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Matrix Effects on the Detection of Milk and Peanut Residues by Enzyme-Linked Immunosorbent Assays (ELISA)

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MATRIX EFFECTS ON THE DETECTION OF MILK AND PEANUT RESIDUES BY ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

by

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Food matrices are complex systems of lipids, carbohydrates, and proteins in which interactions between matrix components and allergenic proteins are known to have negative effects on the recovery of allergens when analyzed by ELISA. The purpose of this study was to first evaluate the recovery of milk and peanut residues from multiple food matrices and mixes and to secondly evaluate the use of a modified extraction protocol, sequential extractions, on the recovery of milk and peanut allergens.

Pastry dough matrices and pastry dough mixes incurred with milk were prepared at varying concentrations of flour and evaluated for recovery of NFDM. Secondly, a series of samples were prepared with increasing flour concentrations (wheat, corn, rice, soy flour) while maintaining a constant allergen (milk or peanut) concentration. Evaluation of sequential extractions was done on pastry matrices (wheat, corn, or rice flour) incurred with milk or peanut. Two matrix types, raw dough and baked matrices were analyzed for recovery.

Recovery of NFDM was reduced in wet pastry dough matrices in comparison to dry pastry dough mixes, indicating that the formation of a food matrix influences the detection of allergens. In concentration mixes, upon the addition of each flour type, the recovery of milk residues decreased as the concentration of flour increased whereas the recovery of peanut

residues was not affected by the increasing concentrations of flour. The implementation of sequential extractions yielded additional soluble protein from all matrices analyzed. Interestingly, ELISA detectable protein was only extracted from raw dough matrices. No detectable allergenic protein was extracted from baked pastry matrices.

The formation of a food matrix reduces the detection of milk allergens and reduced recoveries of milk allergens were observed with both glutinous and non-glutinous flour mixes. Peanut residues are less affected in sample mixes of different flour types. The use of a modified extraction procedure improved the recovery of soluble protein (all matrices) and allergenic protein (in raw matrices only).

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"Ohana means family. Family means nobody gets left behind or forgotten."
- Lilo and Stitch

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CHAPTER 1: LITERATURE REVIEW

I. INTRODUCTION

Food is an essential part of daily living and a central component in many social and cultural settings. Foods provide important nutritional factors such as carbohydrates, proteins, lipids, and vitamins all necessary for body functions. Proteins in foods can help support a complete diet but some proteins are also known for their physiological functions. In biological systems proteins can act as enzyme catalysts, antibodies, storage, or protective proteins particularly in the human body (Damodaran, 2007). Unfortunately for some individuals, the consumption of food proteins may result in an adverse reaction mediated by the immune system. An estimated 8% of children and 3-4% of adults suffer from some form of adverse reaction to foods (Gupta et al., 2011; Sicherer and Sampson, 2010). Food allergies are defined as individualistic reactions and affect only those individuals who are sensitized to certain food proteins (Taylor and Hefle, 2006). The diagnosis of a food allergy can reduce the quality of life for a consumer since avoidance diets are the recommended treatment for the prevention of allergic reaction. Adherence to avoidance diets is challenging in the current market since processed foods may contain trace amounts of allergens due to shared processing equipment, improper cleaning procedures, adulteration, or mislabeling of allergenic ingredients (Khuda et al., 2014). To combat accidental allergen exposure, several international and national laws have been enacted by the European Food Safety Agency (EFSA), the United States Food and Drug Administration (FDA), and other organizations to enforce labelling of allergenic ingredients. Some allergens however may go undetected in foods due to interferences between an allergen and food matrix components which can reduce detection by enzyme-

linked immunosorbent assays (ELISAs). ELISAs are the primary detection method used in food allergen analysis and sensitive enough to detect low concentrations of proteins in foods (Monaci and Visconti, 2010). A majority of food matrix studies are designed to assess the recovery of allergens from processed matrices, consequently less attention is given to unprocessed food matrices which may provide additional information regarding overall allergen detection. Complex matrices have repeatedly shown reduced recoveries in a variety of model food systems including cookies, biscuits, and chocolate (Khuda et al., 2014; Monaci et al., 2011). Secondly, the type of processing method can impact overall detection. Differences in recovery values were observed in thermally and non-thermally processed pastry dough model food matrices prepared with milk and analyzed using a variety of commercial ELISA kits (Bly, 2014; Downs and Taylor, 2010). More interestingly, reduced recoveries of milk were apparent in pastry dough samples prior to processing in both studies suggesting matrix interactions may be affecting the reduced detections. Milk and peanut allergens will be the focus of this review, and their detection in various matrices.

II. FOOD SENSITIVITIES

Food sensitivities affect a small proportion of the population and are defined as individualistic abnormal reactions due to the consumption of a particular food (Taylor and Hefle, 2002). For susceptible individuals, food sensitivities are considered a major health concern and require dietary monitoring to avoid accidental ingestion of specific foods. As a result, nearly 20% of the population adjusts their diet to avoid offending foods (Sicherer and Sampson, 2006). Food sensitivities can be classified into two categories, primary and secondary food sensitivities. Primary food sensitivities are the main focus of our

discussion since this classification encompasses both food intolerances and food allergies. Intolerances and allergens are differentiated by their mediation mechanisms employed by the immune system (Taylor and Hefle, 2002). Symptoms associated with food intolerances are less severe and are more easily treated than those associated with food allergies (Taylor and Hefle, 2002). Food allergies often impose life-long implications with varying degrees of symptoms in diagnosed individuals.

Food Intolerances

More often than not, symptoms associated with any form of food sensitivity are classified as a food allergy due to a general misunderstanding between the two types of reactions. True food allergies are immunologically mediated reactions caused by allergenic food proteins whereas food intolerances are non-immunologically mediated reactions and generally have higher threshold levels than those associated with true food allergies (Taylor and Hefle, 2002). Food intolerances can further be classified into three categories: anaphylactoid reactions, metabolic reactions, and idiosyncratic reactions (Taylor and Hefle, 2001).

Symptoms associated with anaphylactoid reactions and true food allergies are nearly identical except no allergen specific antibodies are involved in these reactions (Taylor, 1987). During an anaphylactoid reaction, histamine and other inflammatory mediators are released in response to certain foods, similar to a true food allergy but without the intervention of IgE antibodies. Strawberries are a well-known example of an anaphylactoid reaction. The fruit contains very little protein, therefore the allergy-like symptoms individuals may experience are due to the release of analogous allergy mediators (Taylor and Hefle, 2002). Although rare, some individuals may be sensitized

to the strawberry protein. Data supporting anaphylactoid reactions is deficient since causative agents have not been chemically isolated or identified (Taylor, 1987).

Metabolic food disorders are characterized as an individual's inability to metabolize certain foods or food components stimulating an adverse reaction. These types of reactions are often genetically inherited traits (Taylor, 1987). Lactose intolerance is a commonly encountered example of a metabolic food disorder.

Lactose intolerance is caused by a deficiency in the enzyme β-galactosidase (β-gal) resulting in an inability to digest lactose, the primary disaccharide in milk (Taylor and Hefle, 2001). β-galactosidase is responsible for hydrolyzing lactose into its constituent monosaccharides, glucose and galactose which are absorbed across the epithelial lining of the small intestine. Without the enzymatic activity of β-galactosidase, the intact disaccharide will pass into the colon where bacteria will ferment lactose resulting in diarrhea, gas, and abdominal pain or cramping (Sandine and Daly, 1979). Symptoms of lactose intolerance are not systemic, but isolated to the abdominal region (Taylor and Hefle, 2001).

Idiosyncratic reactions are adverse reactions to food or food components where the reaction mechanisms remain largely unknown (Taylor, 1987). Symptoms associated with idiosyncratic reactions are individualistic and can vary from mild to life threatening due to the broad array of potential mechanisms (Taylor and Hefle, 2002). Sulfite induced asthma is a well-known example of an idiosyncratic reaction caused by foods. Sulfites are naturally present in foods as a result of fermentation or are added by the food industry to prevent enzymatic and non-enzymatic browning, inhibit growth of microorganisms, and provide antioxidant properties (Taylor and Hefle, 2002; Taylor et al., 1986). In

susceptible individuals, ingestion of sulfites can lead to an asthmatic reaction. The mechanism of this reaction is not yet understood, however a clear cause and effect relationship has been demonstrated in numerous case reports and has been further supported by positive double blind placebo controlled food challenges (DBPCFC) to sulfites in foods (Bush and Taylor, 1998; Taylor and Hefle, 2002).

Food Allergies

True food allergies are reactions mediated by the immune system in response to the ingestion of an allergenic protein present in a food. According to the Food and Agricultural Organization of the United Nations (FAO), eight common food groups account for 90% of all food allergic reactions and are referred to as "The Big Eight." The Big Eight allergens include milk, eggs, soy, wheat, fish, crustacean shellfish, peanuts, and tree nuts (FAO, 1995).

The immune system responds to allergenic proteins provoking an allergic response whereas non-immunologically mediated food intolerances are reactions stimulated by other compounds and molecules found in foods (Sicherer and Sampson, 2006; Taylor and Hefle, 2002). Foods contain many proteins however only a small number of these proteins are classified as allergens and minor traces of allergens from a food material are capable of eliciting reactions (Taylor and Lehrer, 1996). Major allergens are defined as proteins that bind to serum antibodies from more than 50% of patients, and these proteins are typically the cause of allergic reactions, although in rare instances patients may be sensitized to minor allergens (Metcalfe et al., 1996; Taylor and Baumert, 2012). Furthermore, food allergies can be classified as immediate type

hypersensitivity reactions or delayed type hypersensitivity reactions based upon the immune system response (Taylor and Hefle, 2002).

Immediate hypersensitivity reactions are mediated by allergen specific immunoglobulin E (IgE) antibodies and characterized by rapid onset of symptoms that can occur minutes to hours post ingestion (Taylor and Hefle, 2001; Taylor, 1987). The mechanism of an IgE mediated reaction occurs in two phases, the sensitization phase and reaction phase. During the sensitization phase, plasma cells produce allergen specific antibodies (IgE) in response to an allergen which then bind to the surfaces of mast cells or basophils (Taylor, 1987; Taylor and Hefle, 2002). The mast cells are now sensitized. No symptoms occur during the sensitization phase (Taylor and Hefle, 2002). After the sensitization phase and upon subsequent exposure to an offending food, the allergens cross-link surface-bound IgE antibodies resulting in the degranulation of mast cells, releasing physiological mediators such as histamine, leukotrienes, or prostaglandins into specific tissues or into the bloodstream (Taylor, 1987; Taylor and Hefle, 2002).

Individuals experiencing an IgE-mediated allergic reaction usually exhibit a variety of symptoms which can affect one or multiple organ systems or result in a generalized systemic reaction (Taylor and Hefle, 2006). Affected organ systems include the gastrointestinal tract, the cutaneous system (e.g. skin), and the respiratory tract; generalized anaphylaxis can also occur (Taylor and Hefle, 2002). Table 1-1 lists symptoms associated with an IgE mediated allergic reaction. Symptoms can range from mild to severe and vary among individuals (Taylor and Hefle, 2002). There are no known dosage levels of allergens correlated with symptom severity, therefore physicians

and clinicians recommend complete dietary avoidance of suspected foods (Flinterman et al., 2006).

	Organ system			
	Gastrointestinal	Cutaneous	Respiratory	Generalized
Symptom	Vomitting Nausea Diarrhea Gastroesophogeal reflux Abdominal cramps	Urticaria Eczema Angioedema Prutitus	Asthma Rhinitis Laryngeal edema	Anaphylactic shock

Table 1-1. Symptoms occurring in response to an IgE mediated allergic reaction. Table adapted from Taylor and Hefle, 2006, Introduction to Food Allergies.

Delayed type hypersensitivity reactions are classified as cell-mediated reactions and occur 6 – 24 hours after consumption of a causative food (Taylor and Hefle, 2006). Cell-mediated reactions do not involve recognition of antigen-antibody complexes as in immediate hypersensitivity reactions, but instead involve tissue-bound antigen-sensitized T-cells which release mediators upon recognition of an allergen (Sampson, 1991; Taylor and Hefle, 2001). The significance of cell-mediated reactions is less understood in comparison to immediate-type hypersensitivity reactions of food allergy (Taylor and Hefle, 2006).

Celiac disease is a delayed hypersensitivity reaction manifested by the malabsorption of nutrients induced by proteins sourced from wheat, barley, rye, or other related grains such as triticale, kamut, or spelt (Green and Cellier, 2007; Taylor and Hefle, 2001). Consumption of gluten proteins from grains in sensitive individuals causes damage to the mucosal lining of the small intestine resulting in nutrient malabsorption across the gut epithelium (Taylor and Baumert, 2012). The prolamin gliadin stimulates the production of digestion resistant toxic peptides (α-gliadin) that interact with T-cells

and generate the observed adverse reaction (Green and Cellier, 2007; Taylor and Baumert, 2012). Symptoms include fatigue, muscle cramps, diarrhea, weight loss, and bloating (Taylor and Hefle, 2001). Similar to food allergies, total avoidance of wheat and other grains is required for celiac patients.

Prevalence of Food Allergy

An estimated 5% of adults and 8% of children in the US are diagnosed with food allergy and the diagnosis of food allergy is estimated to be increasing particularly in children (Sampson et al., 2014). From 1997 – 2007, diagnosed food allergies in children increased by 18% (Branum and Lukacs, 2009; Wang and Sampson, 2011). The true prevalence of food allergies is difficult to assess based on variability between study designs (prospective, retrospective, cohort selections), population demographics, and diagnostic method (self-diagnosed, physician diagnosed, clinician diagnosed). Nearly 50% of all children diagnosed with a food allergy are sensitized to peanut (20%) or milk (25%) allergens (Warren et al., 2013). Children commonly outgrow food allergies to milk, egg, wheat, and soy but allergies to peanut, tree nut, fish, and shellfish often persist into adulthood (Wood, 2003). Adults are most often allergic to peanuts or tree nuts from childhood sensitization, but development of allergies to fish and crustacean shellfish are more likely to occur during adulthood (Boyce et al., 2010). Mechanisms of tolerance are largely unknown although studies have indicated exposure to baked allergens and oral immunotherapy treatments have aided in attaining tolerance (Lee et al., 2015; Nowak-Wegrzyn et al., 2008).

In efforts to prevent accidental exposures to food allergens, the United States implemented the Food Allergen Labelling and Consumer Protection Act (FALCPA) in

2006. FALCPA states that allergenic ingredients or ingredients derived from an allergenic source must be declared on the package label (Taylor and Hefle, 2006). This directive along with good manufacturing practices used by the food industry are aimed at reducing the risk of accidental exposures.

Conclusion

Food allergies are of minor concern for a majority of the population, however for some individuals the ingestion of an allergen could lead to life threatening symptoms illustrating the importance of food allergies to public health. The severity of symptoms is variable between patients, and there are no known approved curative treatments available for those diagnosed with a food allergy, therefore avoidance of offending foods becomes essential. Consumers rely on manufacturers to disclose allergens in a given food product, necessitating good manufacturing practices to prevent accidental exposures and protect consumer health.

III. BOVINE MILK

Cow's milk is often referred to as a nearly perfect food source providing many beneficial and nutritional components for human health (O'Mahony and Fox, 2014). Bovine milk is the predominant form of milk produced and consumed globally, with 85% of all fluid milk sourced from dairy cattle (*Bos taurus)* (Bush and Hefle, 1996; Fox et al., 2015). In addition to cow's milk, other mammalian milks are frequently consumed including milk from goats, sheep, humans, and buffalos. However, consumption of these milk types occurs less frequently and is often dependent upon a geographic region (Fox et al., 2015). In the food industry, cow's milk is a versatile food ingredient and the

principal ingredient in several foods including cheeses, yogurt, butter, and ice cream. The proteins in cow's milk (casein and whey) are well known for their functional properties as ingredients or supplements but more importantly are the cause of milk allergy (Fox et al., 2015).

Cow's Milk Composition

In its most simplistic definition, cow's milk can be described as water and milk solids. Water is the primary component in milk and accounts for 85% of the total product providing fluidity characteristics to milk, and is one of the most adjusted components in milk processing (Jenness, 1988).

Milk solids include the fundamental nutritional factors including carbohydrates, fats, proteins, and minerals (Fox et al., 2015; Taylor and Kabourek, 2003). Carbohydrates are the second most prevalent component in milk and predominantly consist of the disaccharide lactose (Fox et al., 2015). Uniquely, milk is the only known biological source of lactose (O'Mahony and Fox, 2014). In addition to lactose, cow's milk contains smaller quantities of other carbohydrates, oligosaccharides or monosaccharides, including glucose, glucosamine, and galactosamine (Fox et al., 2015; Jenness, 1988).

Another major source of energy in milk is derived from the lipid moiety. Lipids provide nearly two times as much energy in comparison to lactose, although both are considered excellent energy sources (Fox et al., 2015). A majority (> 95%) of lipids in cow's milk are triacylglycerols and exist in solution as fat globule membranes (Fox et al., 2015; Jenness, 1988). Minor lipid components include phospholipids, free fatty acids, waxes and sterols (Taylor and Kabourek, 2003). Phospholipids are present in

concentrations less of than 1% but are important in maintaining the structure and functionality of milk fat globule membranes and other lipid membranes.

Over 20 proteins have been identified in cow's milk and can be differentiated into two categories based on their solubility, the caseins and whey proteins (Taylor and Kabourek, 2003). Cow's milk proteins will be discussed in further detail in the following section.

Cow's Milk Proteins

Bovine milk contains 3.0 – 3.5% total protein and consists primarily of two fractions, caseins and whey proteins. The caseins account for 80% of the total protein fraction and whey proteins account for the remaining 20% (Monaci et al., 2006; Wal, 2001). Upon acidification to a pH of 4.6, the isoelectric point of milk, the caseins become insoluble, form aggregates, and precipitate out of solution (Wal, 2001). The whey proteins however remain in solution at pH of 4.6 (Wal, 2001). Cow's milk proteins have high heat stability and good functional properties as concentrated proteins (Fox and Kelly, 2003). Differences in amino acid composition, protein structure in solution, and functional properties differ between the protein fractions and will be highlighted in the following sections.

Caseins

Caseins are the primary protein family present in cow's milk, accounting for 80% of the total milk protein (Wal, 2002). Whole casein exists in solution as an ordered arrangement of four proteins, α_{s1}-, α_{s2}-, β_1-, and κ-casein accounting for 37, 10, 35, and 12% of whole casein (Fox et al., 2015; Wal, 2001). Each individual protein represents a

distinct chemical compound that is encoded by different genes present on the same chromosome (Wal, 2001). Caseins were once described as random coils but have since been re-defined as 'rheomorphic' proteins based on their open and flexible nature and mobile conformation which allow good foaming and emulsification properties (Holt and Sawyer, 1993; Sawyer et al., 2002).

Caseins can be further characterized as calcium sensitive or calcium insensitive. The calcium sensitive caseins include α_{s1}-, α_{s2}-, and β_1-casein, whereas κ-casein is calcium insensitive (Wal, 2001). The calcium sensitive caseins are anionic due to phosphorylation at serine residues allowing them to readily chelate calcium molecules (Fox et al., 2015; Horne, 2009; Monaci et al., 2006). In fluid milk, approximately 30 mM of calcium is present, therefore one would anticipate rapid coagulation between the negatively charged calcium sensitive caseins and the positively charged calcium in solution (Fox and Kelly, 2003). However, caseins in solution will crosslink and form micelles, a protective structure which precludes this interaction from occurring (Fox and Kelly, 2003; Wal, 2001). Micelles are colloidal structures with a hydrophobic interior often termed 'sub-micelle,' and hydrophilic exterior. Assembly of casein micelles is first accomplished by interactions between the hydrophobic regions of caseins, forming a hydrophobic core. Within the interior of the micelle, electrostatic repulsion is present due to phosphoserine residues of caseins (Fox et al., 2015; Horne, 2009). This electrostatic repulsion is reduced by internally binding calcium and forming calcium phosphate molecules (Fox et al., 2015). Upon calcium binding, the anionic charges are offset resulting in micelle stabilization. The calcium insensitive κ-casein provides further stabilization to the micelle by surrounding the hydrophobic submicelle and forming a "hairy layer." The κ-casein C-terminal region

is hydrophilic and protrudes from the micelle generating the appearance of a hairy layer (Fox et al., 2015). The addition of chymosin or other proteases will destabilize the micelle by hydrolyzing the κ-casein fraction resulting in aggregation and casein precipitation (Fox et al., 2015; Horne, 2009). As demonstrated, the micelle structure is a key component in maintaining casein solubility and has been extensively studied for its structural properties (Fox et al., 2015).

Whey proteins

Whey proteins are globular proteins that remain soluble at a pH of 4.6 and can be subdivided into major and minor whey constituents (Wal, 2002). The major whey proteins are β-lactoglobulin (BLG) and α-lactalbumin (ALA) and the minor whey proteins include bovine serum albumin (BSA), lactoferrin (LF), and immunoglobulin (Ig) (Monaci et al., 2006). Whey proteins denature upon heating (90°C for 10 minutes) and are calcium insensitive (Fox et al., 2015).

Major Whey Proteins

BLG accounts for 50% of the whey protein fraction and exists as a 36 kDa dimer and has two intramolecular disulfide bonds and one free cysteine group (Fox and Kelly, 2003; Monaci et al., 2006; Wal, 2002). At a pH of 3.5 or less, BLG dissociates into its constituent monomers (Fox et al., 2015). In its monomeric form, BLG is a polypeptide of 162 amino acids with two prominent genetic variants (A and B) and eleven less frequently recognized variants (Fox et al., 2015; Sélo et al., 1999). Both genetic variants are present in milk at differing ratios based on the breed of cow and greatly influence the properties of BLG since each variant has slight differences in their amino acid sequences (Farrell Jr et al., 2004).

The protein structures of BLG have been thoroughly studied and the secondary, tertiary, and quaternary structures have been well described. BLG is classified as a protein in the lipocalcin family based upon its well-defined tertiary structure (Monaci et al., 2006; Wal, 2001). It is considered one of the best lipid binding proteins and it is readily able to bind retinol, β-carotene, saturated fatty acids, and unsaturated fatty acids (Breiteneder and Mills, 2005). As a result, the lipase activity in BLG is increased (Fox and Kelly, 2003).

ALA is a monomeric protein (14.4 kDa) of 123 amino acid residue with four disulfide bridges and a high affinity for calcium (Monaci et al., 2006; Wal, 2001). The binding of calcium provides stabilization to the secondary structure of ALA (Monaci et al., 2006; Wal, 2001). Interestingly, ALA shares 44% (54 residues) sequence homology with lysozyme (chicken egg white) and an additional 23 residues are similar structurally (Fox et al., 2015). ALA contains no free cysteine although the molecule has four disulfide bridges. The sulfur containing amino acids are present as cystine or methionine (Fox et al., 2015).

Minor Whey Proteins

The minor whey proteins include bovine serum albumin (BSA), lactoferrin (LF), and immunoglobulins (Igs).

BSA is a large protein, 66.4 kDa and accounts for 5% of the total whey protein fraction (Monaci et al., 2006). Its amino acid sequence is 582 residues long and contains 17 disulfide bridges, which are found on the interior of the protein structure (Monaci et al., 2006; Wal, 2001). Monosensitization is known to occur to BSA, and is independent of other milk proteins (Wal, 2001).

LF is also a large protein (76.1 kDa) known for its ability to readily bind iron, but is present in very small proportions (<1%) in most species and consists of a single polypeptide chain (Conneely, 2001; Monaci et al., 2011). The allergenicity of LF has not been well established and it is not considered a major allergen. Furthermore individuals with epitopes to LF have been sensitized to other milk proteins as well, resulting in challenging estimations of LF sensitization (Monaci et al., 2006).

A class of immunoglobulins (Igs) are produced in milk providing protection to the newborns (Fox et al., 2015). Igs in bovine milk include IgA, IgG, and IgM; IgG_1 is the primary Ig produced in bovine milk (Fox et al., 2015). Igs in bovine milk are structurally similar to Igs of humans, with heavy and light chains present in a Y shape structure (Monaci et al., 2006).

Heterogeneity of Milk Proteins

Milk proteins exhibit microheterogeneity among individual proteins which further affects protein functionality and allergenicity (Fox et al., 2015). Micoheterogeneity of milk proteins can occur due to differences in protein structures or environmental influences (Wal, 2001). Genetic polymorphism is a second factor contributing to the variation in milk proteins. BLG is known to have two variants, A and B, which both exist in cow's milk. The variants differ in two amino acids at residues 64 and 118, where variant A has aspartic acid and valine, and variant B has glycine and alanine respectively (Wal, 2001). Other events may include deletion of short amino acid sequences, phosphorylation, glycosylation, and other post-translational modifications all which are known to influence the overall heterogeneity of milk proteins (Wal, 2001). Overall, milk proteins have very heterogeneous structures, with few structural similarities. The

heterogeneity that exists between structures is only further complicated by genetic polymorphism. More importantly, these events can alter the allergenic potential of milk proteins promoting or inhibiting antibody recognition of an allergen (Fox and Kelly, 2003; Wal, 2001).

Adverse Reactions to Cow's Milk

For some individuals, consumption of cow's milk can cause an adverse reaction, either IgE mediated or non-IgE mediated. Lactose intolerance, a non-IgE mediated reaction, is the most referenced example of a metabolic food disorder (Taylor and Hefle, 2002). Allergy to cow's milk is an IgE mediated reaction and occurs more frequently in children compared to adults, and will be the primary focus of this discussion.

Cow's milk allergy (CMA) is an IgE mediated reaction present in nearly one-fifth of food allergic children (19.9%) making it the second most common allergy in children (Warren et al., 2013). 75% of milk allergic individuals are sensitized to more than one milk protein contributing to the observed variability between allergic reaction responses (Bush and Hefle, 1996; Wal, 2002). Children often develop an allergy to cow's milk since it is one of the first antigens introduced into the diet of a child (Taylor and Kabourek, 2003). The presence of cow's milk which is viewed as an antigen by a child's immune system will induce an immune response since their immune systems are not yet fully developed. The onset of CMA in children can be delayed by employing a few preventative measures during infancy. Exclusion of cow's milk from an infant's diet until six months of age or later and prolonged breastfeeding are the two recommended methods for the prevention of childhood CMA (Taylor and Kabourek, 2003). Prolonged breastfeeding provides nutritional supplements and immune factors which further

enhance and strengthen a child's developing immune system (Taylor and Kabourek, 2003).

