8-2016

Development of a Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) for Detection of Macadamia Nut Residues in Processed Food Products

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DEVELOPMENT OF A SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETECTION OF MACADAMIA NUT RESIDUES IN PROCESSED FOOD PRODUCTS

by

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A THESIS

Presented to the Faculty of
The Graduate College of the University of Nebraska
In Partial Fulfillment of Requirements
For the Degree of Master of Science

Major: Food Science and Technology

Under the Supervision of Professor Joseph L. Baumert

Lincoln, Nebraska

August 2016

DEVELOPMENT OF A SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETECTION OF MACADAMIA NUT RESIDUES IN PROCESSED FOOD PRODUCTS

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University of Nebraska, 2016

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Macadamia nut is considered a priority food allergen and undeclared macadamia nut residues pose a potential food safety risk to individuals with macadamia nut allergies. To date, there are a limited number of immunochemical methods that are available for detection of processed macadamia nut residues. Therefore, the aim of this study was to develop a reliable and robust sandwich ELISA for detection of macadamia nut residues. Raw and roasted macadamia nuts were used for immunization in three different species of animals (sheep, goat, and rabbits). Macadamia nut specific IgG antibodies produced by each animal were tested for their specificity and monitored by determining their titer values. Rabbit antiserum was used as the capture reagent while goat antiserum was used as the detector reagent in the optimized sandwich ELISA. These antisera recognized both raw and roasted macadamia protein equivalently. Potential matrix interference was evaluated in sugar cookies, vanilla ice cream, and dark chocolate to assess the overall sensitivity of the ELISA. The cookie and ice cream matrices did not significantly affect the sensitivity of the developed ELISA ($p<0.05$). The dark chocolate matrix decreased

macadamia nut protein extraction and overall ELISA sensitivity; however, the addition of NFDM or fish gelatin into the extraction buffer enhanced the extraction efficiency which allowed for an estimated limit of quantification of 1 ppm macadamia nut in three matrices. Potential cross-reactivity was assessed in 86 food ingredients. Pure extracts from a few ingredients (all-spice, cinnamon, cloves, paprika, Brazil nut, poppy seeds, oregano, nutmeg, and cherries) showed low level binding but would not significantly affect the accuracy of the ELISA. The sensitivity and efficiency of the developed ELISA was evaluated further through macadamia incurred sugar cookies and vanilla ice cream. The overall high percentage recovery of macadamia nut residues in both model foods shows that the developed ELISA can sufficiently and reliably detect macadamia nut proteins in processed foods, and will provide a useful tool to support the validation of allergen control procedures within food companies or regulatory compliance.

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my family for their unconditional support and encouragement throughout my graduate program. Each thing they do for me is a stair I climb to reach the platform where I now stand. They taught me to never give up, no matter where lives takes me, when one door closes, another one opens, and God works in incredible mysterious way. I hope I can continue to make them proud with my future endeavors.

My sincere gratitude to my advisor Dr. Joe Baumert for his benevolent guidance, knowledge and support. Thank you for allowing me to grow and learn throughout my academic years; through you, I've gain innumerable lessons and valuable insights. I would also like to thank my committee members, Dr. Steve Taylor, Dr. Vicky Schlegel, and Dr. Randy Wehling who provide input and advices to my thesis research and writing.

Special thanks to Shyamali for always being there for me, a kind soul that is so generous in sharing her knowledge, one that pushes me when I needed a kick, and one that picks me up when I falls. I am grateful for knowing you and blessed for the friendship. Thank you for making my graduate journey an enjoyable memory that I can take along with. To Shi Min, a kind hearted friend that always lends her helping hand and is always a good listener. Thank you for making the office a livelier and fun place to work at.

My extended appreciation to Yen, who is always one call away, one that always goes a long way to lend her help, and one that never abandoned me when I fall and break. I am so proud to call you the best friend and thanks for sharing all the laughter and tears together.

I would like to thank Ferlie, Julie, Deb, Lynn, Seon, Pat, Jamie and all the FARRP staff for all your assistance and endless help throughout my time here. To my fellow lab mates, Rachel, Abby, Kaye, Michael, thank you for the research advice and friendship.

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I. INTRODUCTION

Food is an essential part of life that provides the body with sufficient nutrients such as carbohydrate, proteins and other macro and micro nutrients which allow us to perform functional needs. It has also fundamentally become the key part for most social and cultural events. Moreover, food has become the center of many billion dollar industries (National Restaurant Association, 2015) as food chains have expanded significantly during the past few decades.

Normally nutritious foods that are safe for consumption by a vast majority of individuals do not apply to the population of people with food allergic reactions. Food allergy is a growing concern as it has increasingly been found to cause adverse effects and potential fatalities in both adults and young children. The prevalence of food allergy appears to have increased over the past few years. Young children that are affected by adverse immune responses to foods increased from 5% to 8% and from 3% to 5% in adults within a three year period (Sicherer & Sampson 2010; Sicherer & Sampson 2014). The incidence of severe food allergies to tree nuts and other foods has also increased over the past decades among the European populations, and the latest statistics was showing an approximation of 6% of allergies (Johnson et al., 2014; Nwaru, et al., 2014; Nwaru et al., 2014). A study was carried out to assess the prevalence of food allergy among infants in a China province, Chongqing, which found that over the course of the 10-year study (1999-2009), the prevalence of food allergy had increased from 3.5% to 7.7% (Hu et al., 2010). Data from a another study with a total of 38480 children showed that 38.7% of

children with food allergy had experienced severe allergic reactions, and 30.4% had history of multiple food allergies (Gupta et al., 2011). The most common foods that infants are sensitized to include milk and egg, where prevalence range up to 3% and 1.7%, respectively, based on oral food challenges (Boye, 2012). However, infants who are sensitized to milk and egg are more likely to outgrow their allergies. This explains the trend that school age children aged 5 and above have an overall lower prevalence of food allergy as compared to younger children based on a global survey (Prescott et al., 2013).

Strict dietary restrictions and complete avoidance of the offending foods are the best way to prevent allergic reactions as no effective treatments are available for food allergy yet. Nevertheless, even with all the precautions and prevention, unintended ingestion and undeclared allergenic residues may still provoke adverse allergic reactions. Several countries in the world have enacted allergen labeling laws which require clear declaration of priority food allergens on pre-packaged food labels (Gendel, 2012). In the U.S., the Food Allergen Labeling and Consumer Protection Act (FALCPA) of 2004 (Public Law 108-282, Title II) which was enacted by Congress requires all food products that contain major food allergens as ingredients or processing aids to be labeled in plain English terms in the ingredient statement or in a ‘Contains’ statement on the pre-packaged food label (FDA, 2004). A good quality label on pre-packaged foods label will provide consumers with a credible source of information and keep them aware of ingredients from allergenic sources (Mills et al., 2004). While FALCPA and other labeling regulations address the need to clearly disclose priority allergens when used as an ingredient or processing aid in the product formulation, the laws do not address when labeling should be used in the event of unintended allergen presence in food products due

to cross-contact. The food industry is allowed to voluntarily use precautionary allergen labeling (PAL) to communicate potential risk to allergic consumers, provided the PAL is not used in lieu of good manufacturing and allergen control practices (DunnGalvin et al., 2015). Consumer's lack of vigilance on adhering to avoidance of products with various PAL terms such as "may contain" could increase the risk of food allergic reaction (Allen et al., 2014; DunnGalvin et al., 2015). Remington and colleagues (2013) conducted a quantitative risk assessment on 352 products and found that 8.6% of food products with advisory labeling had detectable peanut compared to 37.5% of peanut was detectable on products with peanut listed as minor ingredients. The result of this study is similar to those that were conducted in year 2005 and 2009. In addition, a study with 569 samples of cookies and chocolate purchased from the European market found that positive test was detected at a higher frequency in the presence of precautionary labeling and the likelihood of containing undeclared allergen is higher in chocolate than in cookies (Pele et al., 2007). Data from another study indicated that allergenic residues were detected in 5.3% of foods products that contain advisory labeling and this allergen contamination phenomenon happened more frequently in smaller scale companies than large manufacturers (Ford et al., 2010).

The commercially available immunological assay, Enzyme-Linked Immunosorbent Assay (ELISA), is the preferred method used for validation of allergen control practices and the detection of allergenic residues by manufacturing companies and regulatory agencies (Poms et al., 2004). To date, there are a limited number of immunochemical methods that are available for detection of processed macadamia nut

residues. Therefore, the aim of this study is to develop a reliable and robust sandwich ELISA for detection of macadamia nut residues.

II. ADVERSE REACTIONS TO FOODS

a) Food Allergy

Food hypersensitivity refers to any adverse reactions upon intake of food or ingredients (Johnson et al., 2015). True food allergic reactions are immune mediated (Taylor & Hefle, 2006). However, these are often confused with other non-immune mediated responses to food such as food intolerances, toxic food reactions and metabolic food reactions to cite a few (Lomer, 2015). Food allergies occur when the immune system reacts abnormally to components which usually are naturally occurring proteins present in the food. The immunological response involves either cell mediated (non IgE) mechanisms (delayed hypersensitivity) or specific IgE-mediated mechanisms (immediate hypersensitivity) (Muraro et al., 2014).

