Journal of Food Science and Technology, 13-23.

O'Keeffe, R.B., Fox, P.F., Daly, C., 1976. Contribution of rennet and starter proteases to proteolysis in Cheddar cheese. Journal of Dairy Research 43, 97-107.

O'Sullivan, M., Fox, P.F., 1990. A scheme for the partial fractionation of cheese peptides. Journal of Dairy Research 57, 135-139.

Quesnel, V.C., 1968. Fractionation and properties of the polymeric leucocyanidin of the seeds of Theobroma cacao. Phytochemistry 7, 1583-1592.

Rank, T.C., Grappin, R., Olson, N.F., 1985. Secondary Proteolysis of Cheese During Ripening: A Review. Journal of Dairy Science 68, 801-805.

Sampson, H.A., 2004. Update on food allergy. Journal of Allergy and Clinical Immunology 113, 805-819.

Sicherer, S.H., Sampson, H.A., 2010. Food allergy. Journal of Allergy and Clinical Immunology 125, S116-S125.

Singh, T., Fox, P., Højrup, P., Healy, A., 1994. A scheme for the fractionation of cheese nitrogen and identification of principal peptides. International Dairy Journal 4, 111-122.

Singh, T.K., Fox, P.F., Healy, A., 1995. Water-soluble peptides in Cheddar cheese: isolation and identification of peptides in the diafiltration retentate of the water-soluble fraction. Journal of Dairy Research 62, 629-629.

Singh, T.K., Fox, P.F., Healy, Á., 1997. Isolation and identification of further peptides in the diafiltration retentate of the water-soluble fraction of Cheddar cheese. Journal of Dairy Research 64, 433-443.

Skripak, J.M., Matsui, E.C., Mudd, K., Wood, R.A., 2007. The natural history of IgE-mediated cow's milk allergy. J Allergy Clin Immunol 120, 1172-1177.

Skripak, J.M., Nash, S.D., Rowley, H., Brereton, N.H., Oh, S., Hamilton, R.G., Matsui, E.C., Burks, A.W., Wood, R.A., 2008. A randomized, double-blind, placebo-controlled study of milk oral immunotherapy for cow's milk allergy. J Allergy Clin Immunol 122, 1154-1160.

Sousa, M.J., Ardö, Y., McSweeney, P.L.H., 2001. Advances in the study of proteolysis during cheese ripening. International Dairy Journal 11, 327-345.

Trayer, H.R., Trayer, I.P., 1988. Fluorescence resonance energy transfer within the complex formed by actin and myosin subfragment 1. Comparison between weakly and strongly attached states. Biochemistry 27, 5718-5727.

Upadhyay, V.K., McSweeney, P.L.H., Magboul, A.A.A., Fox, P.F., 2004. Proteolysis in cheese during ripening, in: P.L.H.M.T.M.C. Patrick F. Fox, P.G. Timothy (Eds.), Cheese: Chemistry, Physics and Microbiology. Academic Press, pp. 391-VIII.

Wal, J.-M., 2002. Cow's milk proteins/ allergens. Annals of Allergy, Asthma and Immunology 89, 3-10.

Wal, J.-M., Bernard, H., Clement, G., Chatel, J.-M., Adel-Patient, K., 2001. Characterization of allergens in milk. Current Allergy and Clinical Immunology 14, 4-6, 8-10.

Wallace, J., Fox, P., 1998. Rapid spectrophotometric and fluorimetric methods for monitoring nitrogenous (proteinaceous) compounds in cheese and cheese fractions: a review. Food Chemistry 62, 217-224.

Warburg, O., Christian, W., 1942. Insulation and Crystalisation of the Fermenting Process of Enolase. Biochem Z 310, 384-421.

CHAPTER 5. SURVEY OF RETAIL CHEESES: THE EFFECT OF DIFFERENT METHODS OF FERMENTATION ON THE DETECTION OF MILK PROTEIN RESIDUES BY ENZYME-LINKED IMMUNOSORBENT ASSAY

1. Abstract

Milk is one of the most important allergenic foods in the United States and around the world and contains several major antigens with differing susceptibilities to proteolytic enzymes. The extent of proteolysis in cheese varies as a result of conditions during manufacture and ripening. Proteolysis has the potential to degrade allergenic epitopes that are important for residue detection and elicitation of allergic reactions. Enzyme-linked immunosorbent assays are not currently validated for use in detecting residues in hydrolyzed or fermented products. Five retail cheeses produced using different styles of fermentation were investigated for detectable milk protein residues with four commercial ELISA kits. Pasta-filata, propionic acid-fermented, surface-mold ripened, internal mold ripened, and surface bacterial-ripened cheeses were assessed. The Neogen Veratox® Total Milk and r-Biopharm RIDASCREEN® Fast Casein kits are capable of detecting milk residues in all cheeses evaluated. Only kits that employ antibodies sensitive to κ-casein are capable of detecting milk residues in blue cheese that exhibits extensive proteolysis. The other two ELISA kits, Neogen Veratox® Casein and ELISA Systems™ Casein can detect milk residues in cheeses other than blue-veined varieties. ELISA results cannot be quantitatively compared among kits. The quantitative reliability of ELISA results in detection of cheese residues is questionable, but these methods are sufficiently robust to use as a semi-quantitative indication of proper allergen control or the validation of cleaning programs in industry settings.

2. Introduction

Proteolysis is arguably one of the most important biochemical processes for texture and flavor development in cheese (Fox and McSweeney, 1996). Some cheeses, like Mozzarella, are minimally ripened and are served fresh. Other cheeses, such as blue-veined varieties, are

subjected to extensive proteolysis. However, proteolysis during the manufacture and ripening of cheese can potentially destroy milk protein epitopes that are important for detection of allergenic residues or elicitation of reactions in milk-allergic consumers.

Cow's milk is one of the most dominant food allergens, especially among infants and children in the United States (Sicherer, 2011a). Cow's milk is composed of two main groups of proteins; whey and caseins. The caseins comprise roughly 80% of total milk proteins, while whey proteins compose the remaining 20% (Wal, 2001b). While two whey proteins, β-lactoglobulin and α-lactalbumin play major roles in milk allergy, these proteins are drained from the curds during the production of cheese and have minimal presence in finished cheeses. The caseins, α-, β, and κ-casein, are retained in the cheese; casein is also a major milk allergen (Wal, 2001b).

There are several important proteolytic enzymes in cheese that contribute to ripening. The two most important enzymes for primary proteolysis are chymosin and plasmin. These enzymes are reviewed more in depth in Chapter 4 of this thesis. Chymosin is added to milk during the initial stages of cheese making. This enzyme initially drives the disruption of milk micelles and causes the coagulation of milk into curd by hydrolyzing a specific bond in κ-casein; Phe_{105}-Met_{106}. Whey proteins and the soluble fragment of κ-casein f(1-105) are drained from curds during manufacture. Plasmin is an indigenous milk protease and exhibits the highest level of activity in the later stages of cheese ripening. Primary proteolysis of all cheese varieties involves hydrolysis of α_{s1}-casein by chymosin and β-casein by plasmin (Fox and McSweeney, 1996). The levels of proteolysis by chymosin and plasmin depend on the variety of cheese and conditions of ripening.

Additional bacteria and mold cultures are often added to cheese during production. These cultures are employed specifically to produce characteristic flavors and textures during

ripening through the action of unique enzymes (Fox et al., 2004; Rank et al., 1985; Upadhyay et al., 2004).

The extent and patterns of proteolysis in cheese are used as indicators of product quality and maturity. Proteolysis of cheese has been evaluated in numerous ways and has been thoroughly reviewed (Bansal et al., 2009; Fox, 1989; Fox and McSweeney, 1996; Fox et al., 2004; McSweeney and Fox, 1997; Sousa et al., 2001; Upadhyay et al., 2004). The relationship between style of fermentation and the presence of intact milk residues and allergenic epitopes has not been evaluated thoroughly.

Different degrees of proteolysis among retail cheeses indicate the possibility that allergenic epitopes are also differentially degraded in various cheeses. This may provide issues for milk allergic consumers, especially those that try to avoid foods that may contain allergenic proteins.

This research seeks to determine if commercially-available milk ELISA kits are capable of detecting residues in five types of retail cheeses with differing degrees of proteolysis. Measurement of the extent of proteolysis in cheese may suggest further application for ELISA kits as indicators of cheese quality and maturity. Additionally, results may indicate whether concern is warranted for kit manufacturers, the food industry, or food-allergic consumers associated with the detecting trace levels of cheese in food products.

3. Materials and Methods

Cheese Procurement and Sample Preparation

Five varieties of commercial cheese were purchased from a local retail establishment. Cheese varieties included Mozzarella (BelGiosio Cheese Inc. Green Bay, Wisconsin), Emmentaler (Emmi, Lucerne, Switzerland), Blue (Maytag Dairy Farms, Newton, Iowa),

Limburger (Landhaus, Monroe, Wisconsin), and Brie (Martin-Collet, Normandy, France). Approximately 1 lb of each cheese was purchased.

Individual pieces of cheese were sliced in half so as to provide a full representation of aging throughout the wedge or wheel. Brie cheeses aged in wheels may have different degrees of proteolysis throughout the slices in which the cheese is sold (Upadhyay et al., 2004). Where necessary (Brie and Emmentaler), rinds were removed from cheeses. All cheese samples were partitioned into approximately 1 cm x 1 cm cubes. 30g of each cheese was finely ground using a Spex 6850 CentriPrep Freezer/Mill (Metuchen, NJ). Freezer-milled samples were placed into plastic zip-top bags and were frozen at -20^{O}C until time of analysis.

Preparation of Extracts for SDS-PAGE and ELISA Analysis

For protein concentration assays and SDS-PAGE analysis, 5 g of each freezer-milled cheese was mixed with 20 mL of 5% sodium chloride solution in a 50 mL Falcon™ tube, using a pulse-vortex technique (BD Biosciences, San Jose, CA). Cheeses were extracted with horizontal shaking for 60 minutes hour at room temperature (22^{o}C) on a Barnstead Thermolyne LabQuake Shaker (Thermo Fisher Scientific, Rockford, IL). After extraction, the samples were centrifuged for 30 minutes at 3020 x g at 10^{o}C in an IEC Centra MP4R Centrifuge (International Equipment Company, Needham Heights, MA). The aqueous layer was removed to a new 50 mL Falcon™ tube, and pellets and fat layers were discarded (BD Biosciences, San Jose, CA). Aqueous layers were frozen at -20^{o}C until time of analysis.

For preparation for ELISA analysis, 20 g of each finely-ground freezer-milled cheese was defatted using a cold hexane extraction. 180 g of cold hexane (-20^{o}C) was poured over 20 g of each freezer-milled cheese in a 500 mL Erlenmeyer flask. Flasks were briefly mixed using a swirling technique, covered, and placed in an ice bath on a Barnstead Thermolyne LabQuake Horizontal Shaker (Thermo Fisher Scientific, Rockford, IL) in a fume hood. Samples were

allowed to extract for 30 minutes with horizontal shaking. The hexane and fat layer was decanted into an appropriate waste collection container. Another 180 mL of cold hexane was added to each flask. The swirling technique and 30 minute extraction were repeated. The hexane and fat layer was decanted, and the cheese was suspended in a final wash of 180 mL of cold hexane and was allowed to extract for a final 30 minutes. The final cheese and hexane extraction were swirled to mix and filtered by gravity filtration through a fluted Whatman Size 1 Filter paper in a glass funnel. The filter paper and retained cheese were removed from the funnel, laid flat, and were allowed to dry overnight in a fume hood. Dried and defatted cheese samples were transferred to plastic zip-top bags and stored at -20^{o}C until the time of analysis. Used hexane was disposed according to directions provided by the Office of Environmental Health and Safety at the University of Nebraska-Lincoln.

Defatted cheese samples were prepared for ELISA analysis by dissolution in 5% sodium chloride. 1 g of each defatted cheese was mixed with 9 mL of 5% NaCl by pulse-vortex technique in a 15 mL Falcon™ tube. Solutions were horizontally shaken at 90% max speed for 3 hours on a Barnstead Thermolyne LabQuake Horizontal Shaker (Thermo Fisher Scientific, Rockford, IL). Solutions were centrifuged at 3,020 x g for 30 minutes at 10^{o}C. The aqueous layer was decanted to a new 15 mL Falcon™ tube and pellets and fat layers were discarded. Samples were frozen at -20^{o}C until time of analysis.

Protein Concentration of Extracts

Micro- Lowry, Microtannin, and A_{280} protein assays were used to measure the concentration of protein in cheese extracts. Extracts were prepared as described above for SDS-PAGE. The Micro-Lowry assay was performed according to standard protocol and adapted for use in a 96-well microtiter plate (Lowry et al., 1951). The Lowry assay is a colorimetric dye-binding assay that quantitates protein in a sample through copper-protein complex formation. A

2 mg/mL standard solution of Bovine Serum Albumin (BSA) was diluted to build a standard curve of 0-200 μg/mL (Pierce Scientific, a division of Thermo Scientific, Rockford, IL). Briefly, a solution of 2% sodium carbonate in 0.1 N sodium hydroxide was generated, and referred to as solution A. A 0.5% cupric sulfate solution in deionized, distilled water (d_2H_2O) was also constructed and called solution B. For a 96-well microtiter plate, 23 mL of solution A was added to 0.46 mL solution B and mixed well by a pulse-vortex method. The A + B solution was referred to as solution C. Solution D constituted a 1 N solution of Folin Reagent. For a 96-well microtiter plate, approximately 2.4 mL of solution D was required. The assay was performed on cheese extracts at several levels of dilution. Samples were diluted with 5% sodium chloride to fall within the constructed standard curve. 40 μL of each dilution level was applied in duplicate to a 96-well plate. BSA standard solutions (0-200 μg/mL) were applied in triplicate. 200 μL of Lowry Solution C was added to each well using a multi-channel pipette and the plate was incubated at room temperature (22^{o}C) for 10 minutes. After the incubation, 20 μL of Lowry solution D was added to each well of the plate using a multi-channel pipette. The plate was mixed for 30 seconds on a horizontal plate shaker and the plate was incubated at room temperature for an additional 30 minutes. The absorption was read at 490 nm with a BioTek EC808x Microplate Reader and evaluated with KC Junior Software (Winooski, VT).