For milk allergic individuals, tolerance to cow's milk is often achieved during childhood, accounting for the decreased prevalence of CMA in adults, cow's milk allergy is therefore considered a transient allergy. In a majority of children, tolerance was observed on average by age four and approximately 60% of children aged 12 years or older were tolerant based on a reports of retrospective analyses (Ahrens et al., 2012; Nicolaou et al., 2014; Skripak et al., 2007; Warren et al., 2013). Due to acquired tolerance, CMA occurs in less than 1% of the adult population (Bush and Hefle 1996; Taylor and Kabourek, 2003). Children diagnosed with non-IgE mediated CMA are likely to acquire tolerance sooner than those with IgE mediated CMA, yet less is understood about the mechanisms resulting in tolerance (Nicolaou et al., 2014; Skripak et al., 2007).

Avoidance diets are the most commonly physician recommended management strategy for diagnosed food allergies. Alternatively, oral immunotherapy has been recommended as a management method of food allergies, especially in cases involving CMA (Nowak-Wegrzyn and Fiocchi, 2009). Recent studies have demonstrated inclusion of baked milk in the diets of children diagnosed with CMA have promoted tolerance (Leonard et al., 2015; Nowak-Wegrzyn et al., 2008). In one study, 68% of milk allergic children exhibited tolerance to baked milk after consuming two model foods in succession, (1) a muffin (1.3 g milk/muffin, baked at 350°F for 30 minutes) and (2) waffle (1.3 g milk/waffle, baked at 500°F for 3 minutes (Nowak-Wegrzyn et al., 2008). The authors posit that extensive heating alters the conformation of milk proteins reducing antibody detection in vivo and allergenicity (Nowak-Wegrzyn et al., 2008). Secondly,

other studies have demonstrated extensive heating is known to alter the detection of milk in pastry matrices (Bly, 2014; Downs and Taylor, 2010). Inclusion of baked milk in the diets of children may be a safer alternative to the traditional oral immunotherapy methods potentially leading to tolerance (Nowak-Wegrzyn and Fiocchi, 2009). Individuals with a persistent milk allergy are likely sensitized against the linear epitopes of milk as opposed to conformational epitopes. The structure of linear epitopes remains intact upon heating whereas a loss of structure occurs in conformational epitopes, reducing antibody detection (Chatchatee et al., 2001).

Conclusion

Cow's milk is a popular global food commodity due to its nutritional value and valuable functional ingredient applications within the food industry. Variability between the casein and whey protein fractions allows for extensive applications of milk proteins which are used in a multitude of food processing applications. For some individuals, consumption of cow's milk may lead to adverse reactions, either immunologically or non-immunologically mediated. As a whole, cow's milk proteins are a diverse set of proteins capable of eliciting allergic reactions in sensitized individuals leading to varying degrees of allergic responses. Children are more likely to be affected by CMA than adults, due to the acquisition of tolerance and most are able to consume milk and milk products by adulthood.

IV. PEANUT

Allergy to peanut (*Arachis hypogaea*) is the most common food allergy present in children and adults in westernized countries and those suffering from a peanut allergy often experience the most severe symptoms associated with food allergies. Once a native plant of South America, peanuts are now widely cultivated in warm temperate climate regions including the tropics and subtropics (Duke, 1981). The most widely cultivated peanut crops include the Virginia, Spanish, and runner varieties (Burks et al., 1998; Duke, 1981). Historically termed ground-nut or earth-nut, peanuts are legumes in the Fabacaea family which also includes peas, beans, and lentils (Becker and Jappe, 2014; Hourihane, 2011).

According to the World Health Organization International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Committee, a total of seventeen peanut allergens (Ara h 1-17) to date have been identified, though only a few of these are considered major peanut allergens (WHO/IUIS Allergen Nomenclature Sub-Committee, 2016). The major peanut allergens include Ara h 1, Ara h 2, Ara h 3, and Ara h 6 (Bernard et al., 2007; Burks et al., 1998; WHO/IUIS Allergen Nomenclature Sub-Committee, 2016). Allergenic peanut proteins are all seed storage proteins; Ara h 1 and 3 are globulin proteins from the cupin superfamily and Ara h 2 and 6 are 2S albumins of the prolamin superfamily (Becker and Jappe, 2014; Burks et al., 1998).

Peanut Composition

Peanuts are a nutritionally important seed crop and contain ~25% protein by weight making it comparable to other protein rich foods including beef and fish (Hourihane, 2011). Considerable amounts of ten essential amino acids can be sourced

from peanuts (Hoffpauir, 1953), enhancing the overall nutritional profile of peanut. Peanuts also contain a large proportion of fat with approximately 50% of the peanut fraction consisting of unsaturated fat (Hourihane, 2011). The lipid portion is composed primarily of long chain fatty acids, making it similar to other vegetable cooking oils (Hoffpauir, 1953). Carbohydrates account for 12 – 15% of the total peanut fraction and include starch, cellulose, pectin, and sucrose (the only sugar molecule present) (Hoffpauir, 1953). China and India are the leading producers of peanuts globally, followed by the US (Sampson, 2002). In the US, four types of peanuts are grown for consumption: the Runner, Virginia, Spanish, and Valencia cultivars (Koppelman et al., 2016). The Runner (79%) and Virginia (20%) varieties are the two predominant peanut crops produced in the US. The Runner variety is primarily used to produce peanut butter, whereas the Virginia crop is used for snack mixes and other peanut packaged snacks (Koppelman et al., 2016)

Major Allergenic Peanut Proteins

Ara h 1

Ara h 1 is a 7S vicilin recognized by IgE antibodies from serum of >90% peanut allergic individuals and was one of the first major peanut allergens characterized (Burks et al., 1998; Shin et al., 1998). It exists as a stable homo-trimer or oligomer in a compact structural arrangement with a total monomeric molecular mass of 64.5 kDa (Shin et al., 1998; van Boxtel et al., 2006). Epitope identification revealed the presence of 23 linear binding epitopes and four immunodominant epitopes in Ara h 1 using peptide sequencing techniques (Shin et al., 1998). Over 80% of sera obtained from patients with peanut specific IgE were able to recognize the immunodominant epitopes (Burks et al., 1997;

Burks et al., 1998; Shin et al., 1998). Further evaluation of the immunodominant epitopes revealed substitution of a single amino acid within the center of an epitope resulted in reduced IgE binding reactivity, and this effect was more pronounced when a hydrophobic residue was substituted (Shin et al., 1998). No common sequence motifs are present among all identified epitopes and only one cysteine residue is present within the Ara h 1 molecule (Shin et al., 1998).

The primary sequence of Ara h 1 shows 46% homology with phaseolin, another legume seed storage protein, suggesting Ara h 1 is a highly structured molecule based on sequence similarity (Koppelman et al., 1999). Further structural analysis by far-UV CD spectroscopy analysis indicated a high level of secondary structures and a distinct tertiary fold (Koppelman et al., 1999). The secondary structures of Ara h 1 are comprised of 31% α-helices, 36% β-sheet and 33% random coil (Koppelman et al., 1999).

Ara h 1 is stable to extensive thermal processing but is highly susceptible to degradation by digestive enzymes including trypsin, chymotrypsin, and pepsin. Koppelman et al. (2010) describes the relatively rapid digestive hydrolysis of Ara h 1 at high concentrations of pepsin. Digestion of Ara h 1 using 0.1 U pepsin per microgram of substrate resulted in peptide fragments with masses ranging from 20 – 50 kDa (Koppelman et al., 2010). The arrangement of Ara h 1 in a trimeric structure provides protection to the epitopes from digestive enzymes resulting in multiple linear epitopes present on a given peptide fragment after digestion (Maleki et al., 2000). Although the protein has been digested, the peptides are still capable of eliciting a reaction in vivo since several of the linear epitopes remain intact.

Similarly, the linear epitopes of Ara h 1 remain unchanged after exposure to high heat and extensive thermal processing applications (Koppelman et al., 1999). Heating of purified Ara h 1 at different temperatures resulted in minor structural changes of Ara h 1, but did not alter the IgE binding capacity suggesting the protein epitopes are primarily linear and few conformational epitopes are involved in reactions to Ara h 1 (Koppelman et al., 1999).

Ara h 3

Ara h 3 is classified as an 11S seed storage protein in the glycinin family based upon amino acid sequence homology to other legumins. It is a hetero-multimeric protein with an overall molecular mass of ~400 kDa composed of ~65 kDa monomers (Koppelman et al., 2003; Rabjohn, Helm, et al., 1999). The monomeric subunits of Ara h 3 are composed of acidic (40-45 kDa) and basic (25 kDa) subunits linked together by a disulfide bond (Becker and Jappe, 2014; Koppelman et al., 2003). A third 14 kDa protein fraction may be present originating from the basic subunit (Koppelman et al., 2003). Ara h 3 is synthesized as a single polypeptide chain and cleaved post-translationally by endopeptidase enzymes into its acidic and basic subunits, which then combine to form the monomeric units (Shewry et al., 1995; Wen et al., 2007). Four linear IgE epitopes have been identified in Ara h 3 and are recognized by 45% of peanut allergic patients. These epitopes are found on the acidic subunit and three of the epitopes are found in all isoforms of Ara h 3 (Rabjohn, Burks, et al., 1999).

Similar to Ara h 1, Ara h 3 is highly susceptible to digestion as described by Koppelman et al., (2010). Using high concentrations of pepsin (760 ug/ml pepsin and 250 ug/ml Ara h 3), the Ara h 3 subunits were rapidly degraded to peptide fragments after

15 seconds of digestion. Lower applied pepsin concentrations (7.6 ug/ml pepsin and 250 ug/ml Ara h 3) were examined and resulted in medium length peptides after 4 minutes of digestion, but as the digestion time increased the peptides were further digested into fragments of 20 kDa or less (Koppelman et al., 2003).

Originally, Ara h 3 and Ara h 4 were isolated separately and established as two individual peanut proteins. Upon re-analysis and comparison of the two amino acid sequences, it was revealed the two shared 91% sequence homology, confirming Ara h 3 and Ara h 4 are isoallergens (Koppelman et al., 2003). In 2012, the WHO/IUIS Allergen Nomenclature Subcommittee reclassified the two proteins as Ara h 3.01 and 3.02 respectively (Becker and Jappe, 2014).

Ara h 2

Ara h 2 is another major allergen found in peanut and classified as a 2S albumin in the prolamin superfamily (Becker and Jappe, 2014; Burks et al., 1992). It is a 17.5 kDa conglutin type glycoprotein. Ten IgE binding epitopes have been identified in Ara h 2, and three epitopes were classified as immunodominant (Burks et al., 1995; Stanley et al., 1997). The immunodominant epitopes are located at residues 27 – 36, 57 – 66, and 64 – 74 (Burks et al., 1995; Stanley et al., 1997).

Two isoforms of Ara h 2 have been identified, Ara h 2.01 (16.6 kDa) and Ara 2.02 (18.1 kDa); Ara h 2.02 has an additional twelve amino acid insert beginning at residue 75 and contains a third copy of the immunodominant epitope DPYSPS (Chatel et al., 2003). It was hypothesized that Ara h 2.02 was more reactive than Ara h 2.01 due to an extra copy of the immunodominant epitope (DPYSPS). However, the two were determined to be antigenically similar based on results of a competition assay comparing

the inhibition of IgE reactivity from Ara h 2.01 and 2.02 (Hales et al., 2004). In addition, the binding capacity of each isoform was analyzed using sera from peanut specific patients to determine if differences in allergenicity were present. Sera IgE was bound in high concentrations by both isoforms, 77% and 81% for Ara h 2.01 and Ara h 2.02 respectively (Hales et al., 2004). The digestive stability of reduced and non-reduced Ara h 2 was assessed using simulated digestive conditions with the enzymes trypsin, chymotrypsin, and pepsin. Non-reduced Ara h 2 was determined to be an extremely stable protein that retained its native structure after exposure to high pepsin concentrations (10 U pepsin per microgram substrate) (Koppelman et al., 2010; Sen et al., 2002). However, the reduced form of Ara h 2 is less stable to these same digestive processes. After exposure to proteases, a 10 kDa peptide fragment was observed and the fragment is resistant to further proteolysis by digestive enzymes (Sen et al., 2002). The differences apparent between the reduced and non-reduced forms of Ara h 2 suggests the disulfide bonds are critical in maintaining the native Ara h 2 protein structure, and the protein is more easily digested after it has been reduced (Koppelman et al., 2010; Sen et al., 2002).

Ara h 6

More recently, Ara h 6 has been identified as a major allergen in addition to the previously identified allergens Ara h 1, 2, and 3. Ara h 6 is a 2S albumin and thus shares many similar structural and functional features with the related protein Ara h 2 (Koppelman et al., 2010; Lehmann et al., 2006).

By comparing the primary amino acid sequences of Ara h 2 and 6, the two proteins share 56% sequence homology (Becker and Jappe, 2014). Ara h 2 has eight

cysteine residues, whereas Ara h 6 has ten cysteine residues (Becker and Jappe, 2014). Ara h 6 is highly cross reactive with Ara h 2 and known to elicit comparable allergic reactions with high severity (Asarnoj et al., 2012). Additionally, Ara h 6 exhibits similar digestion patterns by generating a stable 10 kDa fragment in reducing conditions after digestion (Asarnoj et al., 2012). These factors have suggested Ara h 6 be included as a diagnostic marker for peanut allergy alongside Ara h 2 (Koid et al., 2014). A majority of peanut specific IgE responses are directed against Ara h 2 or 6 since these proteins are stable against denaturation processes and heat treatments. As previously described, Ara h 1 and 3 are less stable against similar digestive processes (Koppelman et al., 2010).

Although these two proteins are extremely similar, there are a few key differences. A majority of epitopes between Ara h 2.01 and 6 are similar; Ara h 6 has two unique epitopes and Ara h 2.01 has five unique epitopes (Otsu et al., 2015).

Prevalence of Peanut Allergy

Peanut is the most prevalently diagnosed food allergy in children and an estimated 25% of food allergic children are sensitized to peanut (Gupta et al., 2011). Approximately 20% of children will outgrow a peanut allergy and become peanut tolerant by adulthood, especially those with low serum IgE levels to peanut while the remainder will remain sensitized throughout a lifetime (Hourihane et al., 1998; Sampson, 2002; Skolnick et al., 2001). The persistence of peanut allergy further explains the high rate of adults diagnosed with a peanut allergy. A population study utilizing three cohorts of children (Cohort 1: 1989, Cohort 2: 1994 – 1996, Cohort 3, 2001 -2002) in the UK indicated an increase in diagnosed peanut allergy (Venter et al., 2010). The diagnosis of clinical peanut allergy for cohorts 1, 2, and 3 were 0.5, 1.4, and 1.2%, respectively, with a

pattern of gradual increase over time (Venter et al., 2010). A separate study demonstrated a similar trend in a randomized phone survey questioning parents about children diagnosed with peanut allergy. The authors indicated a steady increase of peanut allergy in children, 1997 (0.4%), 2002 (0.8%), and 2008 (1.4%) (Sicherer et al., 2010).

It was once thought that the prevalence of peanut allergy in children is increasing due to the early introduction of peanut and/or peanut products into the diet of a child (Whitaker et al., 2005). A more recent study has indicated inclusion of small doses of peanut in the diet may actually reduce a child's risk of developing a peanut allergy. This notion previously negates the recommendation of excluding allergenic foods from an infant's diet. In 2015, Du Toit et al., published a study that analyzed the prevalence of peanut allergy in two groups of infants (age 4 – 11 months at start of study, age 60 months at end of study). Study subjects were divided into two groups, a peanut consumption group and a peanut avoidance group, where the consumption group was instructed to consume a puffed peanut snack. At the end of the study, the consumption group had significantly less children diagnosed with a peanut allergy in comparison to the avoidance group (Du Toit et al., 2015). The novel information provided by this study may alter the way an allergenic food is incorporated into an infant's diet, however more studies may be necessary to understand the working mechanisms behind the decreased development of peanut allergy.

Conclusion

The increasing diagnosis and persistent nature of peanut allergy are two factors that contribute to the reaction severity observed in allergic patients. Peanut allergy affects children and adults at nearly equivalent rates since peanut allergy is a non-

resolving and persistent allergy. Several proteins are associated with peanut allergy, however some proteins are more suitable as analytical targets than others. Ara h 1 and Ara h 3 were first thought to be the most potent allergens, however more recently Ara h 2 and Ara h 6 have been suggested as the most potent and stable allergens. The digestion resistant nature and extreme thermal stability of Ara h 2 and 6 have suggested the proteins would make excellent analytical targets for detection methods. Detection of a readily stable target is beneficial especially in complex food systems or extensively processed foods; however these traits are undesirable by the food allergic consumer because reactions to Ara h 2 and 6 are often severe.

V. ALLERGEN DETECTION METHODS

Due to the increasing population of individuals diagnosed with a food allergy, detection of allergenic residues is becoming a critical component in food manufacturing quality control programs to protect food allergic consumers from accidental exposures. Food allergies themselves have become a notable food safety concern among food companies, regulatory agencies, and consumers (Taylor et al., 2009). Several established methods have been implemented for detecting and quantifying allergens. The most often employed methods in food allergen analysis include immunochemical methods, (ELISA, lateral flow devices), DNA based methods (PCR, RT-PCR), and mass spectroscopy (MS) methods (Torok, Hajas, Bugyi, et al., 2015).

Most foods are subjected to some form of food processing, which can potentially influence overall detection and/or allergenicity (Torok, Hajas, Bugyi, et al., 2015). Secondly, many food allergens are present in trace amounts requiring the use of very

sensitive methods (Poms, Klein, & Anklam, 2004). Detection methods must be designed to sufficiently detect target analytes in complex matrices, extensively processed foods, and in trace amounts (Taylor et al., 2009). More importantly, selection of the proper analytical method is essential for detection and quantitative analysis of a given material (Taylor et al., 2009).

DNA Based Methods

Detection of food allergens using DNA based methods relies on the extraction of DNA from contaminating food sources. These methods are highly sensitive and specific and can provide confirmatory data on the presence of a food allergen (Poms, Anklam, & Kuhn, 2004). DNA sequences are amplified using polymerase chain reaction (PCR) techniques compared to established DNA sequences of known allergens and properly identified (Monaci et al., 2006; Poms, Anklam, & Kuhn, 2004).

It is important to note the distinguishing factors between DNA detection methods and immunochemical detection methods. Immunochemical methods detect allergenic proteins, whereas DNA-based methods detect DNA sourced from an allergenic food (Poms, Anklam, & Kuhn, 2004). DNA is used as a marker for residue of an allergenic material, and does not indicate the presence of an allergenic protein (Monaci et al., 2006; Poms, Anklam, & Kuhn, 2004). DNA methods can be effective in detecting allergens in extensively thermally processed foods which are known to alter IgE epitopes and decrease immunoassay efficiency (Poms, Anklam, & Kuhn, 2004).

Mass Spectrometry Methods

The use of mass spectrometry (MS) methods in food allergen analysis has increased in recent years due to advances in instrumentation and data informatics resulting in improved detection of allergens. These methods are sensitive, reliable, and can provide an accurate assessment of allergenic proteins present in a food (Johnson et al., 2011). MS methods rely on the detection and analysis of peptide fragments generated from the protein of interest or intact proteins and subsequent comparison to a protein sequence database (Lane, 2005; Poms, Klein, & Anklam, 2004).

A general proteomic approach in food allergen analysis includes sample fractionation usually by enzymatic digestion, protein solubilization and separation, and identification using a mass spectrometer (Monaci et al., 2015; Monaci and Visconti, 2009).

All MS systems consist of three core components: an ion source, one or more mass analyzers, and a detector (Lane, 2005). The combination of different mass analyzers, detectors, and proteomic approaches further expands the ability to detect allergens in complex food systems or allergens present in low levels. Albeit an efficient detection method, routine application of MS would be a costly analytical procedure therefore further development of efficient and reliable detection methods should be pursued. The use of MS methods as a confirmatory tool is excellent and can provide further support to immunochemical or DNA methods.

ELISA Methods

Immunochemical detection methods, including ELISA and lateral flow devices (LFD), are protein based assays and the most commonly selected analytical methods in food allergen analysis. LFD's are qualitative measures of allergen concentration, whereas ELISA kits provide quantitative information of allergen concentration (Taylor and Baumert, 2015). ELISAs will be the primary focus of this section since they are frequently used in routine quantitative analysis (Gomaa and Boye, 2013; Poms, Klein, & Anklam, 2004).

Immunoassays are used because they provide several advantages over other analytical methods including their simplistic format, target specificity and sensitivity, reliability, and rapid analysis time (Poms, Klein, & Anklam, 2004; Yeung et al., 2006). Two ELISA formats have been developed for the detection of food allergens, the competitive and sandwich (Yeung et al., 2006). The sandwich ELISA is used most often and designed to detect intact allergenic proteins or larger protein fragments. The competitive ELISA is used in certain situations when protein levels are exceptionally low or specific peptides are being targeted (Immer and Lacorn, 2015).

Sandwich ELISAs require two antibodies, a capture antibody and an enzyme labelled conjugate antibody to detect allergens (Immer and Lacorn, 2015; Yeung, et al., 2006). The first antibody is coated onto microtiter well plates and immobilized, this is the capture antibody and is responsible for binding an allergen and forming the antigen-antibody complex (Immer and Lacorn, 2015). Capture antibodies must be targeted against stable and readily detectable peptides. Sandwich ELISAs generally use polyclonal antisera targeted against a combination of allergenic proteins to produce assay

antibodies (Taylor and Baumert, 2015). After establishing the antigen-antibody complex, a second enzymatically labelled conjugate antibody is added, forming the 'sandwich.' The enzymatically labelled conjugate antibody will produce a measurable colorimetric product, directly proportional to allergen concentration when the substrate is added (Yeung et al., 2006). An enzyme substrate, most often horseradish peroxidase (HRP) or alkaline phosphatase, is added to promote development of the colored end product. The allergen concentration can be determined by obtaining the optical density and calculating the allergen concentration of samples in comparison to the standard curve (Yeung et al., 2006). In a sandwich ELISA, two allergen binding sites must be present on an allergen to allow binding of both capture and detection antibodies. The use of paired polyclonal antibodies allows for the development of sensitive and specific ELISA methods which can sufficiently detect food allergens in a variety of matrices.

The competitive ELISA is second type of ELISA which differs slightly in assay format but provides greater sensitivity and a lower limit of detection than a sandwich ELISA. In a competitive ELISA, the antigen and antibody are first incubated together in a buffered solution and then plated onto the antigen coated wells (Immer and Lacorn, 2015). If an allergen is present in a sample, it will bind to the antibody in solution and preclude antibody binding to plate coated antigen. If no allergen is present, antibodies will bind to plate adsorbed antigen. After incubation, an enzymatically labelled detector antibody (conjugate), for the plate adsorbed antigen-antibody complex is added. Upon addition of an enzymatic substrate, a colorimetric product will generate if the antibody complex is bound to plate coated antigens (Yeung et al., 2006). The formation of a colorimetric product is inversely proportional to the allergen concentration.

Although the ELISAs differ in format, some factors are consistent between assay types. Detection of antigens is based on protein solubility during extraction and only extracted proteins are capable of being detected by the assay (Poms, Klein, & Anklam, 2004). Additionally, the efficacy of an immunoassay is highly dependent upon the quality of capture antibodies and antigenic targets (Immer, 2006). Each ELISA kit can differ in the capture and detection antibodies manufactured, which will affect the overall kit accuracy.

ELISAs have many advantages over other analytical methods such as assay reliability, sensitivity to low concentrations, and target residue specificity (Yeung et al., 2006). Many ELISA kits are capable of detecting and quantifying low concentrations of proteins, ranging from 2.5 – 25 ppm (Taylor et al., 2009). Protein concentrations less than 20 ppm will likely not elicit a reaction in a food allergic individual (Taylor et al., 2009). Therefore the ability to detect proteins at such low levels provides increased protection to a food allergic consumer.

Though a widely used tool for analysis, there are a few disadvantages when using ELISAs. ELISAs are highly specific, often targeting one or multiple allergenic epitopes or proteins which decreases the probability of obtaining a false positive result (Taylor et al., 2009). However, cross-reactivity can yield positive results in food samples that do not contain any allergenic material (Koerner et al., 2013). Proteins originating from the same family with similar antibody accessible epitopes may result in a positive outcome using ELISA (Bublin and Breiteneder, 2014). Consequently, during the development of an ELISA, the absence of cross reactivity must be validated by analyzing the assay

against a wide array of foods and ingredients to insure against false positives (Abbott et al., 2010).

Even though ELISA methods are robust against a variety of foods, detection of allergens is still imperfect. The inability to detect allergens causes reports of false negative results, which are more likely to occur than false positives (Taylor et al., 2009). False negative results increase a consumer's chance of experiencing an unwarranted accidental exposure. Hydrolysis, digestion, and insolubility are known factors which increase the likelihood of obtaining false negative results. Peptide fragments generated by hydrolysis or partial digestion may still retain immunoreactive properties and elicit a reaction in vivo and go undetected by ELISA antibodies. Moreover, allergenic peptides present in insoluble proteins can be released in vivo due to the action of digestive proteases in the stomach originating from insoluble proteins (Taylor et al., 2009). Poor protein extraction due to insolubility can also generate false negatives. Similarly, insoluble proteins retain allergenicity and are capable of eliciting reactions. Extensive food processing can induce physical, chemical, or biochemical changes to allergenic proteins further altering antibody detection (Thomas et al., 2007). Likewise, interactions occurring due to a food matrix or processing can reduce solubility and ultimately detection of allergenic proteins when analyzed by ELISA (Gomaa and Boye, 2013). Changes due to food processing will be discussed in further detail in a later section.

An inherent issue with ELISA's is the lack standardization among commercially available kits. There are no officially approved ELISA kits for allergen analysis defined by the US Food and Drug Administration (FDA) (Immer, 2006; Torok, Hajas, Bugyi, et al., 2015). Due to the lack of reference materials for assay development, kit antibodies

can be targeted against a variety of targets and best suited for different applications. In addition, materials used for calibration of assay standards is proprietary information to the developer and often not disclosed to the end user, commonly resulting in an added calibration factor when calculating the final results (Torok, Hajas, Bugyi, et al., 2015). Commercial kits can be calibrated against specific proteins, soluble protein, total protein, or whole food (Taylor et al., 2009). Using milk as an example, a kit could potentially be calibrated using ppm casein, ppm milk protein, or ppm total milk (NFDM) (Taylor et al., 2009).

Not all ELISAs are built equally and effects due to processing or interactions within a food matrix can inhibit adequate detection of target residues. Therefore careful consideration must be taken when selecting an ELISA for analytical procedures. It is highly recommended to validate an ELISA against the target allergen and food matrix if being used for routine analysis (Taylor et al., 2009). It is also suggested to test several different kits using various concentrations of the target analyte within the matrix of interest (Monaci et al., 2011). The target analyte must be homogenously distributed within a matrix to ensure appropriate measurement of allergen concentration and overall kit performance (Taylor et al., 2009). Kit efficacy can also be measured using "spike-and-recovery methods" where a known amount of allergen is homogenously distributed within a material and analyzed for recovery. Lastly, one must accurately assess a kit based on its final reporting units and overall purpose.