Immediate hypersensitivity usually involves the formation of allergen-specific IgE antibodies during the sensitization phase. Subsequent ingestion of the offending food allergen initiates the elicitation phase of an allergic reaction by cross-linking two surface bound IgE antibodies on mast cells and basophils (Mekori, 1996). Signs of an immediate hypersensitivity reaction can develop within few minutes or up to an hour following the intake of offending food, whereas, symptoms associated with delayed hypersensitivity reactions could take 24 hours or longer to develop (Taylor & Hefle, 2001). Symptoms that are commonly associated with IgE-mediated food allergy vary from mild (nausea, diarrhea, vomiting, eczema, asthma) to severe anaphylactic reactions (Sampson, 2001).

The prevalence of IgE mediated food allergy is estimated to range between 3 – 6% of total population worldwide (Sicherer, 2011) . However, dietary habits and environmental factors across populations may result in differences in the prevalence of a specific food allergy (Taylor et al., 1999). Estimates of prevalence include a wide range of methods to collect data including self-reported allergy, oral food challenges, telephone surveys and others. A study performed by Muraro et al. (2014) demonstrated that incidence of self-reported food allergy was 6 times higher than those of challenge proven allergy. Self-diagnosis and misconceptions on the definition of a true food allergy can lead to higher rate of allergy reported (Sloan & Powers, 1986). Children tend to have a higher prevalence of overall food allergy (8%) as compared to adults (5%) (Sicherer & Sampson, 2014). Children who are allergic to milk, eggs, and soy tend to outgrow their allergies, which could partly attribute to the trend of higher allergy occurrences in younger children. In some cases, early exposure and repeated exposure may increase oral tolerance over time (Strobel & Mowat, 1998). The Learning Early about Peanut Allergy Study (LEAP) team showed that among high risk infants, sensitization to peanut can be prevented through early oral introduction to peanuts (Du Toit et al., 2015). However, peanut and tree nut (TN) allergies are often severe and rarely outgrown, only about 9% of children outgrow peanut allergy. The likelihood of outgrowing peanut and TN allergies is even lower especially with children who are sensitized to one or more types of TN (Fleischer et al., 2005; Sicherer et al., 2003).

More than 160 foods are capable of causing allergic reactions and most allergens are proteins that are resistant to heat and processing (Bannon, 2004; Hefle et al., 1996). Approximately 90% of IgE-mediated food allergies are caused by the “Big 8” food

groups (FAO Technical Consultation on Food Allergies, 1995) which include peanut, tree nuts, egg, cow's milk, fish, crustacean shellfish, soybean, and wheat (FDA, 2015). Food groups that provoke the vast majority of severe to fatal anaphylactic reactions are known to be peanut, tree nut, fish and crustacean shellfish (Bock et al., 2001; Kemp et al., 1995). It is estimated that more than 100 deaths that occur each year in the U.S. are associated with IgE-mediated food allergies (Sampson, 2003)

b) Mechanism of Food Allergy

As previously mentioned, food allergy refers to an adverse immunological reaction in response to exposure of an allergen (Johnson et al., 2015). The immune system reacts to proteins present in the allergenic food thereby recognizing the protein(s) from the offending source as a foreign component. The immune system responds to these 'foreign invaders' by initiating an immune response which leads to the development of protein specific IgE antibodies that help to protect the host. The primary function of IgE in the body is to facilitates protective immunity fighting off parasitic infection (Burton & Oettgen, 2011).

Adverse reactions take place in a process with two different phases namely the sensitization phase and the elicitation phase, respectively. During the sensitization phase, the immune system will begin to recognize certain food proteins as harmful substances for unknown reasons and will mobilize an immune response. The specific food protein(s) (allergen) will be captured by antigen presenting cells such as dendritic cells that are present in the intestines, respiratory tract or under the skin. The food allergen is broken into small peptide fragments and presented by the major histocompatibility complex class

II (MH-II) and recognized by naive CD4 + T cells (Kumar et al., 2012). CD4 (+) T-lymphocytes produce cytokines such as IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 that are believed to play an integral role in the allergic response (Bellanti, 1998). This stage takes place without any symptoms as opposed to the elicitation phase where allergic symptoms develop during an active allergic response to the offending allergenic food.

An IgE-mediated food reaction is commonly associated with immediate onset of symptoms and the two main components that facilitate this reaction are the high affinity receptor, fragment crystallizable epsilon region R1, (FcεR1) and the low affinity receptor FcεRII (CD23) that are found on mast cells and basophils (Gould & Sutton, 2008). During early sensitization, allergen-IgE complexes are captured by CD-23 and transported from the epithelium into the mucosa where the complexes bind to FcεR1 on both mast cells and dendritic cells. In the mucosa, plasma cells synthesize IgE and are transported by CD-23 from mucosal side into the gut lumen where they capture allergen and binds to CD-23 to be delivered back into the mucosa. Following that, inflammation caused by the IgE-mediated allergic reaction in the mucosa damages the intestinal epithelium, which allows free allergens to pass between cells and binds to IgE-sensitized dendritic and mast cells, intensifying the food allergy reaction (Gould & Sutton, 2008). Subsequent exposure to the same allergenic food(s) allows the allergenic protein(s) to cross-link two or more IgE antibodies bound to the surface of mast cells and basophils, resulting in the aggregation of FcεR1 (Burton & Oettgen, 2011). This interaction stimulates the degranulation of mast cells and basophils, releasing mediators from the granules containing potent inflammatory substances into blood stream and tissues of the

host (Taylor & Baumert, 2012). The elicitation stage is responsible for the rapid onset of adverse reactions which could take place within minutes.

Various types of mediators have been identified to be associated with adverse reactions caused by IgE mediated food allergy such as prostaglandins, leukotrienes, and β-hexosaminidase. Histamine is known to be the primary mediator among others in causing an immediate hypersensitivity reaction. Binding of histamine to its receptors Histamine 1 (H1) and Histamine 2 (H2) causes symptoms due to contraction of gastrointestinal tract and airway muscles, increase in production of mucus, secretion of gastric acid, etc. While in the skin, the H1 receptor stimulation can also mediate symptoms that include pruritus and urticaria (White, 1990).

III. MACADAMIA NUT

a) Macadamia Nut Nutrition and Consumption

Macadamia nut, also known as Queensland nut, is named after its native land of plantations, Queensland Australia. Australia holds a 45% share of all macadamia nut production in the world, making it the world's largest producer of macadamias followed by Hawaii and South Africa (USDA, 2015). Macadamia nut has been widely consumed globally and statistical data show that the production of macadamia kernel has increased by 70% in the past 10 years with more than 44,000 metric tons of crops being produced in 2014 (International Nut & Dried Fruit, 2015). Additionally, there has been a 51% increase in the world's macadamia export over the past 9 years (2004-2012). In 2013, the United States exported 1475 metric tons of shelled macadamia nuts and South Africa remained as the top exporter (International Nut & Dried Fruit, 2015).

Macadamia nut belongs to the family of *Proteaceae* and consists of 9 species, only 2 of which are edible (Ripperton et al., 1938). These commonly consumed species are *Macadamia intergrifolia* and *Macadamia tetraphylla* but the production is mainly focused on M. *intergrifolia* for commercial purposes. The smooth shelled M. *intergrifolia* is chosen instead of M. *tetraphylla* because of its higher oil content (Australian Government, 2015). In addition, the lower sugar content in M. *intergrifolia* decreases the browning of the kernels due to the Maillard reaction when roasted as compared to M. *tetraphylla* (California Rare Fruit Growers, 1997). High oil and low sugar content are the major factors in determining the quality of macadamia nuts.

Macadamia nut seeds contain 75.8% of fat, 7.91% of protein, 8.6% dietary fiber and 1.36% water (USDA, 2000). Of the 75.8% total fat, 59.3% is composed of monounsaturated fat which mostly consists of oleic acid with small amount of palmitoleic acid and erucic acid (Gebhardt & Thomas, 2002; Venkatachalam & Sathe, 2006). Minerals such as calcium, potassium, and iron that are beneficial to health are also present in macadamia nut seeds (Schlörmann et al., 2015). Macadamia nut has the highest total fat among all other tree nuts, but the high fraction of monounsaturated fats are believed to positively influence human health by lowering the low density lipoprotein (LDL) and increasing high density lipoprotein (HDL), thus improving overall serum lipid profiles (Curb et al., 2000).

Tree nut consumption has significantly increased in recent years, due in part to the association of tree nuts with improved quality of health and high nutrient value (O'Neil et al., 2015). It is recommended in the FDA Qualified Health Claims guidance to incorporate about 1.5 ounces of macadamia into the daily diet (Griel et al., 2008). Several

studies indicated that inclusion of macadamia nut had significantly reduced triglycerides and serum cholesterol levels by approximately 20.9% and 7.9%, respectively (Colquhoun et al., 1996; Curb et al., 2000).

b) Macadamia Allergy and Characterized Allergens

Macadamia nut is included as one of the major tree nut allergens in "Big Eight" food groups that are collectively known to be the most common allergenic food sources involved in the elicitation of allergic reactions. Compared to other tree nuts such as walnut, cashew, and almond, the prevalence of macadamia nut allergy is much lower (Lerch et al., 2005). Macadamia nut is categorized as one of the tree nuts that is less commonly associated with adverse allergic reactions (Roux et al., 2003). Based on a food allergy study involving 5149 participants, less than 5% reported that their tree nut reaction was attributed to macadamia nut (Sicherer et al., 2001). Walnuts were most often implicated in inducing an allergic reaction, accounting for 34% of the reports (Sicherer et al., 2001). A smaller study involving 101 tree nut-allergic patients reported a similar finding for the prevalence of allergy to various tree nuts (Fleischer, 2007).