The Microtannin protein precipitation assay was also performed on extracts prepared for SDS-PAGE. The Microtannin assay relies on the precipitation and suspension of protein, driven by the formation of phenol-tannic acid complexes (Mejbaum-Katzenellenbogen and Dobryszycka, 1959; Trayer and Trayer, 1988). Two solutions were required for the assay, tannin reagent and gum Arabic solution. Tannin reagent, a solution of 10% w/v tannic acid in 2% phenol v/v in 1 N HCl, was produced. A 0.2% w/v solution of gum Arabic was also prepared. For the assay, samples were diluted to fall within the standard curve range, 0-75 μg/mL BSA using 5% sodium chloride. 1 mL of tannin reagent was added to 1 mL of each unknown sample

in duplicate borosilicate glass disposable culture tubes. Samples were briefly mixed using a pulse-vortex technique and were allowed to incubate for 10 minutes at room temperature (22^{o}C). 1 mL 0.2% gum Arabic solution was added to each tube, mixed by the pulse vortex method, and the absorbance of precipitated protein in standards and samples was read in disposable cuvettes with 10 mm path length in a NanoDrop 2000c Spectrophotometer at 520 nm (ThermoScientific, Rockford, IL).

The UV absorbance of extracts prepared for SDS-PAGE was measured at A_{280}. The A_{260}/A_{280} ratio was used to correct the estimated protein content by applying the Warburg-Christian method. Because proteins containing tyrosine, tryptophan, and disulfide bonds and ribonucleic acids absorb light at 280 nm, and only nucleic acids absorb at 260 nm, the A_{260}/A_{280} ratio will remove the contribution of nucleic acids from the concentration estimate. A_{280} was measured using the pedestal function of a NanoDrop 2000c Spectrophotometer. The Warburg-Christian equation is listed below.

Equation 5.1. Warburg-Christian Method for determining protein concentration from A280

$$(A_{280}) \times (correction\ factor) = mg/mL\ protein$$

Correction factors were determined from corresponding A_{260}/A_{280} ratio values in a standard table (Warburg and Christian, 1942).

Electrophoresis

Results from the protein quantitation assays were used to determine the necessary volume of sample per well. Because published literature does not indicate the superiority of any assay over the others for accurately determining the concentration of soluble proteins in cheese extracts, the results for the Micro-Lowry, Microtannin, and A_{280} assays were averaged and used to calculate a general estimate of concentration for electrophoretic purposes only. Samples were

prepared under reducing conditions (Laemmli, 1970). Briefly, 25 µL of each extracted sample was mixed with 25 µL of Laemmli sample buffer containing 5.4% dithiothreitol and was boiled in a 100^{O}C water bath for 5 minutes. Reduced samples were allowed to return to room temperature and were centrifuged for 5 minutes at 16,200 x g in a ThermoScientific Legend Micro 17 centrifuge (ThermoScientific, Rockford, IL). Protein separation was performed in a Bio-Rad Mini-Protean® Tetracell electrophoresis unit using 18% Tris-HCl precast polyacrylamide Ready Gels® for Mini-Protean® systems, 10 wells, 30 µL maximum volume per well (Bio-Rad Laboratories, Hercules, CA). 5 µL of Precision Plus Protein™ Dual X-tra Standard was loaded in the first lane of each gel to serve as a molecular weight marker (Bio-Rad Laboratories, Hercules, CA). Samples were loaded on gels in two different concentrations; 10 µg/lane and 15 µg/lane. Empty lanes were loaded with 5 µL of sample buffer to improve electrophoretic profiles.

Gels were run at 200 volts for approximately 30-40 minutes, or until the dye front reached the bottom of the gel. Gels were removed from their cassettes, rinsed in several changes of d_2H_2O, and fixed in 1x fixing solution for 30 minutes. Gels were rinsed in several changes of d_2H_2O and stained overnight using the Coomassie Brilliant Blue R-250 Staining System (Bio-Rad Laboratories, Hercules, CA). After staining, gels were allowed to destain for approximately 3-4 hours, with 3-4 changes of destain solution, or until the desired level of destaining was reached. Gels were rehydrated for 10 minutes in a solution of 25 mM Tris and 192 mM Glycine at pH 8.3 (Bio-Rad Laboratories, Hercules, CA). Gels were then imaged at F=8.0, Z=30.0 with UV/ Fluorescence in a Kodak Gel Logic Imaging System equipped with Carestream Molecular Imaging Software (Eastman Kodak, Rochester, NY).

ELISA Analysis

Four commercial ELISA kits were used. Neogen Veratox® Total Milk and Casein ELISA kits were obtained from Neogen® Corporation (Lansing, MI, USA). ELISA Systems™

Casein kits were obtained from ELISA Systems (Windsor, Queensland, Australia). R-Biopharm RIDASCREEN® Fast Casein (Darmstadt, Germany) kits were obtained from Pi Bioscientific (Seattle, WA, USA). Kit information regarding the levels of detection and quantitation (LOD and LOQ, respectively), composition of manufacturer-provided standard solutions, and kit reporting units are displayed in Table 5.1.

Table 5.1. Commercial Milk ELISA kit sensitivities. Limits of Detection (LOD) and Limit of quantitation (LOQ) are listed for each kit.

Kit	LOD	LOQ	Standards	Reporting Units (ppm)
r-Biopharm Fast Casein	0.12 ppm	0.5 ppm	casein	casein
Neogen Veratox Total Milk	1 ppm	2.5 ppm	NFDM	NFDM
Neogen Veratox Casein	1 ppm	2.5 ppm	NFDM	NFDM
ELISA Systems Casein	0.5 ppm	1.0 ppm	a-casein	skim milk powder

Prior to sample extraction according to the ELISA procedure, defatted and suspended samples were diluted an additional 1:100 in 5% sodium chloride. Extractions were performed according to manufacturer-provided protocol for 1 mL of liquid sample. Briefly, 1 mL of liquid sample was extracted in a recommended amount of heated (60^{O}C) extraction buffer in a disposable Falcon™ tube. Samples were briefly mixed using a pulse-vortex technique and were extracted in a 60^{O}C shaking water bath for a set amount of time. Next, samples were cooled to room temperature and 1 mL of each extraction was centrifuged for 5 minutes at 16,200 x g in a ThermoScientific Legend Micro 17 centrifuge (ThermoScientific, Rockford, IL). Additional dilutions were performed as necessary for samples to fall within the dynamic range of each kit (Appendix B). All samples were extracted in triplicate.

ELISA assays were performed according to protocols provided. Briefly, 100 µL of each manufacturer-provided standard and extracted sample were applied to antibody-coated wells of a microtiter plate. All samples were applied to triplicate wells. Plates were allowed to incubate for the appropriate amount of time (10-15 minutes, depending on each kit). At the end of incubation,

plates were inverted and sample was poured out of the wells. Wells were filled with wash buffer, plates were inverted, and the process was repeated for the appropriate number of washes. At the final wash, plates were inverted and excess wash buffer was tapped out onto an absorbent paper towel.

Next, 100 µL of enzyme-labeled conjugate antibody solution was added to each well and plates were incubated for 10-15 minutes, according to manufacturer's instructions. Plates were washed as described above and excess buffer was removed. 100 µL of substrate solution was added to each well and plates were incubated in the dark for a final 10-15 minutes to allow a colorimetric reaction to occur. In the four kits evaluated, the intensity of color development is directly related to the concentration of detected protein residue in the sample. 100 µL of acidic stop solution was added to each well to prevent additional color development. The absorbance of each well was read at the manufacturer recommended wavelength using a BioTek Eon Microplate Spectrophotometer equipped with Gen5 v 2.0 Software (Winooski, VT).

Data Analysis

Data was analyzed using Microsoft Excel, GraphPad Prism v 4.03, and SAS v 9.2. Appropriate curve fits for ELISA kits were determined using PROC GLIMMIX regression analysis in SAS v 9.2. All kit standard curves were appropriately modeled using a quadratic response curve. Optical density readings for unknown samples were fit to the curve and results were interpolated. Where appropriate for comparisons, ELISA kit results were converted to relevant units using equations 5.2 and 5.3.

Equation 5.2. Conversion detected concentration of ppm NFDM to ppm casein

$$ppm\ casein = \frac{0.35\ ppm\ milk\ protein}{1\ ppm\ NFDM} \times \frac{0.8\ ppm\ casein}{1\ ppm\ milk\ protein} \times detected\ ppm\ NFDM$$

Equation 5.3. Conversion of detected ppm casein to ppm NFDM

$$ppm\ NFDM = \frac{1\ ppm\ milk\ protein}{0.8\ ppm\ casein} \times \frac{1\ ppm\ NFDM}{0.35\ ppm\ milk\ protein} \times detected\ ppm\ casein$$

4. Results

Protein Concentration Determination Assays

Results obtained with the three protein concentration assays are listed below (Tables 5.2-5.4, Figure 5.1). Micro-Lowry assay results are displayed in Table 5.2 and Figure 5.1. The amount of sodium chloride-soluble protein is lowest in Mozzarella cheese, and highest in Brie cheese. The %CV for all samples except Mozzarella cheese is quite high, indicating variability in solubility, sample homogeneity or assay performance.

Table 5.2. Micro-Lowry Protein Assay of Retail Cheeses.

Micro-Lowry Protein Assay[a]			
Sample	**Concentration (µg/µL)**	**Std. Dev**	**%CV**
Mozzarella	7.6	0.2	3%
Emmentaler	15.2	2.5	17%
Blue	10.4	3.8	36%
Limburger	11.0	3.9	35%
Brie	25.0	7.9	32%

[a] Cheeses were extracted in 5% sodium chloride.

Results of the Microtannin assay are listed in Table 5.3 and Figure 5.1. Assay results indicate that Mozzarella and Emmentaler cheese display similar protein concentration (11.1 ± 0.7 and 11.1 ± 0.1 µg/µL protein, respectively). Blue cheese displays a very low protein concentration in extracts, while Brie displays the highest concentration of the extracts evaluated (1.2 ± 0.02 µg/µL protein and 19.1 ± 0.6 µg/µL protein, respectively). The Limburger cheese extract contains approximately 13.7 ± 0.8 µg/µL protein, according to the Microtannin assay

(Table 5.3). Samples evaluated with the Microtannin assay have a much lower %CV than samples evaluated with the Micro-Lowry method.

Table 5.3. Microtannin Protein Assay of Retail Cheeses.

Microtannin Protein Assay			
Sample	**Concentration (µg/µL)**	**Std. Dev**	**%CV**
Mozzarella	11.1	0.7	6%
Emmentaler	11.1	0.1	1%
Blue	1.2	0.0	1%
Limburger	13.7	0.8	6%
Brie	19.1	0.6	3%

[a] Cheeses were extracted in 5% sodium chloride.

A_{280} data with the Warburg-Christian Correction applied are displayed in Table 5.4 and Figure 5.1. Mozzarella and Emmentaler cheese display the two lowest concentrations of protein in the extracts as measured by the A_{280} assay (3.6 ± 0.1 and 4.9 ± 1.4 µg/µL protein, respectively). Limburger cheese has the highest protein concentration in the extracts with 16.5 ± 1.3 µg/µL protein, followed by Blue and Brie cheese, at concentrations of 13.5 ± 1.3 and 10.4 ± 1.8 µg/µL protein, respectively. The %CVs observed in the assay are lower than the levels observed in the Micro-Lowry assay.

Table 5.4 Absorbance at 280nm of Retail Cheeses with Warburg-Christian Correction.

A_{280} Protein Assay			
Sample	**Concentration (µg/µL)**	**Std. Dev**	**%CV**
Mozzarella	3.6	0.1	2%
Emmentaler	4.9	1.4	29%
Blue	13.5	1.3	10%
Limburger	16.5	1.3	8%
Brie	10.4	1.8	18%

[a] Cheeses were extracted in 5% sodium chloride.

Excluding data obtained during the analysis of Mozzarella extracts, the Microtannin assay exhibits the lowest variation in detection across all other cheese samples.

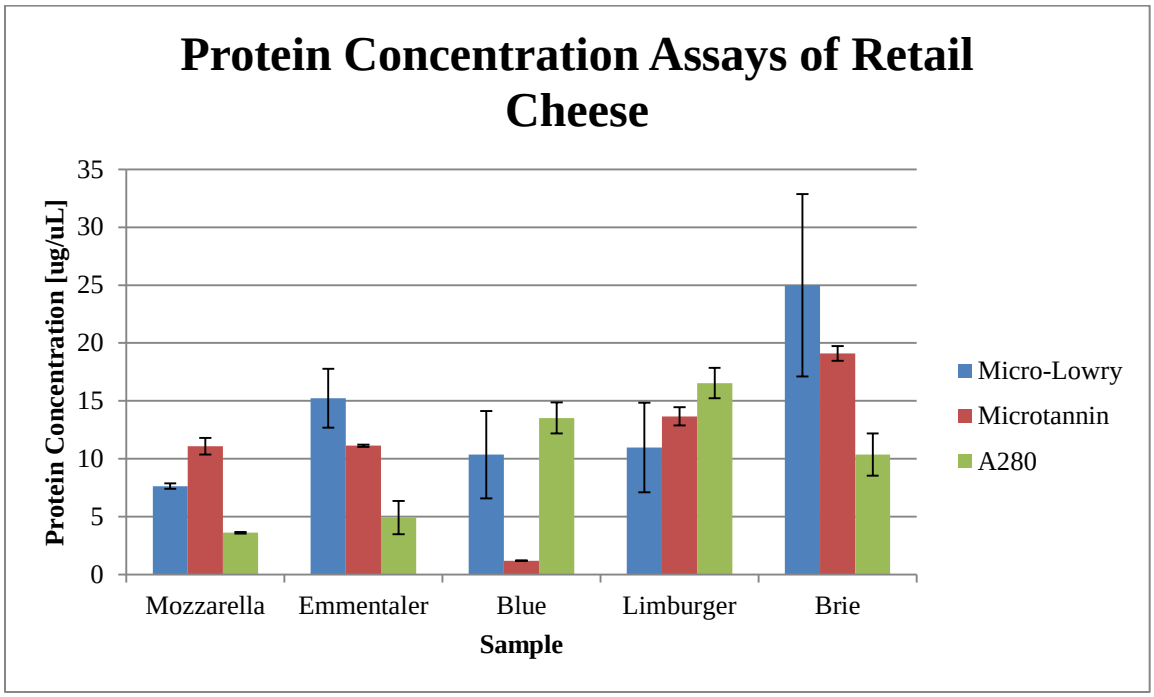


Figure 5.1. Cumulative results of protein assays for retail cheese samples. Error bars represent standard deviation. All sample extracts were solubilized in 5% NaCl prior to the assay.