Conclusion

ELISAs are a highly effective tool in the detection of allergens and provide good sensitivity and specificity based on assay format and target analyte. They are currently

the most developed detection method for a majority of allergens and widely used in the food industry and research facilities. The rapid analysis time, ease of use, and reliability, and ruggedness to detect allergens in various food matrices and processing methods make ELISAs advantageous over other more laborious and costly methods. However, the interpretation of results is important and based upon the kit calibration materials and is an important factor when selecting kits. The Japanese government (Ministry of Health, Labor, and Welfare) is the only regulatory agency currently with official ELISA methods for allergen detection (Akiyama et al., 2011). Whereas in other countries ELISA kits have been approved for use as confirmatory tools. For labelling purposes and cleaning validations, any suitable kit can be selected so long as they are a sufficient and adequate detection method. The lack of standardization materials for ELISA development leads to different calibration materials among kit manufacturers causing discrepancies between results obtained from kits manufactured by different companies. Even though ELISA kits provide many advantages to the detection of food allergens and are widely used, understanding the differences between kits is important when selecting the best analytical method.

VI. PROCESSING EFFECTS ON THE DETECTION OF FOOD ALLERGENS

In general, most foods undergo some form of processing which provides a beneficial or desired function to the end product (Thomas et al., 2007; Torok, Hajas, Horvath, et al., 2015). Processing methods are used for a multitude of reasons including reduction of microbial populations, enzyme and/or toxin inactivation, sensory quality enhancement, or procurement of processing byproducts (oils, isolates) (Thomas et al.,

2007). Processing can affect the allergenicity of proteins by altering interactions with allergen-specific IgE antibodies or by inducing other changes to proteins that will alter access to the IgE antibodies. Processing can also affect the detection of residues of the allergenic food using ELISAs that depend upon the interaction of food-specific IgG antibodies with proteins from the allergenic food. Because allergenicity depends of binding to IgE antibodies and detection depends upon binding to IgG antibodies, a change in detection does not invariably result in a similar change in allergenicity.

Effect of processing on allergenicity (IgE)

Food allergens are extremely stable molecules so the use of processing to eliminate allergenicity is often not feasible. Additionally, most individuals are sensitized to multiple allergenic proteins in a food which most often have both conformational and linear epitopes (Fiocchi et al., 2004). Thus in some instances, processing can have differential effects on the allergenicity of a food depending upon which specific proteins are responsible for sensitization to a specific food for a given individual. Thus, processing may alleviate allergenicity for one individual but not another even though they are sensitized to the same food.

Changes in the structures of allergenic proteins due to processing can enhance or reduce allergenicity depending on the length of processing time and the type of processing method. Alteration of target IgE-binding epitopes as a result of processing is one of the main issues regarding protein allergenicity (Verhoeckx et al., 2015). Contrastingly, some processes can generate new epitopes, “neo-epitopes,” and may increase the allergenicity of a protein.

Conformational epitopes are the most likely implicated epitopes to be affected by processing due to their susceptibility to denaturation due to heating, low pH, and enzymatic digestion resulting in decreased detection by IgE (Järvinen et al., 2001; Nowak-Wegrzyn and Fiocchi, 2009). Linear epitopes are stable towards many food processing operations, with the exception of hydrolytic processes, and remain intact after food processing (Thomas et al., 2007). Hydrolysis of linear epitopes will digest the protein into partial fragments which are generally rendered unrecognizable by IgE (Thomas et al., 2007). Formation of protein aggregates due to intermolecular disulfide bonding may alter epitope recognition by IgE antibodies. Using milk as an example, BLG contains conformational epitopes and will readily denature under high heat conditions, reducing its detection by IgE (80°C) (Wal, 2002). In addition, denaturation of BLG will promote intermolecular disulfide bonding with other food proteins in a matrix upon heating and form aggregates, reducing its allergenicity (Thomas et al., 2007). The allergenicity of BLG is greatly reduced after extensive thermal processing destroying some epitopes, however not all epitopes are destroyed therefore BLG may retain some allergenicity (Bloom et al., 2009; Nowak-Wegrzyn and Fiocchi, 2009).

On the other hand and equally important, is the formation of "neo-epitopes." Neo-epitopes, sometimes referred to as neo-allergens, are the formation of new epitopes of an allergen in a processed foods and can enhance or reduce allergenicity in sensitized individuals. Dry roasting peanuts is known to induce the formation of neo-epitopes and enhance allergenicity. Increased binding to IgE was reported against heat treated Ara h 2 to peanuts caused by heating are known to induce the formation of neo-epitopes in Ara h 2 (Nowak-Wegrzyn and Fiocchi, 2009). Neo-epitopes have also been documented in

shellfish as a result of glycation due to extensive heating (Nowak-Wegrzyn and Fiocchi, 2009; Thomas et al., 2007). Food in which the Maillard reaction has taken place due to extensive heating processes have a higher probability of generating neo-epitopes as opposed to other food processing operations (Thomas et al., 2007). Interactions within a food matrix can also lead to formation of neo-epitopes and ultimately prevent digestion of proteins and allergenic epitopes are retained.

Differences in sensitization patterns may be present among different types of processed foods. Exposure to an allergen and the preparation method are two factors contributing to overall sensitization (Wen et al., 2007). Milk and peanut have various preparation methods largely influenced by cultural locations. Although most milk is consumed after pasteurization, allergenicity to raw milk cannot be excluded (Verhoeckx et al., 2015). Peanut, on the other hand, is processed using many different methods dependent upon geographic location.

In China there is less incidence of peanut allergy than in the United States, this phenomenon is attributed to contrasting preparation methods used regularly in the two countries (Hill et al., 1997). In China, boiling and frying are the most commonly used methods for preparing peanuts, whereas in the US peanuts are dry roasted (Beyer et al., 2001). In a study conducted by Beyer et al., the allergenicity of boiled, fried, and roasted peanuts was evaluated by SDS-PAGE and immunoblotting. Results of the SDS-PAGE gel indicated the Ara h 1 fraction is less abundant in boiled and fried peanuts than in dry roasted peanuts (Beyer et al., 2001). Secondly, immunoblots using sera of peanut allergic patients showed decreased binding towards fried and boiled peanuts was present, and the most binding was observed between IgE and roasted peanut extracts (Beyer et al., 2001).

In summation, these results indicate increased allergenicity is present dry roasted peanuts as opposed to boiled or fried.

Effect of processing on detection (IgG)

Some of the same processing effects on proteins can likewise alter binding of the proteins to IgG antibodies used in detection of allergen residues by ELISA. Protein structure dictates protein solubility, therefore changes in protein structure caused by processing will have an overall impact on detection and assay performance (Abbott et al., 2010). The sensitivity of selected detection methods will likely decrease due to implications caused by processing (van Hengel, 2007). Changes in target IgG binding epitopes due to processing is a key factor to consider when evaluating protein allergenicity (Verhoeckx et al., 2015). Processes which decrease the detection of epitopes by IgG antibodies can result in false negatives. Similarly, conformational epitopes are altered due to processing effects (heating, low pH, enzymatic digestion) which results in decreased detection by IgG during ELISA detection (Järvinen et al., 2001; Nowak-Wegrzyn and Fiocchi, 2009). As previously discussed linear epitopes are less effected by food processing applications and remain intact, with the exception of hydrolysis (Thomas et al., 2007). But, IgG antibodies are more often directed at conformational epitopes (Thomas et al., 2007). Processing methods can broadly be categorized into thermal and non-thermal processing (Thomas et al., 2007). Thermal processing is accomplished using either moist heat (microwave cooking, boiling, steaming) or dry heat (oven roasting, oil roasting) (Sathe et al., 2005). Non-thermal processing methods include milling, fermentation, proteolysis, high pressure pasteurization (HPP), germination, or ultrafiltration (UF) (Sathe et al., 2005). Overall,

these changes can result in decreased protein solubility, reduced extractability, loss of conformational epitopes, or aggregation (Faeste et al., 2007; Poms, Klein, & Anklam, 2004; Thomas et al., 2007). These factors are known to decrease assay performance and potentially result in false negative results. Additionally, the combination of processing applications will only multiply these effects and further complicate detection.

Conclusion

Processing generally results in decreased detection and allergenicity of food allergens, however many of these food allergens are resistant to processing and remain potent stimulants of the immune system. Destruction of conformational epitopes, protein aggregation, and hydrolytic processes have been demonstrated to reduce allergenicity. Although, some processes may induce formation of neo-epitopes increasing allergenicity such as dry heating of peanuts. Understanding the influence of food processing applications is important in identifying risk factors for food allergic individuals and more important in the selection of adequate detection methods.

VII. FOOD MATRICES

Food matrices are complex systems of fats, carbohydrates, proteins, minerals, and other compounds that are known interact with one another and influence the properties of allergens. Factors including matrix composition, pre-processing interactions, and processing induced interactions can influence the overall detection of allergens. Understanding these interactions could further benefit development of detection methods and provide greater sensitivity and specificity of analytical tools. Secondly it can

improve knowledge of protein interactions in foods. The most common way to analyze food matrix interactions is by preparing model foods such as cookies, biscuits, or pastry doughs with known amounts of allergen and determining recovery by ELISA or another analytical method, sometimes referred to as incurred samples (Taylor et al., 2009).

Less is understood regarding the influences of food matrix interactions as opposed to food processing effects where numerous research efforts have been focused. As the food supply and food products become more diverse, the types of food matrices also increase in diversity. Matrix types can be primarily classified into three categories: solid, liquid, or paste like matrices (Taylor et al., 2009). Furthermore, different types of matrices can have profoundly different influences on allergens and detection.

Glutinous Matrices

Solid type matrices can include flour based or other grain based materials. Most often, these matrices are glutinous and include foods such as cookies, breads, and cracker type matrices in which unidentified allergens may be present. Studies focused on the detection of allergenic residues in glutinous matrices have indicated lower recoveries of allergens before processing (Bly, 2014; Bugyi et al., 2010; Downs and Taylor, 2010; Monaci et al., 2011). Many of these same studies also evaluated the recovery of allergens after processing operations including thermal and non-thermal treatments (Bly, 2014; Bugyi et al., 2010; Downs and Taylor, 2010). Thermal processing yielded a distinct decrease in recovery of target allergens, however the extent of thermal processing contributes greatly to overall detection. The lowest observed recovery values when analyzed by ELISAs were obtained from glutinous pastry matrices after baking or frying (Bly, 2014; Downs and Taylor, 2010). These studies have reported similar reduced

recoveries of allergens from glutinous matrices prior to thermal processing (e.g. baking) indicating interactions occurring within a matrix are inhibiting detection and overall quantitation (Bly, 2014; Downs and Taylor, 2010). The comparison of results have proven difficult due to differences in ELISA kits, kit standards, and preparation of model foods used for analysis although overall trends and patterns can be assessed regarding the interactions in glutinous food matrices.

The presence of wheat and the formation of gluten has been attributed to reduced allergenicity both in vivo and in vitro. In OFCs designed to assess baked egg tolerance, egg allergic individuals given a wheat flour baked muffin passed the OFC at greater rate than those given the rice flour baked muffin (Lanser et al., 2015). Overall, children were more tolerant to wheat flour muffins than rice flour muffins indicating wheat flour may provide protective benefits to baked egg in OFCs and reduced antigenicity (Lanser et al., 2015). In vitro studies have provided further support to in vivo studies. Shin et al., 2013 indicated decreased IgE reactivity to both egg white proteins (ovalbumin and ovomucoid) in prepared mixtures of egg white and wheat flour baked for 30 minutes, whereas no loss in IgE reactivity was observed in heated egg white samples (Shin et al., 2013). The authors attribute the observation to aggregation mechanisms occurring between egg white and wheat flour during the heating process (Shin et al., 2013). Aggregation mechanisms may secondly be masking allergenic epitopes reducing detection by IgE antibodies.

Non-glutinous Matrices

It is just as important to evaluate non-glutinous matrices due to the increasing popularity of gluten-free diets. Non-glutinous matrices are composed of a variety of flours, starches, or powders which can all interact with allergens and influence overall

detection. In one study which analyzed allergen recovery by ELISA from non-glutinous cookies (buckwheat flour, rice flour, sorghum flour, tapioca starch, sugar, salt, sodium bicarbonate, baking soda, sunflower oil, and water) showed adequate recoveries of target allergens (casein, egg, gluten, soy) before baking, but observed significantly reduced recoveries after baking for all allergens (Gomaa and Boye, 2013). This phenomenon is similar to the observations in glutinous cookie matrices. A second study compared non-glutinous cereal bars (rolled oats, rice crisp, corn flour) for recovery of milk, egg, or peanut before and after baking using commercial ELISA kits (Parker et al., 2015). Recovery results indicate good recoveries of each allergen prior to baking (dough state), however after baking reductions in recovery were apparent for all allergens analyzed (Parker et al., 2015).

Liquid Matrices

Less attention and research has been focused on allergen recovery from liquid matrices, however interactions are still possible (although the popularity of liquid foods is increasing). Allergen extraction from liquid matrices poses a less challenging task in comparison to solid matrices. Distribution of allergens in liquid matrices is uniform unless the allergen is present as a particle (Taylor et al., 2009). To assess processing effects using a model liquid food matrices, milk prepared in a phosphate buffered saline solution was evaluated. Recovery of milk from a liquid matrix (phosphate buffered saline solution) before processing yielded >80% recovery when analyzed by nine different milk ELISA kits (Bly, 2014). After thermal processing ELISA kits were still capable of detecting milk, however thermal processing reduced final recoveries by approximately 40-50% of original milk concentration (Bly, 2014).

Conclusion

Differences in matrix compositions can interfere with overall allergen detection. In general, solid matrices are a more challenging matrix to extract proteins from due to their complex nature and ingredient composition. Contrastingly, the recovery of allergens from aqueous matrices is less complex in minimally processed foods. The addition of extensive thermal processing has indicated drastically reduced recoveries of allergens in samples. Detection of allergens in extensively processed foods then becomes even more challenging because two intricate systems, the food matrix and processing operations are influencing protein structures consequently affecting detection. Although the issue seems daunting comparisons and general trends can be broadly assessed among studies. Understanding the complexity of a food matrix and/or food processing can aid in selection of the most suitable detection method.

VIII. SUMMARY

The detection of allergenic food residues is becoming more advanced with improved detection methods, new instrumentation, and improvement of existing detection methods. The effects of processing on allergen recovery and stability are still being investigated. Processing induced changes may mask or alter allergenic epitopes making them less detectable by current detection methods. These epitopes are still potentially reactive, but remain undetected thus posing a significant risk to consumers. In addition, the complex nature of processed foods which consist of several different ingredients and can confer many different matrix types further complicates detection. Furthermore, the level of processing is known to cause reduced detection of allergens in

model food systems, however potential interactions occurring within a matrix could also be a factor in reduced detection. ELISAs are the current detection method used by regulatory agencies, food manufacturers and research facilities, but pose the risk of reporting false negatives due to changes in the immunoreactive epitopes. The detection of allergens relies heavily on the solubility of proteins, which may also be altered due to processing. A better working knowledge of how food matrix interactions affect detection by ELISAs will aid in the understanding of matrix induced interactions as well as processing induced interactions.

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CHAPTER 2:
DETECTION OF MILK AND PEANUT RESIDUES IN MODEL PASTRY DOUGH MATRICES AND SAMPLE MIXES

ABSTRACT

Reduced recoveries of allergens have been reported in processed glutinous food matrices when analyzed using enzyme-linked immunosorbent assays (ELISA). Interactions occurring in the food matrix due to thermal and non-thermal processing are likely inhibiting the detection of allergens by ELISA. However, it is unknown if reduced detection is a result of processing effects or matrix interactions occurring prior to processing. The objective of this study was to examine interactions occurring between unprocessed food matrices and residues of the allergenic food, milk and peanut, using commercial ELISAs and describe the type(s) of interaction causing reduced detection. Model food matrices containing non-fat dry milk (NFDM), wheat flour, salt, shortening, and water were prepared with varying levels of flour (100, 80, 60, 40, 20, 0% flour), substituting wheat flour with wheat starch when necessary. Analogous non-glutinous (dry mix) matrices incurred with NFDM were prepared without shortening and water (dry flour, and/or starch, salt). Secondly, samples of a known allergen (NFDM or peanut) concentration and increasing concentrations of flour (0, 0.0025, 0.025, 0.25, 2.5, 25, 30, 35, 40, 45, 50, and 55%) were prepared. Neogen Veratox® Total Milk or Neogen Veratox® Peanut ELISA kits were used for analysis. Statistically significant differences ($p \leq 0.05$) were found between the recovery of milk from glutinous and non-glutinous matrices; recovery was lower in glutinous matrices (77 ± 19%) compared to non-glutinous matrices (117 ± 19%). In the concentration analyses, recovery of milk was

lowest (66 ± 15%) at the highest level of flour (55%) flour whereas recovery of peanut was adequate (98 ± 11%). Reduced recoveries of allergenic residues by ELISA, particularly milk, are observed in glutinous food matrices especially at high flour concentrations. Inadequate detection of residues does not imply reduced allergenicity therefore further understanding of the interaction between allergenic proteins and glutinous food matrices is needed to ensure the safety of allergic consumers.

INTRODUCTION

Food allergies affect 3 – 4% of the U.S. population and are caused by the ingestion of allergenic proteins derived from food (Sicherer and Sampson, 2010). Higher prevalence rates of food allergy occur in children, with 4-8% of children diagnosed with a true food allergy whereas only 1-5% of adults have a true food allergy (Weinberg, 2011). Given that food allergies are individualistic reactions, different minimal eliciting doses of a particular allergic food will affect sensitized individuals differently. Consequently, consumers rely heavily on the food industry to employ good manufacturing practices, use adequate cleaning procedures, and properly label all allergen-containing foods. In order to effectively protect consumer health, the Food Allergen Labeling and Consumer Protection Act (FALCPA) was passed in 2004 and requires the labeling of any allergenic ingredient or ingredient derived from a major allergic food (Taylor and Hefle, 2006). On a global basis, eight foods are responsible for >90% of all food allergic reactions and are referred to as ‘The Big 8’ (Taylor and Hefle, 2002; Taylor and Baumert, 2012). These eight foods include milk, eggs, soy, wheat, tree nuts, peanuts, fish, and crustacean shellfish (Joint FAO/WHO Expert Consultation on

Allergenicity of Foods Derived from Biotechnology, 2001). To protect sensitized individuals from an unwarranted allergic reaction, the detection of allergenic residues becomes critically important. However, this becomes challenging when the food matrix is taken into consideration. Food allergic individuals are extremely unlikely to consume allergenic ingredients alone, but rather experience reactions from accidental exposures to allergens contained in food matrices having a wide variety of other components. Cross contact from shared processing equipment leading to hidden allergens within a food matrix is a frequent cause of accidental exposure.

Food matrices are complicated systems of fat, carbohydrates, proteins, water, and various components and are often subjected to one or multiple food processing operation(s). Interactions occurring between the various matrix components may decrease the recovery of residues from allergenic foods present in a food system. Food processing is known to reduce the detection of allergens, especially in the case of thermal processing (Bly, 2014; Downs and Taylor, 2010; Khuda et al., 2012).

The design of food matrix studies is a complex issue and has been addressed in a variety of ways using different model food matrices and detection methods. Only a few model food matrices including cookies, biscuits, or chocolate have been used in such studies. Differences between model food formulations, ingredients, sample preparation, processing methods, and target allergen(s) can all influence the outcome of these studies. In addition, inherent variability among commercial ELISA kits will influence detection and overall recovery. Sampling methods are another critical factor in the assessment of recoveries and proper homogenization of an allergenic material into a model food is another key factor to consider when designing and assessing food matrix studies.

The overall purpose of this chapter was to better understand the interactions occurring in various model food systems by analyzing different matrices and ingredient mixes and their effects on the recovery of milk or peanut residues using commercial ELISA kits. As previously mentioned, food processing operations are known to reduce the overall detection of allergens; therefore, the matrices used in this study were minimally processed and only subjected to mixing operations.

MATERIALS AND METHODS

Preparation of Wheat Starch – Sugar Matrix

Wheat starch (Midsol-50 Wheat Starch, MGP) and NFDM (Darigold low-heat non-fat dry milk powder, Caldwell, ID) were evaluated for their interactive effects by preparing sample mixes of different ratios of wheat starch and powdered sugar while maintaining a constant NFDM concentration. The sample mix compositions are summarized in Table 2-1. Powdered sugar (C and H Cane Sugar) was prepared by grinding pure cane sugar in a coffee grinder (Mr. Coffee precision coffee mill, Model IDS77) on speed 'fine' for 30 seconds, followed by thoroughly scraping the bowl and lid. The grinding process was repeated once more. After individual batches (~100 g) were ground in the coffee grinder, the batches were combined in the bowl of a Kitchen-Aid stand mixer (Kitchen-Aid Ultra Power 4.5 Quart Tilt Head Stand Mixer, Model KSM95) and blended for 20 minutes on speed 'stir'. Powdered sugar was used as a substitute material in cases where the matrix did not contain starch.

Table 2-1. Summary of wheat starch and powdered sugar samples prepared at 250 ppm NFDM to assess interactive effects between wheat starch and NFDM.

Wheat starch % (w/w)	Wheat starch (g)	Powdered sugar (g)	Concentrated NFDM spike mix in wheat starch, (2500 ppm) (g)	Concentrated NFDM spike mix in powdered sugar, (2500 ppm) (g)	Final NFDM concentration in samples (ppm)
100	90	0	10	0	250
80	70	20	10	0	250
60	50	40	10	0	250
40	30	60	10	0	250
20	10	80	10	0	250
0	0	90	0	10	250

Milk was added to the sugar-starch matrix at a concentration of 250 ppm non-fat dry milk on a wet basis (µg per gram of total mass) by first preparing a concentrated spiking stock material of 2,500 ppm NFDM (µg per gram of total mass on a dry basis) in wheat starch by mixing 498.75 g wheat starch and 1.25 g NFDM in an 11-cup Kitchen-Aid food processor (Model Number KFP600) with the multi-purpose blade attachment. The mixture was blended for two minutes, followed by thoroughly scraping the sides and lid of the bowl. The mixing and scraping procedure was repeated four times. An appropriate amount of wheat starch was replaced by this spike material in the starch-sugar matrix to obtain a final concentration of 250 ppm NFDM in sample mixes. For 0% (w/w) wheat starch samples, a NFDM concentrated spiking material was prepared in powdered sugar by the same method previously described. In that case, an appropriate amount of powdered sugar was replaced in the matrix by the concentrated spiking material.

All concentrated spiking materials were analyzed for homogeneous distribution of NFDM after mixing. Homogeneity analysis was performed by taking nine subsamples from the concentrated spiking mix and analyzing each of the subsamples for recovery of milk using the Neogen Veratox® Total Milk ELISA kit. The average recovery of all nine samples must be within 15% of the desired 250 ppm spike level and the overall coefficient of variation (CV, %) is required to be $\leq$ 15%.

To mix samples, ingredients were weighed according to the formulations described in Table 2-1 on an analytical balance and placed into the bowl of a coffee grinder (Mr. Coffee precision coffee mill, Model IDS77) and mixed for 15 seconds on speed 'fine'. After mixing, the sides of the bowl and lid were scraped. The mixing process was repeated one time and samples were stored in zip-top bags in the freezer (-15°C) until needed for further analysis.

Samples were prepared in duplicate. Control samples (0 ppm NFDM) were prepared similarly omitting the spiking material.

ELISA Analysis

All samples and controls were analyzed using Neogen Veratox® Total Milk ELISA kits. The kit manufacturer supplied all assay components including buffers and reagents. Duplicate extracts for all samples were independently prepared, and each extract was plated into triplicate wells. The extraction and assay procedures for ELISA analysis were performed as written by the kit manufacturer. In summary, a 5.0 gram sample was extracted in 125 mL extraction buffer in a 60°C shaking water bath for 15 minutes. After extraction, samples were allowed to settle and cool to room temperature. A 1 mL aliquot was removed from each extract and centrifuged at 13,000 x g for 5

minutes in a microcentrifuge (ThermoScientific Sorvall Legend Micro 17). The dynamic range of the Neogen Veratox® Total Milk ELISA is 2.5 – 25 ppm expressed as NFDM. If dilution of extracts was necessary, samples were diluted using the kit extraction buffer. Samples and standards (100 µL) were plated onto antibody coated wells and allowed to incubate for 10 minutes at room temperature. After incubation, plates were washed with the appropriate assay washing buffer. The enzyme labelled conjugate antibody solution (100 µL) was added and plates were incubated at room temperature (10 min) and washed as previously described. The enzymatic substrate (100 µL) was added, generating a colorimetric product, and incubated as before. After incubation with the substrate, the colorimetric reaction was ceased by the addition of stop solution (100 uL) provided by the kit manufacturer. The absorbance values of samples and standards were determined using a plate reader (ELX808 Ultra Microplate Reader) set at 650 nm.

The quantitative results (Neogen Veratox® Software v 3.0.2, Neogen Corporation, Lansing, MI) for samples were determined using software provided by the manufacturer. A standard curve was generated based on the observed optical density and the corresponding sample values were calculated using the equation generated by the standard curve. The r^2 value for the standard curve was required to be ≥ 0.98. If this criteria was not met, the assay was performed again. The lower limit of quantitation for the Neogen Veratox® Total Milk ELISA kit is 2.5 ppm NFDM.

Statistical Analysis

Percent recoveries were determined using the following formula: percent recovery = ((measured ppm NFDM/incurred ppm NFDM)*100). SAS 9.4 was used to perform statistical analysis and results were determined to be significant if $p \leq 0.05$. Analyses were

done using a least squares means experimental design with the Dunnett adjustment for multiple comparisons.

Preparation of Wheat Flour Pastry Dough Model Foods

Two model foods were prepared to assess recovery in this series of experiments, a wet pastry dough matrix and a dry pastry dough mix. The wet pastry dough matrix consisted of a well-developed glutinous network and the dry pastry dough mix was a dry powder mix, with no glutinous network formed.

Preparation of wet pastry dough matrix

A wet pastry dough matrix was adapted from Downs and Taylor (2010) to serve as the complex glutinous matrix to assess interactions between the matrix and the target allergen, NFDM. The following formula was used to prepare the wet pastry dough matrix samples: 57.1% Gold Medal unbleached all-purpose wheat flour, 19.5% Crisco® all-vegetable shortening, 1.5% Morton® iodized salt, 21.9% Type I reagent grade water (Barnstead E-pure). All ingredients, except water, were obtained from a local grocery store.

The wet pastry dough matrix samples were prepared at various concentrations of wheat flour, where wheat flour was substituted with wheat starch as appropriate. Samples were prepared at 100, 80, 60, 40, 20, and 0% (w/w) wheat flour in the pastry dough mix.