Until recently, a limited number of macadamia proteins have been described but little was known about the potential allergenicity of these (or other) macadamia nut proteins. None of the macadamia nut allergens have been fully characterized yet (Ghorbani & Morgan, 2010). According to a study by Ghorbani & Morgan (2010), four macadamia nut proteins, all known to be in the structural family of 2S albumins were visualized after running SDS-PAGE under reducing conditions. However, this study did not conclude that these proteins are allergenic. In another study performed by Sutherland

and colleagues (1999), an IgE reactive protein of the approximate molecular weight of 17.4 kDa that was present in both raw and roasted macadamia nut was detected using serum from a macadamia-allergic patient but not in the control serum giving indication that this protein at least has the potential to sensitize individuals. Furthermore, two additional peptide fragments were identified and termed *Macadamia integrifolia* antimicrobial protein, MiAMP1 and MiAMP2c, respectively. Both peptides were isolated from the seed kernel of macadamia nut which was known to be rich in the vicilin seed storage proteins. The MiAMP1 peptide was estimated to be 8.1 kDa and was known to contain antimicrobial activity that aids in helping transgenic plants to be less prone to diseases (Marcus et al., 1997). The second peptide that poses inhibitory activity MiAMP2c, comprise of 45 amino acids residues. This 7S globulin (vicilin) was discovered as an antimicrobial peptide and functions as part of a defense mechanism against pathogenic fungi but no study to date have been performed to study the role of these peptides in food associated allergy (Marcus et al., 1999).

Another study conducted using a pooled of sera composed of 26 polyvalent allergic individuals and IgE immunoblot result shows that there are two IgE-binding macadamia proteins present at 45 kDa and 12kDa respectively. The 45 kDa band was detected under both reducing and non-reducing conditions while the 12 kDa protein was detected only under non-reducing condition (Herbst at al., 2010).

IV. MANAGING ALLERGENS IN THE FOOD INDUSTRY

In the food industry, undeclared allergen residues can occur due to a variety of factors including the use of shared production equipment to process products that contain allergenic ingredients versus those that do not contain these same allergens, cross-contact from cleaning tools, ingredients or products that are incorrectly labeled or packaged, or from sources of raw material that may contain unknown ingredients (Besler et al., 2002). Even trace amounts of allergenic residues could elicit an allergic reaction especially in individuals with heightened sensitivity (Taylor et al., 2002). As such, the food allergen labeling laws in many countries have established a de facto zero threshold so food manufacturers must mitigate the opportunity for cross-contact to occur and assure that no detectable allergen residue is present in products that do not declare these allergens. Detection of undeclared food allergen residue could result in a product recall from the market and can indirectly results in legal and economic consequences to the manufacturing company. Certainly the consequences of the presence of undeclared allergens can be deleterious to food-allergic consumers and may lead to serious adverse health consequences.

a) Analytical Methods for Detection of Allergen Residues in Foods

Allergen detection methods have been and are still in continuous implementation to help protect allergic consumers by quantifying any trace of possible allergen present in food products. Reliable methods used for detection and validation should be highly specific, sensitive, and robust. Radio-allergosorbent test (RAST) and enzyme-allergosorbent test (EAST) inhibition methods are used to detect allergens using human IgE antibodies from allergic individuals. However, these two methods are not the

preferred methods for quantification of food allergens in a food manufacturing settings as serum IgE from allergic patients varies from one individual to another and the use of human sera would present an unwanted potential biological hazard in a food production facility. In addition, individuals may possess multiple sensitivities so IgE targeted against multiple proteins from food sources may be present in the serum giving rise to potential false positive detection of the food allergen residue of interest (Besler at al., 2002).

The ATP/bioluminescence test and the general protein test such as Folin-Lowry method are used as surrogates for general sanitation and serve as a verification method for equipment cleanliness with ATP being more commonly used than the general protein test (Taylor et al., 2006). However, these two methods are not ideal for allergen detection as they do not specifically detect allergenic protein sources. ATP tests are used to measure adenosine triphosphate while general protein methods determined the total protein content from all sources instead of targeting specific allergenic protein.

Protein based immunoassays and DNA based polymerase chain reaction (PCR) are the two main methods used for determination of residues from allergens (López-Calleja et al., 2015). A recent study has been carried out on the development of real time PCR to detect macadamia DNA residue in processed food products (López-Calleja et al., 2015). The method had high accuracy with a detection limit of 0.1 mg kg^{-1} of macadamia nut with the ability to detect macadamia residues in most of the commercial food products that were analyzed making this technique one of the more sensitive choices in food inspection and detection (López-Calleja et al., 2015). Nevertheless, the main limitation of PCR is that this method is used to quantify DNA but does not detect proteins from the source of interest. Also, lower specificity was observed with PCR when a highly

conserved region of DNA was used to design primers. Moreover, efficiency can vary with different extraction techniques (Hanna et al., 2006).

ELISA and mass spectrometry (MS) are the most commonly used protein based techniques for detecting allergenic residues in foods. Mass spectrometry (MS) is a reliable method for allergen detection by quantitatively determining the presence of peptide sequence(s) or protein from an allergenic food product. The enzyme, trypsin, is used to digest the sample followed by separation of peptides using liquid chromatography (LC) coupled with quantification by multiple reaction monitoring (MRM) and electrospray ionization (ESI). The accuracy and reliability of MS detection relies on the signal peptide chosen and therefore should be based on the stability and abundance of the allergen of interest (Costa et al., 2014). However, the cost per analysis of this employed method is much higher than other available methods and requires highly trained analysts to perform.

On the other hand, ELISA continues to be the most reliable method in detecting food allergen residue as compared to other available methods. Antibodies used for these immunoassays are usually allergen specific IgG antibodies that are raised in animals such as rabbits, goats, sheep, chicken, and mice. The causative agents in food allergy are proteins and ELISA is the primary method that actually detects allergen proteins rather than DNA or ATP which act as a surrogate marker for the presence of allergen residues. The high specificity and sensitivity of ELISA makes it a preferred method in validating and detecting allergens by food manufacturers and regulatory agencies (Asensio et al., 2008).

b) Enzyme-linked Immunosorbent Assays (ELISAs) Formats

Competitive ELISA and sandwich ELISA are the two main ELISA formats used to quantify protein and potential allergens in foods. When targeting small proteins for detection, competitive ELISAs are generally the most appropriate choice while the sandwich ELISA is the most commonly preferred method when it comes to food allergen detection (Poms et al., 2004). Sandwich ELISAs are currently the most reliable and routinely used technique by the food industry and regulatory agencies for analyzing foods and for monitoring the safety of food products (Poms et al., 2005). By developing a sensitive, robust and practical immunochemical method, the risk of allergic reactions can be minimized through a better detection of trace amounts of allergen. ELISA is quick, highly specific to the allergenic food (protein) of interest and is a sensitive analytical method with adequate analysis time (Taylor et al., 2009). The highly sensitive and rapid sandwich ELISA is developed by quantifying specific binding of antigen and antibody. The efficiency of the coated capture antibody to bind to the antigen(s) of interest is a determinant factor of ELISA's specificity and sensitivity (Dixit et al., 2010). The immobilized capture antibody will recognize the protein epitope at the surface of antigen where binding takes place. Following that, the secondary antibodies that are labeled with an enzyme binds to the antigen forming a "sandwich". This antigen-antibody complex will catalyze a reaction resulting in the formation of a colored product when a suitable substrate is added. The intensity of the colored product is directly proportional to the amount of antigen present (Berg et al., 2002).

On the other hand, the competitive ELISA is an immunoassay that measures the ability of the unlabeled analyte (sample antigen) in competing with the labeled analyte

for the antibody binding site. Unlike the noncompetitive/sandwich ELISA, a lower signal in competitive ELISA indicates a higher concentration of the target proteins present in the sample. Thus, the intensity of the colorimetric product is inversely proportional to the amount of antigen present in the test samples (Greenfield, 2012). Several competitive ELISA have been developed for the detection of food allergens such as hazelnut, peanut and cow's milk (Koppelman et al., 1999; Holzhauser & Vieths, 1999; Mariager et al., 1994). The most noteworthy application of a competitive ELISA is for the detection of residual gluten proteins that may remain after fermentation during the brewing process. Sandwich ELISAs detect largely intact proteins that have two available antibody-binding epitopes to complete the 'sandwich' for accurate detection. Fermentation, however, will hydrolyze the intact protein into smaller peptide fragments and may disrupt some of the antibody binding epitopes thereby limiting the detection of the gluten residue in a sandwich ELISA. Since the competitive ELISA relies upon antibody binding to a single epitope on the gluten protein/peptide, this format may provide increased accuracy of gluten residue detection in products that have undergone some degree of fermentation (Haas-Lauterbach et al., 2012).

c) Detection of Macadamia Nut Residue

Analytical methods used for the detection of macadamia nut residues are limited compared to other tree nuts such as almond, walnut, and hazelnut. To date, no study has been published on the development of an ELISA for detection of macadamia nut residues. A limited number of commercially available ELISA methods for the detection of macadamia nut residue are available; however, no peer-reviewed references are

available which outline the performance of these methods for detection of macadamia nut residue in processed foods.