Electrophoresis

SDS-PAGE profiles of cheese extracts were observed. The gel loaded with 15 µg protein/lane is depicted in Figure 5.2 as determined by averaging the concentrations of the three protein assays. See Methods section for more information.

Intact α-, β-, and κ-casein bands migrate near the 20-25 kDa molecular weight marker (Wal, 2001b). Intense casein bands appear in extracts of Mozzarella, Limburger, and Brie cheeses (lanes 2, 5, and 6, respectively). Emmentaler cheese displays some faint bands in the casein migration region (Lane 3, Figure 5.2, 20-25 kDa). There are low molecular weight traces of protein, although there are no definitive bands present below 10 kDa. In SDS-PAGE

evaluation of Limburger cheese extracts, intense bands are observed both in the casein region (20-25 kDa) and between 10-15 kDa (Lane 5, Figure 5.2). These bands appear in a similar region of the whey proteins, β-lactoglobulin and α-lactalbumin, which migrate near 18.3 and 14.2 kDa, respectively (Wal, 2001b). However, these bands may also correspond to degradation products of larger molecular weight milk proteins. In Brie extracts, intense bands are observed in the casein migration region (Lane 6, Figure 5.2). Additionally, heavy bands are also observed in the region between 10-15 kDa, which may correspond to β-lactoglobulin and α-lactalbumin residues. Casein bands are not apparent in the Blue cheese extract, as shown in Lane 4. The low presence of bands in the gel emphasizes the low level of intact protein in the sample. Blue cheese appears to exhibit the most extensively proteolysis among the retail cheese samples evaluated.

Low molecular weight bands in any lane may correspond to peptide fragments of larger molecular weight proteins that result from proteolysis occurring during cheese manufacturing. Some banding below 10 kDa is also observed, likely corresponding to breakdown products of higher molecular weight milk proteins. Light shading appears in low molecular weight regions of the gel, especially in Mozzarella, Limburger and Brie extracts (lanes 2, 3, and 6).

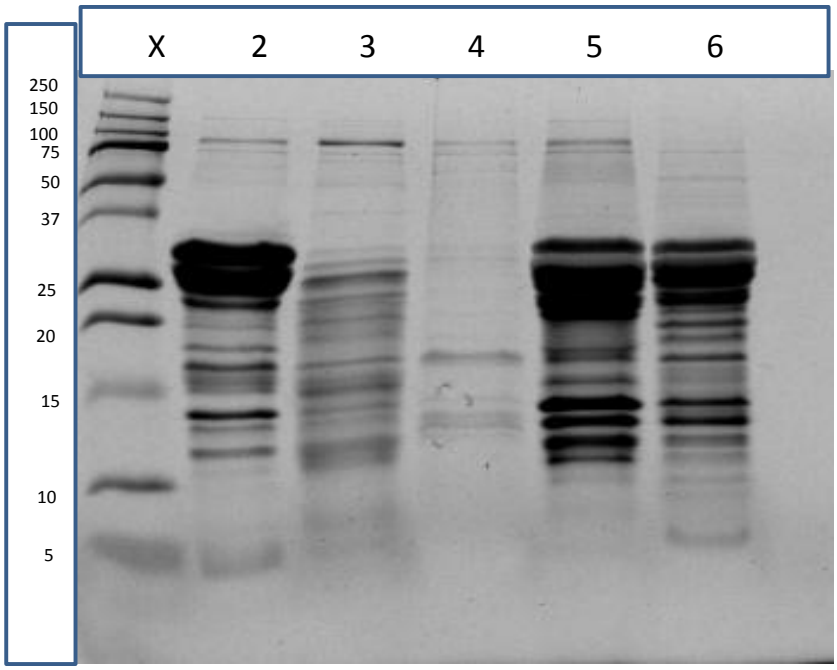


Figure 5.2. SDS-PAGE gel of 5% sodium chloride soluble cheese extracts; 15 µg protein per lane. Lane X: molecular weight ladder. Lane 2: Mozzarella; Lane 3: Emmentaler; Lane 4: Blue; Lane 5: Limburger; Lane 6: Brie. Gels were run at a constant voltage of 200V for approximately 35 minutes.

ELISA Analysis

To compare the results of all four commercial ELISA kits for milk residues, the results were converted, where necessary (Neogen Veratox® Total Milk and Casein kits), to ppm casein using Equation 5.2. All cheeses contained detectable milk residues with all cheeses except with the two casein ELISA methods for Blue cheese.

In the r-Biopharm RIDASCREEN® Fast Casein kit, Brie cheese displayed the highest amount of detectable milk residue at 39,000 ± 4,000 ppm casein (Table 5.5), while Blue cheese displayed the lowest detectable milk protein residue at 16 ± 1.6 ppm casein. All samples contain detectable milk residues.

Table 5.5. r-Biopharm RIDASCREEN® Fast Casein ELISA analysis of retail cheeses

r-Biopharm Fast Casein ELISA Results			
Sample	Detected Average ppm Casein	Std. Dev.	%CV
Mozzarella	21000	2500	12%
Emmentaler	4400	330	8%
Blue	16	1.6	10%
Limburger	2700	130	5%
Brie	39000	4000	10%

The analysis of retail cheeses with the Neogen Veratox® Total Milk kit displayed similar results to the r-Biopharm RIDASCREEN® Fast Casein kit. The Blue cheese extract displays the lowest concentration of milk residues (410 ±40 ppm casein, Table 5.6). Brie and Mozzarella cheese display the highest detectable concentration of milk residues in the extracts at 7,300 ± 1,500 ppm casein and 7,000 ± 460 ppm casein, respectively. The highest level of variation is observed with the Neogen Veratox® Total Milk kit during the analysis of Brie cheese extracts, at 21% CV.

Table 5.6. Neogen Veratox® Total Milk ELISA analysis of retail cheeses.

Neogen Veratox® Total Milk ELISA Results			
Sample	Detected Average ppm Casein	Std. Dev.	%CV
Mozzarella	7000	460	7%
Emmentaler	2300	210	9%
Blue	410	40	10%
Limburger	6200	250	4%
Brie	7300	1500	21%

The Neogen Veratox® Casein kit detects the highest concentration of milk residues in Limburger cheese extract at 21,000 ± 2,900 ppm casein (Table 5.7). Milk residue is not detected in Blue cheese extracts using the Neogen Veratox® Casein kit. The lower limit of quantitation

for this kit is 2.5 ppm NFDM or approximately 0.7 ppm casein). Mozzarella displays the second highest level of detected milk residues of the cheeses evaluated (12,000 ± 1,200 ppm casein). The highest variation in extracts is observed in Brie cheese, at 24% CV.

Table 5.7. Neogen Veratox® Casein ELISA Analysis of Retail Cheeses.

Neogen Veratox Casein ELISA Results			
Sample	Detected Average ppm Casein	Std. Dev.	%CV
Mozzarella	12000	1200	10%
Emmentaler	4200	460	11%
Blue	BLQ[a]	.	.
Limburger	21000	2900	14%
Brie	2100	500	24%

[a]BLQ indicates a reading below the level of quantitation for the kit. See Table 5.1 for limits of quantitation.

The ELISA Systems™ Casein kit detected high levels of milk residue in all cheese extracts except for Blue cheese where no detectable casein was found. The limit of quantitation (LOQ) in this kit is 1 ppm NFDM (approximately 0.28 ppm casein, see Table 5.1). The highest levels of milk residue were observed in Mozzarella and Limburger cheese at 40,000 ± 2,300 and 33,000 ± 2,100 ppm casein, respectively (Table 5.8). Low levels of variation were observed among all cheese extracts, with the highest %CV at approximately 6% for both Mozzarella and Limburger cheese. Comparatively, this kit displays the lowest level of variation among all the kits evaluated (less than 6%, see Table 5.8).

Table 5.8. ELISA Systems™ Casein results of retail cheese analyses.

ELISA Systems™ Casein ELISA Results			
Sample	Detected Average ppm Casein	Std. Dev.	%CV
Mozzarella	40000	2300	6%
Emmentaler	14000	90	1%
Blue	BLQ[a]	.	.
Limburger	33000	2100	6%
Brie	14000	160	1%

[a]BLQ indicates a reading below the limit of quantitation for the kit. See Table 5.1 for limits of quantitation.

While both the r-Biopharm RIDASCREEN® Fast Casein and the Neogen Veratox® Total Milk kits primarily detect residues of κ-casein (Chapter 3), they provide different estimates of the levels of milk residues present in the cheese extracts. The r-Biopharm Casein kit detects higher levels of milk residues than the Neogen Veratox Total Milk kit for Mozzarella, Emmentaler, and Brie cheeses (21,000 ± 2,500 ppm casein, 4,400 ± 330 ppm casein, and 39,000 ± 4,000 ppm casein versus 7,000 ± 460, 2,300 ± 210, and 7,300 ± 1,500 ppm casein, respectively) (See Tables 5.5 and 5.6). Only two kits, Neogen Veratox® Total Milk and r-Biopharm RIDASCREEN® Fast Casein, are capable of detecting milk residues in Blue cheese extracts (410 ± 40 and 120 ± 5 ppm casein, respectively, see Tables 5.5 and 5.6). The ELISA results confirm that Blue cheese has the highest level of proteolysis among the evaluated cheeses.

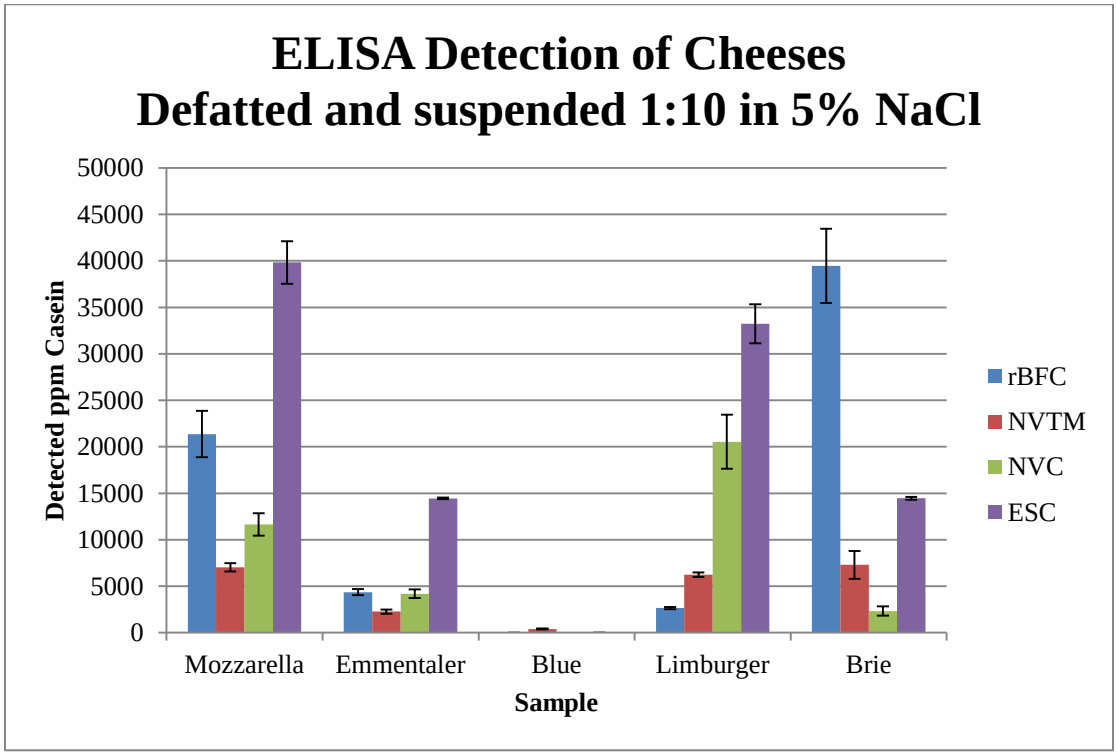


Figure 5.3. Commercial Milk ELISA detection of retail cheeses. rBFC: r-Biopharm RIDASCREEN® Fast Casein; NVTM: Neogen Veratox® Total Milk; NVC: Neogen Veratox® Casein; ESC: ELISA Systems™ Casein. NVTM and NVC values converted to ppm NFDM using Equation 5.2. Error bars represent standard deviation.

5. Discussion

Previous analyses with purified protein residues revealed the sensitivities of commercial milk ELISA kits. Relevant information regarding kit sensitivities to milk residues is displayed in Table 5.9. Briefly, the r-Biopharm RIDASCREEN® Fast Casein kit primarily detects κ-casein. The Neogen Veratox® Total Milk kit is mainly sensitive to κ-casein but also displays some sensitivity to α- and β-casein. The Neogen Veratox® Casein kit detects α- and β- casein with equal sensitivity (approximately 100% of the level of purified casein present). The ELISA Systems™ Casein kit only detects α-casein.

Table 5.9. Sensitivities of ELISA kits to purified casein fragments. For example, The r-Biopharm RIDASCREEN® Fast Casein kit reports the detection of approximately 10% of the level of β-casein present and 100% of the level of κ-casein present.

	Level of Detection		
Kit	α-casein	β-casein	κ-casein
r-Biopharm RIDASCREEN® Fast Casein	0%	10%	100%
Neogen Veratox® Total Milk	10%	10%	50%
Neogen Veratox® Casein	100%	100%	0%
ELISA Systems™ Casein	90%	0%	0%

Mozzarella and other pasta-filata cheeses display minimal proteolysis. This type of cheese is produced with minimal ripening (0-3 weeks) and is favored for its characteristic stretchy texture and fresh dairy flavor (Fox and McSweeney, 1996; Hutkins, 2008). As Mozzarella is cooked and stretched at high temperatures, chymosin is extensively denatured prior to ripening. Proteolysis in Mozzarella cheese is therefore dominated by the activity of plasmin on β-casein (Upadhyay et al., 2004). None of the ELISA kits evaluated provide similar estimates of the concentration of milk residues remaining in Mozzarella cheese. The extent of proteolysis in Mozzarella, although limited, is likely best estimated by the Neogen Veratox® Casein kit, as it displays sensitivity to epitopes of β-casein. Due to the deactivation of chymosin during the cooking step of Mozzarella, kits that detect α-casein, such as the ELISA Systems™ Casein or the

Neogen Veratox® Casein kit, likely provide the best estimates milk residue concentration for this cheese as α-casein is likely to remain more intact.