All wet pastry dough matrix samples were prepared at a concentration of 250 ppm NFDM. NFDM was incorporated into sample mixes from a 2,500 ppm concentrated spiking material of NFDM in wheat flour. The concentrated spike material was prepared

as previously described. An appropriate amount of flour was replaced by the spike material to obtain a final concentration of 250 ppm NFDM in the pastry dough samples. For 0% (w/w) wheat flour samples, a NFDM concentrated spiking material was prepared in wheat starch in the same method previously described. An appropriate amount of wheat starch was replaced by the starch-based concentrated spiking material.

The wet pastry dough matrices were prepared by combining the wheat flour, concentrated spiking material, and wheat starch in the bowl of a 4-cup Kitchen-Aid food processor fitted with the multi-purpose blade and blended for two minutes. After blending, the sides and lid of the bowl were scraped. Blending and scraping was repeated three additional times. Salt was added to the mixture and blended for an additional two minutes, followed by scraping. Shortening was added and incorporated using five one-second pulses. The sides and lid were scraped, and the pulsing process was repeated. Lastly, water was added and incorporated using five-one second pulses which was followed by scraping of the bowl and lid and repeating the pulsing process. Wet pastry dough matrices were refrigerated (4°C) for two hours. After resting, the dough was rolled evenly, ~3 mm thickness, using a pasta roller and cut into smaller pieces using a pizza roller. Samples were frozen (-15°C) until needed for analysis. A set of control samples was prepared similarly at 0 ppm NFDM.

Preparation of dry pastry dough mix

Dry blend pastry dough mixes were prepared using a modified wet pastry dough matrix formula. In the dry pastry dough mixes, no shortening or water was added to the food matrix, which resulted in a dry mix. The sample mix composition consisted of 98.5% wheat flour and 1.5% salt. Samples were prepared using the same flour to starch

ratios as wet pastry dough matrices (100:0, 80:20, 60:40, 40:60, 20:80, and 0:100% w/w of flour:starch). All dry blend pastry mixes were prepared at a concentration of 250 ppm NFDM (µg per gram of total mass on a wet basis) from a 2,500 ppm NFDM in flour- or starch-based concentrated spiking material.

Wheat flour, concentrated spiking material, and wheat starch were placed into the bowl of an 11-cup Kitchen-Aid food processor and blended for two minutes, followed by scraping of the bowl and lid. The blending and scraping process was repeated three times. Salt was added and mixed for an additional two minutes. After mixing, samples were stored in the freezer (-15°C) until needed for analysis. Control samples (0 ppm NFDM) were similarly prepared. All samples and controls were prepared in duplicate.

ELISA Analysis

ELISA analysis was performed as previously described. All samples were extracted in duplicate and plated into triplicate wells. The percent recovery of NFDM was calculated by dividing the observed ppm NFDM of the concentrated spike matrices by the expected ppm NFDM added to the matrix material (250 ppm NFDM) and multiplying this value by 100 to obtain a percentage.

Statistical Analysis

For the percent recovery calculation, the added level of NFDM was determined for each sample based upon the average of nine samples obtained from the concentrated spiking material used to prepare each sample for analysis. SAS 9.4 software was used for the statistical analysis. The recovery values were compared using unstructured differences of least square means design using Tukey's adjustment for p-values. Factors

were determined to be significant if $p \leq 0.05$. Recovery values were compared between wet pastry dough matrices and dry pastry dough mixes at each flour concentration prepared. No comparisons were made between samples of different concentrations, only between different matrix types of the same wheat flour concentration.

Preparation of Wheat Flour Mixes with Known Concentrations of Milk or Peanut Allergens

Sample mixes of wheat flour (Gold Medal unbleached all-purpose wheat flour), powdered sugar (C and H Cane Sugar), and NFDM (Darigold low-heat non-fat dry milk powder, Caldwell, ID) or peanut flour (Light Roasted Peanut Flour, Golden Peanut Company, Alpharetta, GA) were prepared according to the formulations in Table 2-2. The concentration of wheat flour in relation to the concentration of the target allergen was examined by preparing samples with known allergen concentrations and incrementally increasing wheat flour concentrations. Concentrated spiking materials of 250 ppm NFDM or peanut flour were prepared as described earlier. NFDM or peanut flour were distributed in powdered sugar by combining 999.75 g powdered sugar and 0.25 g NFDM or peanut flour. The concentrated spiking materials were mixed for five minutes on speed 'stir' in a Kitchen-Aid stand mixer (Kitchen-Aid Ultra Power 4.5 Quart Tilt Head Stand Mixer, Model KSM95) equipped with the paddle attachment. After mixing, the sides and bottom of the bowl were scraped. The mix-and-scrape process was repeated four times, for an elapsed mixing time of 25 minutes. The concentrated spiking mixtures in powdered sugar were validated for homogeneity of NFDM or peanut flour using Neogen Veratox Total Milk or Neogen Veratox® Peanut ELISA kits.

Table 2-2. Composition of sample mixes prepared using different concentrations of wheat flour while maintaining a known allergen concentration

Concentration of flour (%)	Flour added (g)	Concentrated spike mix in powdered sugar (NFDM or peanut flour) (250 ppm) (g)	Final allergen[1] concentration in samples (ppm)
0	0	100	250
0.0025	0.0025	99.9978	250
0.025	0.025	99.975	250
0.25	0.25	99.75	249
2.5	2.5	97.5	244
25	25	75	188
30	30	70	175
35	35	65	163
40	40	60	150
45	45	55	138
50	50	50	125
55	55	45	113

[1]The allergen indicates the target analyte for a given ELISA kit. For, Neogen Veratox® Total Milk ELISA the allergen is represented as ppm NFDM. For peanut, the allergen is represented as ppm peanut in the Neogen Veratox® Peanut ELISA.

Flour was added to the powdered sugar-spike mix at the following percent concentrations: 0, 0.0025, 0.025, 0.25, 2.5, 25, 30, 35, 40, 45, 50, and 55% in the total mix (Table 2-2). Batch sizes were kept constant (100 g) for each sample prepared.

- The percentage of wheat flour added (Table 2-2) can be represented in equivalent ppm wheat flour values: 0% (0 ppm), 0.0025% (25 ppm), 0.025% (250 ppm), 0.25% (2,500 ppm), 2.5% (25,000 ppm), 25% (250,000 ppm), 30% (300,000 ppm), 35% (350,000), 40% (400,000 ppm), 45% (450,000 ppm), 50% (500,000 ppm), and 55% (550,000 ppm)

Appropriate amounts of flour and concentrated spike material with target allergen were weighed according to the formulations in Table 2-2. Ingredients were combined in a coffee grinder (Mr. Coffee precision coffee mill, Model IDS77) and mixed on speed 'fine' for fifteen seconds. After mixing, the sides and lid were thoroughly scraped. Mixing was repeated one time. Samples were stored in the freezer (-15°C) until needed for further use. All samples and controls (0 ppm NFDM) were prepared in duplicate.

ELISA Analysis

Samples were extracted in duplicate for ELISA analysis and plated into triplicate wells. The ELISA procedure was described earlier; the Neogen Veratox® Total Milk and Neogen Veratox® Peanut ELISA kits were used. Percent recoveries of NFDM or peanut were calculated based on the known concentration of NFDM or peanut in samples and observed recovery values determined by ELISA for each target allergen, respectively.

Statistical Analysis

Results were analyzed for statistical significance using SAS 9.4 software and a least square means experimental design with Tukey's adjustment applied for multiple comparisons. Values were determined to be significant if $p \leq 0.05$.

Preparation of Alternative Flour Mixes with Known Concentrations of Milk or Peanut Allergens

Mixtures of non-glutinous flours, powdered sugar, and the target allergen (NFDM or peanut) were prepared analogously to the concentrated spiking mixes with wheat flour, powdered sugar, and target allergen as described earlier. Alternative non-glutinous flours selected for analysis were corn flour (Bob's Red Mill Stone Ground Whole Grain Corn Flour), rice flour (Bob's Red Mill Stone Ground White Rice Flour), and soy flour (Bob's

Red Mill Stone Ground Whole Grain Soy Flour). Table 2-3 summarizes sample compositions; flour was added to samples up to 25%. Samples and controls were prepared in duplicate.

ELISA Analysis

For ELISA analysis, all samples were extracted in duplicate and plated in triplicate wells. The assay was performed as described previously. Percent recoveries were determined for each sample and allergenic target prepared.

Statistical Analysis

Statistical analysis was performed as previously described, using least squares means experimental design with Tukey's adjustment for multiple comparisons and determined to be significant if $p \leq 0.05$.

Table 2-3. Composition of sample mixes prepared using different concentrations of alternative flours while maintaining a known allergen concentration

Concentration of flour (ppm)	**Flour added (g)**	**Concentrated spike mix in powdered sugar (NFDM or peanut flour) (250 ppm) (g)**	**Final allergen[1] concentration in samples (ppm)**
0	0	100	250
25	0.0025	99.9978	250
250	0.025	99.975	250
2500	0.25	99.75	249
25000	2.5	97.5	244
250000	25	75	188

[1]The allergen indicates the target analyte for a given ELISA kit. For, Neogen Veratox® Total Milk ELISA the allergen is represented as ppm NFDM. For peanut, the allergen is represented as ppm peanut in the Neogen Veratox® Peanut ELISA.

Preparation of Alternative Flour Pastry Dough Matrices

A series of wet pastry dough matrices with corn or rice flour were prepared in the same manner as wet wheat pastry dough matrices described earlier. The same flour-to-starch ratios (100:0, 80:20, 60:40, 40:60, 20:80, and 0:100) were prepared by substituting corn starch (Argo 100% Corn Starch) and rice starch (Hol-Grain All Purpose Gravy Thickener, 100% Rice Starch) for their respective flours when necessary. Concentrated spiking mixes were prepared at 2,500 ppm NFDM as previously described and wet pastry dough matrices were prepared at a concentration of 250 ppm NFDM by substituting appropriate amounts of the concentrated spiking material. After mixing, samples were not placed through the pasta roller since matrices were unable to form a cohesive dough mass especially at the lower flour concentration levels. All samples and controls (0 ppm NFDM or peanut) were prepared in duplicate.

ELISA Analysis

For ELISA analysis, samples were analyzed with their respective ELISA kits (Neogen Veratox® Total Milk or Neogen Veratox® Peanut) as described previously Samples were extracted in duplicate and plated in triplicate wells. Percent recoveries were calculated based on the concentration of the allergenic spiking material and the observed recovery obtained from the ELISA kit.

Statistical Analysis

Statistical significance was determined using a least square means experimental design with Tukey's adjustment for multiple comparisons and analyzed using SAS 9.4 software. Results were determined to be significant if $p \leq 0.05$.

RESULTS

Evaluation of Ingredients for use in Model Foods

Wheat starch and powdered sugar were evaluated for the presence of milk residues using the Neogen Veratox® Total Milk ELISA. Wheat flour and powdered sugar were evaluated for the presence of peanut residues using the Neogen Veratox® Peanut ELISA. Wheat starch, corn flour, rice flour, and soy flour were also evaluated for the presence of gluten using the Neogen Veratox® R5 Gluten ELISA. The nitrogen content of each flour was determined by Dumas nitrogen analysis using a LECO FP-528 Protein/Nitrogen Determinator (LECO Corporation, St. Louis, MO).

The wheat starch, wheat flour, and powdered sugar (sucrose) were verified to contain no detectable milk residues using the Neogen Veratox® Total Milk ELISA. NFDM was homogeneously distributed in the concentrated spiking materials as shown by ELISA (CV < 15%). Thus, the mixing procedure was deemed adequate.

As illustrated in Figure 2-1, excellent recoveries of NFDM were obtained at all wheat starch-powdered sugar ratios analyzed. When compared to the control (0% wheat starch), no significant differences in milk recovery were determined among the various concentrations of wheat starch. At 100% wheat starch, recovery was 102 ± 14.3%, indicating that wheat starch does not inhibit or promote the detection of NFDM in this system of ingredients. Furthermore, at 0% wheat starch (100% powdered sugar) no decrease was observed in the recovery of milk indicating that powdered sugar does not inhibit the detection of milk. Relatively low standard deviations (<20%) were obtained for all samples analyzed. Therefore, we can conclude that these ingredients do not negatively affect the recovery of milk.

Wheat Flour Pastry Dough Model Foods

Wheat starch was also analyzed for gluten using the Neogen Veratox® R-5 gliadin ELISA kit and recovery was determined to be 140.0 ppm gluten.

The concentrated spiking mixes of NFDM dispersed in wheat flour or wheat starch were each determined to be homogeneously distributed with a CV% < 15% using Neogen Veratox® Total Milk ELISA kits.

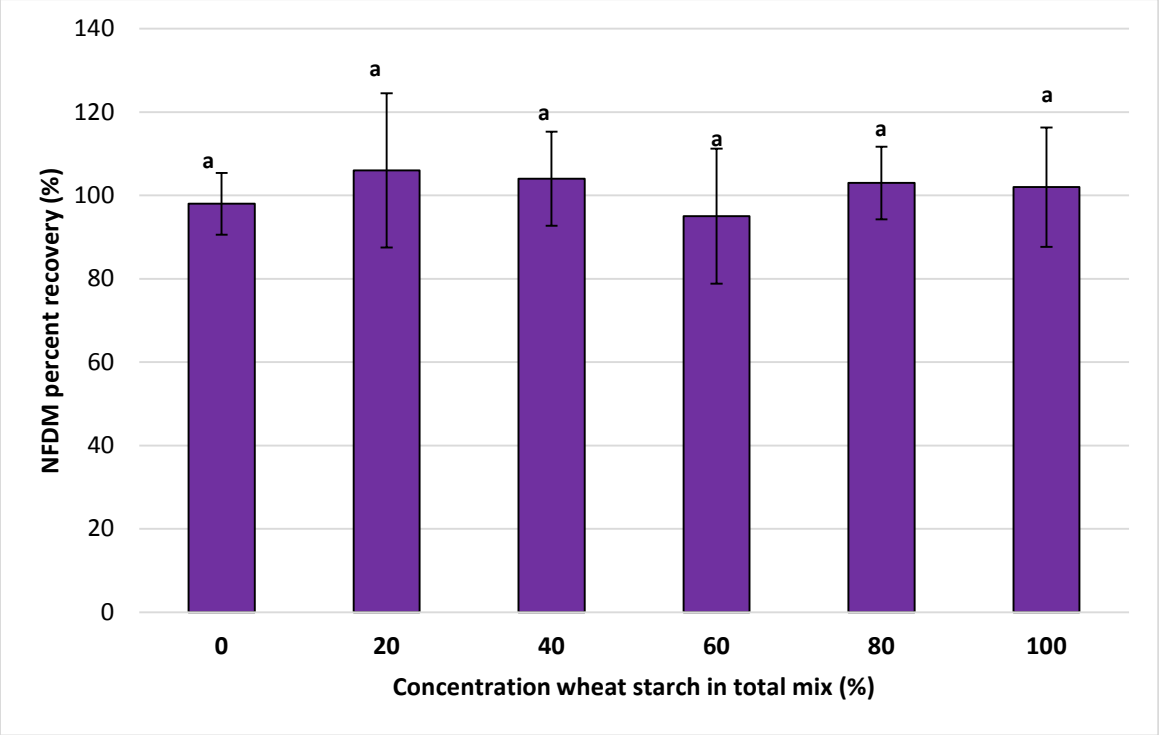

Figure 2-1. Percent recovery and standard deviation of NFDM from samples prepared at various ratios of wheat starch and powdered sugar. Statistical significance ($p \leq 0.05$) was determined using Dunnet Test using 0% wheat starch as the control.

The recovery of milk from dry pastry dough mixes was always greater in comparison to wet pastry dough matrices at corresponding levels of flour (Figure 2-2). Significant differences between dry and wet matrix types were present at each flour concentration prepared. Statistical comparisons were made only between dry and wet pastry dough samples of the same flour concentration. Moreover, the lowest obtained recovery of milk from dry pastry mixes was 100 ± 14.2% indicating detection was not inhibited in these mixes (wheat flour, salt, milk).

In wet pastry dough matrices prepared at 20% wheat flour, the lowest flour concentration, a decrease in the recovery of milk proteins was observed when compared to 0% wheat flour wet pastry dough matrices. The reduced detection of milk in wet dough pastry matrices is likely caused by the formation of a gluten network after the addition of water (Bly, 2014). Furthermore, no general trend was present at the differing concentrations of wheat flour, indicating that while the formation of the glutinous network causes reduced detection, the reduction does not appear to be dependent on the wheat flour concentration in wet pastry dough matrices.

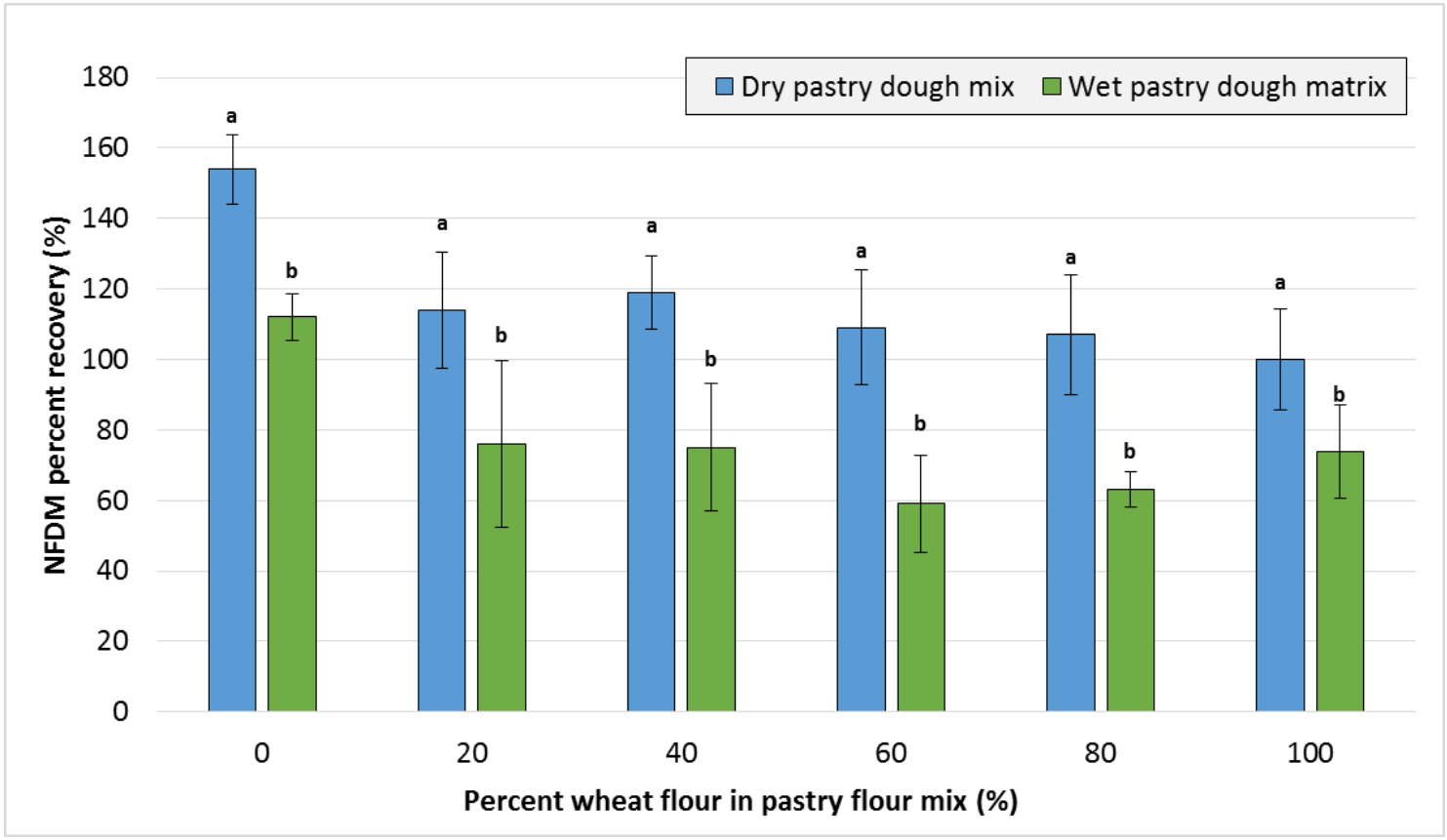

Figure 2-2. Percent recovery of NFDM from dry pastry dough mixes and wet pastry dough matrices at varying wheat flour concentrations as determined by Neogen Total Milk Veratox® ELISA kits. Statistical comparisons were made between dry and wet pastry mixes at the same flour concentrations using an unstructured least square means comparison and Tukey's adjustment and $p \leq 0.05$ for significance.

Wheat Flour Concentration Mixes with Known Concentrations of Milk or Peanut Allergens

A series of concentration experiments were conducted to determine if a particular concentration of wheat flour in a food system resulted in decreased detection of allergens using commercial ELISA kits. The concentrated spiking materials of powdered sugar and NFDM or peanut were validated as homogenous using Neogen Veratox® Total Milk or Neogen Veratox® Peanut ELISA with a CV% $\leq$ 15. Sample batch sizes were maintained at a constant weight, therefore the allergen concentration in each batch was calculated based upon the concentration of wheat flour added as described in Table 2-2. The recovery values were normalized to percent recovery of NFDM or total peanut.

As demonstrated by previous experiments, the powdered sugar does not interfere with allergen detection in wheat starch matrices. Therefore, the same principle was considered true in these experiments.

Milk

Overall, a general decline was observed in NFDM recoveries as the wheat flour concentration increased in samples (Figure 2-3). Significant differences in recovery were determined between samples of the highest and lowest wheat flour concentrations. At wheat flour concentrations of 25% flour or less, recoveries were approximately 100% indicating the interactions influencing decreased milk protein detection in complex matrices are minimized at these levels. Recoveries of NFDM began to decline when concentrations of flour were $\geq$ 30% in individual samples, suggestive of component interactions.

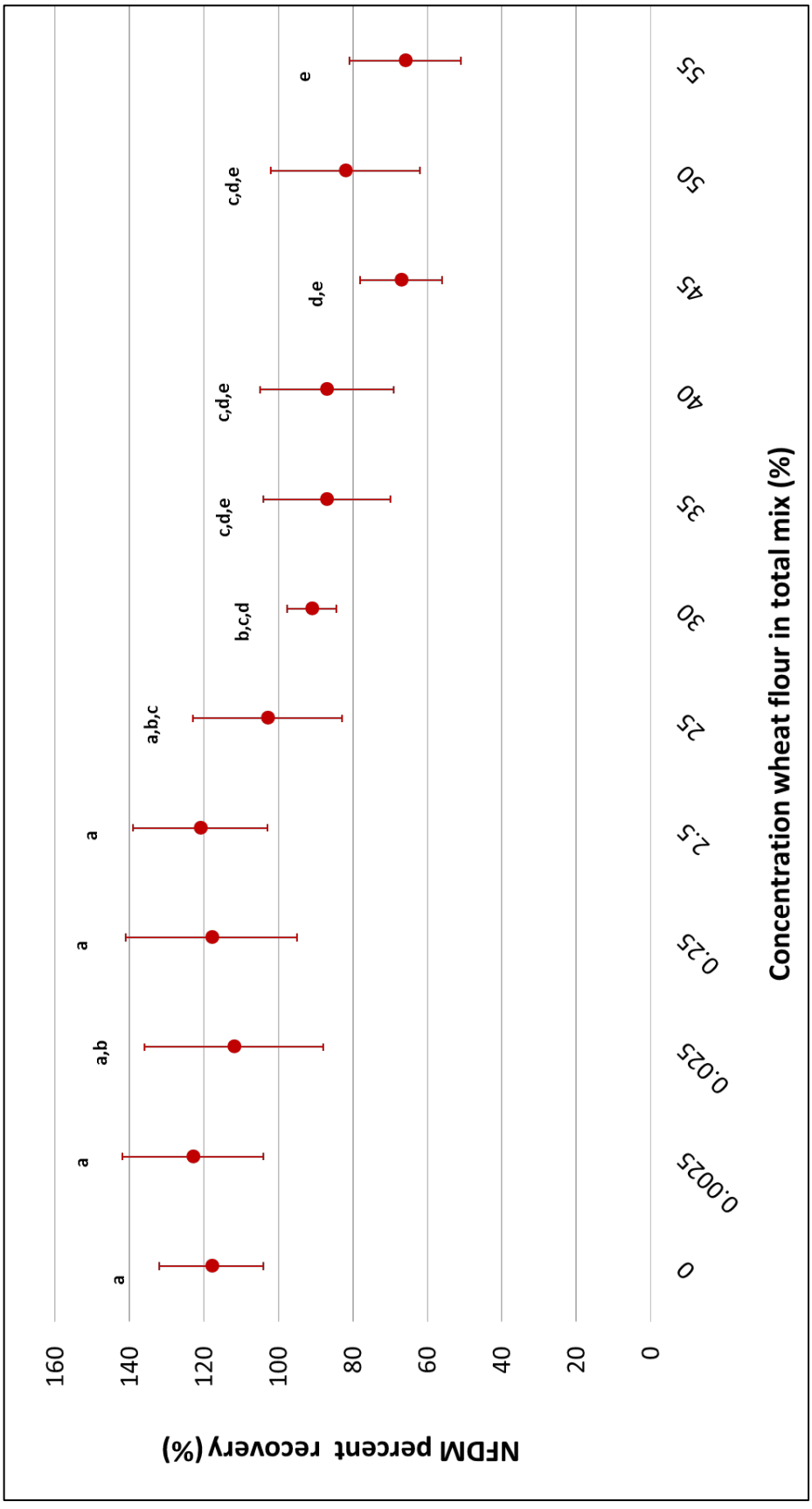

Figure 2-3. Recovery of NFDM from sample mixes of powdered sugar and increasing concentrations of wheat flour. Statistical analysis was done using least square means analysis with Tukey's adjustment and determined significant if $p \leq 0.05$.

Peanut

A second identical concentration experiment was performed in which peanut flour was selected as the target allergen. As indicated by Figure 2-4, recovery of peanut was excellent at nearly all analyzed levels of wheat flour, however slight decreases in peanut recovery were present at the higher levels of wheat flour. All observed recovery values were > 80%. The expected standard error/coefficient of variation for commercial ELISA kits is 20% (Abbott et al., 2010), therefore our results are within the acceptable limits of variation for commercial ELISA kits. Statistical analyses indicate significant differences among sample levels, especially when comparing the highest and lowest concentration levels although the highest and lowest levels are not significantly different.