The performance of two DNA-based methods has been published to date. Brežná and colleagues (2009) used a novel real-time polymerase chain reaction approach to detect DNA from macadamia nuts in food with a practical detection limit of 0.02% (w/w) or 200 ppm macadamia nut. This study also evaluated the performance of the method against DNA fragments isolated from other tree nuts and a variety of plant species to determine the specificity of the PCR method. Specificity is achieved by using a primer set that would only allow the DNA from the food source of interest to be amplified (van Hengel, 2007). The results from this study show that PCR for detection of macadamia nut DNA residue is highly specific where only samples containing macadamia showed positive results while the rest of the non-macadamia samples were all negative. However, the sensitivity of this employed method is at a moderate level when compared to what was deemed to be a high level of sensitivity in an analytical method for detection of food allergen in general (Brežná et al., 2009). A more recent study on the development of real time PCR in detecting traces of macadamia nut residue in 214 commercial foodstuffs was found to be highly sensitive with a limit of detection of 0.1 mg kg^{-1} (López-Calleja et al., 2015). Nonetheless, the use detection of DNA as a surrogate marker for detection does draw into question whether or not the actual protein(s) from the allergenic source of concern are present. The absence of DNA does not guarantee the absence of proteins or vice versa.

Liquid chromatography in combination with tandem mass spectrometry (LC-MS/MS) is another alternative for allergen detection by identifying the peptide

sequence(s) from an allergenic food product. Sealey-Voyksner et al. (2016) developed a multi-allergen detection for peanut and tree nuts by utilizing LC-MS/MS. At least two peptides for peanut and each of the tree nuts were selected and used for analysis. This method has a detection limit of 0.1 ppm and managed to detect macadamia allergen in food products containing macadamia nut. LC-MS/MS is particularly helpful when it comes to detecting multiple food allergens in a single analysis. Despite the advantages of high specificity and the ability to detect multiple allergens in a single run, mass-spectrometry does require costly equipment and highly trained analysts to perform which are a disadvantages compared to ELISAs.

While no single assay is available to potentially test for all allergens, a commercial multiplex kit that utilizes the multianalyte profiling xMAP technology is now able to detect up to 14 allergens including macadamia nut and gluten simultaneously with a limit of detection < 5 ng/ml or 0.005 ppm (Cho et al., 2015). This particular test is beneficial when testing for multiple food allergens in one analysis rather than performing multiple extractions and analytical tests using the traditional sandwich ELISA format. Food companies with multiple allergies in their facilities or commercial labs and regulatory labs that may need to analyze samples for a panel of allergen residues are likely to find these tests useful. However, the possibility of cross-reactivity, especially among tree nuts, would need to be thoroughly evaluated to mitigate the chance of false-positive responses in such assays.

d) Potential Macadamia Nut Cross-Reactivity

Concurrent allergen sensitivity to multiple tree nuts is relatively common in diagnostic and prevalence surveys that have been reported. Clinical reviews indicate that this is especially true among tree nuts that are derived from the same or closely related botanical families (Goetz et al., 2005). An individual that is allergic to one tree nut might also be sensitized to one or more other tree nuts. Cross-reactivity arises when homologous proteins are found in two different food sources which leads to allergic responses to both foods (Frank, 2002). A study conducted by Teuber & Peterson (2010) demonstrated IgE binding cross-reactivity between macadamia nut proteins and walnut proteins, primarily the proteins found of 2S albumin and legumin seed storage structural families. While IgE binding on an immunoblot demonstrates potential cross-reactivity, it does not definitively identify the potential relevance for clinical cross-reactivity between the tree nuts or independent co-sensitization in certain individuals to macadamia nut and walnut. Furthermore, IgG antibodies directed against macadamia nut proteins were found to cross-react with almond proteins in one study that outlined the development of a sandwich ELISA used for detection of almond residues in processed foods (Hlywka et al. 2000). In this study almond-specific IgG antibodies raised in sheep recognized proteins extracted from other tree nuts including macadamia nut in addition to that of almond. Hazelnut and macadamia nut proteins have also been shown to have similar epitopes and exhibit partial cross-reactivity with macadamia as the serum of a macadamia-allergic patient showed moderate IgE binding to hazelnut protein (Sutherland et al., 1999).

V. ELISA DEVELOPMENT

a) Antibody Production

Acquiring suitable antibodies is the first crucial step in developing a successful immunoassay that is sensitive, specific, and reliable. Antibodies are known to be the host immune proteins which contain the principal effector of adaptive immune system that serves as the first response (Lipman et al., 2005). Antibody reagents can be developed from either monoclonal or polyclonal antibodies. Monoclonal antibodies are produced by a single B lymphocyte and have high specificity as the antibody binds specifically to a single epitope on the specific protein of interest, forming an antigen-antibody complex. Therefore, the chance of cross reactivity is lower compared to polyclonal antibodies. On the other hand, the risk of cross-reactivity in polyclonal antibodies is comparatively higher since the epitope that the antibody is directed against is less precisely defined and various polyclonal antibodies could bind to multiple epitopes on a single antigen (Karaszkiewicz, 2005). Generally, antigens are highly complex where they consist of a number of epitopes which will be recognized by lymphocytes. The resulting antibody response is known as polyclonal and polyclonal antibodies have higher sensitivity. Polyclonal IgG antiserum is usually raised in animals such as rabbit, sheep, and goat. A numbers of antibodies will be generated and present in the antiserum and these antibodies might bind to different epitopes on the same antigenic protein. Polyclonal antibodies are usually the preferred choice for detection of allergenic residues in foods that undergo processing because multiple epitopes on the allergenic protein(s) will be recognized by the polyclonal antisera. If the food proteins undergo conformational change in the protein structure or one linear epitope is hydrolyzed, additional epitopes will remain and allow for detection of the residue of interest. With monoclonal antibodies, if a processing

induced change in the epitope occurs, the immunochemical method will miss the detection of the allergen residue of interest thereby reducing the reliability of the method (Lipman et al., 2005).

Selection of the target proteins from the allergenic source should matched as closely as possible to the target food tested to ensure high detection efficiency in the developed ELISA. The processed form of the allergenic protein(s) is usually the more ideal and favorable choice when it comes to producing polyclonal antibodies because the antibodies that are produced will be better at detecting allergens in processed foods. Crude extract of the allergenic food is typically used for development of antibodies as the main concern of the food industry is to determine whether there are any proteins from the allergenic source of interest that may be present as opposed to looking specifically for a specific clinically characterized allergenic protein. Nevertheless, concern in regards to sensitivity and specificity arises due to the presence of other components besides the analyte such as fats, carbohydrate, and polyphenols which might interfere with the immune response in the animal as well as in the developed analytical method. Using a specific allergenic protein that had undergone processing on the other hand will result in a higher sensitivity and specific antibodies in the absence of interfering components. The likelihood of cross-reacting with other sources will also be significantly lower. However, the down side of having to use processed samples is that processing steps might add complexity to the assay which could possibly reduce the robustness of the developed method (Karaszkiewicz, 2005).

Upon determination of the animal species to use for the immunization, the selected antigen is injected into these animals through subcutaneous and/or intramuscular

routes for antibody production, a process known as immunization. The antigen is usually accompanied by the use of an adjuvant during the immunization period to help boost the immune response in animals by increasing the immunogenicity. In addition to improved overall antibody titer with the addition of adjuvant, it also results in production of antibodies that have higher affinity for the target antigen (Reed et al., 2013). There are different classes of adjuvant available and choices will be made depending on the type of application. However, in the case of immunization, the most commonly used adjuvants are Complete Freund's Adjuvant (CFA) and the Incomplete Freund's Adjuvant (IFA).

The primary injection of immunogen is suggested to be at a lower dose because starting the injection with high dose may induce tolerance. Utilizing lower doses of immunogen initially has been shown to induce higher affinity and avidity of the antibodies that are produced. Booster injections are usually performed within three weeks following the first injection to maintain antibody with high titers. (Deshpande, 1996; Koppelman & Hefle, 2006). The amount used for booster injections is generally 10-50 % of the primary immunization dose because this favors the class switching of antibody from IgM to IgG with increasing affinity as booster immunizations continue. For the first injection, immunogen is prepared with CFA, where the oil emulsion contains nonionic detergent with heat-killed *Mycobacterium tuberculosis* bacteria whereas the immunogen for the following booster injections are prepared with IFA where the oil emulsion is without the heat-killed mycobacteria to avoid adverse responses in the laboratory animals (InvivoGen, 2015).

Throughout the immunization period, antisera produced by animals are closely monitored to ensure consistent production of antibody with high titers. The immunization

performance and antibody quality is determined by quantifying the mid-linear portion of the titration curve using the indirect ELISA (Hefle et al., 2001). After careful consideration on production bleeds from each animal, individual bleeds that have consistent and high titer values are often pooled to form a homogenous antiserum that is then used in the development of an ELISA. It is crucial to select antigen-specific antibody that will be used as capture and detection antibody, so that the developed immunoassay will be sensitive, specific, and robust.

b) Optimizing Assay Operating Conditions of ELISA

Following the primary step of production of high quality antibodies, the next step in ELISA development is optimizing reagents and conditions which are crucial in achieving the optimum performance of the assay. Factors that need to be taken into consideration include solid phase support, sample preparation, types of buffers used as well as time and temperature for extraction and at each step of the ELISA (Mire-Sluis et al., 2004).

The surface to which antigen and antibodies are attached to should be a strong carrier, and one that is most commonly used in ELISA is the 96-well polystyrene microtiter plate, which is known for its high throughput and versatility. Additionally, the overall efficiency also relies upon the capacity of protein in attaching to the surface of the plate. High titer antibodies tend to have greater binding capacity as compared to the lower titer counterparts. Furthermore, antiserum should be loaded onto the plate at a concentration that is not too high that proteins get overcrowded nor too low which could lead to an insufficient amount of antibodies present to bind with antigen and also

increases the potential for non-specific binding to the plate if open sites are not properly blocked. At a highly concentrated antiserum level where wells are overloaded with proteins, proteins tend to dissociate more easily from the plate as the bonds that hold interactions between proteins are weaker than the hydrophobic interaction between proteins and the plate. This phenomenon will therefore lower the binding efficacy of proteins (Kemeny & Challacombe, 1988).