Emmentaler and other Swiss-type cheeses develop characteristic flavors and air pockets, or 'eyes' through the action of propionic acid-producing bacteria. Emmentaler cheese typically is made using three starter cultures: *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *Propionibacterium freudenreichii* subsp. *shermanii* (Hutkins, 2008). Because these cheeses are cooked at a high temperature (about 55^{o}C) during manufacture, many of the indigenous and added proteases are deactivated prior to ripening. Emmentaler cheese is described to have decreased proteolytic activity compared to other cheeses that are aged for similar lengths of time (Sousa et al., 2001). Research has revealed that primary proteolysis in Emmentaler cheese is characterized by high plasmin activity and corresponding degradation of β-casein (Grappin et al., 1999; Sousa et al., 2001). Chymosin is extensively denatured during the high-temperature cooking step and displays minimal proteolytic activity in Swiss-type cheeses (Fox and McSweeney, 1996). As with Mozzarella, the extent of proteolysis in Swiss-type cheeses is best characterized using the Neogen Veratox® Casein kit. The assessment of remaining milk residues for food safety purposes is probably best estimated in Emmentaler and Swiss-type cheeses by kits that detect α-casein, specifically the Neogen Veratox® Casein and ELISA Systems™ Casein kit.

Extensive proteolysis occurs during manufacturing of Blue cheese and blue-veined varieties of cheese due to the proteolytic action of *Penicillium roqueforti*. In mature Blue cheese, α- and β-casein are completely hydrolyzed (Fox and McSweeney, 1996; Upadhyay et al., 2004). Brie cheese is ripened by a mold of the same genus used in the ripening of blue-veined cheese; *Penicillium camemberti*. Both *P. roquefortii* and *P. camemberti* secrete aspartyl and metalloproteinases, with targeted specificity for α_{s1}- and β-casein, respectively (Spinnler and Gripon, 2004). Following sporulation of *P. roqueforti* in Blue cheese at approximately 15 days of ripening, proteolytic activity becomes dominated by extracellular proteases and the protein

degradation profile is dramatically changed (Spinnler and Gripon, 2004). Proteolysis is more extensive in Blue cheese than Brie (Upadhyay et al., 2004; Zarmpoutis et al., 1997). This research indicates that both Blue and Brie cheese may be best detected by ELISA kits with kits specific for κ-casein. Results demonstrate that the Neogen Veratox® Total Milk and r-Biopharm RIDASCREEN® Fast Casein kit are both capable of detecting κ-casein residues in Brie and Blue cheese. Substantial milk residues in Brie cheese are detected using all ELISA kits showing that proteolysis of α- and β-casein is incomplete during manufacture of Brie cheese. Milk residues of Blue cheese are only detected with the kits that target κ-casein epitopes which confirms that proteolysis of α- and β-casein is extensive in Blue cheese.

Limburger cheese is a bacterially smear-ripened cheese, employing *Brevibacterium linens* to develop its characteristic odor and flavor. Although only low numbers of *B. linens* can be isolated from the surface of ripening Limburger cheese, its presence is crucial for proper development of characteristic organoleptic properties. *B. linens* primarily targets α_{s1}- and β-casein. All kits detect milk residues in Limburger cheese at high level. Although proteolysis in Limburger cheese has not been extensively studied, this research indicates incomplete proteolysis of α- and β-casein in this cheese. Sufficient concentrations of reactive milk residues exist in the Limburger extracts to detect with current commercial milk ELISA methods and SDS-PAGE.

The results of the three protein assay methods (Microtannin, Micro-Lowry and A_{280}) did not correlate with the level of proteolysis among the evaluated cheese samples. Two of the assays, the Micro-Lowry and $A_{280,}$ rely on intact protein for determining the protein concentration in the samples. The Microtannin assay also has the capability of detecting free amino acids. All assays determine the concentration of soluble protein in an extract. However, these methods were not helpful in assessing the extent of proteolysis in the various cheeses. Higher levels of proteolysis should correspond to increased levels of free amino acids in cheese extracts (Kuchroo and Fox, 1982).

While cheeses display increased characteristics of ripening over time (soluble nitrogen, free amino acids, etc.), the level of degradation does not always correspond to age. The levels of proteolysis observed in cheeses are driven by the activity of proteinases. While Parmesan-Reggiano cheese is often aged for several years or more, Blue cheese is considered to have a higher degree of proteolysis, even though it is aged for only about 3 months (Upadhyay et al., 2004). The activity of enzymes, both indigenous and exogenous, drives the patterns and extent of proteolysis in cheese. Patterns of proteolysis in different varieties of cheese are developed by enzymatic activity. Enzymatic activity does not remain consistent in cheeses throughout aging.

Biochemical changes that occur during ripening are capable of altering the pH of cheeses and affecting the solubility of proteins in solution. Partly due to the dramatic differences in pH among cheese varieties and the resulting effects on solubility and extraction of milk proteins, a universal method to evaluate proteolysis has not been established or widely adopted. Protein concentration assays do not appear to appropriately indicate the level of proteolysis in cheese. The cheeses with higher levels of proteolysis do not necessarily exhibit increased levels of free amino acids or soluble protein in extracts as analyzed with the Microtannin, Micro-Lowry, and A_{280} assays.

The various milk ELISA methods appear to have potential uses in monitoring the proteolysis of various cheeses during ripening because of their different sensitivities to various casein fractions. In cheeses where chymosin is inactivated by heat and subsequent proteolysis is dominated by plasmin activity (Mozzarella and Emmentaler), the specificity of the Neogen Veratox® Casein kit for β-casein may provide a specialized approach to monitor proteolysis during ripening. Additionally, the combination of ELISAs specific to κ-casein such as Neogen Veratox® Total Milk and r-Biopharm RIDASCREEN® Fast Casein kits with ELISAs sensitive to α- and β-caseins such as Neogen Veratox® Casein kit may offer an approach to monitor proteolysis in cheeses subjected to more extensive proteolysis such as Brie and Blue.

Currently available commercial ELISA kits are capable of detecting milk residues in most cheese extracts although only certain ELISA methods are capable of detecting residues in blue-veined and other mold-ripened cheeses. Thus milk ELISAs can be used in the food industry to monitor cross contact where equipment is shared between formulations containing cheese and other non-milk formulations. However, the selection of the most appropriate ELISA methods for such analyses may depend upon the variety of cheese in the formulation.

Although cheese manufacturing seems to lower or even eliminate the concentrations of the major allergenic casein fractions from milk, the effects of fermentation and ripening of cheese on its residual allergenicity cannot be determined by ELISA methods. Proteolytic fragments of casein that are not reactive with the antibodies in the ELISA kits might still have the ability to react with milk-specific IgE antibodies in milk-allergic individuals and provoke reactions. Oral challenge studies with cheese would be needed to evaluate their allergenicity. However, the dramatic loss of α- and β-casein residues in blue-veined cheeses suggests that they might have diminished allergenic activity.

6. References

Bansal, N., Piraino, P., McSweeney, P.L.H., 2009. Determination of Proteolysis in Cheese. Handbook of Dairy Foods Analysis, 405.

Fox, P.F., 1989. Proteolysis During Cheese Manufacture and Ripening. Journal of Dairy Science 72, 1379-1400.

Fox, P.F., McSweeney, P.L.H., 1996. Proteolysis in cheese during ripening. Food Reviews International 12, 457-509.

Fox, P.F., McSweeney, P.L.H., Cogan, T.M., Guinee, T.P., 2004. Cheese: Chemistry, Physics and Microbiology: General Aspects. Elsevier Science.

Grappin, R., Beuvier, É., Bouton, Y., Pochet, S., 1999. Advances in the biochemistry and microbiology of Swiss-typecheeses. Le Lait 79, 3-22.

Hutkins, R.W., 2008. Microbiology and technology of fermented foods. Wiley. com.

Kuchroo, C., Fox, P., 1982. Soluble nitrogen in Cheddar cheese: Comparison of extraction procedures. Anglais 37, 331-335.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J biol chem 193, 265-275.

McSweeney, P.L.H., Fox, P.F., 1997. Chemical methods for the characterization of proteolysis in cheese during ripening. Lait 77, 41-76.

Mejbaum-Katzenellenbogen, W., Dobryszycka, W.M., 1959. New method for quantitative determination of serum proteins separated by paper electrophoresis. Clinica Chimica Acta 4, 515-522.

Rank, T.C., Grappin, R., Olson, N.F., 1985. Secondary Proteolysis of Cheese During Ripening: A Review. Journal of Dairy Science 68, 801-805.

Sicherer, S.H., 2011. Epidemiology of food allergy. Journal of Allergy and Clinical Immunology 127, 594-602.

Sousa, M.J., Ardö, Y., McSweeney, P.L.H., 2001. Advances in the study of proteolysis during cheese ripening. International Dairy Journal 11, 327-345.

Spinnler, H.-E., Gripon, J.-C., 2004. Surface mould-ripened cheeses. Cheese: Chemistry, physics and microbiology 2, 157-174.

Trayer, H.R., Trayer, I.P., 1988. Fluorescence resonance energy transfer within the complex formed by actin and myosin subfragment 1. Comparison between weakly and strongly attached states. Biochemistry 27, 5718-5727.

Upadhyay, V.K., McSweeney, P.L.H., Magboul, A.A.A., Fox, P.F., 2004. Proteolysis in cheese during ripening, in: P.L.H.M.T.M.C. Patrick F. Fox, P.G. Timothy (Eds.), Cheese: Chemistry, Physics and Microbiology. Academic Press, pp. 391-VIII.

Wal, J., 2001. Structure and function of milk allergens. Allergy 56, 35-38.

Warburg, O., Christian, W., 1942. Insulation and Crystalisation of the Fermenting Process of Enolase. Biochem Z 310, 384-421.

Zarmpoutis, I., McSweeney, P., Fox, P., 1997. Proteolysis in blue-veined cheese: an intervarietal study. Irish journal of agricultural and food research, 219-229.

CHAPTER 6. THE DETECTION OF MILK RESIDUES IN ENZYME-MODIFIED CHEESES USING COMMERCIAL ENZYME-LINKED IMMUNOSORBENT ASSAY KITS

1. Abstract

Proteolysis in foods poses a challenge for the detection of protein residues with enzyme-linked immunosorbent assays (ELISAs). Proteolytic enzymes degrade food proteins that are targeted by such assays and results can be misleading. Cross-contamination of food products with residues of foods or ingredients from allergenic sources may go undetected with commercial ELISA kits if those foods or ingredients have been subject to proteolysis. Thus, commercial ELISA kits may not be useful in such situations to verify and monitor product safety and allergen control policies, although this has not been formally evaluated. This research seeks to evaluate the capabilities of commercial milk ELISA kits to detect milk residues in a variety of enzyme-modified cheeses. While few studies have been performed on the allergenic relevance of fermented cheese proteins to elicit reactions in allergic consumers, the widespread use of cheese in packaged foods and the prevalence of milk allergy render cheese an important food ingredient for analysis. Five enzyme-modified cheese (EMC) samples were analyzed with SDS-PAGE and four commercially- available milk ELISA kits. ELISA kits have differing antibody specificities and not all commercial assays are able to detect milk residues in EMC samples. All EMCs display high levels and unique patterns of proteolysis. The r-Biopharm RIDASCREEN® Fast Casein kit displays superior detection of milk residues in EMC extracts compared to other evaluated kits. Alternatively, the ELISA Systems™ Casein kit was unable to detect milk residues in any of the EMC samples evaluated. While the application of ELISA kits for detecting milk residues in foods as a result of the use of EMCs as food ingredients can still be recommended, the selection of a suitable milk ELISA kit is paramount and dependent upon results obtained with positive control samples.

2. Introduction

Enzyme-modified cheeses (EMC) are intensely-flavored and inexpensive derivatives of cheese. EMCs are subjected to intense processing techniques that lead to dramatic changes in structure, composition, biochemical characteristics, texture, and flavor (Guinee and Kilcawley, 2004). They are typically applied to food products in small amounts to add or augment cheese flavor.

EMCs are produced through the incubation of cheese or curd with proteolytic and lipolytic enzymes (Kilcawley et al., 1998). EMCs are typically produced in slurry-type systems, but can be distributed and used in various product formulations in slurry or dried powder forms. The intensity of cheese flavor in EMCs typically ranges from 15-30 times higher than the level of flavor in natural cheese (Kilcawley et al., 1998).

The application of EMCs for use in packaged foods is extensive and includes use in many products, including cheese substitutes, cheese powder, soups, sauces, dips, dressings, and snack coatings (Guinee and Kilcawley, 2004). EMC flavors have been produced to resemble many cheeses including Cheddar, Blue, Swiss, Mozzarella, Parmesan, Gouda, Camembert and others (Guinee and Kilcawley, 2004; Moskowitz and Noelck, 1987).

The manufacture of EMCs begins with traditionally-produced cheese curds of the desired variety. Water and emulsifying salts are added to the curd and the mixture is blended to break up the protein curd matrix and increase the surface area for enzyme activity. The cheese paste is pasteurized to inactivate any residual enzyme activity from the manufacture of the cheese curds. Proteases, peptidases, and lipases are added to the slurry to hydrolyze caseins and fat. Typically, the selected proteases are derived from bacteria and molds, primarily of the genera *Bacillus* or *Aspergillus* (Guinee and Kilcawley, 2004). Because *Bacillus* proteases lack the debittering activity of aminopeptidases, *Aspergillus* or *Lactococcus lactis* derived proteolytic enzymes are

also added to degrade any bitter peptides generated during the production of EMCs (Guinee and Kilcawley, 2004). Lipases also play a critical role in producing characteristic cheese flavors and have been extensively reviewed elsewhere (Guinee and Kilcawley, 2004; Kilcawley et al., 2002; Kilcawley et al., 1998). The enzymes and cheese mix are incubated and slowly agitated for several days (1-4) at a moderate temperature (25^{o}C-45^{o}C) (Guinee and Kilcawley, 2004). After the incubation, the slurry mix is pasteurized and homogenized again to eliminate residual enzyme activity and preserve the developed flavors before storage. Depending on the desired application, the slurry can be dried to a powder to increase shelf life, stability, and mixing capabilities.

Individual manufacturers do not typically reveal the source or specificity of enzymes used to produce EMCs. The combinations of enzymes, their activities, and specificities used to produce the unique characteristics of individual EMCs are proprietary information.