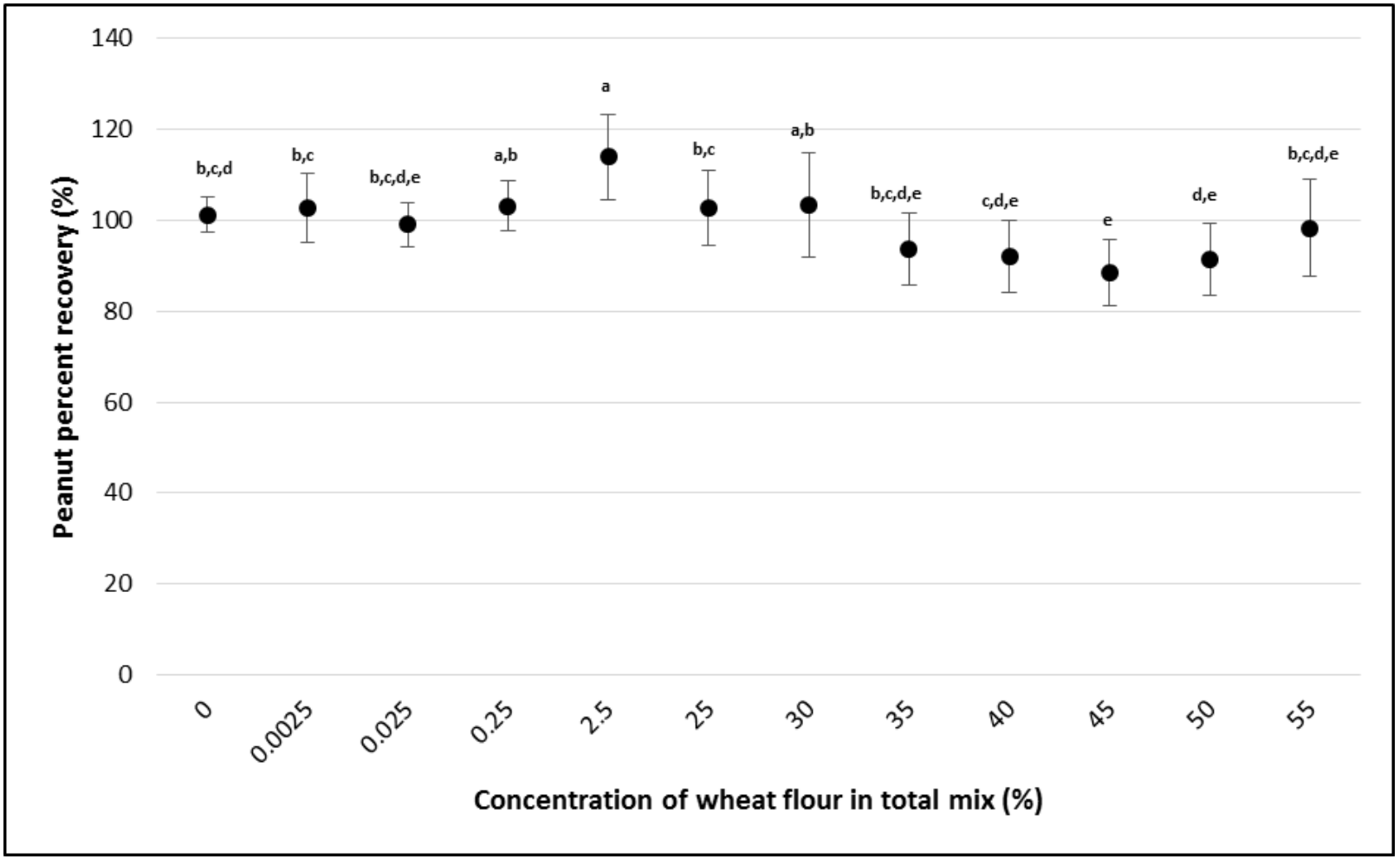

Figure 2-4. Percent recovery of peanut from samples mixes of powdered sugar and increasing concentrations of wheat flour. Statistical analysis was done using least square means analysis with Tukey's adjustment and determined significant if $p \leq 0.05$.

Alternative Flour Concentration Mixes Prepared with Milk and Peanut Allergens at Known Concentrations

The recovery of milk and peanut allergens were evaluated in mixes prepared with alternative non-glutinous flours including corn, rice, or soy flour incurred with milk or peanut allergens prepared in independent sample mixes. Levels for analysis ranged from 0 to 25% added flour increasing on a logarithmic scale to assess the recovery of target allergens. All concentrated spiking materials were validated as homogenous with a CV $\leq$ 15% prior to sample preparation using their respective kits.

The protein contents of the various flours ranged from 6.3 g/100 g (rice) to 34.1 g/100 g (soy) as shown in Table 2-4.

Table 2-4. Nitrogen conversion factors used for total protein determination by LECO Dumas method.

Flour	Protein (gram/100 gram sample)	Nitrogen conversion
Wheat	10.7 ± 0.1	5.70
Corn	9.3 ± 0.2	6.25
Rice	6.3 ± 0.20	5.95
Soy	34.2 ± 0.53	5.71

Milk

In mixes prepared with non-glutinous flours, recovery trends of milk differed among the three alternative flours (corn, rice, and soy), as seen in Figure 2-5. In both corn and rice flour, comparable recovery patterns of milk were observed. At flour concentrations less than 25%, recovery of milk was $\geq$85% for both corn and rice flour samples. However at 25% corn flour, a decline in milk recovery was apparent.

Soy flour samples exhibited a different trend in recovery of milk allergens. At 0.25% flour or less, recovery of milk from soy flour samples was $\geq$ 85%. Interestingly, at 25,000 and 250,000 ppm soy flour, recovery of milk was considerably reduced. As the soy flour concentration increased, the percentage recovery of NFDM decreased. Milk recoveries at the two highest concentrations of soy flour were determined to be significantly different from each other, and from the lower concentration samples of soy flour.

Soy flour contained the most protein (34.2% protein) among the three flours analyzed and displayed the most dramatic changes in the recovery of milk proteins. The protein content of corn flour was 9.3% and at 25% ppm corn flour samples, reduced recoveries were also observed. Lastly, rice flour contained the least amount of protein (6.3%), and did not exhibit a decrease in recovery even at the highest concentration of flour (25% ppm rice flour). No dough matrix was formed, therefore the interactions occurring are resultant of direct interactions between the selected flour and NFDM. It is anticipated further reductions in recovery would be observed if the study were expanded to include additional higher flour concentrations.

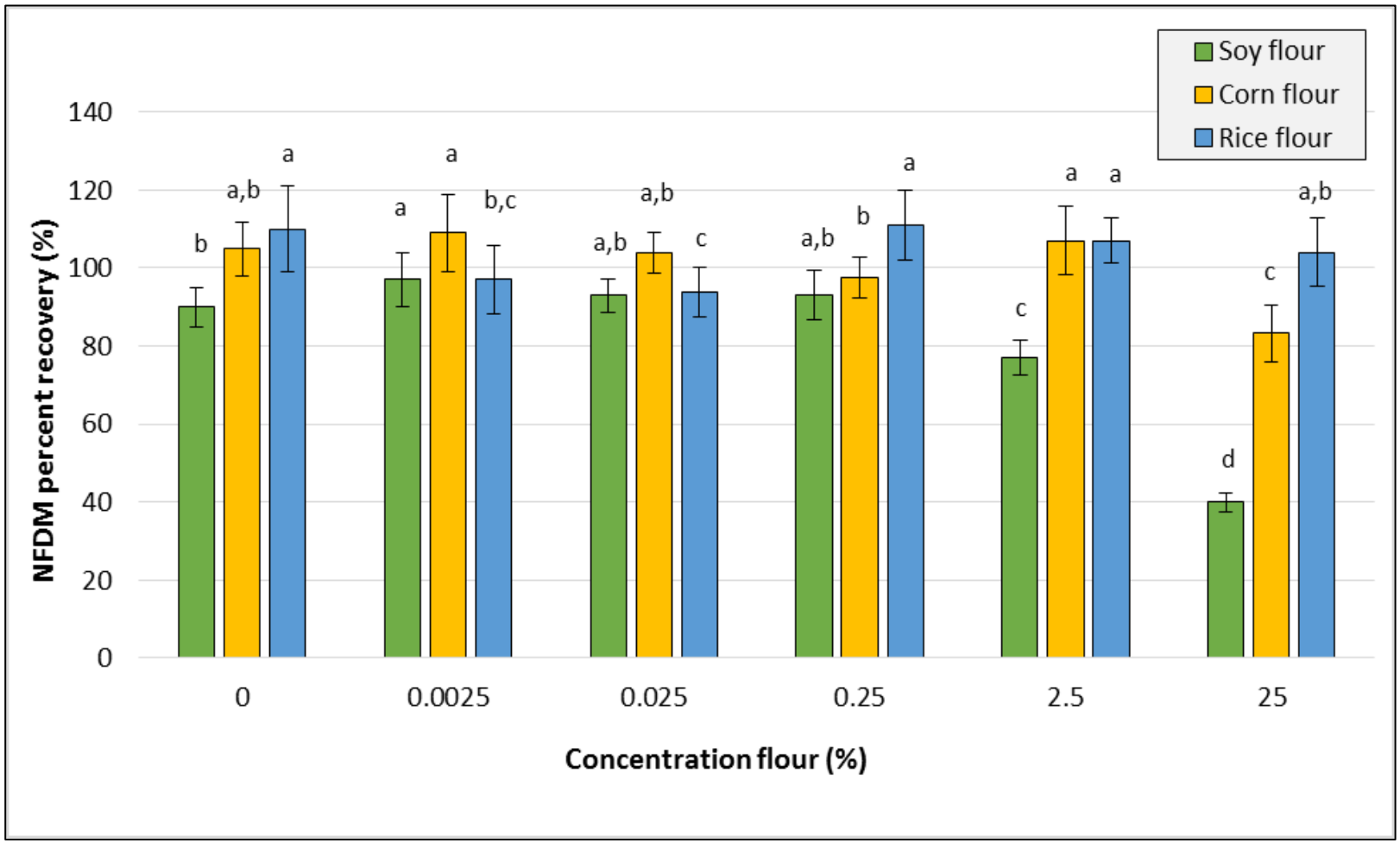

Figure 2-5. Percent recovery of NFDM from sample mixes prepared with powdered sugar and increasing concentrations of non-glutinous flours (soy, corn, or rice flour) Statistical comparisons were made among samples prepared with the same flour at the differing concentrations using an unstructured least square means design with Tukey's adjustment and concluded significantly different if $p \leq 0.05$. No within level comparisons were made between different types.

Peanut

In contrast to milk, the recovery of peanut proteins was consistent at all levels analyzed for all mixes with the exception of the highest added concentration of rice flour, where a slight decrease in recovery was observed; although the highest value is not statistically different from the lowest value (Figure 2-6). Although, the highest value is not statistically different from the lowest value. The lowest recovery of peanut protein was observed at the highest concentration of rice flour (25%), the flour with the lowest protein content. Additionally, samples prepared with soy flour and peanut contained the highest protein contents indicating the Neogen Veratox® Peanut ELISA kits are not dramatically affected by the overall protein content.

Alternative Flour Pastry Matrices Prepared with Milk

Alternative pastry matrices were prepared with corn and rice flours to assess potential mechanisms of reduced detection in dough matrices. For both wheat and corn flour pastry matrices the recovery of NFDM was greater at low flour concentrations and generally declined as higher levels of flour were prepared (Figure 2-7). Alternatively, the recovery of milk from pastry matrices prepared with rice flour were drastically lower in comparison to wheat and corn flour matrices. Secondly, no general trend could be determined based upon flour concentration and recovery. The formation of a dough matrix in rice flour pastry matrices was more affected than the other prepared pastry matrices.

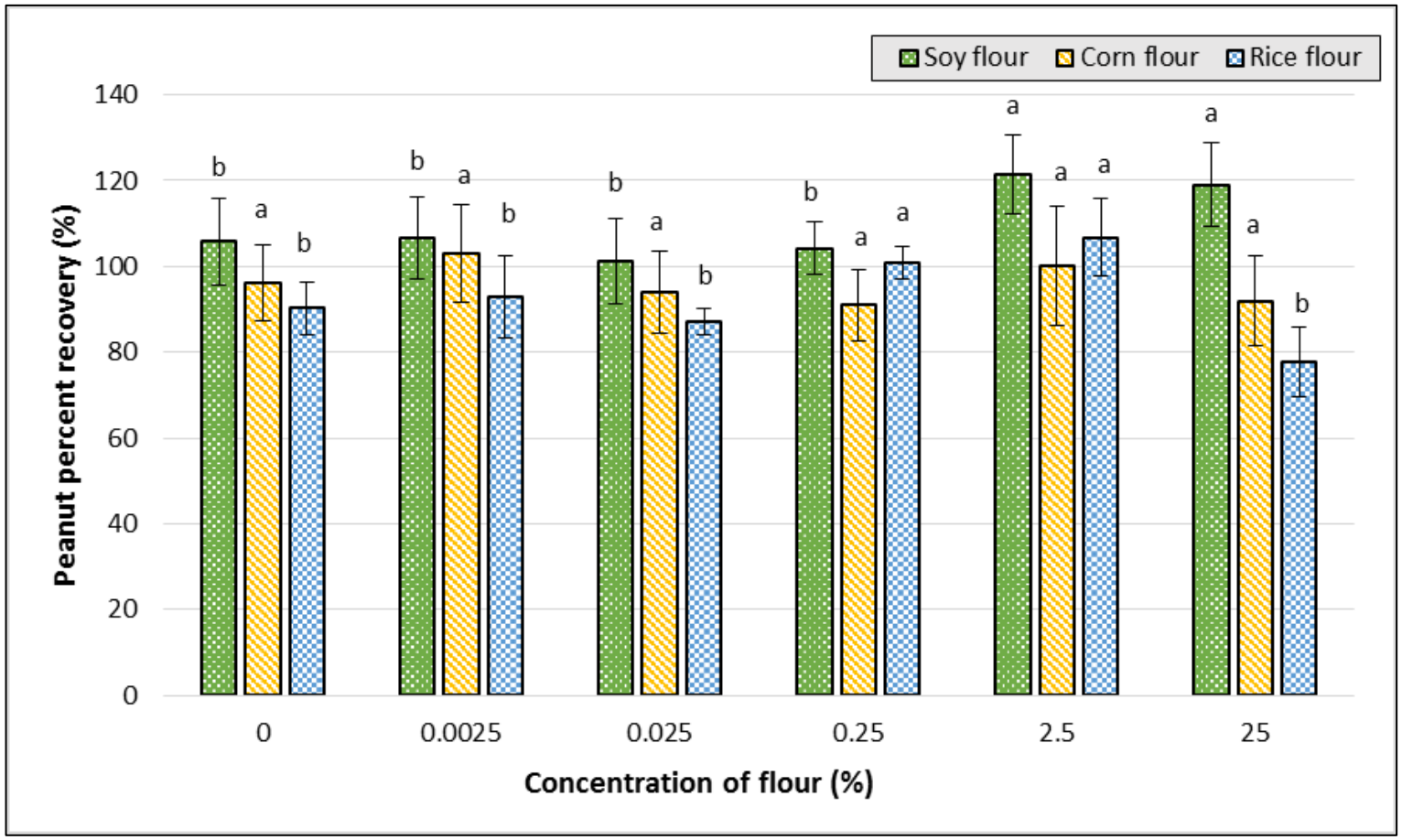

Figure 2-6. Percent recovery of peanut (ppm total peanut) from sample mixes prepared with powdered sugar and increasing concentrations of non-glutinous flours (soy, corn, or rice flour). Statistical comparisons were made among samples prepared with the same flour at the differing concentrations using an unstructured least square means design with Tukey's adjustment and concluded significantly different if $p \leq 0.05$. No within level comparisons were made between different types.

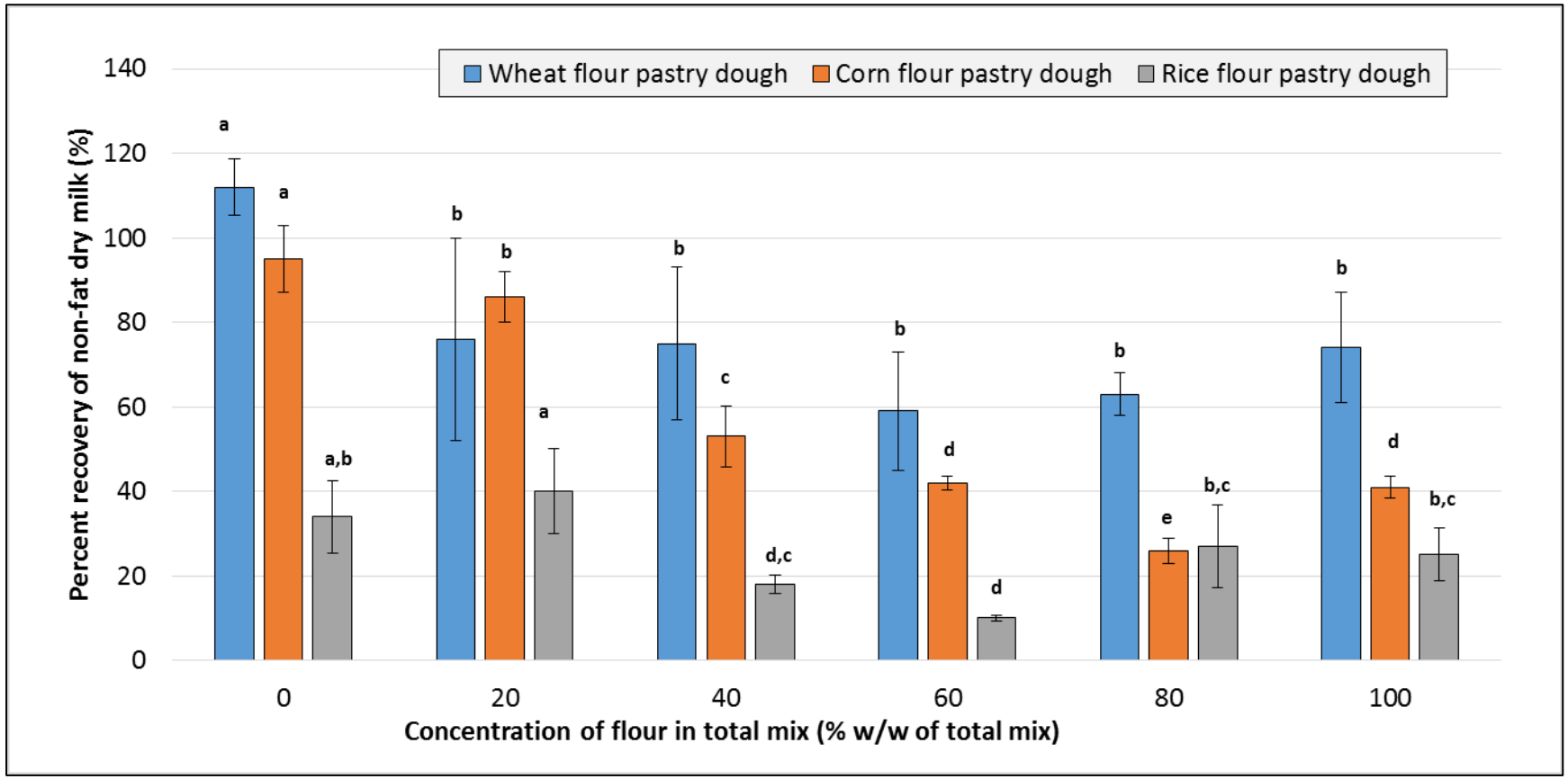

Figure 2-7. Recovery of NFDM from wet pastry dough matrices prepared with alternative non-glutinous flours (corn and rice flours) at different concentrations of flour. Wheat flour wet pastry dough matrix recovery values were obtained from the previous experiment (Figure 2-2). Statistical differences were determined between pastry matrices prepared with the same flour type. No comparisons were made between samples of different flours.

DISCUSSION

Evaluation of Ingredients for use in Model Foods

The use of powdered sugar was validated as a substitute material based on its solubility and lack of interference with other matrix components. Sucrose is readily soluble in water and other polar solvents which are capable of hydrogen bonding (Bubník and Kadlec, 1995). The sucrose molecule has 14 hydrogen atoms and eight hydroxyl groups resulting in high water solubility and sucrose hydration (Bubník and Kadlec, 1995). The excess of water (125 mL) in relation to powdered sugar (≤100 g) in the model food system indicates the sucrose molecules will be readily hydrated and solubilized. At 60°C, the solubility of sucrose in water is equal to 2.88 g sucrose/1 mL water (Bubník and Kadlec, 1995). Therefore in 125 mL of extraction buffer ~360 g of sucrose can be sufficiently solubilized. Although other components (wheat starch, NFDM) are present in this model food system, sucrose appears to be readily solubilized due to an excess of extraction buffer.

Equally important is the role of wheat starch in food functionality. Wheat starch can influence cooking properties, product texture, water holding capacity, and pasting properties (Maningat and Seib, 2010). Wheat starch is obtained by a gluten washing process (Slotter and Langford, 1944). Differences in processing methods and wheat variety will influence the final protein content of wheat starch (gluten or non-gluten containing proteins) (Day et al., 2006). Wheat starch is a semi-soluble granular ingredient capable of absorbing 39 – 87% of water by weight upon heating resulting in swollen and hydrated starch granules (Rasper and DeMan, 1980). Based upon the obtained recoveries, we can effectively conclude that milk recovery is not inhibited by

the swelling and hydration process occurring in wheat starch during extraction. Secondly, any interactions between powdered sugar and wheat starch do not result in decreased recoveries of NFDM.

Wheat Flour Pastry Dough Model Foods

Wheat starch was selected as the substitute for wheat flour since numerous gluten-free baking mixes and products are formulated using a combination of starches. In order to obtain gluten free status, the Food and Drug Administration (FDA) requires <20 ppm gluten present in a given food (Food and Drug Administration, 2013). Based on these guidelines, the wheat starch used in these experiments was not gluten free. Most available native wheat starches contain ≤200 ppm gluten, with a majority <150 ppm (Collin et al., 2004). Furthermore, individuals with celiac disease or gluten-sensitivities are able to safely consume modified and native wheat starches without experiencing abnormal immune responses (Peräaho et al., 2003). The wheat starch was determined acceptable for use in these experiments since the amount of gluten formed in pastry matrices after mixing would be minimal. Additionally, interactions between wheat starch and NFDM were determined negligible as demonstrated in the previous experiment of wheat starch and powdered sugar ratios.

Downs and Taylor (2010) observed a similar decrease in recovery of NFDM in pastry doughs incurred at different concentrations of NFDM. They reported an average 80% recovery of NFDM was obtained from unprocessed wet pastry dough matrices incurred at 250 ppm NFDM analyzed by Neogen Veratox® Total Milk ELISA kits (Downs and Taylor, 2010). Even lower recoveries of NFDM were obtained by Bly (2014) with a 27% recovery of NFDM observed in unprocessed pastry dough analyzed

using commercial ELISA kits. Contrastingly, Monaci et al. (2011) reported no significant reductions in the recovery of milk proteins in cookie dough matrices when analyzed by the same total milk ELISA kit, Neogen Veratox® Total Milk. This observation could be due to differences in mixing time, dough preparation, and dough resting time, or the extent of network formation prior to analysis (Monaci et al., 2011). In all three studies, the model foods were later subjected to processing which ultimately resulted in even lower detection and quantification of milk proteins by commercial ELISA kits (Monaci et al., 2011). As illustrated by Figure 2-2, the dough type (wet vs. dry) had the greatest impact on recovery of milk allergens, suggesting that the formation of glutinous complexes results in decreased recoveries of milk proteins.

Bugyi et al. (2010) measured the recovery of milk allergens from a model cookie matrix at various stages of sample preparation. Recoveries were determined at each of the following stages: the dry mix, dough matrix, and baked cookie matrix and were analyzed using the BioKits Casein assay, an indirect competitive ELISA (Bugyi et al., 2010). They concluded that no differences in the recovery of milk proteins were present between the dry powdered mix stage and dough stage although, after baking, a significant difference in the recovery of NFDM was apparent (Bugyi et al., 2010). In comparing the two studies, our observations differ from those reported by Bugyi et al. 2010. One key difference between these studies is the ELISA format and analytical target of each assay. In our study, a total milk sandwich ELISA was used which selects for total milk proteins. In the study by Bugyi et al. 2010, an indirect ELISA detecting casein was selected. Differences in the analytical targets and assay type (sandwich vs. competitive) may be contributing factors to the contrasting observations. Another study reported differences

in the detection of milk residues from unprocessed sugar cookie doughs when using multiple ELISA kits with different analytical targets (Khuda et al., 2012). In kits designed to target caseins, recovery was approximately 100%, whereas recovery of beta-lactoglobulin (BLG) was highly variable and either underestimated or overestimated in sugar cookie dough (Khuda et al., 2012). Variations between preparation methods of model foods can also be a contributing factor in the detection of incurred allergens. Although these food systems differ in their preparation methods, similarities can be drawn among those which are similar. The unprocessed wet pastry dough matrices were adapted from the pastry dough matrices prepared by Downs and Taylor, (2010) and Bly (2014) allowing for a more direct comparison between these studies. However, differences exist among these results in the recovery of milk proteins. Differences may be attributed to the extent of dough mixing, working, (dough rolling), or dough resting. Additionally, differences could exist due to variation between kit production lots.

The ingredient composition may also affect the overall recovery of milk. In wheat starch, there are over 100 surface associated proteins and other internal proteins with enzymatic functions (Maningat and Seib, 2010). Based on the previously presented results, interactions occurring between wheat starch associated proteins and NFDM are negligible, further supporting interactions between milk proteins and other matrix ingredients are causing the reduced detection.

Previous studies have suggested reduced recoveries resulted from decreased protein solubility caused by protein–protein or protein–non-protein interactions, where protein solubility decreased based upon the processing operation (Bly, 2014; Downs and Taylor, 2010; Faeste et al., 2007). The effects of thermal processing are well established

and known to decrease protein solubility and reduce overall detection. Our experiments indicate interactions may be occurring prior to processing and further processing steps promote these interactions generating even more reduced recoveries.

In another model food system prepared with gluten, (pasta composed of wheat flour and egg white), measurable decreases in the antigenic activity of egg white ovomucoid were observed during each preparation step (mixing, kneading, resting, cutting, boiling) (Kato et al., 2001). After dough resting, the measured concentrations of OM were greatly reduced suggesting the physicochemical or structural changes occurring in the dough are responsible for the insolubilization of OM (Kato et al., 2001). The authors suggest that polymerization occurs during the dough preparation phases resulting in reduced recovery of OM; subsequent processing (e.g. baking) phases then further insolubilize proteins (Kato et al., 2001). Wheat flour consists primarily of starch (60-75%) and gluten proteins (10-15%) after the removal of the bran and germ layers by milling (Shewry and Halford, 2002; Shewry, 2009). Gluten proteins will interact and form a visco-elastic dough due to the addition of water; the gluten network is stabilized by disulfide and hydrogen bonds (Shewry and Halford, 2002). During dough mixing, these disulfide bonds will be repeatedly broken and reformed promoting interactions with the food matrix and allergenic proteins in the matrix (Shewry and Halford, 2002). Our findings are in agreement with these studies and further support the observation of decreased detection in wheat flour dough food systems.