To assure accurate and reliable analysis of antigen that may be present in samples, each step in preparing for the final test samples to be determined in developed ELISA from processed foods plays an important factor. This is especially crucial with samples that are highly processed and contain complex matrices. Phosphate buffered saline (PBS) usually works well in simple extraction for most food samples, but additional additives might need to be coupled with the PBS buffer when attempting to extract proteins from food matrices that may have high concentrations of tannin and other phenolic compounds or those with high fat content. Additives that are typically used and have been shown to effectively remove interfering components by improving the extraction efficiency include non-fat dry milk (NFDM), fish gelatin, and Tween 20 (Costa et al., 2015; Keck-Gassenmeier et al., 1999). Prior to extraction, samples need to be properly ground and mixed to ensure the homogeneity of the potential allergen residue in the sample matrix. Non-homogeneous mixtures may result in inconsistent measurement and are more prone to error during analysis. In addition, a sufficient number of subsamples from each sample should be taken during the validation phase of an ELISA so that the variability of both the assay and the sample preparation can be accurately assessed. This will also ensure

that the test results are consistent and provide a reliable result each time the assay is run (Lipton et al., 2000).

Different buffers will be used throughout the process of running an ELISA such as the coating, blocking, washing, and extraction buffers. The choice of the buffer to be used at each step will depend on types of buffer and their respective conditions such as pH and temperature that provides the optimal result in the developed ELISA. Coating buffer helps stabilize the antibody or antigen that is used to coat the ELISA plate and the most commonly used buffer for coating is carbonate or bicarbonate buffer at pH 9.6 (Bio-rad, 2016). Blocking buffer serves as an agent in preventing non-specific binding by blocking out the unoccupied areas on the microtiter plate where protein/antibody has not bound (ImmunoChemistry Technologies, 2016). Proper blocking will also aid in reducing the background signal and this significantly improves the accuracy of the immunoassay. The two common reagents used in blocking buffers are detergents and proteins. Tween 20 is an example of a detergent that is used to temporarily block the surface and can be washed away following each washing step. Unlike detergent, proteins provide permanent blockage to the surface of the plate and thus only need to be added once after the microtiter plate is coated with the capture molecule. Protein blockers also helps stabilize and space out the biomolecules bound to the plate, which reduces denaturation problems and steric hindrance associated with solid phase assays. Bovine Serum Albumin (BSA), fish gelatin and non-fat dry milk are examples of protein-based blocking buffer additives (Gibbs, 2001).

On the other hand, phosphate buffered saline, PBS at pH 7.4 is most frequently used as washing buffers and in simple extraction. In terms of extraction, the exact same

buffer might extract a high amount of protein from one food type but not others. Therefore, it is important to analyze which buffers work best on extracting protein from the specific allergenic source of interest as well as extracting these proteins from the desired test sample matrices. The period of time at which samples are extracted will also affect the extraction efficiency. A majority of allergens were found to be efficiently extracted within an hour of extraction time while only some allergens were seen to be persistent over in a day long extraction period (Portnoy et al., 1993). The incubation period which allows the interaction between antibody and antigen, proteins and microtiter wells, as well as time allocated for the enzymatic reaction to takes place for color development should also be analyzed and optimized. It was found that the optimum times for antigen-antibody binding range from a one to two hour time frame (Crowther, 2002). Furthermore, other conditions such as temperature and pH were also found to significantly impact the amount of protein being extracted. Typically, plates are coated at 4°C overnight and incubated at 37°C for the successive steps. Studies have shown that buffers operating at neutral to basic pH, ranging from pH 8 to 11, yield the optimal results (Poms et al., 2004).

VI. SUMMARY

Food allergies are a growing concern worldwide and the prevalence has been reported to be increasing, especially in children. Proteins are the major cause of food allergy and these proteins are usually highly resistant to heat and proteolysis. The majority of the IgE-mediated food allergies are caused by the Big 8 food group, which includes peanut, tree nuts, egg, cow’s milk, fish, crustacean shellfish, soybean, and wheat. Symptoms of an allergic reaction to foods can vary from mild nausea, diarrhea,

and eczema, to more severe symptoms such as asthma and anaphylactic reactions involving multiple organ systems including the cardiovascular system.

Macadamia nut is one of the tree nuts that is less frequently associated with adverse food reactions compared to almond, walnut, and cashew. Nevertheless, the consumption of macadamia nut is on the rise because of its potential nutritional benefits that helps improve the overall health profile. To date, a few macadamia proteins have been identified and the clinical significance of these potential allergens has not been thoroughly characterized yet. Studies have demonstrated IgE binding to macadamia nut proteins associated with the 2S albumin and 7S storage globulins structural family of proteins that have been characterized in several other foods.

There is currently no cure for food allergy, thus the best way of prevention is to practice complete avoidance of offending food, paying close attention to allergen labeling declarations in the ingredient panel as well as adhering to avoidance of products bearing precautionary allergen labeling. Additionally, consumers with food allergies should have injectable epinephrine always available. However, with all the precautions stated, the unintended incorporation of allergens into packaged and processed foods could provoke an allergic reaction. Therefore, it is crucial to have reliable detection methods available that help assess the presence of allergenic residues. ELISA is currently the preferred method and a routinely used technique by the food industry and regulatory agencies in detecting and monitoring the safety of food products. In addition, the food industry also relies on ELISA to help investigate food allergy incidents and in complying with allergen labeling regulations. Hence, the objective of this study was to develop a sensitive, specific, and robust sandwich ELISA for detection of macadamia nut residues in

processed foods. The developed assay is expected to be able to facilitate allergen control measures, assist food companies in complying with FDA guidelines and help lower the risk of a food allergic reaction in macadamia allergic individuals, so they could enjoy wider food choices and a better quality of life.

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CHAPTER 2: CHARACTERIZATION OF SOLUBLE MACADAMIA NUT PROTEINS AND PRODUCTION OF POLYCLONAL ANTIBODIES AGAINST RAW AND ROASTED MACADAMIA NUT

I. INTRODUCTION

Food allergy is a growing concern as it has reportedly increased in prevalence in recent years and can be associated with severe or even fatal reactions in both adults and young children. The increase in prevalence of food allergy has resulted in heightened awareness of this public health issue where preventive measures and regulations have been implemented to assure the safety of allergic consumers. Labeling is very crucial as it is the main source of information on the presence of allergenic ingredients in pre-packaged food products. Consumers with food allergies rely on proper and accurate labeling to ensure their safety (Mills et al., 2007). Of the 5.3% of the population that self-reported food allergy in one survey of adults in the United States, it was found by a majority of respondents that one of the key issues in managing their allergy condition was interpretation of product labels (Vierk et al., 2007). The Food Allergen Labeling and Consumer Protection Act (FALCPA) of 2004 was enacted in an effort to provide more clarity and transparency on pre-packaged food labels that contain one or more of the priority allergens (Gendel & Zhu, 2013). Despite the implementation of FALCPA and allergen control programs in food processing facilities, undeclared food allergens remain as one of the primary causes of most voluntary recalls associated with FDA inspected product with the most common cause of these recalls associated with incorrect packaging or labeling errors (Gendel & Zhu, 2013).

It has been estimated that perhaps as many as 150-200 deaths occur per year due to food allergies with many of these fatalities occurring in young children (Patel et al.,

2011). Data from US Centers for Disease Control and Prevention show that the prevalence of food allergy among children ranging from 0 to 17 years has increased from 3.4% in 1999 to 5.1% in 2011 (1.7% increase) (Sicherer & Sampson, 2014). Tree nuts are listed as one of the leading causes of severe allergy symptoms and life threatening anaphylaxis among the Big 8 food groups, which include tree nuts, peanuts, egg, milk, fish, crustacean shellfish, wheat, and soybean (Jarvinen, 2011). Walnut, cashew, and pecan have been reported as the most common implicated tree nuts among allergic individuals which account for approximately 30%, 30%, and 14% of all reactions respectively and are often associated with moderate to severe allergic reactions (Fleischer et al., 2005; Sicherer et al., 2001). Among 141 children with peanut and cashew nut allergy, it was found that children allergy to cashew nut more frequently experience severe clinical reactions as compared to those with peanut allergy (Clark et al., 2007). Thus, a robust, specific, and sensitive analytical method is needed for the detection of allergenic residues in food products. Enzyme-linked Immunosorbent Assays (ELISAs) are currently the most reliable and routinely used analytical methods by the food industry and regulatory agencies for monitoring the safety of food products (Poms et al., 2004). The sandwich ELISA is an immunochemical method that commonly uses polyclonal antisera containing IgG antibodies specifically directed against proteins from the allergenic source of interest. Typically, two different species of animals with polyclonal antibodies directed against proteins (antigens) from the allergenic source of interest will be used in the development of a sandwich ELISA and each antisera contains numerous antibodies that have the ability to collectively recognize multiple epitopes found on the various proteins. These multivalent interactions between antibodies and antigens results

in a system that is more tolerant to minor changes in protein structure and epitopes that may occur on some proteins as a result of processing (i.e. if one epitope is disrupted, there are other antibodies available that will recognize other protein epitopes from the source of interest). Polyclonal antibodies are also more favorable as compared to monoclonal antibodies as they tend to be less susceptible to changes in pH and salt concentration (Lipman et al., 2005).