Although EMCs undergo extensive proteolysis during production, intact proteins and protein fragments (peptides) may persist in the final product. It is unknown whether these degraded protein fragments retain allergenic activity. Current commercially-available immunoassay methods for allergen detection may not be sufficiently sensitive to detect residues in foods subjected to extensive proteolysis, although this has not been previously studied. Analyses of ELISA capabilities to detect milk residues in retail cheeses revealed that residues are not detected in cheese that are subjected to extensive proteolysis with all evaluated ELISA kit methods. If similar detection deficiencies occur with ELISA methods with EMCs, these methods may not be useful in the validation of allergen control regimens in manufacturing settings that use enzyme-modified cheeses.

The widespread use of EMCs in the food industry suggests the potential exists for cross-contamination of milk residues, especially in manufacturing settings. Additional risk to the allergic consumer may accrue if current analytical methods are unable to detect milk residues

from EMCs that display extensive proteolysis. This research seeks to highlight the capability of commercial ELISA kits for detecting milk residues in EMCs subjected to extensive proteolysis.

3. Materials and Methods

Sample Procurement and Preparation

Five varieties of slurry-form enzyme-modified cheese were kindly donated by Givaudan Corporation (Cincinnati, Ohio). The samples included four types of EMC of the Cheddar type and one sample of EMC of the skim milk type. Information provided on the cheese sample labels is listed in Table 6.1. Designations for the samples are listed in column 1of Table 6.1 and will be used throughout the chapter. Cheeses were warmed, stirred, and were distributed into several small containers. Aliquots were stored at -20^{o}C until the time of analysis.

Table 6.1. Enzyme-Modified Cheese Samples

Designation	Sample	Lot Number
EMC-A	Skim Milk Cheese- Proteolytic- (protease, peptidase)	6309326606
EMC-B	Cheddar Cheese- Mild (protease, lipases)	6309227906
EMC-C	Cheddar Cheese- Medium Sharp (proteases, lipase)	6310404110
EMC-D	Cheddar Cheese- Sharp (protease, lipases)	6310506872
EMC-E	Cheddar Cheese- Proteolytic (protease)	6310509009

Briefly, approximately 30 g of each EMC was thawed and a thin layer was spread evenly within a plastic zip-top bag. Bags were laid horizontally and rapidly frozen in a -80^{o}C freezer for approximately 24 hours. Frozen samples were fractured into small pieces and were finely ground using a Spex 6850 CentriPrep Freezer/Mill (Metuchen, NJ). Milled samples were placed into plastic zip-top bags and transferred to a -20^{o}C freezer for storage until the time of analysis.

For samples intended for SDS-PAGE, approximately 5 g of each sample was weighed into a 50 mL Falcon™ tube (BD Biosciences, San Jose, CA). 20 mL of 5% sodium chloride solution was added to each tube and the samples were mixed by a pulse-vortex technique for

approximately 10 seconds. Samples were laid horizontally on a Barnstead Thermolyne LabQuake Shaker and were extracted for 60 minutes at room temperature with shaking (Thermo Fisher Scientific, Rockford, IL). After extraction, samples were centrifuged at 3,020 x g for 30 minutes at 10^{o}C using an IEC Centra MP4R Centrifuge (International Equipment Company, Needham Heights, MA). The aqueous layer was removed to a new 50 mL Falcon™ tube and the pellet and fat layer were discarded. Extracts were frozen at -20^{o}C until the time of analysis.

EMC samples were prepared for ELISA analysis by defatting using a cold hexane extraction procedure. Approximately 20 g of freezer/milled EMC cheese was weighed into a 500 mL glass Erlenmeyer flask. 180 mL of cold hexanes (-20^{o}C) was poured over the samples. Flasks were briefly swirled to mix and placed in an ice bath on a Barnstead Thermolyne LabQuake Horizontal Shaker in a fume hood. Flasks were shaken for 30 minutes. The hexane and fat layer was decanted into an appropriate waste disposal container. Another 180 mL of cold hexane was added to the flasks. Flasks were again swirled to mix and returned to the shaking ice bath for an additional 30 minute incubation. The hexane and fat layer was again decanted and a final wash of 180 mL of cold hexane was added to the flask, swirled, and mixed with shaking for 30 minutes. The final solution was swirled to mix and then gravity filtered using a fluted Whatman size 1 filter paper in a glass funnel in a fume hood. The filter paper and retained sample were removed from the funnel after draining and laid flat to dry overnight in the fume hood. After samples were dried, they were crushed and finely ground using a Mr. Coffee IDS77 Coffee Grinder. Dried and defatted samples were placed in plastic zip-top bags and stored at -20^{o}C until the time of analysis.

Defatted samples for ELISA analysis were solubilized in 5% sodium chloride prior to ELISA extraction. Briefly, 1 g of dried and defatted cheese was weighed into a 15 mL polypropylene Falcon™ tube and 9 mL of 5% sodium chloride was added. Tubes were mixed using a pulse-vortex technique. Samples were solubilized with shaking on a Barnstead Thermolyne LabQuake Horizontal Shaker for approximately 3 hours. Extracts were centrifuged

in an IEC Centra MP4R Centrifuge for 30 minutes at 10°C and 3,020 x g. The aqueous layer was removed to a new 15 mL Falcon™ tube, and the pellet and fat layer were discarded.

Determination of Protein Concentration

The soluble protein concentration of extracts prepared for SDS-PAGE was evaluated using the Micro-Lowry, Microtannin and A_{280} assays. A brief review of these methods is provided (see Chapter 5 for detailed discussion on these methods).

The Micro-Lowry assay was performed according to Lowry et al. (1951) with modifications for application in a 96-well microtiter plate. The Lowry protein assay is a colorimetric dye-binding assay that relies on the addition of Folin-Ciocalteu reagent to samples to quantitate the concentration of soluble protein. The reduction of iron complexes with protein to facilitate a colorimetric reaction that can be read with a spectrophotometer. The standard curve was constructed with diluted solutions of Bovine Serum Albumin (BSA) in the range of 0-75 μg/mL, and the concentration of unknown samples was interpolated from the standard curve.

The Microtannin assay was performed according to procedure developed by Mejbaum-Katzenellenbogen and Dobryszycka (1959) and modified by Trayer and Trayer (1988). Our Microtannin procedure was modified slightly to adapt for use in a 96-well microtiter plate format. In the assay, protein is precipitated using a solution of tannic acid. Proteins, peptides, and free amino acids containing phenol groups react with tannic acid and precipitate out of solution. Precipitated proteins and fragments are suspended in solution with the addition of a dilute gum Arabic solution. The detection of protein in the assay is due to reflectance of the precipitated protein that is held in solution. Like the Micro-Lowry assay, BSA is used to construct a standard curve in the range of 0-200 μg/mL. The concentration of unknown samples is interpolated from the standard curve.

The A_{280} assay is one of the simplest assays used for determining protein concentration in samples. The absorbance of solubilized samples is read at 280 nm and is expressed in units of BSA equivalents. Because ribonucleic acids also absorb at 280 nm, the Warburg-Christian method was applied to results to correct the data (Warburg and Christian, 1942). Because only ribonucleotides absorb radiation at 260 nm, the ratio of A_{260}/A_{280} can be applied to the data to determine the contribution of protein to the results and provide a more accurate estimate of protein concentration. The absorbance of extracts at A_{280} was read using a Nanodrop 2000c Spectrophotometer (ThermoScientific, Rockford, IL).

Electrophoresis

Extracts prepared as described above were used for SDS-PAGE. Extracts were prepared under reducing conditions according to Laemmli (1970). Briefly, 27 mg of dithiothreitol (DTT) was weighed into a 1.5 mL Safe-Lock™ Eppendorf microcentrifuge tube. 500 mL of Laemmli sample buffer was added to the DTT and mixed thoroughly using a pulse-vortex technique (Bio-Rad Laboratories, Hercules, CA). 25 µL of each sample extract was pipetted into a 1.5 mL Eppendorf tube and mixed with 25 µL of the Laemmli sample buffer and DTT mixture. Samples were boiled for 5 minutes in a 100^{o}C water bath followed by cooling to room temperature. Samples were then centrifuged at 16,200 x g for 5 minutes in a ThermoScientific Legend Micro 17 centrifuge (ThermoScientific, Rockford, IL).

Four gels were run, each with approximately 10, 15, 50, and 100 µg of protein loaded in each lane. Load volumes were estimated by averaging the results from the Micro-Lowry and A_{280} assays. Published research has not indicated the superiority of either protein assay in the detection of cheese protein or EMC protein. The Microtannin results were excluded from the estimate of load volume because the results were dramatically lower than those observed with the other assays. Protein separation was performed using 18% Tris-HCl precast polyacrylamide

Ready Gels® for Mini-Protean® systems (Bio-Rad Laboratories, Hercules, CA). The first lane of each well was loaded with 5 µL of Precision Plus Protein™ Dual X-tra Standard (Bio-Rad Laboratories, Hercules, CA) to serve as a molecular weight marker. Unused lanes on the gels were loaded with 5uL of sample buffer to improve electrophoretic profiles.

Gels were run in a Bio-Rad Mini-Protean® Tetracell electrophoresis unit at a constant voltage of 200V for approximately 35-45 minutes, or until the dye front reached the bottom of the gel. After running, gels were removed from their plastic cassettes, rinsed in several changes of d_2H_2O, and fixed using 60% trichloroacetic acid (w/v) and 17.5% 5-sulfosalicylic acid (w/v) diluted 1:5 with distilled, reverse osmosis water (Sigma-Aldrich, St. Louis, MO) for 30 minutes. Fixed gels were rinsed in several changes of d_2H_2O and stained overnight in Coomassie Brilliant Blue R-250 staining system (Bio-Rad Laboratories, Hercules, California) with shaking on a Barnstead Thermolyne LabQuake Horizontal Shaker. After staining, gels were destained for 3-4 hours in several changes of Coomassie Brilliant Blue R-250 Destaining Solution, or until the desired level of destaining was reached (161-0438, Bio-Rad Laboratories, Hercules, CA). The destain solution used is a proprietary formula with estimated content of 50-100% water, 20-35% methanol, and 10-20% acetic acid. Gels were briefly rehydrated for approximately 10 minutes in a solution of 25 mM Tris and 192 mM Glycine at pH 8.3 and imaged with UV/Fluorescence using a Kodak Gel Logic Imaging System equipped with CareStream Molecular Imaging Software (Eastman Kodak, Rochester, NY).

Enzyme-Linked Immunosorbent Assays

The commercial ELISA kits used for analysis of EMCs in this study included the Neogen Veratox® Total Milk, Neogen Veratox® Casein, ELISA Systems™ Casein, and r-Biopharm RIDASCREEN® Fast Casein kits. Neogen Veratox® kits were obtained from Neogen Corporation (Lansing, Michigan). The ELISA Systems™ Casein kit was obtained from the

manufacturer (Queensland, Australia). r-Biopharm® kits (Darmstadt, Germany) were obtained from a distributor, Pi Bioscientific (Seattle, Washington). Enzyme-linked immunosorbent assays (ELISAs) were carried out according to product inserts provided by each kit manufacturer. Both the Neogen Veratox® Casein and Total Milk kits report data in units of parts per million of non-fat dry milk (ppm NFDM). The ELISA Systems™ Casein and r-Biopharm RIDASCREEN® Fast Casein kits report results in units of ppm casein. For comparison purposes, the Neogen® results were converted to ppm casein using Equation 6.1.

EMC samples were prepared for ELISA analysis as described above. Defatted EMCs were suspended 1:10 in 5% sodium chloride solution prior to extraction with ELISA kits. 1 mL of each EMC sample was extracted in triplicate according to instructions provided by each manufacturer. Briefly, 1 mL of aqueous EMC was pipetted into a polypropylene Falcon™ tube. The appropriate amount of pre-warmed (60^{o}C) kit-specific extraction buffer was added to each sample and mixed using a pulse-vortex technique. Samples were extracted in a 60^{o}C shaking water bath for the appropriate amount of time according to each kit's protocol. Samples were cooled to room temperature and a 1 mL aliquot of each extraction was centrifuged in a 1.5 mL Eppendorf microcentrifuge tube in a Thermo Legend Micro 17 centrifuge at 16,200 x g for 5 minutes.

ELISAs were performed according to manufacturer instructions. Briefly, 100 µL of each EMC extraction was applied to antibody-coated wells in triplicate. Standard solutions of known concentration were provided with the kits and were also applied to antibody-coated wells. Well plates were incubated for 10-15 minutes, depending on manufacturer protocol. After the incubation, wells were washed several times by filling each well with a dilute solution of PBS-Tween and inverting the plates to dump the residual buffer. This was repeated several times, as recommended by the manufacturer. After all washes, plates were inverted onto a clean paper towel and excess wash buffer was tapped out. 100 µL of enzyme-labeled conjugate antibody was

added to each well and the plates were incubated for an additional 10-15 minutes. Plates were washed and tapped dry as described previously with PBS-Tween wash buffer. 100 µL of substrate solution was added to each well and plates were incubated for a final 10-15 minutes while a colorimetric reaction occurred. 100 µL of acidic stop solution was added to each well to prevent further enzymatic activity. The resulting absorbance of each well was read at the appropriate wavelengths using a BioTek Eon Microplate Spectrophotometer equipped with Gen5 v 2.0 Software (Winooski, VT). Standard curves were constructed using the absorbances of the standard solutions. The concentration of residues in unknown samples was interpolated from the constructed curve.

Data Analysis and Criteria for Acceptable Performance of ELISA kits

Standard curves for the Micro-Lowry and Microtannin assays were constructed in GraphPad Prism v4.03. The concentrations of the soluble milk protein in EMC samples were interpolated from the standard curves and values were compiled in Microsoft Excel. For ELISA analysis, the appropriate curve fits were determined in SAS v 9.2 using PROC GLIMMIX regression analysis. All ELISA standard curves were appropriately modeled using a quadratic response curve. The absorbance values of unknown samples were fit to the curves and the appropriate concentration was interpolated. Where necessary, conversions were applied to compare kit data. Equations 6.1 and 6.2 were used to transform NFDM concentrations to casein, and vice versa.