In addition to reduced ELISA detection of milk protein allergens present in complex matrices, these same matrices play a significant role in oral food challenges (OFC). In oral food challenges (OFC) performed in egg-sensitive children, tolerance to

baked egg was assessed in wheat flour and non-wheat flour (rice flour) muffins (Lanser et al., 2015). Results of the OFC revealed 30% of children failed OFCs to wheat flour muffins, and 60% of children failed an OFC when administered the rice flour muffin (Lanser et al., 2015). These results suggest interactions between egg proteins and baked glutinous food matrices provide a protective effect from the allergen in sensitized individuals (Lanser et al., 2015). These same types of interactions are less prevalent in muffins prepared with rice flour, therefore less of a protective effect exists in these samples. Other studies focusing on OFC's revealed similar results. Children challenged to baked or extensively heated milk in a glutinous food matrix demonstrated acquired tolerance to milk (Leonard et al., 2015; Nowak-Wegrzyn et al., 2008)

Equally important is the selection of the target analyte detected by the ELISA kit. In thermally processed samples, Khuda et al. (2012) observed significant decreases in recovery of BLG whereas casein recovery was stable, suggesting casein would be a better ELISA target in thermally processed samples. The commercial ELISA kit selected for analysis is an additional factor to consider when assessing recovery.

Analysis of a liquid model food matrix indicated no significant decreases in recovery of NFDM using a variety of commercial ELISA kits (Bly, 2014). These results further support that the formation of a glutinous matrix and/or physicochemical interactions occurring within a matrix affect overall recovery of NFDM.

Wheat Flour Concentration Mixes with Known Concentrations of Milk or Peanut Allergens

Milk

As the concentration of wheat flour increased, the amount of milk allergens recovered correspondingly decreased. An interesting comparison can be made between samples prepared at the highest flour concentration (55%) and the dry pastry dough mix samples. The recovery of NFDM from samples prepared at 55% wheat flour was 66 ± 15%. Interestingly, in the dry pastry dough mix (page 89) prepared with 100% wheat flour, the recovery was 100 ± 14.2%. The concentrations of flour in these two samples were comparable: 55% for the wheat flour mixture and 57.1% wheat flour for the dry pastry dough mix sample, respectively. The addition of salt (1.5%) in dry pastry dough mixes may have contributed to the decreased recovery in the sample concentration mixtures. In the absence of salt, recovery of NFDM was decreased by 30% in nearly analogous sample mixes. The sample concentration mixes (550,000 ppm wheat flour) consisted of wheat flour, powdered sugar, and NFDM whereas the dry pastry dough mixes were composed of wheat flour, salt, and NFDM.

In the process of dough formation, the presence of salt has been shown to delay the development of gluten networks in wheat doughs. Wheat doughs prepared without salt (wheat flour and water) will form glutinous networks more rapidly than those prepared with salt (McCann and Day, 2013). After two minutes of mixing, doughs prepared without salt began to form gluten networks and network formation was nearly complete after six minutes of mixing (McCann and Day, 2013). Contrastingly, doughs prepared with 2% salt did not begin to form a gluten network until approximately six minutes of mixing time (McCann and Day, 2013). The formation of gluten is driven by

interactions between glutenin proteins upon the addition of water and dough working procedures. The hydration of gluten proteins drives protein interactions and network formation (Shewry et al., 2002). These results indicate that the presence of salt reduces interactions occurring before hydration between wheat flour and milk proteins without the addition of water, demonstrating the disruptive effect of salt on protein–protein or other protein–non-protein interactions. At low concentrations of salt, protein-protein interactions are stabilized, however at concentrations >1 M, salt will disrupt protein-protein interactions (McCann and Day, 2013).

In all model matrices and mixes analyzed with wheat flour and milk, reduced recoveries were apparent. Interactions between casein and gluten proteins may be occurring, especially in matrices where dough working procedures took place. Caseins, the principle protein component from milk, are a unique complex of proteins as well. They exist in micelle structures, allowing them to be soluble in aqueous solutions. Caseins also lack defined secondary and tertiary structures but are rather classified as rheomorphic proteins (Holt and Sawyer, 1993). They also contain high contents of proline, similar to gluten proteins.

The interaction of these two complex protein systems may result in decreased detection due to binding events that occur causing modifications to the epitopes targeted by ELISA methods. However further investigation into this protein system should be done to characterize the interaction(s).

Peanut

The recovery of peanut from wheat flour concentration mixes was less affected by the concentration of flour in each sample. Interactions resulting in reduced detection are

minimized or not present in these sample mixes. Secondly, it can be stated the kit targets are less affected by matrix components, indicating sufficient kit performance for peanut residues by the Neogen Veratox® Peanut ELISA kit.

Comparison of Allergen Recovery of Wheat Flour Concentration Mixes

Comparison of the two concentration experiments with wheat flour and milk or peanut as the target allergens indicate recovery of peanut was less affected by the concentration of wheat flour than milk, especially at the higher levels of wheat flour analyzed.

The addition of salt into sample mixes, which were not formed into doughs, enhances the recovery of milk allergens (dry pastry dough mixes). However without salt, the overall recovery of milk was reduced as the concentration of flour increased as demonstrated by Figures 2-3 and 2-4.

Alternative Flour Mixes Prepared with Milk and Peanut Allergens at Known Concentrations

Milk

Reduced recoveries of milk were determined in soy flour mixes at 2.5 and 25% added soy flour and in corn flour mixes prepared at 25% added corn flour. Due to the high concentration of protein in the sample mixes prepared, the Neogen Veratox® Total Milk ELISA kit may be less efficient in detection of allergenic residues at high concentrations of protein. According to Ivens et al., (2016), the Neogen Veratox® Total Milk detects proteins from the κ-casein fraction and does not detect whey proteins from milk.

Several reasons could account for the reduced recovery of milk proteins as detected by ELISA at high protein concentrations. Possibly, the target allergenic epitopes are masked due to the high protein concentration in each sample, since no dough working occurred. Alternatively, protein aggregation induced during the extraction procedure may lead to reduced recoveries at high concentrations of proteins, especially for milk targets.

Epitope masking may potentially be involved in the reduced detection of milk residues, however our studies were not designed to conclusively determine this phenomenon. Grimshaw et al. (2003) reported a high fat food matrix concealed allergenic epitopes and upon entering the digestive system, the epitopes elicited severe allergic reactions with minimal warning symptoms. In our sample mixes, soy flour is a high fat flour in comparison to wheat, corn, and rice flours and the fat may play a role in the reduced recovery of milk allergens.

Peanut

Oppositely, lower percent recoveries of peanut from rice flour mixes secondarily suggests the overall protein content does not inhibit the efficacy of the ELISA kits used in this study. Potential aggregation mechanisms occurring between peanut flour and rice flour may be the cause of the observed lower recoveries

As with the wheat flour concentration samples, it is important to distinguish that no dough working occurred in these samples. Samples were prepared as dry mixes and hydrated only during protein extraction procedures. The effect of dough working may influence the detection of allergenic proteins due to physical and chemical changes occurring within a food matrix. In wheat doughs, dough formation is driven by hydration and polymerization of gluten proteins and the entrapment of starches and other components

(McCann and Day, 2013). The interactions occurring in the mixing phases may be a contributing factor to the observed reductions in detection as well as the concentration of flour in relation to concentration of allergenic proteins. The amount of kneading in doughs also contributes to the decreased detection. Doughs that are kneaded for longer than 20 minutes showed more reduced detection of egg than doughs kneaded for less amounts of time (Kato et al., 2001). In the experiments described here, the effects were dependent on the nature of the proteins. Greater effects occurred in milk by comparison to peanut and with wheat flour compared to other flour sources.

Protein extraction is critical when analyzing foods for allergen content. Based on the previously described experiments, protein-protein interactions may play a role in the reduced detection of allergens, however this is dependent upon the sample composition, target analyte, and kit selected for analysis. In sample mixes prepared with milk, good recoveries were obtained until high quantities of protein were added into a food system, whereby percentage recovery values decreased accordingly. When samples were prepared with peanut and assessed for recovery, the overall sample protein content did not affect the recovery of peanut as observed previously in samples incurred with NFDM.

Alternative Flour Pastry Matrices Prepared with Milk

A series of pastry matrices were prepared with corn and rice flour to evaluate recovery of milk residues from non-glutinous pastry matrices. Unlike wheat flour, corn and rice flour are incapable of forming a glutinous matrix due to the lack of gliadin and glutenin proteins derived from wheat. Since gluten is the predominant protein in wheat, it is anticipated interactions occurring between milk and gluten proteins result in decreased detection by ELISA. Corn flour is rich in prolamin proteins, specifically zein

proteins (α, β, γ, δ-zein) (Chanvrier et al., 2005). These proteins have been described as extremely hydrophobic. Starch is the primary component in corn flour accounting for approximately 70-80% of the total product (Shukla and Cheryan, 2001). Rice flour contains multiple seed storage proteins including albumins, prolamins, globulins, and glutelins (Juliano and Hicks, 1994). Rice flour also has a large proportion of carbohydrates, therefore potential interactions between milk residues may be causing the observed reductions in recovery (Sivaramakrishnan et al., 2004).

Proteolytic enzymes may be present and active in the corn and rice flours used in the prepared pastry matrices potentially affecting assay performance. During the milling of all purpose and refined flours, this outer layer is removed (Ito et al., 2009). However in whole grain flours, the outer layer is not removed and is instead milled (Ito et al., 2009). Proteases present in the outer layer remain in the whole grain flour, and may negatively affect the capture and detection antibodies of an immunoassay.

However, based upon the results in Figure 2-7, other interactions occurring between allergenic residues and other non-glutinous proteins or matrix components may be influencing reduced detection of milk proteins given that the recovery of milk from rice flour pastry matrices were the lowest in comparison to wheat and corn flour pastry matrices. Reduced recoveries could be attributed to more complex interactions occurring in corn flour between starches, proteins, and the fatty phase. In rice flour, more complex interactions between the carbohydrates, proteins, and fatty phase may be the cause of the reduced recovery. The addition of the fatty phase, water, and dough working procedures are all contributing factors to the decreased detections in pastry matrices prepared with corn and rice flour. This effect is more apparent in the rice flour pastry matrices than in

wheat or corn flour prepared samples in the recovery of milk allergens. As noted previously, fat may play a role in epitope masking or protein extraction.

Upon comparison of all sample matrices and mixes analyzed in this study, apparent differences occur in kit performance and target allergen recovery due to interactions occurring within a food system. The importance of assay design in commercial ELISA kits plays a vital role in the assessment of allergenic protein recoveries. Differences in antibody type, specificity, and/or calibration materials can influence the results of an ELISA (Diaz-Amigo, 2010). The selection of adequate antibody targets is important for the detection of proteins (Immer and Lacorn, 2015). Antibodies can be monoclonal or polyclonal and directed against linear or conformational epitopes (van Hengel, 2007). Conformational epitopes are more susceptible to changes caused by processing as opposed to linear epitopes which are stable towards processing operations (van Hengel, 2007). Certain kits (e.g. Neogen Gliadin R5 ELISA kit) have multiple extraction procedures tailored to the type of food matrix containing the allergen.

Proteins may be rendered insoluble due to extensive processing (heating, baking, frying), however the formation of aggregates and complexes due to dough mixing and matrix interactions may result in insoluble aggregates (Poms et al., 2006). Any modification to an epitope may result in decreased binding efficiency of the antigen-antibody complex and report false negative results.

The total milk kits are designed to detect both casein and whey fractions and employ polyclonal antibodies. Kit manufacturers generally do not disclose the specific details regarding antibody targets, therefore variability can exist between kits produced by different manufacturers. Understanding the differences and limitations of ELISA kits

will greatly affect the overall allergenic assessment of a food. The ELISA kit used in this study (Neogen Veratox® Total Milk ELISA) principally detects κ-casein (Ivens et al., 2016), a protein found in both the casein and whey fractions. Other milk ELISA kits have different target milk proteins and thus matrix effect on the detection and milk protein recovery may be different (Ivens et al., 2016).

The peanut kits used in this study were targeted against total peanut, indicating multiple peanut proteins are being targeted. According to Jayasena et al. (2015), the Neogen Veratox® Peanut ELISA kit primarily detects Ara h 3 followed by Ara h 1. Ara h 1 and Ara h 3 are the two most abundant peanut proteins, and both are classified as globulins (Becker and Jappe, 2014).

Immunoassays will only detect extracted proteins which remain soluble in the extraction buffer solution. Inefficient protein extraction will impact the overall results of immunoassays. In commercial ELISA kits, an extraction additive is added during extraction procedures to promote protein extraction and maintain protein solubility. Extraction additives are often other protein sources (e.g. fish gelatin, NFDM) and function to bind compounds such as tannins or polyphenols which may interact with proteins and decrease the extraction and detection of target allergenic proteins (Keck-Gassenmeier et al., 1999). In this study, the target milk protein (κ-casein principally) is either trapped in the matrix or aggregated into insoluble complexes. The extraction additives used in the Neogen Veratox® Total Milk ELISA failed to resolve extraction issues. However, this effect was not so pronounced in the recovery of peanut proteins from similar matrices. The observed reduction in recoveries may partly be caused by

protein insolubility. Additives should always be validated by kit manufacturers to ensure no cross reactivity or inhibiting effects are present.

As a function of overall protein content, the peanut kits are less affected by food matrices of high protein content. Interestingly, in high protein samples prepared with milk, recovery begins to decline as a food becomes more protein rich. This suggests total protein content may affect the efficacy of ELISA kits used in this study.

CONCLUSIONS

Numerous factors play a role in the recovery and detection of allergenic residues from various food matrices by ELISA analysis. The overall recovery of milk from wheat flour pastry matrices was reduced, however in non-glutinous matrices made with identical components, the recovery of NFDM was not inhibited. Our results suggest that the formation of a glutinous complex and concurrent interactions between milk and matrix components impacts the overall detection of milk by commercial ELISA kits. Furthermore, analysis of sample mixes prepared with increasing flour concentrations revealed a decrease in the recovery and detection of milk residues as the concentration of wheat flour increased. Additional mixes were prepared using non-glutinous flours (corn, rice, soy) which revealed decreased recoveries of milk in correlation with the protein content of the flour. Soy flour had the highest protein content and the lowest recovery of milk, whereas rice flour contained the lowest protein and exhibited the highest recovery of milk. In contrast, pastry dough matrices incurred with milk and prepared using corn or rice flour indicated lower recoveries of milk in comparison to wheat flour pastry dough matrices. The formation of a glutinous complex is only one contributing factor to a

seemingly complicated problem. The dough working operations and addition of fat and water to non-glutinous flours resulted in decreased recovery and detection.

The analytical targets detected by the commercial ELISA kits also influence the recovery of milk proteins. The Neogen Veratox® Total Milk ELISA kit primarily detects the κ-casein protein from milk, however the use of a different kit targeting whey proteins (BLG, ALA) as opposed to casein(s) may lead to different observations (Ivens et al., 2016). Sample matrices with reduced recoveries of milk (wheat flour pastry matrices, alternative flour pastry matrices, wheat flour concentration mixes) indicate an interaction between κ-casein and the sample matrix is occurring and impacting the overall performance of the selected immunoassay.

For example, excellent recoveries of milk from unprocessed dough matrices when using a commercial ELISA kit targeting caseins have been previously reported (Bugyi et al., 2010; Khuda et al., 2012). Contrastingly, kits designed to detect whey proteins over- or under- estimate milk protein in dough matrices. The analysis of unprocessed pastry matrices reported reduced recoveries when total milk kits were selected as the analytical method (Bly, 2014; Downs and Taylor, 2010; Khuda et al., 2012). To further assess kit performance, studies to analyze dough matrices with ELISA kits designed to detect casein or whey proteins should be done to aid in the determination of matrix interactions and kit targets.

Commercial ELISA kits are designed to detect specific epitopes and different kits may target different epitopes or combinations of epitopes by employing polyclonal antibodies. The recovery of milk proteins was more affected than the recovery of peanut proteins in matrices containing various concentrations of wheat and alternative flours.

The detection of allergens becomes impaired in different matrices and mixes due to poor extraction or detection of proteins or epitopes targeted by the commercial kits. Secondly, the overall detection of milk declined as the overall protein content increased in sample mixes. To better understand this observation, additional studies should be performed to assess the overall effect of protein concentration in a given model food system.

The necessity of improved detection methods, especially for milk cannot be understated since processing has been shown to further decrease the recovery of milk allergens by altering the structure of conformational epitopes (Downs and Taylor, 2010; Nowak-Wegrzyn and Fiocchi, 2009).

In contrast, the recovery of peanut from independently prepared sample mixes with increasing concentrations of wheat, corn, rice, or soy flour, indicated no reductions in overall recovery. The overall recovery of peanut was less affected by protein concentration and the various sample mixes prepared. This suggests limited matrix interactions occur between peanut and matrix components. Secondly, the ELISA kit (Neogen Veratox® Peanut) used for analysis is proficient at detecting peanut residues in less complex matrices.

The same principle can be applied to other allergenic targets and commercial ELISA kits for detection of allergenic proteins. Interactions between matrix components and the principle allergenic protein target are negligible in peanut (Ara h 3), however as demonstrated by the results presented here, detection was reduced using a total milk assay. Further studies using different commercial ELISA kits for specific milk proteins to detect allergenic residues would likely report different results based upon the target analyte.

In addition, evaluation of other kits (e.g. soy, almond, egg) for their primary ELISA targets and subsequent interactions with matrix components may lead to an improved understanding of protein interactions and performance characteristics of ELISAs in complex matrices. Evaluation of kits would provide information regarding the primary target of an assay, which can in turn can be applied when assessing complex matrices for recovery of incurred allergenic proteins.

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CHAPTER 3:
EVALUATION OF SEQUENTIAL EXTRACTIONS ON PROTEIN QUANTIFICATION USING MODEL FOOD MATRICES

ABSTRACT

Incomplete recovery of allergenic proteins has been documented in both processed and unprocessed food matrices resulting in reduced detection using commercial immunoassays (ELISA). Extraction of proteins for immunoassays is critically important in the overall detection of food allergens since detection relies on efficient extraction procedures. The purpose of this study was to evaluate a modified extraction procedure, sequential extractions, for recovery of allergenic proteins. After the initial extraction, the supernatant was removed and stored, followed by resuspension of the precipitant with extraction buffer and then repeating the extraction process a second and third time. Glutinous (wheat flour) and non-glutinous (corn or rice flour) pastry matrices (flour, salt, shortening, water) incurred with non-fat dry milk (NFDM) or peanut flour were prepared for analysis. Dough (unprocessed) and baked (processed) matrix samples were analyzed using the sequential extraction methodology. All extracts were analyzed for total soluble protein content (GE Healthcare 2-D Quant kit) and allergenic protein (Neogen Veratox® Total Milk or Neogen Veratox® Peanut ELISA kits). Using the sequential extraction method, additional soluble protein was able to be detected for all matrices (dough and baked). ELISA detectable protein was quantified in all dough pastry matrices (NFDM and peanut) for each extract, however no detectable ELISA protein was detected in baked pastry matrices. In all assays, the first extract contained the highest concentration of total soluble protein (2-D Quant) and ELISA detectable protein. In the

second and third extractions, protein was still recovered and quantified although in lesser quantities. The use of sequential extractions improved the overall soluble protein in all matrices and allergenic protein obtained from raw dough matrices.

INTRODUCTION

Immunoassays are the most commonly used analytical technique for the detection of food allergens (Monaci and Visconti, 2010; Torok et al., 2015). The overall performance of an immunoassay is influenced by several factors including matrix composition, extent of processing, antibody targets, and extraction procedures. Several studies have demonstrated that the composition of a food matrix plays a significant role in overall kit performance and allergen detection (Bly, 2014; Downs and Taylor, 2010; Parker et al., 2015). The addition of processing only reduces the ability to detect allergens in these complex systems due to epitope modifications or protein denaturation, which further reduces the reliability of immunoassays. Furthermore, detection antibodies are designed to target one or multiple allergenic proteins, resulting in inconsistencies in the reported values when comparing commercial ELISA kits.

Extraction procedures are a key factor to consider when developing or performing immunoassays or any other analytical methods that require an extraction step. If allergenic proteins are unable to be extracted, they will not be detected by the immunoassay. Many times these undetected allergens are present at low concentrations and therefore extraction methods must be suitable to detect target proteins at low concentrations (van Hengel, 2007). Secondly, only those proteins which are soluble will be extracted as opposed to the insoluble proteins. Insoluble proteins are still capable of causing an allergic reaction in vivo, however may go undetected when using

immunoassay methods (Taylor et al., 2009). Extraction buffers should efficiently solubilize and extract target allergens necessitating the validation of protein extraction procedures (Abbott et al., 2010). Other factors influencing protein extraction include buffer composition, pH, and extraction time and temperature. These are all variables that must be taken into consideration during the development of an analytical method.

The necessity of improved extraction procedures becomes apparent due to reduced detection of milk allergens in unprocessed pastry matrices. The focus of this study was to evaluate a modified extraction procedure, sequential extractions, to determine if improvements in overall protein quantification and ELISA detectable protein could be made when analyzing incurred complex food matrices using two different target allergens (NFDM or peanut).

MATERIALS AND METHODS

Preparation of Model Foods

A series of wet pastry dough matrices were prepared with wheat, corn, or rice flour using an adapted formulation from Downs and Taylor (2010), and as previously described in Chapter 2. Pastry matrices were prepared at a 100% flour concentration and incurred with either 250 ppm NFDM (Darigold low-heat non-fat dry milk powder, Caldwell, ID) or 250 ppm peanut flour (Light roasted peanut flour, 12% fat, 50% protein, Golden Peanut Company, Alpharetta, GA). Each pastry dough matrix was prepared with one allergenic target (NFDM or peanut). Control samples for each flour type were similarly prepared with no incurred allergen. Samples were stored at -15°C until needed for further analysis.

Preparation of Baked Model Foods

A 20 g subsample of dough was taken from the prepared wet pastry dough matrices (wheat, corn, or rice flour incurred with NFDM or peanut) for baking procedures. The pastry dough was rolled to an even thickness of ~3 mm using a rolling pin and cut into 2 cm x 2 cm squares. Squares were placed onto a lined baking tray and baked for 30 minutes at 190°C in a conventional oven (Groen Combi-oven, model CC10-E).

Preparation of Sample Extracts

Extracts were prepared using a modified extraction procedure based on the Neogen Veratox® ELISA kit(s) protocol. A series of sequential protein extractions for each sample was conducted. All buffers and reagents were provided by the kit manufacturer. No modifications were made to extraction buffers in order to maintain compatibility with the ELISA kits. In summary, a 1 gram sample of pastry dough was extracted in 25 mL of extraction buffer (0.01 M phosphate buffered saline, PBS) in a 60°C shaking water bath for 15 minutes. Samples were centrifuged (Beckman GS-15R centrifuge) for 10 minutes at 3800 x g at room temperature. After centrifugation, 20 mL of the supernatant was removed and stored as extract 1 (X-1) at 4°C. Upon partial removal of the supernatant, the remaining pellet was re-extracted by adding 20 mL of extraction buffer followed by vortexing to redistribute the pellet in buffer. Extract 2 (X-2) was obtained in the same manner as extract 1. The extraction procedure was repeated an additional time resulting in extract 3 (X-3). After obtaining the individual extracts (X-1, X-2, and X-3), a pool of the extracts was made by combining 4 mLs of each extract resulting in a total extract (X-T).

In summary, four extracts were obtained from each raw and baked dough sample for each flour type and allergen combination.

ELISA Analysis

Extracts from all samples and controls were analyzed using the Neogen Veratox® Total Milk or Neogen Veratox® Peanut ELISA kits. All assay components, including buffers and reagents, were included in the Veratox® ELISA kits. Triplicate extracts were independently prepared for all samples, and each extract was plated into duplicate wells. For example, raw wheat flour pastry dough matrix incurred with 250 ppm NFDM was extracted three times, replicates A, B, and C. These three extract replicates were further extracted using the sequential extraction procedure previously described. The assay procedures for ELISA analysis were performed as written by the kit manufacturers. If dilution was necessary in order to fit within the assays' standard curve, samples were diluted using the kit extraction buffer. A 100 µL portion of samples and standards were plated into antibody coated wells and allowed to incubate for 10 minutes at room temperature. After incubation, plates were washed with the appropriate assay washing buffer. The conjugate solution (100 µL) was added and plates were incubated (10 mins.) and then washed. The enzymatic substrate (100 µL) was added and allowed to develop a colorimetric product during the final incubation (10 mins.). After incubation with the substrate, the colorimetric reaction was terminated by adding the stop solution (100 µL) provided by the manufacturer. The absorbance values of samples and standards were determined using a plate reader (ELX808 Ultra Microplate Reader) set at 650 nm.

For each extract (i.e. X-1), the CV% must be less than 20% and the standard curve was required to have an r^2 value $\geq$ 0.98. If these criteria were not met, the assay

was performed again. ELISA results are presented as ppm milk protein or ppm peanut protein.

Total Soluble Protein Quantification

Total soluble protein quantification was performed on all extracts prepared for ELISA analysis using the GE Healthcare 2-D Quant kit (GE Healthcare Bio-Sciences, Pittsburgh, PA). Instructions for analysis were provided by the manufacturer and a brief summary is provided herein. The assay is based on the binding of copper to proteins that have been precipitated and re-suspended in solution (Chassaigne et al., 2007). A standard curve was prepared using bovine serum albumin (BSA) ranging from 0 – 50 µg protein and standard solutions were prepared at the following concentrations: 0, 10, 20, 30, 40, and 50 µg protein. Sample tubes and standards were prepared by pipetting an appropriate amount of sample extract or standard into individual tubes. In each tube, 1 – 50 µL of sample protein extract can be added. The precipitant (trichloroacetic acid, TCA) was added to each tube, vortexed, and incubated for 2 minutes followed by the addition of the co-precipitant (acetone) and additional vortexing. Tubes were centrifuged (ThermoScientific Sorvall Legend Micro 17) at 10,000 x g for 5 minutes. The supernatant was decanted and completely removed. The copper solution and de-ionized water were added to each tube and briefly vortexed. Lastly, the working color reagent was added and incubated at room temperature for 15 minutes resulting in the formation of a colorimetric product. The absorbance was measured using a plate reader (ELX808 Ultra Microplate Reader) at 490 nm for all samples and standards. The quantity of protein in each sample was calculated using a linear regression best fit calibration curve produced with each assay.

The standard curve was required to have an $r^2 \geq 0.98$. If the r^2 value was less than 0.98, the assay was performed again.