The efficiency of the extraction buffer in extracting target soluble proteins is crucial for the development of a sensitive and robust ELISA. So, various conditions have to be taken into consideration such as pH, concentration of salt, and extraction time and temperature. Several studies have shown that extraction at neutral pH is more effective than acidic or alkaline solutions. The process of heating was found to help improve the separation of proteins and optimize the conditions for solubilization (Wang et al., 2012).

The objective of this study was to develop highly specific polyclonal IgG antibodies against raw and roasted macadamia nut proteins for the development of a robust sandwich ELISA for detection of macadamia nut residues in processed foods.

II. MATERIALS AND METHODS

a) Macadamia Nut Immunogen Preparation

Both shelled raw and roasted macadamia nuts were purchased from bulk.com (Boston, MA). Six hundred grams of each whole raw and roasted macadamia nut were washed in reverse osmosis (RO) water and thoroughly dried with multiple layers of paper towels to remove any extraneous residue that may have been present on the surface of the nuts. A total of 6 washing and drying cycles were carried out followed by air drying under a fume hood for 24 hours. The macadamia nuts were then ground into a fine particle size using a freezer mill (SPEX 6850). The total protein content of ground macadamia nuts was determined by the Dumas nitrogen method using a LECO thermogravimetric system (LECO Corporation, St. Louis, MO). A portion of the raw and roasted ground macadamia nut was used for subsequent immunization of laboratory animals to produce polyclonal antibodies as discussed below. The remaining ground macadamia nut materials were stored at -20°C until needed for further analysis.

b) Polyclonal IgG Antibody Production

Three rabbits, a sheep and a goat each were used to generate polyclonal IgG antiserum for raw and roasted macadamia nuts, respectively. A standard immunization protocol (Harlow & Lane, 1988) was adapted for the production of polyclonal antibodies at Covance Research Products (Denver, PA). For the initial immunization, each rabbit was injected subcutaneously with 250 µg of macadamia protein at multiple sites while goat and sheep were injected with 1500 µg. The protein quantity was based on the total protein content as measured by the Dumas method. The ground nut paste (including the

protein fraction) was suspended in Freud's Complete Adjuvant (FCA) to form an emulsified slurry. For each rabbit, booster injections were administered at 21 day intervals by mixing 125 µg macadamia nut protein in Freund's Incomplete Adjuvant (FIA) for the first booster and then TiterMax Classic on the second booster through a subcutaneous route. A rotation of adjuvants was used for these subsequent boosters (two months with FIA then following a month later with TiterMax Classic). At day 10 and 24 following each booster injection, test or production bleeds were collected to monitor the production of macadamia nut-specific IgG antibodies. As for the goats and sheep, the exact same protocol was used except that 750 µg macadamia nut protein was used during booster immunizations. Test bleeds from the goats and sheep were collected 10 days post booster injection to monitor the antibody production.

c) Extraction of Soluble Macadamia Proteins

Protein solubility is an important factor towards accurate and reliable results. Optimizing extraction enables better expression of soluble protein, which helps generate good titer values and precise assessment of antisera as well as development of a sensitive and robust ELISA. To evaluate the extraction efficiency of raw and roasted macadamia nut proteins, various extraction buffers were utilized and tested. These included:

- 0.01 M PBS with 0.5 M NaCl (pH 7.2)
- 0.01 M PBS with 1.5 M NaCl (pH 7.2)
- 0.01 M Tris HCl (pH 7.5)
- Borate Saline Buffer (BSB; 0.1 M Boric Acid + 0.025 M Sodium Borate) (pH 8.5)

- 0.025 M Tris HCl with 1.5 M NaCl (pH 7.5)
- 2 M NaCl solution (pH 6.8)

A comparison on extraction efficiency of soluble macadamia nut proteins was conducted at both room temperature (RT) and at 60 ºC. Prior to protein extraction, both raw and roasted macadamia nuts were prepared as described previously. Thoroughly ground raw and roasted nut samples were extracted at 1:10 ratio (w/v) at room temperature and at 60 ºC in a shaking water bath for 1 hour. The heated extracts were allowed to equilibrate for 10 minutes followed by centrifugation (using Sorvall® Legend ™ RT, Kendro Laboratory Products, Newton, CT) at 3020 x g for 30 minutes at 10 ºC. The supernatant was then collected, aliquotted and frozen at -20°C until needed for further analysis. The Lowry method (Lowry et al., 1951) was used to estimate the soluble protein content of each sample extract. Of all of the extraction buffers that were evaluated, 0.01 PBS with 0.5M NaCl (pH 7.2) was found to be the optimum buffer for extracting both raw and roasted macadamia nut proteins at both temperatures, with extraction at 60 ºC for 1 hours showing the best result (Table 2.1). Based upon this analysis we chose to use the 0.01 PBS, 0.5M NaCl (pH 7.2) extraction buffer for all subsequent experiments outlined in this chapter. The extractions were carried out at 60 ºC for 1 hour.

Extraction Buffers	Soluble Protein Content of Raw Macadamia Nut (mg/ml)		Soluble Protein Content of Roasted Macadamia Nut (mg/ml)	
	RT*	60ºC	RT	60ºC
0.01 M Tris HCl (pH 7.5)	7.0	7.0	6.3	7.5
BSB (pH 8.5)	5.3	6.1	7.4	7.3
0.01 M PBS w/ 0.5 M NaCl (pH 7.2)	7.5	9.0	7.4	11.0
0.01 M PBS w/ 1.5 M NaCl (pH 7.2)	6.1	7.5	7.7	9.1
0.025 M Tris HCl w/ 1.5 M NaCl (pH 7.5)	6.4	9.1	6.9	11.0
2 M NaCl solution (pH 6.8)	3.6	8.5	7.0	10.8

Table 2.1 Soluble protein content of raw and roasted macadamia nut as estimated by the Lowry method.

*RT- Room Temperature

d) Titer Determination

Antisera produced by each animal were evaluated and monitored by determining the titer values at 3 different soluble protein concentrations coated on the microtiter plates, 10 µg/ml, 1 µg/ml, and 0.1 µg/ml, respectively. As described by Hefle et al. (2001), a non-competitive ELISA was performed by coating 3 different dilutions macadamia nut extract with coating buffer (0.5M carbonate bicarbonate, pH 9.6) in a 96 well microtiter plate (NUNC-Immuno™ MaxiSorp™ 96-MicroWell™ plates, Nagle Nunc Intl., Rockester, NY, USA). The plate was then incubated overnight at 4°C after 100 µl of solution was dispensed into each well. The plates were then washed four times with conjugate buffer consisting of 0.025 M PBS (0.005 M NaH2PO4, 0.02 M Na2HPO4, 0.85% NaCl, pH 7.4 containing Tween 20) using an automated microplate washer (AM60, Dynex Technologies, Inc., VA). 350 µl of blocking buffer [0.025 M PBS, 0.1% gelatin (300 bloom, porcine, Sigma-Aldrich Co., St. Louis, MO) pH 7.4] was added into each well followed by a 1 hour incubation at 37 ºC. Plates were then washed again with the plate washer using the BLOCK setting which consist of four cycles and each cycle with (100, 200, 300, and 100 µl) of wash buffer dispensed respectively in each well, and then 10-fold serial dilutions (with conjugate buffer) of macadamia nut-specific rabbit, goat, or sheep antibody was added (100 µl) to each well of the plate and incubated at 37 ºC for 2 hours. Following another washing cycle, each well was filled with 100 µl of conjugate buffer containing a 1:5000 dilution of alkaline phosphatase-conjugated anti-species IgG antibodies [rabbit anti-goat IgG, rabbit-anti-sheep IgG, and goat anti-rabbit IgG (Immunopure®, Pierce Biotechnology, Inc., Rockford, IL] and incubated for 2 hours at 37 ºC. After the washing step, 100 µl of enzyme substrate solution, p-nitrophenyl

phosphate (p-NPP SigmaFast™ Tablets, Sigma Chemical Co., St. Louis, MO), was added into each well followed by a 30 minute incubation at RT in a dark environment. To stop the enzymatic reaction, 100 µl/well of 1 N NaOH was added and the absorbance was measured using a microplate reader (ELx808 Ultraplate, BioTek Instruments, Inc., Winooski, VT) at 405 nm. GraphPad Prism® v.4.03 software (GraphPad Prism® software, Inc., San Diego, CA) was used to construct the titration curve of macadamia nut-specific IgG antibodies.

e) Protein Recognition of Animal Antisera for Raw and Roasted Macadamia Nut

i) 1-D Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

1-Dimensional SDS-PAGE was used to evaluate the protein profiles under reducing conditions as described by Fling & Gregerson (1986) using a Bio-Rad Mini Protean® Tetracell (Bio-Rad Laboratories, Hercules, CA). The soluble proteins of raw and roasted macadamia nuts were separated on the polyacrylamide precast resolving gels (15% Tris-HCl; 8.6cm x 6.8cm x 1.0mm; Bio-Rad Laboratories, Hercules, CA) based on their molecular weight. To prepare protein samples, a 1:1 ratio of macadamia nut extract and Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue; Bio-Rad Laboratories) containing 54 mg dithiothreitol (DTT Cleland's Reagent; Bio-Rad Laboratories) were mixed and heated in a boiling water bath for 5 minutes, then allowed to cool down to room temperature. The mixture was then centrifuged at 16200 x g for 5 minutes. The supernatant was collected for subsequent loading into the wells of the SDS-PAGE gel. Running buffer was prepared by diluting 10X Tris/Glycine/SDS Buffer (Bio-Rad Laboratories) with RO water in a 1:10 ratio. Before running the gel, 5 µl of standard molecular weight markers (Precision Plus Protein Dual Color Standards, Bio-Rad Laboratories) and 15 µg of the protein extract from each sample were loaded into separate wells. Electrophoresis was run at 200 volts for 30 minutes. Fixing solution was prepared by diluting the 5X Concentrate (60% (w/v) 77 trichloroacetic acid and 17.5% (w/v) 5-sulfosalicylic acid, Sigma-Aldrich, Inc.) with RO water in a 1:5 ratio. The gel was then submerged in the prepared fixing solution and gently rocked for 30 minutes to fix the proteins to the gel and followed by overnight

staining with Coomassie Brilliant Blue R-250 Staining Solution (Bio-Rad Laboratories). The gel was destained on the following day to remove residual dye by adding Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad Laboratories). A Kodak Gel Logic 440 imaging system (Eastman Kodak Company) and the corresponding Kodak 1D v.3.6.5 software (Kodak Scientific Imaging System, New Haven, CT) was used to capture an image of each gel.