Equation 6.1. Converting ppm NFDM to ppm casein

$$ppm\ casein = \frac{0.35\ ppm\ milk\ protein}{1\ ppm\ NFDM} \times \frac{0.8\ ppm\ casein}{1\ ppm\ milk\ protein} \times detected\ ppm\ NFDM$$

Equation 6.2. Converting ppm casein to ppm NFDM

$$ppm\ NFDM = \frac{1\ ppm\ milk\ protein}{0.8\ ppm\ casein} \times \frac{1\ ppm\ NFDM}{0.35\ ppm\ milk\ protein} \times detected\ ppm\ casein$$

Previous work has documented that commercial ELISA kits perform differently and often inconsistently both within and among kit lots (see Chapters 2 and 3). Instability of kit components, especially standard solutions used to construct standard curves, contribute to high levels of variation among ELISA results. Although universal criteria for validation of commercial ELISA kits has been suggested, it has not been widely implemented (Abbott et al., 2010). For the purposes of this research, variation will be measured and expressed as percent coefficient of variation (%CV). Acceptable intra-sample variation is limited to %CV ≤ 20%. Additionally, an $R^2 \geq 0.98$ was required for acceptance of the standard curve constructed from the manufacturer-provided standards. If %CV conditions were not met, the assay was repeated to confirm variation. Any assay with an $R^2 < 0.98$ was rerun and previous data was not used.

4. Results

Determination of Protein Concentration

Cumulative results of the protein assays are displayed in Figure 6.1.

Results of the Micro-Lowry assay are listed in Table 6.2. The highest protein concentration as determined by the Micro-Lowry Assay is 25.8 ± 0.7 μg/μL protein and was observed in EMC-A (skim milk cheese, Table 6.2). The lowest concentration of soluble protein was found in the EMC-E (proteolytic Cheddar Cheese) sample (11.0 ± 5.0 μg/μL protein). A high %CV was observed among the protein concentrations measured in EMC-E samples (approximately 46%, Table 6.2).

Table 6.2. Micro-Lowry Analysis of EMC Extracts

Micro-Lowry Protein Assay			
Sample	Concentration (μg/μL)	Std. Dev	%CV
EMC-A	22.6	2.4	11%
EMC-B	25.8	0.7	3%
EMC-C	15.8	3.0	19%
EMC-D	20.6	5.4	26%
EMC-E	11.0	5.0	46%

Substantially lower soluble protein concentrations were observed in the EMC extracts using the Microtannin Assay (Table 6.3). The highest level of protein was observed in the EMC-D extract (1.0 ± 0.4 μg/μL protein, Table 6.3). The lowest concentration of protein was measured in EMC-E, cheddar cheese with protease (0.1 ± 0.01 μg/μL protein). High variation was observed in EMC-D, the sharp Cheddar cheese sample produced with protease and lipase.

Table 6.3. Microtannin Analysis of EMC Extracts

Microtannin Protein Assay			
Sample	Concentration (μg/μL)	Std. Dev	%CV
EMC-A	0.6	0.07	11%
EMC-B	0.4	0.01	1%
EMC-C	0.5	0.01	1%
EMC-D	1.0	0.36	35%
EMC-E	0.1	0.01	6%

In the A_{280} assay, the highest concentrations of protein were found in EMC-E and EMC-A, at 15.4 ± 0.4 μg/μL and 15.1 ± 0.3 μg/μL, respectively (Table 6.4). EMC –E is the proteolytic cheddar, while EMC-A is the proteolytic skim milk cheese. The lowest level of protein was detected in EMC-D, corresponding to the Sharp Cheddar Cheese. Acceptable levels of variation were observed among analysis of replicates (%CV≤ 20%).

Table 6.4. A_{280} Results from EMC Extracts

A_{280} Protein Concentration			
Sample	Concentration (µg/µL)	Std. Dev	%CV
EMC-A	15.1	0.3	2%
EMC-B	8.4	0.3	4%
EMC-C	10.6	0.9	8%
EMC-D	7.8	1.2	16%
EMC-E	15.4	0.4	3%

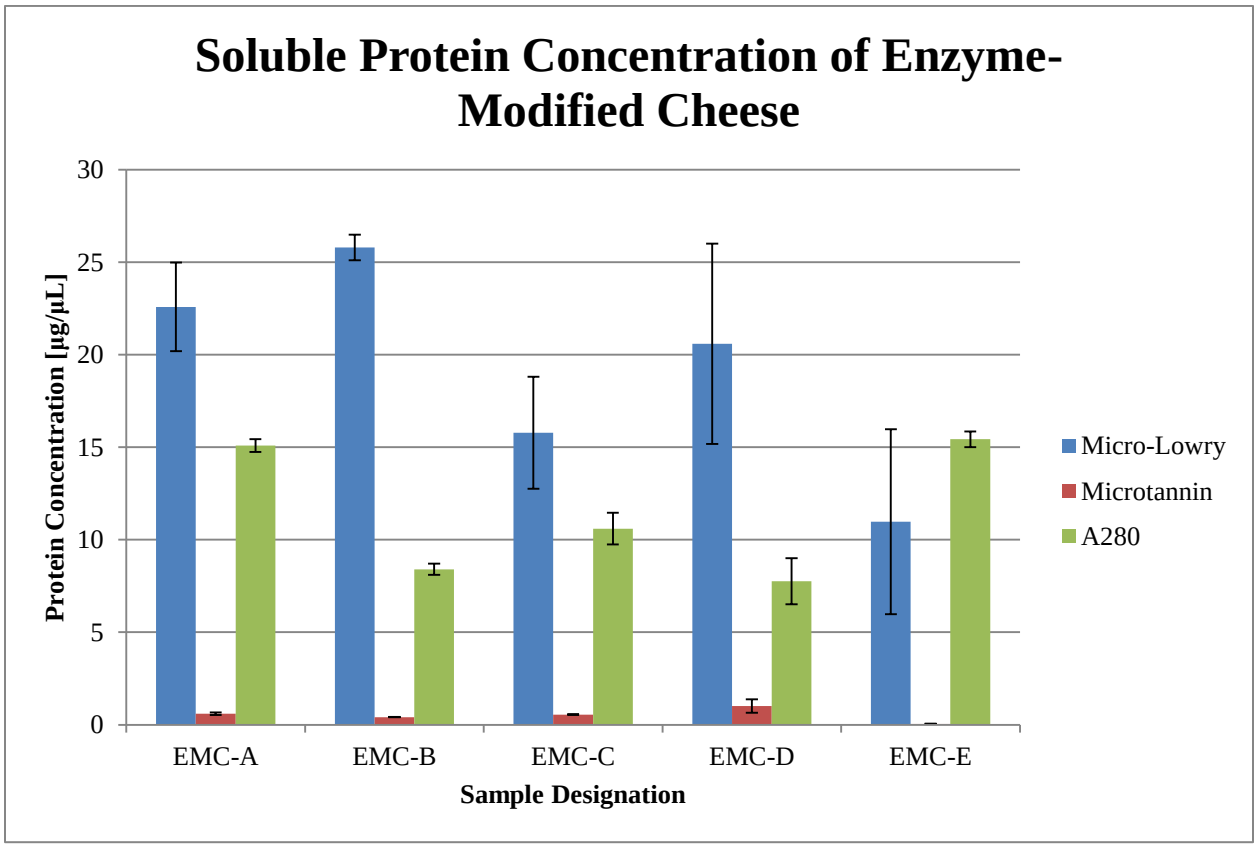


Figure 6.1. Cumulative Results of Protein Concentration for EMCs. Error bars represent standard deviation.

Electrophoresis

Electrophoresis was performed as described above. Images of gels loaded with 15 µg and 100 µg of protein per lane are depicted below in Figures 6.2 and 6.3. In Figure 6.2, some very

faint bands are visible in lanes 2-5, corresponding to EMC- A, B, C, and D. Only two bands (estimated MW of 13 kDa) in lanes 4 and 5) were consistently observed among cheeses. No bands are observed in lane 6, EMC-E, the proteolytic Cheddar made with only protease. None of the bands in any sample appear particularly intense, and it is difficult to judge and compare the level of proteolysis among EMC samples based on the gels alone. The lack of observed bands in lane 6 would indicate that this EMC may have undergone the most proteolysis. The highest molecular weight protein band is observed at about 30 kDa in Lane 5, corresponding to Sharp cheddar cheese produced with protease and lipase. Although some slight shading appears in the gel below 10 kDa, this is likely a remnant of staining, rather than the presence of low molecular-weight peptides.

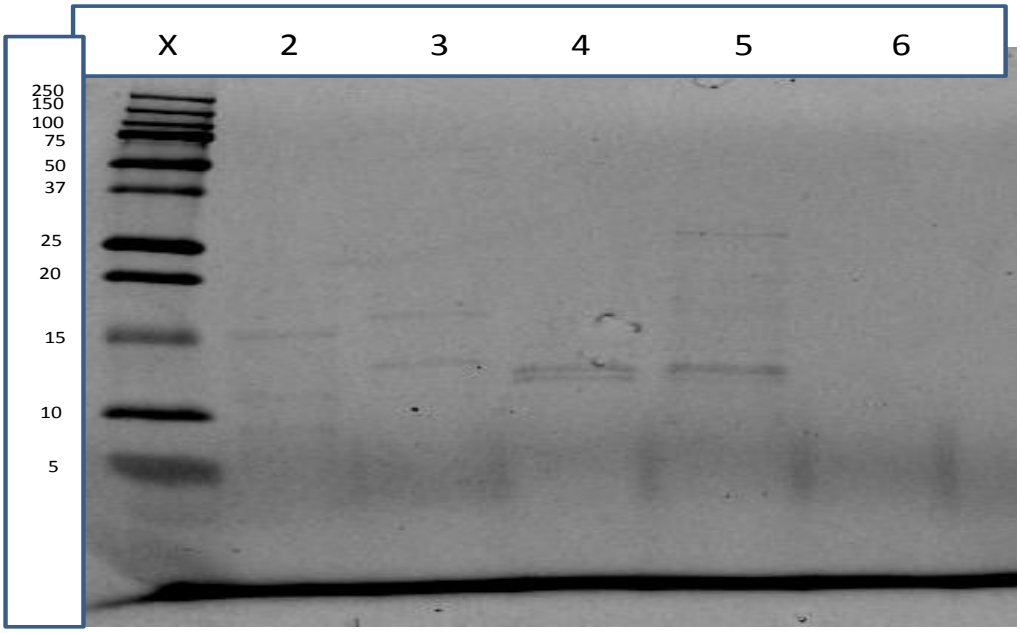


Figure 6.2. Enzyme-Modified Cheese 15 µg of protein loaded per lane. LaneX: Molecular weight marker, Lane 2: EMC-A, Lane 3: EMC-B, Lane 4: EMC-C, Lane 5: EMC-D, Lane 6: EMC- E. SDS-PAGE run under reducing conditions.

The gel loaded with 100 µg of protein per lane improves the intensity of protein bands in the EMC extracts (Figure 6.3). Several residual bands are observed in lanes 3 and 6, corresponding to EMC-A (skim milk cheese) and EMC-D (Sharp Cheddar EMC), respectively.

The bands that appear in lanes 3 and 4 in Figure 6.2 appear more intense in Lanes 4 and 5 of Figure 6.3. In lane 7, no bands are apparent for the EMC-E sample, corresponding to the Proteolytic Cheddar extract. Shadowed staining is observed at ranges below 5 kDa in lanes 3-6. This staining may correspond to low molecular weight peptides. Unlike the staining observed in the region below 10 kDa in Figure 6.2, the staining observed in Figure 6.3 is unequal across lanes. Both EMC-A and EMC-D appear to have minor bands migrating closely within the casein region (20-25 kDa) (Wal, 2001b). Two closely-migrating heavy bands occur in both lanes 5 and 6 near 13 kDa. While these bands could correspond to the whey protein α-lactalbumin, the majority of the whey proteins are drained from the cheese curd prior to EMC production. α-lactalbumin bands are documented to migrate near 14 kDa (Wal, 2001b).

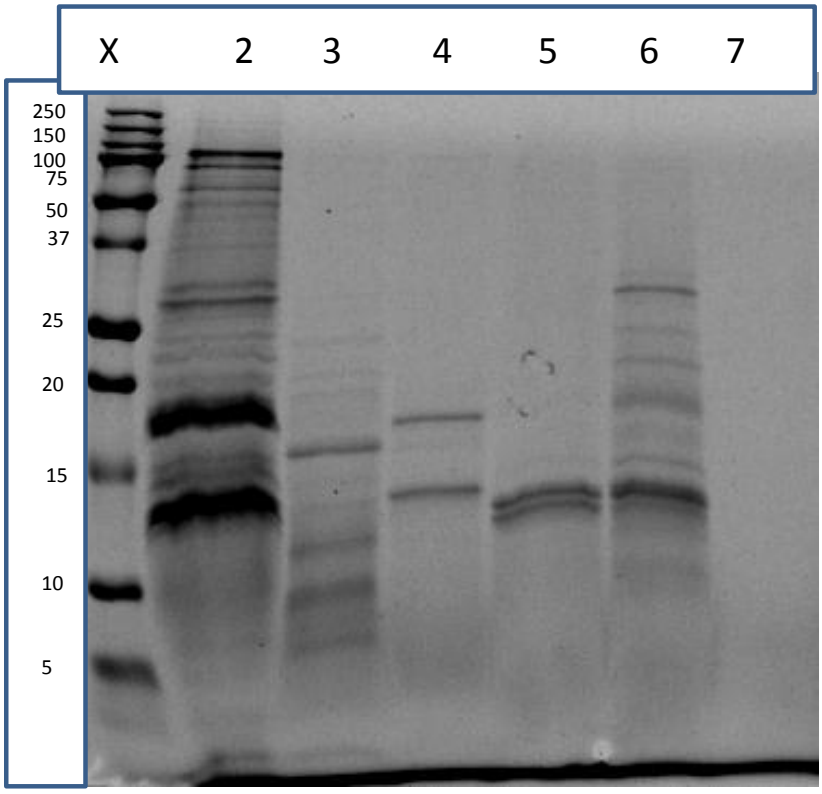


Figure 6.3. Enzyme-Modified Cheese, 100 µg of protein loaded per lane. Lane X: Molecular weight marker. Lane 2: Blue Cheese control. Lane 3: EMC-A, Lane 4: EMC-B, Lane 5: EMC-C, Lane 6: EMC-D, Lane 7: EMC-E. SDS-PAGE run under reducing conditions.

Loading the Tris/Glycine gel with 100 μg of protein per lane facilitated the observation of low intensity protein bands. The high amount of protein required to load in the wells in order to observe bands in EMC samples suggests that the protein fragments produced during the manufacture of EMCs are rapidly degraded by additional proteolysis and other enzymes. The profiles observed in lanes 4 and 5 (EMC-B (Mild Cheddar) and C (Medium Sharp cheddar), respectively) display little diversity. Two definite bands are observed in each lane; some other potential bands may be present. The EMC samples in lanes 3 and 6 (EMC-A and EMC-D) display a diversity of peptide products and may indicate that these EMCs are produced using many enzymes with various specificity.