Normalized Results

In order to effectively compare the results of the ELISA and protein quantification assays, the reporting units of both analytical methods were converted into identical units. Ultimately, the final reporting units were mg/kg protein for both assays. The normalization calculations were done only on raw pastry matrices. Quantifiable results from ELISA and protein quantification assays were necessary to complete the normalization calculation. No ELISA values were obtained for the control matrix samples or from baked matrix samples, therefore they are not included here.

The total extracts are represented in two forms as shown in Figure 3-1, 3-2. The total extract (e.g. X-T) represents the observed value reported by each individual assay as determined by the plate reader. The summed total extract (e.g. X-T (sum)) represents the summation of extracts -1, -2, and -3. This sum represents the theoretical or expected value to be determined by the ELISA.

The Neogen Veratox® Total Milk ELISA kit reports results as ppm NFDM. The total protein content of the low heat non-fat dry milk used in these experiments was 37.5% as determined by Dumas nitrogen analysis using a LECO FP-528 Protein/Nitrogen Determinator (LECO Corporation, St. Louis, MO). Therefore, the reported ppm value was multiplied by a factor of 0.375 to obtain ppm milk protein.

The same principle was followed to normalize the results of the peanut pastry matrices. For peanut, the reporting units of the Neogen Veratox® Peanut ELISA are ppm total peanut. Based on the assumption that peanuts contain ~25% peanut protein, the

ppm total peanut was converted to ppm peanut protein by a multiplication factor of 0.25 (Oerise et al., 1974). The ppm values are equivalent to ug/g or mg/kg peanut protein. Therefore, the final reporting units used in our comparative analysis was ppm peanut protein (mg/kg).

The results of the total soluble protein quantification assay were given in µg/µl which is equivalent to mg/ml. The reported values were converted to total mg protein in each extract accounting for the required volume of buffer used in each extraction step. Extract -1 and -2 required 20 mL of buffer, extract -3 required 25 mL buffer, and extract -4 (pooled total extract) required 65 mL buffer. The obtained mg value is the mg of protein in each extract, however since 1 gram of sample was extracted this ultimately equates to mg protein per 1 gram of sample. The determined mg values were multiplied by a factor of 1,000 in order to attain mg/kg units.

After both reported assay values were converted into comparable units, the results were normalized by dividing the ELISA values by the total soluble protein values of each extract to determine the amount of ELISA detectable protein per total soluble protein in a given extract. The calculated ratio is a unit-less measure allowing for direct comparison of the two assays.

Equation:

$$\frac{\text{ELISA}}{\text{2D}} = \frac{\text{mg/kg ELISA}}{\text{mg/kg total soluble protein}}$$

This study was designed as a pilot study to determine if sequential extractions provide improved allergen residue recoveries and analytical results over traditional

extraction protocols in immunoassays. As a result, the intent of these preliminary experiments was aimed to evaluate if this novel approach would be fit for allergen analysis purposes and thus did not generate sufficient data or power for statistical analysis in these trials. A complete validation study with sufficient statistical power may be envisioned as part of future research projects.

RESULTS

ELISA

All sample extracts were analyzed for allergen content using Neogen Veratox® Total Milk or Neogen Veratox® Peanut ELISA kits, dependent upon the target allergen. The procedure was followed as described previously. Software provided by the kit manufacturer (Neogen Veratox® Software v 3.0.2, Neogen Corporation, Lansing, MI) and GraphPad Prism v.4.03 were used to analyze the ELISA results.

In all raw dough pastry matrices incurred with milk or peanut, a general trend was observed for detectable protein from the allergenic source. The quantity of ELISA detectable protein was greatest in the first extract (X-1), and decreased upon subsequent extractions (X-2 and X-3). The total protein extract (X-T) is a pooled representation of all three extracts, and is comparable to the theoretical sum (X-T (sum)) of the previous extracts when detected by ELISA.

Samples Incurred with Milk (NFDM)

All negative control samples (raw and baked) for each flour type (wheat, corn, and rice) were analyzed and determined to be negative for the presence of milk protein residue (below the kit limit of quantification (LOQ) of 2.5 ppm NFDM or 0.94 ppm milk protein).

Results of the incurred baked pastry matrix samples indicated no detectable milk residues were present for each flour type prepared, all baked samples were below the LOQ for the Neogen Veratox® Total Milk ELISA kit.

Analysis of the incurred samples containing 250 ppm NFDM (93.8 ppm milk protein) revealed detectable milk protein from raw pastry matrices in all flour types (Figure 3-1). Comparison of the first extracts of each matrix type indicated recovery of milk was greatest in rice flour pastry matrices (42.8 ± 0.5 ppm milk protein; 45.6% of expected). Pastry matrices prepared with corn flour reported the lowest recovery of NFDM (19.6 ± 1.1 ppm NFDM; 20.9% of expected) in the first extract in comparison to wheat and rice flour pastry matrices. Recovery of milk from wheat flour pastry matrices in the first extract was 26.3 ± 2.3 ppm NFDM (28% of expected). In the second and third extracts of all dough pastry matrices, additional allergenic protein was extracted, although in lower concentrations than the previous extract(s). The milk residue detected in the total extract for each matrix type incurred with milk was representative of the calculated sum of the previous three extracts. The total extract represents the highest quantity of protein from the allergenic source recovered from the matrix.

As indicated by the results of the first extract, less than half of the incurred protein was recovered. Additionally, a second observation indicates an underestimation of

protein from the allergenic source in the total extract, although the total extract is more representative of the overall allergen content of a sample.

Sample Incurred with Peanut (defatted light roasted peanut flour)

The control negative pastry matrices (raw and baked) for each flour type (wheat, corn, and rice) were analyzed and determined to be negative for the presence of peanut protein residue (below the Neogen Veratox® Peanut ELISA kit's reported limit of quantification (LOQ) of 2.5 ppm peanut or 0.625 ppm peanut protein based upon 25% protein in whole peanuts).

Similarly, no allergenic proteins were detected in baked pastry matrices by the peanut ELISA. All analyzed extracts were below the LOQ of the kits.

For raw dough pastry matrices prepared with 250 ppm peanut flour (125 ppm peanut protein), ELISA detectable protein was present in all flour types (wheat, corn, rice) (Figure 3-2). Reduced recoveries of peanut were observed in all sample matrices. Rice flour pastry matrices yielded the highest recovery of detectable peanut protein (49.5 ± 5.5 ppm peanut protein; 39.6% of expected) in the first extract. Recovery of peanut protein in the first extract was similar in pastry matrices prepared with wheat (38.6 ± 16.3 peanut protein; 31% of expected) and corn flour (45.8 ± 3.95 ppm peanut protein; 37% of expected). Additional protein was detected from the second and third extracts. The concentration of peanut protein detected in the total extract was representative of the calculated sums of the individual extracts. The total extract is the most representative value of protein from the allergenic source in a sample matrix, however this value remains lower than the expected recovery of the incurred material.

The inability to detect allergenic proteins (milk or peanut) from all baked pastry matrices suggests baking alters the allergenic epitopes of milk and peanut ultimately influencing the detection of proteins when using Neogen Veratox® Total Milk or Neogen Peanut Veratox® ELISA kit as discussed in more detail following the discussion of the total soluble protein quantification. Similar results were reported in previous studies (Downs and Taylor, 2010; Poms and Anklam, 2004).

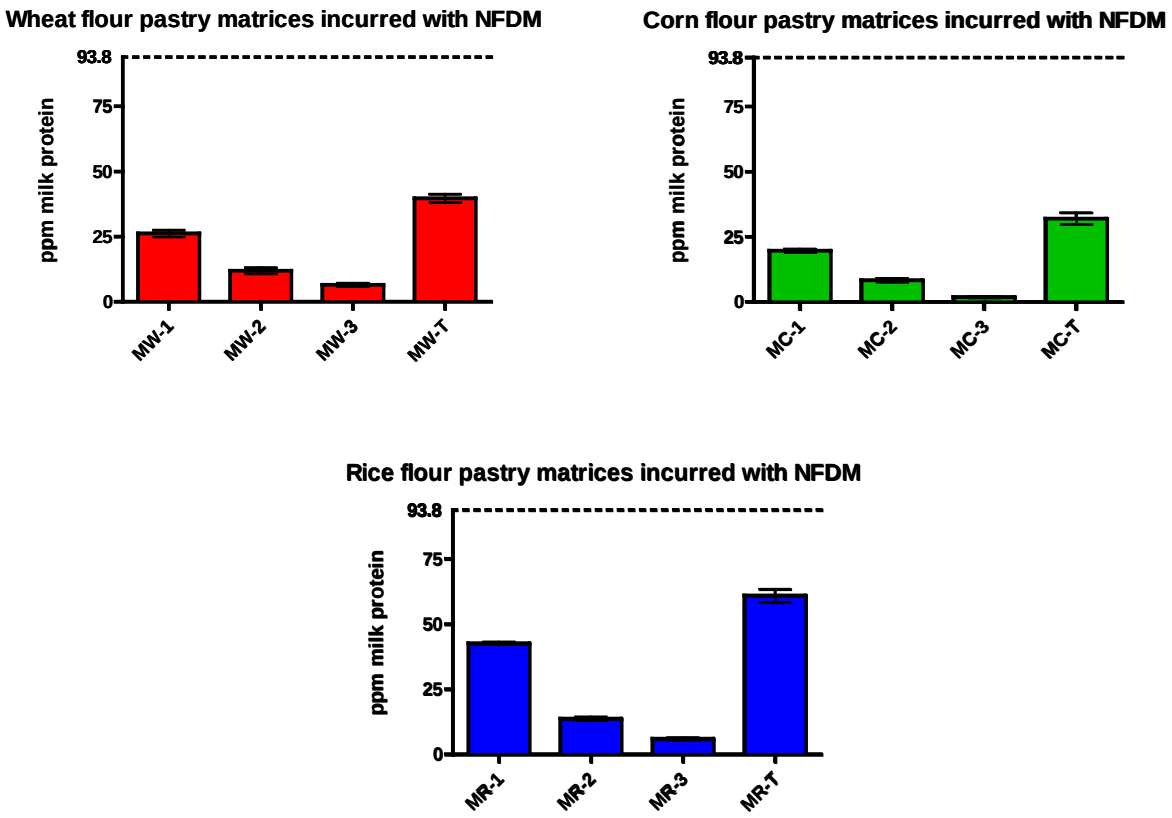

Figure 3-1. ELISA recovery values (percent recovery ± standard error) for sequential extractions of unprocessed wheat, corn, and rice pastry matrices incurred at 93.8 ppm milk protein (250 ppm NFDM). Samples are labeled using the following abbreviations: (MW) milk-in-wheat, (MC) milk-in-corn, (MR) milk-in-rice, with the following 1, 2, 3 or T representing extract 1, 2, 3 or total extract, respectively. The dotted line represents the expected recovery assuming 100% extraction efficiency.

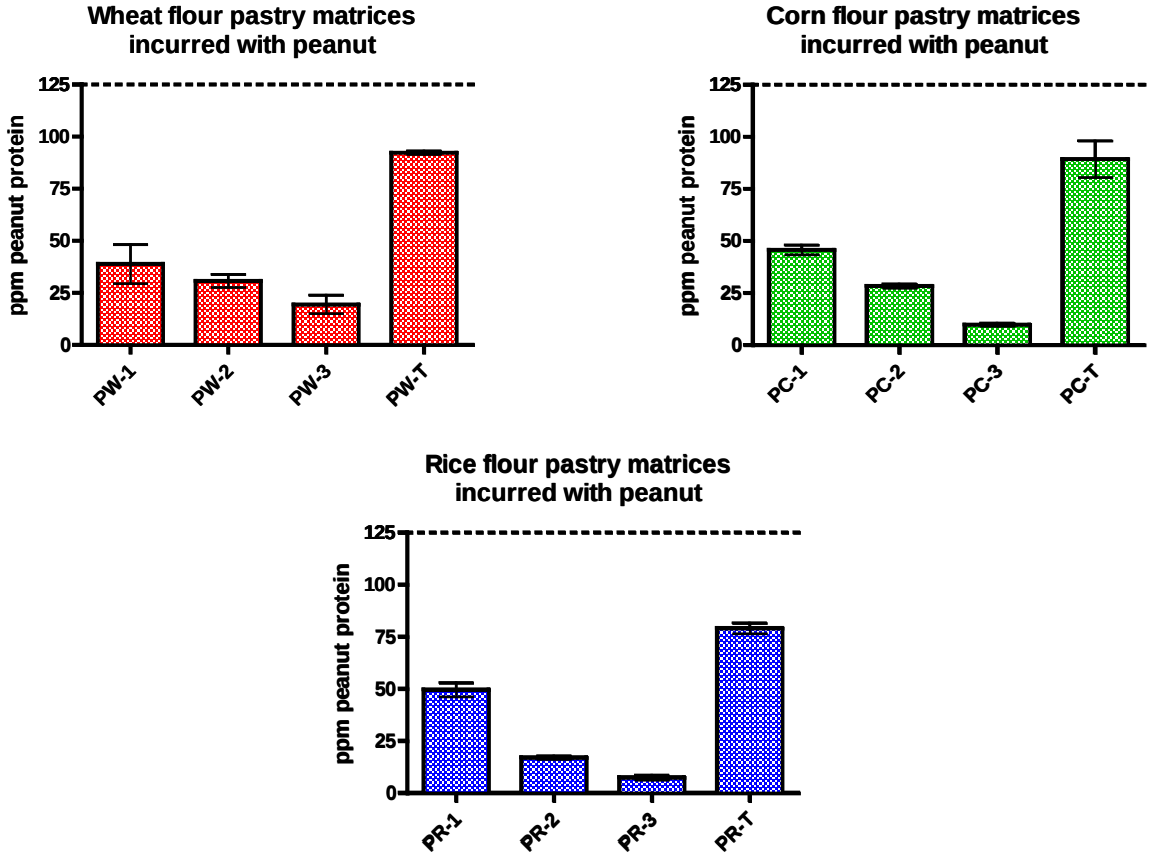

Figure 3-2. ELISA recovery values (percent recovery ± standard error) for sequential extractions of unprocessed wheat, corn, and rice pastry matrices incurred at 125 ppm peanut protein (250 ppm peanut flour). Samples are labeled using the following abbreviations: (PW) peanut-in-wheat, (PC) peanut-in-corn, (PR) peanut-in-rice, with the following 1, 2, 3 or T representing extract 1, 2, 3 or total extract, respectively. The dotted line represents the expected recovery assuming 100% extraction efficiency.

Total Soluble Protein Quantification

The total soluble protein content of each sample extract prepared for ELISA analysis was determined (Table 3-1 for incurred milk samples and Table 3-2 for incurred peanut samples). In all samples analyzed, a general pattern was present in the amount of total soluble protein quantified. The first extract (X-1) consistently contained the largest quantity of protein and subsequent extractions contained decreasing amounts of protein in the second (X-2) and third (X-3) extractions, respectively. The total pooled extract (X-T) that was analyzed using the 2-D Quant kit reported similar protein values to the sum (X-T (sum)) of the individual extracts.

Sample Extract	Mean mg protein ± SD	Sample Extract	Mean mg protein ± SD	Sample Extract	Mean mg protein ± SD
MW-1*	65 ± 18	MC-1*	67 ± 44	MR-1*	51 ± 20
MW-1	58 ± 18	MC-1	53 ± 35	MR-1	41 ± 16
MW-2	24 ± 0.5	MC-2	18 ± 0.8	MR-2	15 ± 8
MW-3	7.7 ± 2	MC-3	5.4 ± 0.3	MR-3	5.3 ± 4
MW-T	123 ± 9	MC-T	104 ± 9	MR-T	75 ± 9
MW-T (sum)	90 ± 14	MC-T (sum)	77 ± 35	MR-T (sum)	62 ± 19
B30-MW-1*	77 ± 9	B30-MC-1*	57 ± 19	B30-MR-1*	46 ± 5
B30-MW-1	62 ± 7	B30-MC-1	46 ± 15	B30-MR-1	37 ± 4
B30-MW-2	15 ± 2	B30-MC-2	14 ± 2	B30-MR-2	12 ± 3
B30-MW-3	3.4 ± 0.8	B30-MC-3	3.1 ± 0.5	B30-MR-3	2.7 ± 2
B30-MW-T	86 ± 6	B30-MC-T	4.3 ± 5	B30-MR-T	77 ± 13
B30-MW-T (sum)	80 ± 8	B30-MC-T (sum)	62 ± 14	B30-MR-T (sum)	52 ± 4
MW-C-1*	61 ± 4	MC-C-1*	69 ± 3	MR-C-1*	69 ± 0.1
MW-C-1	49 ± 4	MC-C-1	55 ± 2	MR-C-1	55 ± 0.1
MW-C-2	18 ± 3	MC-C-2	19 ± 0.2	MR-C-2	17 ± 2
MW-C-3	7.2 ± 2	MC-C-3	7.1 ± 3	MR-C-3	5.7 ± 0.5
MW-C-T	105 ± 5	MC-C-T	117 ± 14	MR-C-T	88 ± 2
MW-C-T (sum)	74 ± 0.8	MC-C-T (sum)	81 ± 2	MR-C-T (sum)	77 ± 1
B30-MW-C-1*	46 ± 9	B30-MC-C-1*	77 ± 7	B30-MR-C-1*	99 ± 26
B30-MW-C-1	37 ± 7	B30-MC-C-1	62 ± 5	B30-MR-C-1	79 ± 21
B30-MW-C-2	14 ± 3	B30-MC-C-2	20 ± 2	B30-MR-C-2	11 ± 0.9
B30-MW-C-3	4.7 ± 0.4	B30-MC-C-3	6.0 ± 0.5	B30-MR-C-3	2.9 ± 0.2
B30-MW-C-T	106 ± 38	B30-MC-C-T	102 ± 10	B30-MR-C-T	82 ± 5
B30-MW-C-T (sum)	55 ± 6	B30-MC-C-T(sum)	87 ± 4	B30-MR-C-T(sum)	93 ± 22

Table 3-1. Total soluble protein quantification values for all sample matrices and extracts incurred with milk and analyzed for total soluble protein using the 2-D Quant kit. Baked samples are represented by a B30 label. MW indicates milk in wheat, MC indicates milk in corn, and MR indicates milk in rice flour pastry matrices. Negative control samples are labeled as –C– while all others represent the samples incurred with NFDM. Extract 1* indicates the expected values if no sequential extractions were done. Extract 1 (X-1) indicates the first sequential extraction, X-2 and X-3, the second and third sequential extractions. X-T indicates the value obtained by pooling equal volumes (4 mL each) from extracts X-1, X-2, and X-3 for each assay. X-T (sum) represents the sum of X-1, X-2, and X-3.

Samples Incurred with Milk (NFDM)

Wheat Flour

The protein content of incurred raw dough wheat flour pastry matrices (250 ppm NFDM; 93.8 ppm milk protein) and negative control matrices (0 ppm NFDM/milk protein) displayed slight differences in the amount of protein in comparable extracts. This same observation was true when comparing incurred and negative control baked pastry matrices. In both matrix types, the incurred pastry matrices revealed slightly higher protein contents than the control, however the only difference is the addition of 93.8 ppm milk protein in the incurred samples. Given only a trace amount of milk protein (93.8 ppm) was added, the protein contents should be nearly similar. The quantity of protein extracted in subsequent extracts was always lower than in the previous extract. In all wheat flour samples, the recovery of proteins was noticeably lower in the second and third extractions, respectively.

A second comparison between raw and baked wheat flour pastry matrices prepared at the same spike level (e.g. 93.8 ppm milk protein in raw and baked matrices) indicated only minor reductions in overall protein content after baking.

Minor differences were present in the protein content quantified between the raw and baked negative control pastry samples (0 ppm milk protein).

Corn Flour

In raw corn flour pastry matrices prepared with milk, the quantity of protein in each extract was similar between incurred (250 ppm NFDM) and control (0 ppm NFDM) matrices. Baked corn flour matrices showed a similar pattern in protein quantification, in which minor differences were observed between the incurred and control matrices.

A second comparison of the raw and baked corn flour pastry doughs of the same spike level indicated baking procedures did not drastically decrease the amount of protein quantified. Altogether, the protein contents of each extract were similar among raw and baked incurred samples. These same observations were also true in the control corn flour pastry matrices, suggesting minimal changes in extractable protein content occurred during baking of the control samples.

Rice Flour

The protein content of raw dough rice flour pastry matrices was similar between the incurred matrices (250 ppm NFDM) and control matrices (0 ppm NFDM). The baked rice flour pastry matrices prepared at 250 ppm NFDM were nearly similar for all extracts except the first extract of the incurred baked dough sample (B30-MR-250-1) of the incurred rice flour matrix. The protein contents in the first extract are expected to be nearly similar among incurred and control samples, since only a small quantity of milk was added to the samples. An error may have occurred while performing the assay, contamination, or inefficient extraction may have caused this observation. This also suggests the protein content of rice flour matrices is less affected by baking processes since there was no noticeable decrease in the amount of protein recovered.

Further comparison of raw and baked pastry matrices prepared at the same spike concentration indicated comparable protein recoveries between the raw and baked rice flour pastry matrices.

Sample Extract	Mean mg protein ± SD
PW-1*	106 ± 21
PW-1	84 ± 16
PW-2	40 ± 5
PW-3	19 ± 2
PW-T	183 ± 27
PW-T (sum)	142 ± 12
B30-PW-1*	93 ± 6
B30-PW-1	74 ± 5
B30-PW-2	34 ± 13
B30-PW-3	14 ± 0.6
B30-PW-T	101 ± 6
B30-PW-T (sum)	103 ± 31
PW-C-1*	89 ± 15
PW-C-1	71 ± 12
PW-C-2	47 ± 2
PW-C-3	22 ± 2
PW-C-T	188 ± 63
PW-C-T (sum)	140 ± 11
B30-PW-C-1*	78 ± 13
B30-PW-C-1	62 ± 11
B30-PW-C-2	26 ± 12
B30-PW-C-3	12 ± 2
B30-PW-C-T	127 ± 4
B30-PW-C-T (sum)	120 ± 46

Sample Extract	Mean mg protein ± SD
PC-1*	113 ± 35
PC-1	91 ± 28
PC-2	43 ± 3
PC-3	13 ± 2
PC-T	170 ± 39
PC-T (sum)	146 ± 28
B30-PC-1*	125 ± 20
B30-PC-1	100 ± 16
B30-PC-2	28 ± 2
B30-PC-3	12 ± 2
B30-PC-T	165 ± 50
B30-PC-T (sum)	140 ± 19
PC-C-1*	104 ± 13
PC-C-1	83 ± 10
PC-C-2	38 ± 2
PC-C-3	10 ± 3
PC-C-T	161 ± 39
PC-C-T (sum)	131 ± 6
B30-PC-C-1*	83 ± 10
B30-PC-C-1	66 ± 8
B30-PC-C-2	25 ± 2
B30-PC-C-3	11 ± 2
B30-PC-C-T	228 ± 81
B30-PC-C-T (sum)	102 ± 6

Sample Extract	Mean mg protein ± SD
PR-1*	78 ± 6
PR-1	62 ± 5
PR-2	38 ± 2
PR-3	13 ± 0.6
PR-T	117 ± 28
PR-T (sum)	112 ± 3
B30-PR-1*	132 ± 50
B30-PR-1	106 ± 40
B30-PR-2	36 ± 4
B30-PR-3	11 ± 5
B30-PR-T	152 ± 38
B30-PR-T (sum)	152 ± 39
PR-C-1*	78 ± 11
PR-C-1	63 ± 9
PR-C-2	37 ± 4
PR-C-3	15 ± 0.5
PR-C-T	153 ± 54
PR-C-T (sum)	121 ± 3
B30-PR-C-1*	83 ± 16
B30-PR-C-1	66 ± 13
B30-PR-C-2	26 ± 7
B30-PR-C-3	12 ± 1
B30-PR-C-T	128 ± 27
B30-PR-C-T (sum)	104 ± 20

Table 3-2. Total soluble protein quantification values for all sample matrices and extracts incurred with peanut and analyzed for total soluble protein using the 2-D Quant kit. Baked samples are represented by a B30 label. PW indicates peanut in wheat, PC indicates peanut in corn, and PR indicates peanut in rice flour pastry matrices. Negative control samples are labeled as –C– while all others represent the samples incurred with peanut flour. Extract 1* indicates the expected values if no sequential extractions were done. Extract 1 (X-1) indicates the first sequential extraction, X-2 and X-3, the second and third sequential extractions. X-T indicates the value obtained by pooling equal volumes (4 mL each) from extracts X-1, X-2, and X-3 for each assay. X-T (sum) represents the sum of X-1, X-2, and X-3.

Samples Incurred with Peanut (defatted light roasted peanut flour)

Wheat Flour

Table 3-2 summarizes the values determined for quantified total soluble protein in peanut matrix extracts.

For incurred wheat flour pastry matrices (250 ppm peanut flour; 125 ppm peanut protein) the amount of protein quantified was similar between extracts from raw and baked samples. Baking did not to appear to alter the overall extractability of the protein in wheat flour pastry matrices since soluble protein contents were similar. Comparable recoveries of protein were obtained from the negative control raw and baked pastry matrices for each extract (0 ppm peanut flour/peanut protein). A difference in protein content was present in the total extract of the incurred pastry matrices, where the raw wheat flour pastry matrices incurred with 125 ppm peanut protein yielded a higher protein content (183 ± 27.1 mg protein) in comparison to the baked wheat flour pastry matrices incurred with 125 ppm peanut protein (101 ± 5.7 mg protein).

Analysis of the negative control wheat flour pastry matrices indicated analogous protein contents for each extract.

Corn Flour

Similar protein contents were determined in all extracts of pastry matrices prepared with corn flour. Minor differences were present between the incurred raw and baked pastry matrices. For control pastry matrices (0 ppm peanut flour/peanut protein), the amount of protein present in each sample was similar. It is important to note the quantity of protein obtained from the first extract of the negative control baked pastry matrices (B30-PC-C-T) was lower than the first extracts of other corn flour samples

which may be caused by poor extraction or experimental error during in the first extract, since the second and third extracts were similar in protein concentration.

Rice Flour

The protein contents of incurred rice flour pastry matrices were highly comparable between raw and baked samples. Furthermore, similar amounts of protein were quantified in negative control rice flour pastry matrices. In the baked samples incurred with peanut flour, the first extract yielded a higher concentration of protein than the other three sample matrices prepared with rice flour. This suggests baking in rice flour pastry matrices does not inhibit the detection of peanut proteins since the protein content was similar between raw dough and baked samples.

Normalization of ELISA and Total Soluble Protein Quantification Results for Assay Comparison

A normalization of ELISA and protein quantification results was performed to directly compare the two assays. The results represent the quantity of ELISA detectable protein in relation to the total soluble protein quantified in each individual extract. Higher values indicate more ELISA detectable protein is present per total soluble protein in a given extract. ELISA values were only obtained for raw pastry matrices therefore the normalization calculations were only done on raw pastry matrix extracts.