ii) IgG Immunoblotting (Western Blotting)

1-D SDS-PAGE was followed by IgE immunoblotting to determine the binding affinity and specificity of the macadamia nut-specific IgG antibodies produced by each animal using methods previously described by Koppelman et al. (2004). Soluble macadamia nut proteins were separated by 1-D SDS-PAGE under reducing conditions as described above. After electrophoretic separation, the gels were then allowed to soak in blotting buffer (1X Tris/ Glycine Buffer, Bio-Rad Laboratories; 20% Methanol, Analytical Grade, Fisher Scientific) for 15 minutes with gentle rocking. Separated protein bands from the gels were transferred onto a polyvinylidene difluoride (PVDF) membrane (Immunoblin-P PVDF membrane, 0.45 µm, Millipore Corporation, Billerical, MA) by electrophoresis which was run at a constant voltage of 100 V for 1.5 hours. Membranes were then washed for 3 times with RO water for 5 minutes to remove any remaining blotting buffer. The membrane was then blocked at room temperature for 2 hours with 0.01 M PBS containing 0.05% Tween 20 (PBS-T, pH 7.4) with 0.2% bovine serum albumin (BSA, RIA grade, USB Corp., Cleveland, OH). Subsequently, pooled animal antisera containing macadamia nut-specific IgG antibodies were diluted 1:10,000 (v/v)

with blocking buffer, added onto the membrane and incubated for an hour at room temperature under gentle rocking. Later, the membranes were washed with wash buffer (0.01 M PBS with 0.05% Tween 20) four times at five minutes each and were then incubated for another hour with the corresponding secondary antibodies. These commercially available secondary antibodies (rabbit anti-goat IgG antisera, rabbit anti-sheep IgG antisera, and goat anti-rabbit IgG antisera) which were conjugated with horseradish peroxidase (Immunopure®, Pierce Biotechnology, Inc., Rockford, IL) were diluted 1:25,000 (v/v) with blocking buffer. After another wash, the membranes were then incubated with DAB (3‘3 – diaminobenzidine) substrate solution (Pierce Technology, Inc., Rockford, IL) at room temperature until the bands were well developed for visualization and image capture. A final wash was done to remove excess substrate and to stop the reaction, which was followed by thorough air drying before capturing the images with a Kodak Gel Logic 440 Imaging System (Eastman Kodak Company) and Kodak Gel Logic ID v. 3.6.5 software (Kodak Scientific Imaging Systems, New Haven, CT).

III. RESULTS AND DISCUSSION

a) Titer Determination

IgG antibodies directed against roasted macadamia nut proteins and produced by the five immunized animals (rabbit NE 338, rabbit NE 339, rabbit NE 340, goat N 1178, and sheep G 786) were monitored by determining their titer values throughout the immunization period. Titers were carried out by performing a non-competitive, indirect ELISA in a 96 well microtiter plate as discussed previously. The enzyme-substrate complex yielded a yellow colored product after the addition of p-NPP and the intensity of

the color is directly proportional to the amount of specific antibodies present in the animal's antisera. GraphPad Prism software was used to generate titration curves by plotting log antibody dilution on the x-axis and absorbance values on the y-axis. The log reciprocal of the mid-linear portion of a titration curve was used to determine the titer value. An example of a titration curve generated from a bleed dated on 8/3/15 of rabbit NE 338, coated with 1.0 µg roasted macadamia nut protein/ml is shown on Figure 2.1. The mid-linear portion of the titration curve corresponds to -4.727, the log antibody dilution and titer value was determined as antilog of 4.727 which equals 53,333. Animals with titer value of 10,000 or more were considered optimum to be use in the development of the sandwich ELISA. The cutoff value of 10,000 was previously established in the FARRP Laboratory as an arbitrary minimum accepted value of the antisera for further use in the development of sensitive sandwich ELISAs.

Production bleeds of each animal were monitored and compared to ensure that the production of IgG antibodies was consistent and that tolerance was not being developed (indicative of declining titer values over time). Figure 2.2 shows the immunization response of the rabbits (NE 338, 339, 340) to roasted macadamia nut while Figure 2.3 shows the immunization response of rabbit (NE 341, 342, 343) to raw macadamia nut. Figure 2.4 shows the immunization of both the sheep and goats to raw as well as roasted macadamia nut, respectively. Macadamia nut-specific IgG antibody produced by each of the rabbits had titer values greater than 10,000 by the first month post initial injection indicating a good immune response to the immunogen. The immune response in the sheep and goats was >10,000 by the second month of the immunization. Overall, all the animals with high titer IgG antibody values show an excellent immune response towards

both raw and roasted macadamia nut and could serve as a good foundation for the development of macadamia nut sandwich ELISA.

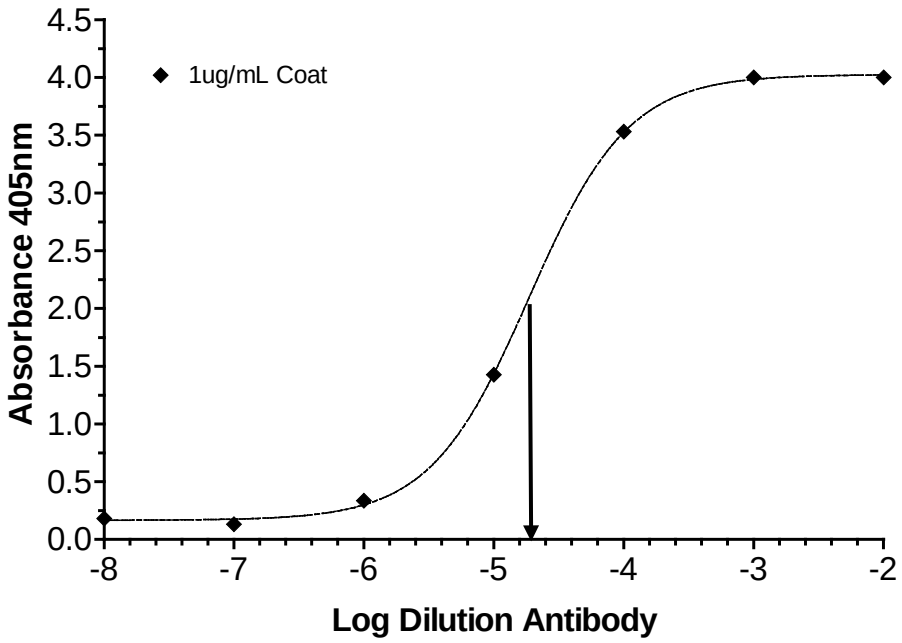

Figure 2.1. Titration curve generated from rabbit NE 338 (bleed date-8/3/15) anti-roasted macadamia antisera coated with 1.0 µg of roasted macadamia protein/ml. The vertical line indicates the mid-linear portion of the curve where the roasted macadamia nut IgG antibody titer was determined. Each data point represents the mean of triplicate readings and the standard error of each point is less than 0.1 AU.

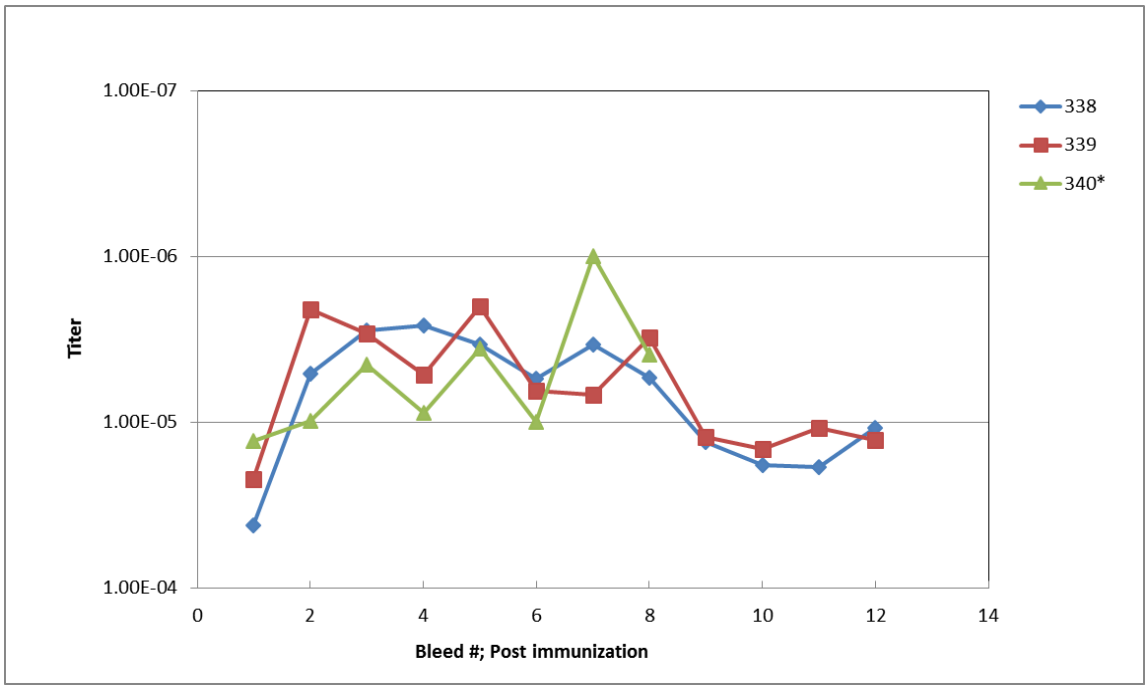

Figure 2.2 Immune response of each rabbit (NE 338, 339, 340) to roasted macadamia nut immunogen. Each of the data points indicates the mean of triplicate readings.