ELISA Analysis

The Neogen Veratox® Total Milk kit results are displayed in Table 6.5 and Figure 6.4. The concentration of milk residues detected by this kit is expressed in ppm NFDM. Equation 6.1 was applied to the data to report results in units of ppm casein. The limit of quantitation for this kit is 2.5 ppm NFDM, corresponding to approximately 0.7 ppm casein. In two of the samples, EMC-C (Medium Sharp Cheddar) and EMC-E (Proteolytic Cheddar), milk residues were not detected. The other three samples, EMC-A (Skim Milk Cheese), EMC-B (Mild Cheddar), and EMC-D (Sharp Cheddar) had detectable milk residues within the range of quantitation for the kit.

Table 6.5. Neogen Veratox® Total Milk ELISA Results for EMCs

Neogen Veratox® Total Milk ELISA Results			
Sample	Detected Average ppm casein	Std. Dev.	%CV
EMC-A	11	1.7	15%
EMC-B	14	0.7	5%
EMC-C	BLQ[a]		
EMC-D	16	4.5	28%
EMC-E	BLQ[a]		

[a]BLQ= Below the limit of quantitation of 2.5 ppm NFDM (0.7 ppm casein)

Neogen Veratox® Casein results are expressed in Table 6.6 and Figure 6.4. The concentration of detected milk protein residues is expressed by the kit in units of ppm NFDM. Equation 6.1 was used to convert the reported level of ppm NFDM in each sample to an equivalent concentration of ppm casein. The limit of quantitation for this kit is 2.5 ppm NFDM, corresponding to approximately 0.7 ppm casein. Three of the EMC extracts did not contain milk residues that were detectable by the Neogen Veratox® Casein ELISA kit. EMC-A (Skim Milk Cheese), EMC-C (Medium Sharp Cheddar) and EMC-E (Proteolytic Cheddar) extracts had results below the level of quantitation (BLQ) for the kit (Table 6.6). The EMC-B and EMC-D extracts had results of 11 ± 0.4 ppm casein and 14 ± 0.9 ppm casein, respectively. The EMC-B and EMC-D extracts containing detectable milk residues exhibited low and acceptable levels of variation (3% and 6% CV, respectively).

Table 6.6. Neogen Veratox® Casein Analysis of EMC Samples

Neogen Veratox® Casein ELISA Results			
Sample	Detected Average ppm casein	Std. Dev.	%CV
EMC-A	BLQ[a]		
EMC-B	11	0.4	3%
EMC-C	BLQ[a]		
EMC-D	14	0.9	6%
EMC-E	BLQ[a]		

[a]BLQ= Below the limit of quantitation of 2.5 ppm NFDM (0.7 ppm casein).

Results of EMC extract analysis using the r-Biopharm RIDASCREEN® Fast Casein kit are listed below in Table 6.7 and Figure 6.4. The kit reports the results in units of ppm casein. The limit of quantitation for this kit is 0.5 ppm casein. While similar to the LOQs for the Neogen Veratox® kits, milk protein residue was detected in all samples using the r-Biopharm RIDASCREEN® Fast Casein kit. All samples displayed acceptable %CVs (see Chapter 2 for more information on determination of criteria for acceptable kit performance). The lowest concentration of milk residue was detected in the EMC-E (Proteolytic Cheddar) extract (12 ± 1.2

ppm casein). The highest concentration of milk residue was detected in the EMC-D extract (22 ± 3.6 ppm casein).

Table 6.7. r-Biopharm RIDASCREEN® Fast Casein Analysis of EMCs

r-Biopharm RIDASCREEN® Fast Casein ELISA Results			
Sample	Detected Average ppm casein	Std. Dev.	%CV
EMC-A	19	0.9	4%
EMC-B	18	0.7	4%
EMC-C	16	1.0	6%
EMC-D	22	3.6	16%
EMC-E	12	1.2	10%

Results determined by the ELISA Systems™ Casein kit are listed in Table 6.8 and Figure 6.4. The LOQ for this kit is 1 ppm skim milk, corresponding to approximately 0.3 ppm casein. The ELISA Systems™ Casein kit reports the results in units of ppm casein, however, the kit was unable to detect any milk protein residues in the EMC extracts. Theoretically, this kit has the most sensitivity to quantify residues of milk protein present in a sample.

Table 6.8. ELISA Systems™ Casein Analysis of EMCs

ELISA Systems™ Casein ELISA Results			
Sample	Detected Average ppm casein	Std. Dev.	%CV
EMC-A	BLQ[a]		
EMC-B	BLQ[a]		
EMC-C	BLQ[a]		
EMC-D	BLQ[a]		
EMC-E	BLQ[a]		

[a]BLQ= Below the limit of quantitation of 1 ppm skim milk powder

In summary, the r-Biopharm RIDASCREEN® Fast Casein kit detected milk residues in all EMC samples, while the ELISA Systems™ Casein kit failed to detect milk residues in any of the samples evaluated. The Neogen Veratox® Total Milk kit detected milk residues in EMC-A,B, and D, corresponding to Skim Milk Cheese, Mild Cheddar, and Sharp Cheddar,

respectively. The Neogen Veratox® Casein kit detected milk residues in only two of the extracts, EMC-B and EMC-D, corresponding to Mild and Sharp Cheddar EMCs, respectively.

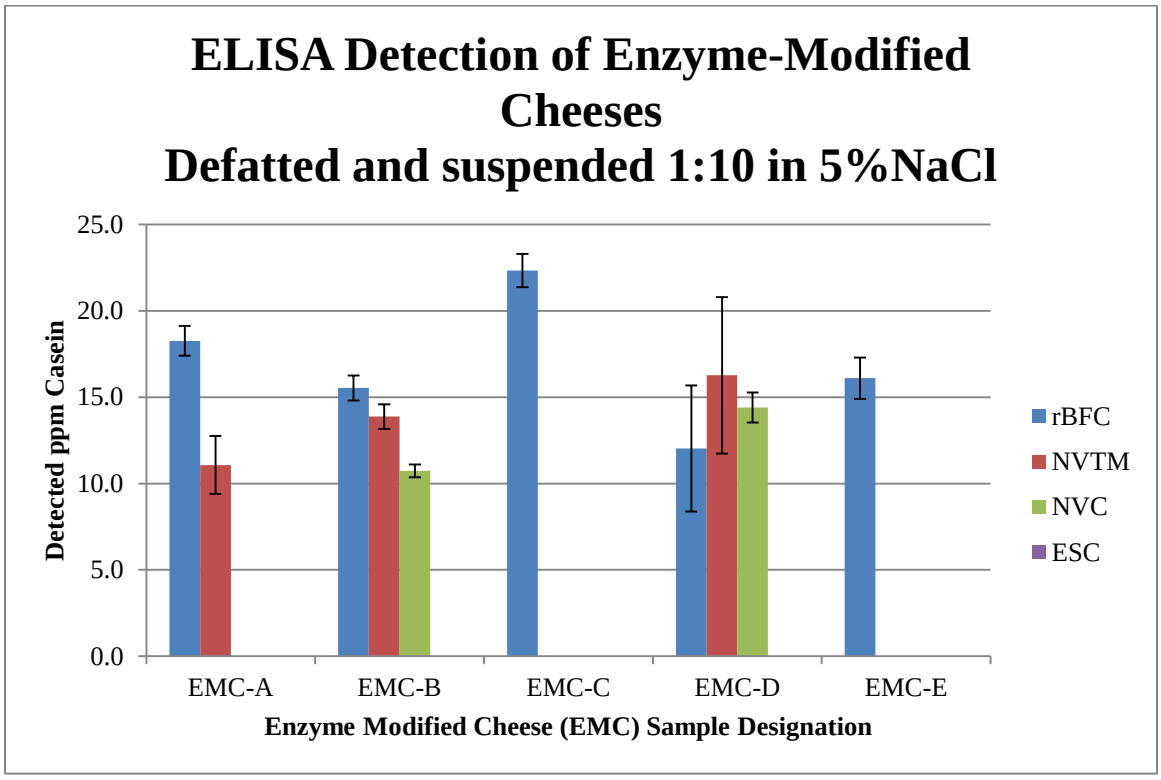


Figure 6.4. Cumulative ELISA Analysis of Enzyme Modified Cheese (EMC) Extracts. rBFC: r-Biopharm RIDASCREEN® Fast Casein kit; NVTM: Neogen Veratox™ Total Milk; NVC: Neogen Veratox® Casein; ESC: ELISA Systems™ Casein. Error bars represent standard deviation.

5. Discussion

The general protein assays conducted in this research give conflicting information regarding the level of proteolysis in samples. No observable trends are present among EMC samples, and the protein assays do not agree on the level of protein or amino acid present in the extracts. The Micro-Lowry assay suggests that the highest protein concentration is observed in EMC-B (Mild Cheddar cheese). The A_{280} assay suggests EMC-E (Proteolytic Cheddar) contains the highest concentration of protein in the extract, while the Microtannin assay suggests EMC-D (Sharp Cheddar) is the most concentrated. In agreement with the Microtannin assay, results from

ELISA analysis suggest that EMC-D (Sharp Cheddar) has the highest level of detected milk protein residues in the extracts in the r-Biopharm RIDASCREEN® Fast Casein, Neogen Veratox® Total Milk and Neogen Veratox® Casein kit. Compared to the other protein assays, the Microtannin assay suggests extracts contain very low concentrations of protein. The actual protein concentration of extracts is unknown. Published research evaluating proteolysis in cheese often employs the Kjeldahl procedure to determine protein concentrations (Creamer, 1991). Additional assays must be performed to confirm the protein concentration of EMC extracts.

Milk protein residues are not detected in all EMC samples with all commercial milk ELISA kits. Results suggest that the r-Biopharm RIDASCREEN® Fast Casein kit is capable of detecting milk residues in all EMC samples evaluated. Additionally, the ELISA Systems™ Casein kit fails to detect milk residues in any EMC sample tested. Previous research identified the specificities of the ELISA kits for individual purified caseins (see Chapter 3). The specificities of the kits are listed in Table 6.9. Commercial ELISA kits exhibit different specificities of detection for each milk protein. The r-Biopharm RIDASCREEN® Fast Casein kit is primarily sensitive to epitopes of κ-casein, but also has some capability to detect residues of β-casein. The Neogen Veratox® Total Milk kit also exhibits sensitivity to κ-casein, but also has some affinity to α- and β-casein residues. The Neogen Veratox® Casein kit detects α- and β-casein equally well, while the ELISA Systems™ Casein kit only detects α-casein.

Table 6.9. Specificities of Commercial ELISA Kits for Detection of Purified Milk Proteins[a]

Kit	α-casein	β-casein	κ-casein
r-Biopharm® Fast Casein	0%	10%	100%
Neogen Veratox® Total Milk	10%	10%	50%
Neogen Veratox® Casein	100%	100%	0%
ELISA Systems™ Casein	90%	0%	0%

[a]Percentages are based on the ability of each kit to detect milk residues in spiked samples of known concentration. See Chapter 3 for detailed equations and explanations.

Knowing the specificities of commercial ELISA kits contributes to the ability to identify trends in the detection of EMC samples. The increased detection of milk residues by the r-Biopharm RIDASCREEN® Fast Casein kit compared to other kits evaluated is likely a consequence of the resistance of κ-casein epitopes targeted by the antibodies in the kit to the proteolytic enzymes used to produce the EMCs. The lowest detection of milk residues reported by this kit is observed in the EMC-E (Proteolytic Cheddar) sample (Table 6.7). According to the limited information provided with the samples, the Proteolytic Cheddar (EMC-E) is produced using a protease enzyme (Table 6.1). Milk residues are detected in all samples by the r-Biopharm RIDASCREEN® Fast Casein kit, indicating that at least some of the κ-casein epitopes targeted by the antibodies in this kit remain intact in all samples, even after exposure to extensive proteolysis with a variety of enzymes.

Alternatively, no detectable milk residues are reported in the ELISA Systems™ Casein analysis of EMC samples (Table 6.8). This kit relies solely on the presence of epitopes of α-casein for detection of milk residues in samples (Table 6.9). Because no milk residues are detected, the results indicate that α-casein is extensively degraded by proteolytic enzymes during the production of EMCs.

Although tempting to attempt to correlate the detection of milk residues in Mild, Medium Sharp, and Sharp Cheddar EMCs (EMC-B, C, and D, respectively), the appropriate information regarding enzyme specificity is lacking. Making the assumption that all three EMCs are produced using enzyme mixtures with identical activity and specificity is unfounded. Viewing the SDS-PAGE profiles of extracts, it is apparent that EMC-B, C, and D are not produced using the same enzymes or protocols (Figure 6.3). The three profiles display starkly different protein profiles with minimal overlap or similarities. While it is possible that the bands are different fragments of the same proteins, they do not migrate similarly. Advanced proteomic analyses are required to identify the bands and confirm their protein origins. In a study of a range of

commercial Cheddar EMCs, it was determined that EMCs display extensive and dissimilar patterns of proteolysis (Kilcawley et al., 2000). HPLC studies revealed that even when electrophoretic protein profiles appeared similar, EMCs were produced using different proteolytic systems (Kilcawley et al., 2000).

In the three ELISA kits capable of detecting milk residues in EMCs, EMC-D (Sharp Cheddar) has more detectable milk protein residues than any of the other samples. In terms of the detected levels of milk protein residues, EMC-D (Sharp Cheddar) may have the most intact epitopes detected by ELISA kits. This correlates well with the observations in the SDS-PAGE analysis where a number of high molecular weight protein bands were present. The fewest detectable milk residues are found in samples EMC-C and EMC-E (Medium Sharp Cheddar and Proteolytic Cheddar, respectively) which also had limited or no proteins bands detected on the SDS-PAGE gel as well.

In EMC-A, the ELISA Systems™ Casein and Neogen Veratox® Casein kit detect the least amount of milk residues (Table 6.6 and 6.8). These kits are sensitive to detecting α-casein and α- and β-casein, respectively (Table 6.9). Detection by the r-Biopharm RIDASCREEN® Fast Casein kit and Neogen Veratox® Total Milk kits suggest that the order of degradation of milk proteins from most degraded to least degraded in EMC-A (Skim milk cheese) is $\alpha > \beta > \kappa$ (Table 6.5, 6.7, 6.9).