Sample Incurred with Milk (NFDM)

The normalized milk protein recovery values are presented in Figure 3-3. Interestingly, different patterns in the normalized values for pastry matrices (wheat, corn, rice) incurred with NFDM were observed.

The raw pastry matrices prepared with wheat flour were nearly equal in their ratios of detectable ELISA protein in the first and second extracts (Figure 3-3; W-1, W-2). As illustrated by Figure 3-3 (extract W-3), the third extract was the most concentrated extract, where the highest concentration of protein from the allergenic source compared to extractable protein was present.

In corn flour matrices, the concentration of detectable ELISA protein was similar among all extracts, and the most concentrated fraction was obtained from the second extraction.

The rice flour matrices exhibited a different pattern in protein recovery, in which the first and third extractions contained the most concentrated fractions of detectable ELISA protein for milk. In matrices prepared with rice flour, we can see detectable ELISA protein is more readily extracted in the first extraction, in comparison to wheat and corn flour matrices.

For all observations, the total extract was generally always slightly lower than the summed theoretical extract. This is attributed to potential variability that could be introduced during pooling and/or aggregation of proteins as a result of pooling.

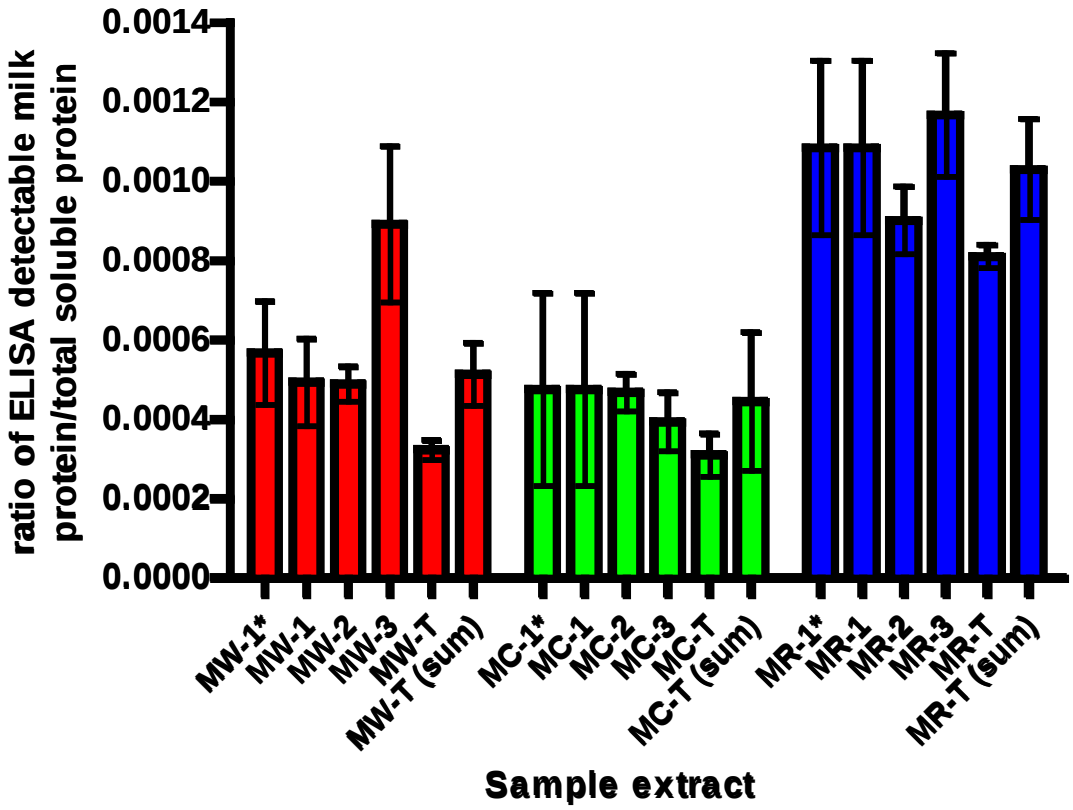

Figure 3-3. Normalized milk protein results for wheat (W), corn (C), and rice (R) flour pastry matrices incurred at 93.8 ppm milk protein (250 ppm NFDM). Values represent the ratio of detectable milk protein by ELISA out of the total soluble protein quantified ± standard error. Extract 1* indicates the expected value if no sequential extractions were done. Extract 1 (X-1) indicates the first sequential extraction, followed by X-2 and X-3, the second and third sequential extractions. X-T indicates the reported value obtained by pooling equal volumes (4 mL each) from extracts X-1, X-2, and X-3 for each assay. X-T (sum) represents the summation of X-1, X-2, and X-3.

Samples Incurred with Peanut (defatted light roasted peanut flour)

A similar pattern in peanut protein recovery was observed in wheat and corn flour pastry matrices incurred with light roasted peanut flour (Figure 3-4). An increase in the detectable protein from the allergenic source upon subsequent extractions in relation to total protein was observed in wheat and corn flour pastry matrices. This demonstrates repeating the extraction process results in an increased amount of detectable ELISA protein that can be obtained. In each extraction procedure, the detectable ELISA protein becomes more concentrated and/or interfering substances in the matrices may be removed in the supernatant and therefore more readily extracted. For both wheat and corn pastry matrices, the highest concentration of detectable allergenic proteins was extracted in the third extraction (Figure 3-4, PW-3, PC-3).

Contrastingly, rice flour pastry dough matrices incurred with light roasted peanut flour exhibited a different pattern upon normalization of the two assays. The first extract contained a majority of the ELISA detectable protein (PR-1). The second extract reported the lowest concentration of ELISA detectable protein, whereas the third extract reported a larger concentration of ELISA detectable protein. Interestingly, between the second and third extracts an increase in the detected allergenic protein is present.

The pattern of recovery observed in rice flour pastry matrices is similar between matrices incurred with milk (NFDM) and peanut (light roasted peanut flour), suggesting that components in the rice flour matrix have less of an interactive effect than the other flours used in matrix preparation.

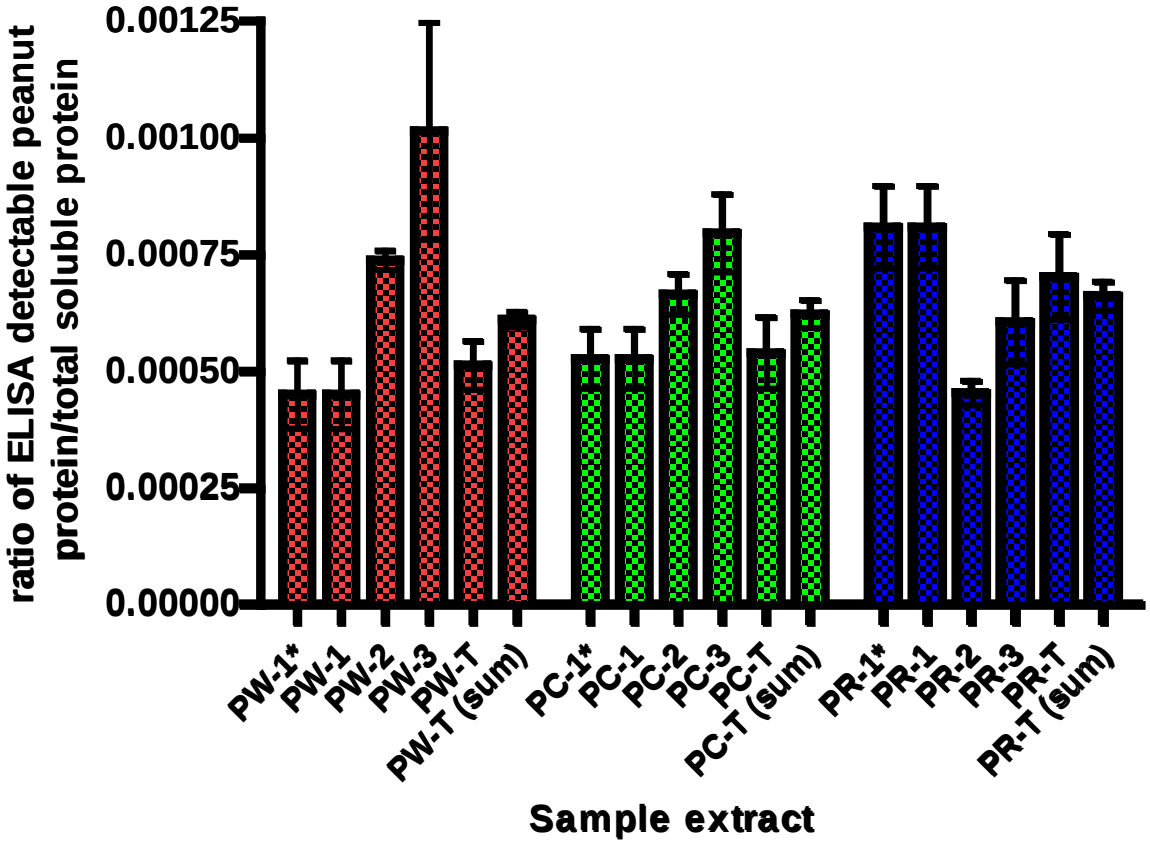

Figure 3-4. Normalized peanut protein results for wheat (W), corn (C), and rice (R) flour pastry matrices incurred at 125 ppm peanut protein (250 ppm peanut flour). Values represent the quantity of detectable peanut protein by ELISA out of the total soluble protein quantified ± standard error. Extract 1* indicates the expected value if no sequential extractions were done. Extract 1 (X-1) indicates the first sequential extraction, followed by X-2 and X-3, the second and third sequential extractions. X-T indicates the reported value obtained by pooling equal volumes (4 mL each) from extracts X-1, X-2, and X-3 for each assay. X-T (sum) represents the summation of X-1, X-2, and X-3.

DISCUSSION

The sequential extraction process yields an overall increase in the quantity of proteins extracted from a given sample extract prepared for ELISA analysis. ELISAs are designed to detect specific proteins in a complex food system containing other compounds (proteins, lipids, vitamins, etc.) and are specific for the detection of protein from the allergenic source of interest (Westphal et al., 2004). However, inefficient protein extraction and matrix interferences can negatively affect the performance of an immunoassay. Typically in food allergen immunoassays, only one extraction step takes place. In our studies this is represented by X-1*, the expected result if only a single extraction were to take place, as with standard ELISA protocol. During protein extraction if the sample material is of solid or particulate form, a centrifugation step takes place in order to separate solid particles from the supernatant and for extract clarification. A majority of the proteins will remain in the supernatant and will be detected by the immunoassay. Although our studies determined additional soluble protein was extracted from the precipitant upon repeated extraction (Tables 3-1 and 3-2). The proteins extracted in X-2 and X-3 are potentially less soluble in comparison to proteins extracted in the first extraction (X-1). Removal of the supernatant of the first extract may remove interfering matrix proteins or other compounds and allow for solubilization of other additional proteins. Proteins insoluble in the first extraction may therefore become more soluble upon subsequent extractions. The preferential extraction of gluten or other non-allergenic proteins in the first extract results in lower recovery values of the target analyte in the first extract.

It is important to note that when comparing raw and baked pastry matrices only minor differences were apparent between the amounts of total soluble protein quantified in comparable extracts. When these same extracts were analyzed by ELISA, detectable protein was only quantified from raw pastry matrices and no ELISA detectable protein was quantified from baked pastry matrices. This further supports the notion that baking and/or extensive thermal processing alters the allergenic epitopes and reduces the overall detection by immunoassays (Downs and Taylor, 2010; Nowak-Wegrzyn and Fiocchi, 2009). Since similar amounts of protein were quantified from both matrix types (raw and baked), this supports interactions are occurring within the matrix during baking and likely during dough preparation.

In all pastry matrices analyzed, glutinous and non-glutinous, recoveries of target proteins from the allergenic sources were decreased in comparison to the expected recovery based on the incurred concentration level.

Zhou et al., (2015) outlined a basic procedure for sequentially extracting proteins and identified five factors influencing protein extraction efficiency. These factors include (1) extraction buffer composition, (2) extraction temperature, (3) time of extraction, (4) centrifugal force, and the (5) liquid-to-solid ratio of a sample. In our study, no modifications were made to the assay components (buffers, additives, etc). The extraction procedure was simply repeated in order to assess the extraction efficiency.

The composition of extraction buffers is the most critical factor in protein extraction. Proteins must be soluble in buffering systems in order to be detected by the selected analytical techniques. Different proteins are capable of being extracted in different buffering systems, therefore a thorough understanding of targeted proteins is

advantageous. Most allergenic proteins can be classified into three categories based on their solubility, the albumins (water soluble), globulins (salt soluble), and prolamins (water and alcohol soluble) (Westphal et al., 2004). Commonly used buffers for extraction of allergenic proteins include phosphate-buffered saline (PBS), hepes-buffered saline (HBS), tris, or carbonate buffers (Chassaigne et al., 2007; Poms, Capelletti, & Anklam, 2004; Poms, Klein, & Anklam, 2004; Westphal et al., 2004). Several protein extraction buffers are saline based with a neutral pH (Monaci and Visconti, 2010).

The pH of extraction buffers must be optimized in order to achieve the best extraction of proteins. One study demonstrated that when using tris-buffered saline, nearly 35% less protein was extracted at pH = 7.4 than at pH = 8.2 where protein extraction was optimized for roasted peanut (Poms, Capelletti, & Anklam, 2004).

Westphal et al. (2004) demonstrated that more peanut protein could be extracted with carbonate buffers as opposed to PBS, HSB, or Tris buffers. Carbonate buffers typically have pHs that can range from 9 to 11. Higher pH buffers (typically above pH 10) are not compatible with immunoassays, therefore these buffer systems are not frequently used for food allergen analysis. PBS (pH = 7.4) is commonly used as an extraction buffer since it provides good protein solubility and a pH compatible with most immunoassays. At pH = 7.4, antigen-antibody binding is promoted, therefore providing a favorable environment for the detection of allergenic proteins (Westphal et al., 2004). At higher pH values, proteins are generally more soluble due to the charge state of a protein. At the isoelectric point, proteins are less soluble and will precipitate out of solution since there is no charge on the protein. A majority of food allergens have acidic isoelectric points (pH ~ 4 – 6) therefore improved extraction occurs at pH ~7 where allergenic

proteins carry a charge and are therefore more soluble due to charge imbalances between the buffer and proteins (Taylor and Lehrer, 1996). The repeated use of PBS in our sequential extraction study improved recoveries of allergenic residues but this buffer system was still not efficient at recovering a majority of the allergenic proteins from the matrices.

Several ELISA kits instruct for protein extraction to occur at 60°C. Protein extraction at 60°C has yielded an increase in the amount of proteins quantified by an BCA assay (Albillos et al., 2011). At lower temperatures (40 – 50°C) protein extraction was less efficient and at higher temperatures (70°C) protein denaturation begins to occur causing proteins to become less soluble and more difficult to extract (Albillos et al., 2011). Therefore, protein extraction at 60°C is preferred due to increased protein solubility before denaturation events (Albillos et al., 2011).

Studies evaluating the use of two different extraction buffers sequentially have demonstrated improved protein recovery from roasted and boiled peanuts (Chassaigne et al., 2007). Protein extraction from roasted peanuts improved when two different extraction buffers were used in sequence (Chassaigne et al., 2007). Different allergenic protein fractions may be soluble in different buffering systems. In general, the most soluble and abundant proteins are detected in a typical immunoassay. Extraction of less soluble proteins and those proteins present in low concentrations is equally important and may provide beneficial information about the quantity and specific allergenic protein(s) of interest. The total quantity of proteins extracted increased upon continued extractions with the first extract containing ~18% of total peanut protein and the second extract containing ~74% (Chassaigne et al., 2007). Tris buffered saline was used first in

extraction followed by an ethanol-water (20:80) mixture (Chassaigne et al., 2007). In extract 1, both Ara h 1 and Ara h 2 were detected, however in extract 2, Ara h 2 was not detected whereas Ara h 1 was detected (Chassaigne et al., 2007).

A similar extraction method was evaluated using defatted peanut flour as a sample matrix and a five step sequential extraction and one extraction buffer (Zhou et al., 2015). For each extraction, additional protein was quantified from the peanut flour. In total, 84% of the peanut protein was extracted, extract 1 yielded 53% total protein and extract 5 generated 1.1% total protein (Zhou et al., 2015). By using a protein rich sample with no matrix interferences, nearly all the protein was extracted.

In our study, sequentially extracting proteins with the same extraction buffer (0.01 M PBS) yielded an increase in the recovery of both total soluble protein and ELISA detectable protein by the kit(s) in raw pastry matrices for both milk and peanut as illustrated in Figures 3-3, 3-4. Likewise, the sequential extraction procedure yielded additional protein in all matrices prepared supporting the sequential extraction protocol. A straightforward extraction protocol was followed in which only one buffer and an extraction additive were added to extract proteins. This extraction procedure was used for compatibility with selected ELISA kits. The vast proportion of protein from flour in relation to the allergenic protein influences the overall protein extraction from the pastry matrices. Extraction of target allergenic proteins from a matrix consisting of other non-allergenic proteins influences the overall protein efficiency.

The normalized values represent the quantity of detectable ELISA protein in relation to the amount of total extracted protein. In pastry matrices incurred with milk, different extracts indicated different concentrations of detectable milk protein in NFDM.

In wheat and rice flour samples prepared with NFDM, the third extract (Figure 3-3; W-3 and R-3) indicated the highest concentration of protein detectable by ELISA. For corn flour pastry matrices prepared with NFDM, the second extract (Figure 3-3, C-2) contained the highest concentration of ELISA detectable milk protein.

In peanut, a similar pattern is present among the normalized protein values for wheat and corn flour pastry matrices, however rice flour presented a slightly different pattern (Figure 3-4). In matrices incurred with light roasted peanut flour, wheat and corn flour matrices presented similar trends in the amount of ELISA detectable proteins per total soluble protein. As presented in Figure 3-4**,** the third extraction procedure yielded the highest concentration of ELISA detectable protein out of the total soluble protein quantified.

Rice flour matrices presented interesting results in overall allergen detection. In rice flour pastry matrices incurred with milk, the first and third extracts (Figure 3-3, R-1 and R-3) yielded the most concentrated fraction(s) of ELISA detectable protein, whereas in rice flour pastry matrices incurred with peanut, the first extract contained the most concentrated fraction of ELISA detectable protein (Figure 3-3, R-1).

This could be an artifact of the overall protein content of a food matrix. Of the three flour matrices prepared, rice flour contained the least amount of protein (See Chapter 2, Table 2-4) suggesting a protein exclusion effect is occurring in the other matrices prepared with corn and wheat flour, both of which have higher protein contents than rice flour. If pastry matrices were prepared with soy flour (34.2% protein), it is anticipated that the allergenic proteins would not be detected entirely in the first extraction, although this theory would need to be analytically confirmed in future studies.

To potentially improve the extraction of allergenic proteins, an extraction protocol should be designed to preferentially extract proteins from the allergenic source of interest. This could be done by altering the extraction buffer or extraction procedures. The implementation of a multi-step extraction procedure (i.e. sequential extractions) has proven to be beneficial in extraction of milk and peanut proteins from a protein rich matrix using the same extraction buffer. Alternatively, the use of sequential extractions and multiple buffers could further improve allergen detection by using different buffer combinations for extraction to maximize the total amount of ELISA detectable proteins.

The implementation of harsher extraction procedures (e.g. denaturants, detergents, reducing conditions, etc.) would yield an increase in protein (Immer and Lacorn, 2015). However, this approach may be ineffective if the allergenic proteins are unable to be sufficiently solubilized in a single extraction step. The use of more extreme extraction conditions has the potential to alter the protein structure and antibody binding epitopes of an allergen resulting in decreased detection. (Baumert, 2014; Immer and Lacorn, 2015). These more extreme environments are less compatible with ELISA kits, and often not employed by commercial ELISA kits (Immer and Lacorn, 2015). DNA based methods use harsher extraction protocols since DNA remains stable under the more extreme extraction conditions intact protein is not necessary for PCR detection (Baumert, 2014; Immer and Lacorn, 2015).

The observed general increase in detectable ELISA protein content is potentially an effect of solvation space. In each extract there is a finite amount of buffer in regards to the amount of sample weighed for analysis. During the first extraction procedure those proteins which are most soluble will be extracted, both protein from the allergenic source

of interest and proteins from the sample matrix. For instance, in the prepared matrices if wheat proteins are more soluble than the competing milk proteins, the wheat proteins will be preferentially extracted. Alternatively if the majority of proteins in a matrix are soluble in the extraction buffer, these proteins will be preferentially extracted over the low concentration of allergenic proteins which are present in lower concentrations. As a result, less of the milk protein is detected by the ELISA kit due to inefficient extraction of these milk proteins. Therefore upon resuspension of the precipitant formed during the first extraction, an increase in the ELISA detectable protein is observed.

Different matrices present different challenges in regards to overall protein extraction. Protein rich matrices appear problematic during the extraction of allergenic proteins. During extraction, those proteins which are more soluble in the extraction buffer (0.01 M PBS) will occupy the buffer space, thereby excluding the amount of available buffer capacity for an allergenic protein to occupy.

A key point to consider from this series of food matrices is comparisons between detectable ELISA proteins and total soluble protein could only be made among the incurred raw dough matrices. Baking resulted in poor detection of proteins from the allergenic sources by ELISA, both milk and peanut. Other than the dough working steps, no additional thermal processing was done on these samples. It can therefore be assumed that the allergenic proteins remain in their native conformational state and are able to be recognized by the ELISA kit antibodies.

This observed phenomenon is likely to be present in other protein rich matrices. Currently, the employed ELISA kits are capable of detecting the designated allergens in a food matrix. However, insufficient quantification is present using only the first

extraction. Thereby if allergens are present at levels less than 250 ppm of the food commodity, the incurred level for these experiments, poor detection and quantification may occur and the probability of obtaining a false negative result increases.

CONCLUSIONS

The detection of allergens from complex food matrices has proven difficult in several cases. The use of sequential extractions for detection of total soluble protein and ELISA detectable protein increased the overall quantity of protein detected by each respective method. Upon subsequent extractions, additional protein was extracted from each food matrix prepared. Previous studies (Chassaigne et al., 2007; Zhou et al., 2015) analyzed protein recovery from less complex matrices, peanuts and peanut flour, and reported increases in the amount of extracted protein in both matrices. Our study reported an increase in overall protein content from complex protein rich matrices (pastry matrices) which were incurred with NFDM or light roasted peanut flour, and subjected to processing. For total soluble protein quantification, quantifiable protein was extracted from all unprocessed (dough) and baked pastry matrices whereas ELISA analysis was only able to detect allergenic proteins in unprocessed pastry matrices.

After baking, no protein for the allergenic sources was capable of being detected by the selected ELISA kits. However, soluble protein was quantified from all sample extracts of the baked matrices suggesting interactions between matrix components and allergenic proteins result in epitope masking or degradation and affecting overall detection.

The formation of a glutinous complex was hypothesized as the cause of reduced detection of allergens in pastry matrices. After further investigation, glutinous matrices are only one factor in the overall reduced detections. Other matrices (corn and rice flour) incurred with milk or peanut proteins also provided indication of reduced detections by ELISA.

Moreover, when the two assays were compared by normalizing the results, subsequent extractions indicated increases in the concentration of ELISA detectable protein out of the total soluble protein of an extract.

By simply repeating the extraction procedure, a more complete profile of allergens can be acquired for a given sample extract. Improvements in the overall quantity of allergenic protein can be obtained when using the sequential extraction method. This approach was applied to complex and protein rich matrices, therefore in order to fully validate its efficacy a variety of matrices and target allergens should be analyzed for recovery.

In complex matrices sequential extractions provide an improvement in the overall determination of allergenic proteins, especially in unprocessed matrices. Processing reduces detection of proteins from important allergenic sources, however soluble protein is still detected. A more representative profile of allergens can be determined when using the sequential extraction method for allergen analysis and quantification.

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SUMMARY

Previous studies have reported reduced recovery of allergens in food matrices after processing. Processing operations are known to reduce the overall recovery of allergens however, interactions occurring prior to processing may preclude these events. In this study, interactions between allergenic proteins and matrix components resulted in decreased recovery of allergens. In particular, reduced recoveries of milk were observed in both unprocessed sample matrices and mixes (glutinous and non-glutinous) when analyzed by ELISA (Neogen Veratox® Total Milk ELISA). Contrastingly, the recovery of peanut from analogous unprocessed matrices and mixes (glutinous and non-glutinous) were less affected when analyzed using a commercial ELISA kit (Neogen Veratox® Peanut). In the concentration mixes prepared, as the concentration of flour increased in a sample mix the recovery of milk residues decreased. The overall recovery of allergenic milk proteins was influenced by the protein content of a given flour. In alternative flour mixes, soy flour (34.2% protein) reported the lowest recovery of milk, followed by corn (9.3% protein) and rice flour (6.3% protein). Furthermore, the formation of a dough matrix (wheat, corn, or rice flour pastry dough matrices) demonstrated reduced recoveries of milk in all pastry matrices prepared. Peanut was less affected by the concentration of protein in the various flours used to prepare sample mixes.

In order to improve the quantitation of allergenic proteins a modified extraction procedure, sequential extractions, was evaluated. The use of sequential extractions demonstrated improved recoveries of target allergenic proteins (milk and peanut) in raw pastry matrices prepared with wheat, corn, and rice flour when analyzed using commercial ELISA kits (Neogen Veratox® Total Milk ELISA, Neogen Veratox® Peanut).

Two protein assays, ELISA and 2-D Quant, were used to quantify the amount of ELISA detectable protein and total soluble protein in each extract prepared, respectively. Upon normalization of the two assays, different extracts yielded more concentrated fractions of ELISA detectable protein in relation to the total soluble protein in each extract. Therefore, the first extract for ELISA analysis which is typically used in commercial ELISA kits, is not fully representative of the total allergenic profile of complex matrices (unprocessed pastry dough matrices). By repeating the extraction procedure, the overall recovery of allergenic residues improves providing a better estimate of the true quantity of allergenic proteins.

In baked pastry dough matrices (processed) no detectable allergenic residues (milk or peanut) were quantified by commercial ELISA kits. Although the total soluble protein from all extracts was similar between the raw and baked pastry matrices indicating baking inhibits the detection of allergenic residues when analyzed using ELISA methods.

Recovery of allergens from complex food matrices has proven challenging in many circumstances, particularly in processed matrices. Our study demonstrates interactions between allergenic residues in unprocessed model food matrices and mixes reports reduced recoveries of allergens. By implementing a modified extraction protocol, the recovery of allergens improves providing a better estimate of the true concentration of allergens in a sample.