In EMC-B (Mild Cheddar Cheese), the order of degradation of milk proteins from most to least degraded is $\alpha > \beta > \kappa$. In EMC-C (Medium Sharp Cheddar) the order of degradation is $\alpha \approx \beta > \kappa$. In EMC-D (Sharp Cheddar), the order is $\alpha > \kappa > \beta$. In EMC-E (Proteolytic Cheddar), the order of degradation is $\alpha \approx \beta > \kappa$.

EMC-C and EMC-E contain the fewest detectable milk residues and also exhibit the order of degradation as $\alpha \approx \beta > \kappa$. In these samples, the α- and β-casein fragments are completely

degraded as seen by the lack of detection in kits that focus exclusively on the detection of these two fragments for quantitation of milk residues (ELISA Systems™ Casein and Neogen Veratox® Casein). While the Neogen Veratox® Total Milk kit has the capability to detect κ-casein in addition to some sensitivity to epitopes of α- and β-casein, no milk residues are reported. However, the r-Biopharm RIDASCREEN® Fast Casein kit detects some milk residues in the samples. This is likely a result of the kit antibodies targeting different epitopes of κ-casein than the Neogen Veratox® Total Milk kit. We can confirm the analysis of the ELISA kits with regards to the lack of detection of milk residues EMC-E samples by viewing the SDS-PAGE gel (see Figures 6.2 and 6.3). Even at extremely high concentrations of loaded protein, no discernible protein bands are visible in the molecular weight regions depicted on the gel (18% Tris/Glycine with the 2.5-250 kDa molecular weight markers).

Milk protein residues from most enzyme-modified cheeses can be qualitatively detected with commercial immunoassays. However, not all commercial ELISA kits are appropriate for the quantitative analysis of milk protein residues in foods where cross-contact of EMC residue may be a concern. The r-Biopharm RIDASCREEN® Fast Casein kit provides improved detection of EMC samples tested in this study, however due to the extensive proteolysis during the production of EMC, quantitative results obtained from this kit (or any of the other kits) may not be 100% accurate. Quantitative ELISA results are subject to the presence of intact target epitopes, as opposed to the actual concentration of milk protein residues. Because EMCs are produced using a variety of enzymes, the capability of ELISA kits to quantify milk protein residues is highly dependent on the specificity of enzymes used to manufacture the cheeses. This study was conducted using concentrated extracts of EMC. Recommendations for commercial kit usage may be different for analysis of trace residues of EMC in food products, as would be observed in situations where cross-contact may be a concern. While the application of ELISA kits for detecting milk residues in foods as a result of the use of EMCs as food ingredients can still be

recommended, the selection of a suitable milk ELISA kit is paramount and dependent upon results obtained with positive control samples. Further research is needed to evaluate the capability of commercial ELISA kits, especially the r-Biopharm RIDASCREEN® Fast Casein kit, to detect milk residues in samples containing trace amounts of EMC.

6. References

Abbott, M., Hayward, S., Ross, W., Godefroy, S.B., Ulberth, F., van Hengel, A.J., Roberts, J., Akiyama, H., Popping, B., Yeung, J.M., Wehling, P., Taylor, S.L., Poms, R.E., Delahaut, P., 2010. Validation procedures for quantitative food allergen ELISA methods: community guidance and best practices. Journal of AOAC International 93, 442-450.

Creamer, L., 1991. Electrophoresis of Cheese. Bulletin of the International Dairy Federation, 14-28.

Guinee, T., Kilcawley, K., 2004. Cheese as an ingredient. Cheese: Chemistry, physics and microbiology 2, 395-428.

Kilcawley, K., Wilkinson, M., Fox, P., 2000. A survey of the composition and proteolytic indices of commercial enzyme-modified Cheddar cheese. International Dairy Journal 10, 181-190.

Kilcawley, K., Wilkinson, M., Fox, P., 2002. Determination of key enzyme activities in commercial peptidase and lipase preparations from microbial or animal sources. Enzyme and microbial technology 31, 310-320.

Kilcawley, K.N., Wilkinson, M.G., Fox, P.F., 1998. Enzyme-modified cheese. International Dairy Journal 8, 1-10.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J biol chem 193, 265-275.

Mejbaum-Katzenellenbogen, W., Dobryszycka, W.M., 1959. New method for quantitative determination of serum proteins separated by paper electrophoresis. Clinica Chimica Acta 4, 515-522.

Moskowitz, G.J., Noelck, S.S., 1987. Enzyme-modified cheese technology. Journal of Dairy Science 70, 1761-1769.

Trayer, H.R., Trayer, I.P., 1988. Fluorescence resonance energy transfer within the complex formed by actin and myosin subfragment 1. Comparison between weakly and strongly attached states. Biochemistry 27, 5718-5727.

Wal, J., 2001. Structure and function of milk allergens. Allergy 56, 35-38.

Warburg, O., Christian, W., 1942. Insulation and Crystalisation of the Fermenting Process of Enolase. Biochem Z 310, 384-421.

7. Acknowledgments

The author would like to acknowledge and express gratitude to Kay Krammer and Pam Gribou of Givaudan Corporation for donating the EMC samples used in this research.

APPENDIX A. ADDITIONAL MATHEMATICAL EQUATIONS AND CONVERSIONS

Equation A.1. Converting ppm α-casein spikes to equivalent ppm casein

$$ppm\ \alpha - casein\ spike\ \times \frac{1\ ppm\ casein}{0.5\ ppm\ \alpha - casein^{a}} = 2\ ppm\ casein$$

[a]Values obtained from estimates for α_{s1}- and α_{s2}-casein from (Wal et al., 2001)

Equation A.2. Converting ppm β-casein spikes to equivalent ppm casein

$$ppm\ \beta - casein\ spike\ \times \frac{1\ ppm\ casein}{0.37\ ppm\ \beta - casein^{a}} = 2.7\ ppm\ casein$$

[a]Values obtained from estimates for β-casein from (Wal et al., 2001)

Equation A.3. Converting ppm κ-casein spikes to equivalent ppm casein

$$ppm\ \kappa - casein\ \times \frac{1\ ppm\ casein}{0.13\ ppm\ \kappa - casein^{a}} = 7.7\ ppm\ casein$$

[a]Values obtained from estimates for β-casein from (Wal et al., 2001)

Equation A.4. Calculating proportions: What proportion of casein is α-casein?

$$proportion\ \alpha - casein = \frac{0.5\ \alpha - casein}{1\ unit\ casein} = 0.5$$

Equation A.5. Calculating proportions: What proportion of milk protein is α-casein?

$$proportion\ \alpha - casein = \frac{0.5\ units\ \alpha - casein}{1\ unit\ casein} \times \frac{0.8\ units\ casein}{1\ unit\ milk\ protein} = 0.4$$

Equation A.6. Calculating proportions: What proportion of NFDM is α-casein?

$$\begin{aligned} proportion\ \alpha - casein \\ &= \frac{0.5\ units\ \alpha - casein}{1\ unit\ casein} \times \frac{0.8\ units\ casein}{1\ unit\ milk\ protein} \times \frac{0.35\ units\ milk\ protein}{1\ unit\ NFDM} \\ &= 0.14 \end{aligned}$$

Equations A.4 –A.6 are also used to calculate proportions of β- and κ-casein in casein, milk protein, and NFDM by substituting the proportions of β- and κ-casein in the bovine milk micelle in the equation in place of the α-casein term (0.5). For β-casein, the value is 0.37 and for κ-casein, the value is 0.13 (Wal et al., 2001).

Equation A.7. Calculating proportions: What proportion of milk protein is BLG?

$$proportion\ BLG = \frac{0.1\ units\ BLG}{1\ unit\ milk\ protein} = 0.1$$

Equation A.8. Calculating proportions: What proportion of NFDM is BLG?

$$proportion\ BLG = \frac{0.1\ units\ BLG}{1\ unit\ milk\ protein} \times \frac{0.35\ units\ milk\ protein}{1\ unit\ NFDM} = 0.035$$

Equation A.9. Calculating proportions: What proportion of milk protein is ALA?

$$proportion\ ALA = \frac{0.05\ units\ ALA}{1\ unit\ milk\ protein} = 0.05$$

Equation A.10. Calculating proportions: What proportion of NFDM is ALA?

$$proportion\ BLG = \frac{0.05\ units\ ALA}{1\ unit\ milk\ protein} \times \frac{0.35\ units\ milk\ protein}{1\ unit\ NFDM} = 0.0175$$

Equation A.11. Calculating conversion factors: WPC34 to ppm milk protein.

$$\frac{0.3255\ g\ protein}{1\ g\ WPC34} \times \frac{100\%\ WP}{WPC34\ protein} \times \frac{1\ unit\ MP}{0.2\ units\ WP} = 1.62$$

Equation A.12. Calculating conversion factors: WPC34 to ppm NFDM

$$\frac{0.3255\ g\ protein}{1\ g\ WPC34} \times \frac{100\%\ WP}{WPC34\ protein} \times \frac{1\ unit\ MP}{0.2\ units\ WP} \times \frac{1\ unit\ NFDM}{0.35\ units\ MP} = 4.63$$

Equation A.13. Calculating conversion factors: WPC80 to ppm milk protein

$$\frac{0.7847\ g\ protein}{1\ g\ WPC80} \times \frac{100\%\ WP}{WPC80\ protein} \times \frac{1\ unit\ MP}{0.2\ units\ WP} = 3.92$$

Equation A.14. Calculating conversion factors: WPC80 to ppm NFDM

$$\frac{0.7847\ g\ protein}{1\ g\ WPC80} \times \frac{100\%\ WP}{WPC80\ protein} \times \frac{1\ unit\ MP}{0.2\ units\ WP} \times \frac{1\ unit\ NFDM}{0.35\ units\ MP} = 11.21$$

Equation A.15. Calculating conversion factors: Sodium Caseinate to ppm milk protein

$$\frac{0.8988\ g\ protein}{1\ g\ NaCas} \times \frac{100\%\ casein}{NaCas\ protein} \times \frac{1\ unit\ MP}{0.8\ units\ casein} = 1.12$$

Equation A.16. Calculating conversion factors: Sodium Caseinate to ppm NFDM

$$\frac{0.8988\ g\ protein}{1\ g\ NaCas} \times \frac{100\%\ casein}{NaCas\ protein} \times \frac{1\ unit\ MP}{0.8\ units\ casein} \times \frac{1\ unit\ NFDM}{0.35\ units\ MP} = 3.21$$

Equations A.11-A.16 were developed using values for percent protein contained in each milk-derived ingredient as listed in Table 3.2. The proteins present in WPC34 and WPC80 were assumed to be 100% whey protein. The proteins present in sodium caseinate were assumed to be 100% caseins. Additional terms are added into the equation in case different estimations of the source of protein are obtained in the future.

APPENDIX B. COMMERCIAL MILK ELISA KIT OPERATING INFORMATION

Table B.1. Sensitivity, range, calibration, and reporting units of commercial milk ELISA kits

Kit	LOD	LOQ	Dynamic Range	Calibrant	Reporting units (ppm)
Casein Kits					
ELISA Systems™ Casein	0.5 ppm	1.0 ppm	1.0-10 ppm	a-casein	skim milk powder
Neogen Veratox® Casein	1 ppm	2.5 ppm	2.5-15 ppm	NFDM	NFDM
R-Biopharm RIDASCREEN® Fast Casein	0.12 ppm	0.5 ppm	0.5-13.5 ppm	casein	casein
BLG Kits					
ELISA Systems™ BLG	0.05 ppm	0.1 ppm	0.1-1 ppm	BLG	BLG
Neogen BioKits™ BLG	2 ppm	2.5 ppm	2.5-40 ppm	whole milk powder	BLG
R-Biopharm RIDASCREEN® Fast BLG	0.19 ppm	0.5 ppm	0.5-13.5 ppm	BLG	BLG
Romer Labs® AgraQuant® BLG	1.5 ppb	10 ppb	10-400 ppb	BLG	BLG
Total Milk Kits					
Neogen Veratox® Total Milk	1 ppm	2.5 ppm	2.5-25 ppm	NFDM	NFDM
R-Biopharm RIDASCREEN® Fast Milk	0.7 ppm	2.5 ppm	2.5-67.5 ppm	milk protein	milk protein

APPENDIX C. RESEARCH SUMMARY

The main outcomes of this research, organized by chapter, are as follows.

Chapter 2

- Milk ELISA kits do not perform equally- and have different reporting units and reference materials
- Milk kits that lack Certificates of Authenticity, have a high %CV and/or a high % difference from the expected values are not recommended for quantitative analysis of milk residues. Kits that are excluded from further chapters include the Morinaga Casein, Morinaga BLG, and Romer Casein kits.

Chapter 3

- Each kit manufacturer uses antibodies of varying specificity and affinity to develop commercial ELISAs. Casein ELISA kits detect various individual fragments of casein, BLG ELISA kits detect BLG, total milk ELISA kits detect various milk proteins, and no currently available commercial ELISA is capable of detecting ALA.
- Milk ELISA kits have targeted specificities, not broad spectrum detection of milk proteins
- Total Milk ELISA kits are capable of detecting milk residues in all milk-derived ingredients, although these estimates are not quantitatively accurate.
- Some residual caseins are present in whey protein concentrates; there may also be some residues of BLG in casein ingredients. This could be an advantage for detection using total milk kits.

Chapter 4

- ELISA methods can detect milk residues in young Cheddar cheese, but the detection signal dramatically decreases during aging. A 90% loss of signal is observed between the youngest and oldest Cheddar cheeses using commercial ELISA methods.

- ELISA kits can detect milk residues from concentrated Cheddar cheese aged up to 2 years, although the capability of these methods to detect trace levels of aged cheese may be a challenge for reliable detection and quantitation.

Chapter 5

- Currently available ELISA methods detect milk residues in most cheese extracts, but only some ELISA methods detect residues in extensively proteolyzed cheese.

- Appropriate selection of milk ELISA kits for accurate residue detection depends on the variety of cheese in the formulation.

Chapter 6

- Not all commercial ELISA kits are appropriate for milk residue detection in EMC samples. Concern is warranted when using ELISA methods for EMC evaluation. Some kits fail to detect milk residues in any of the EMC samples tested; only one kit is capable of detecting milk residues in all samples. A low level of detection is reported in kits that are capable of detecting milk residues in EMCs.

Overall

- Appropriate selection of ELISA kits is crucial to accurate quantitation of milk residues in fermented products.

- Commercial ELISA kits have some ability to detect milk residues in highly concentrated samples of fermented dairy products

- Further work needs to be performed to assess the ability of ELISA kits to detect trace residues of milk from fermented foods in industry-specific conditions.