*Titers for rabbit NE 340 after the 8th bleed ceased due to high background and inconsistent antibodies production.

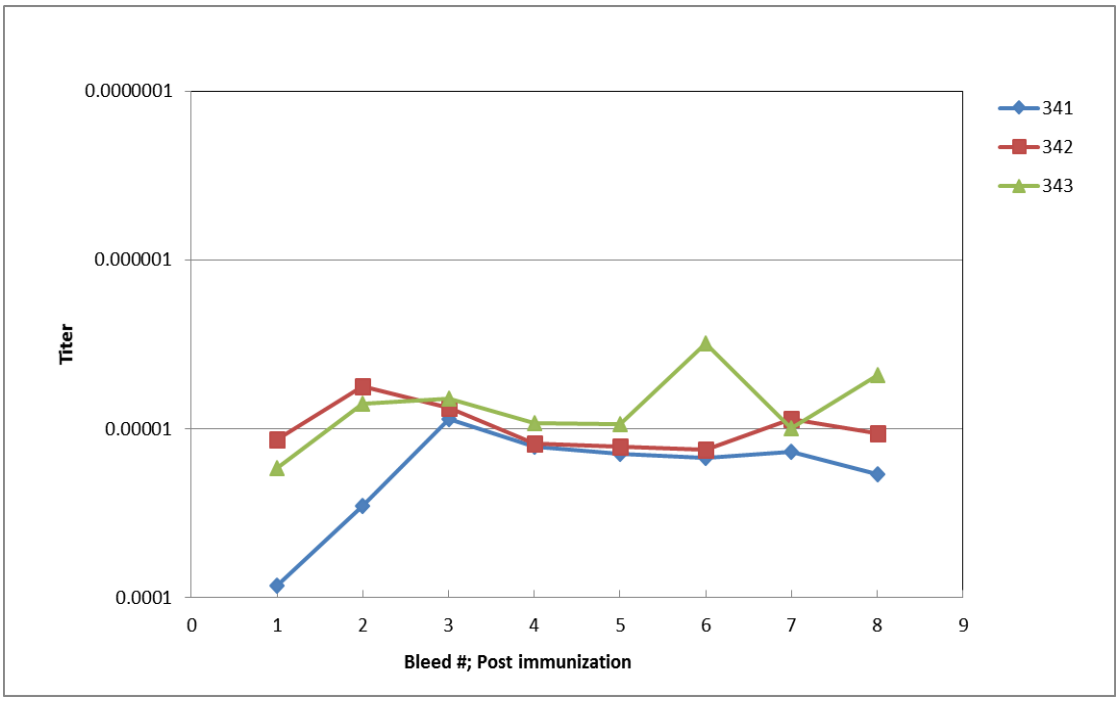

Figure 2.3 Immune response of each rabbit (NE 341, 342, 343) to raw macadamia nut immunogen. Each data point indicates the mean of triplicate readings.

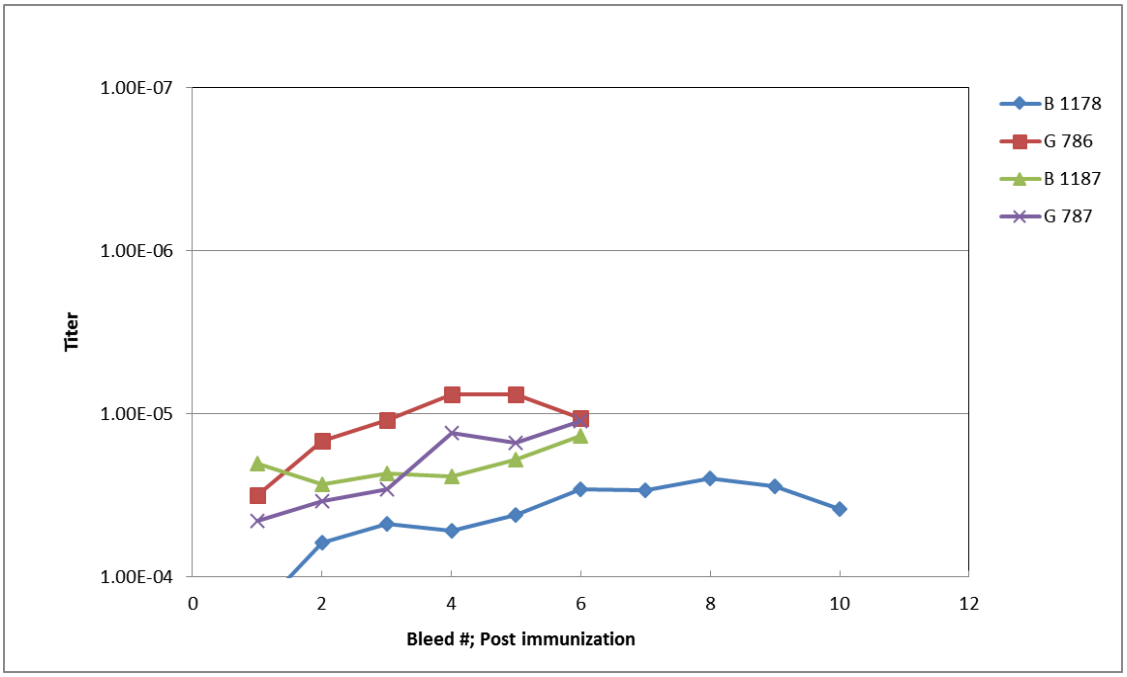

Figure 2.4 Immune response of goat and sheep immunized against raw and roasted macadamia nut immunogen. Each of the data points indicates the mean of triplicate readings.

*Titers for sheep G 786 (roasted) and G 787 (raw) ceased after the 6th bleed due to high background during development of sandwich ELISA when used as both capture and detection antibody

**Titers from goat B 1187, where antibodies were raised against raw macadamia, ceased after the 6th bleed as well.

b) SDS-PAGE and Immunoblotting

1-D SDS-PAGE under reducing conditions in combination with immunoblotting serves as a validation tool in determining the binding affinity and specificity of IgG antibodies directed against raw and roasted macadamia nut proteins. No significant differences were observed in the 1-D SDS-PAGE profiles of the raw and roasted macadamia proteins that were extracted with the various extraction buffers (Figure 2.5). Major polypeptide bands located at 17.4 kDa (Sutherland et al., 1999) and 45 kDa (Herbst et al., 2010) detected in both raw and roasted macadamia nut as discussed in other studies were clearly demonstrated in the gel profile indicating the presence of proteins believed to be associated with allergic responses in macadamia nut individuals. One band at approximately 19 kDa was not detected on both raw and roasted macadamia nut extracts when Tris HCl and Borate Saline Buffer were used as the extraction buffer. When PBS (1.5 M NaCl), Tris HCl + NaCl, and 2 M NaCl were used as buffer, the band at 19 kDa was detected in the roasted macadamia nut extract but only faintly detected in raw macadamia. Based upon the soluble protein analysis using the Lowry procedure and the 1-D SDS-PAGE profiles, the optimum extraction buffer was determined to be the 0.01 M PBS with 0.5 M NaCl as sufficient proteins across a broad range of molecular weights were present in the extracts of both raw and roasted macadamia nut. Similar protein profiles were observed with all extraction buffers that contained 0.5 M NaCl or higher so it appears that the higher ionic strength provided by the additional NaCl is needed to extract macadamia nut proteins across a wide range of molecular weights. We did not observe a significant increase in the quantity of protein or differences in the 1-D protein profiles when 1.5 or 2 M NaCl was included in the extraction buffers. So, 0.01 M

PBS with 0.5 M NaCl also provides the optimum extract buffer from an economic standpoint as well.

Figure 2.6, 2.7, 2.8, and 2.9 show the IgG immunoblot on the reactivity of antisera from 3 species (rabbit, goat, and sheep) raised against roasted and raw macadamia respectively. There was no significant difference observed between immunoblots from both raw and roasted macadamia nut extracts. Protein bands at approximately 8, 15, 17, 20, 26, 37, and 45 kD were clearly recognized by all antisera. However, two bands at approximately 20 and 37 KD for raw and roasted macadamia in immunoblots were strongly recognized by goat and sheep but qualitatively appears to have less recognition by the rabbit anti-macadamia nut IgG antibody. In contrast, a protein at 8 KD was faintly detected in goat and sheep immunoblot profile as compared to those in rabbits which shows an observable band.

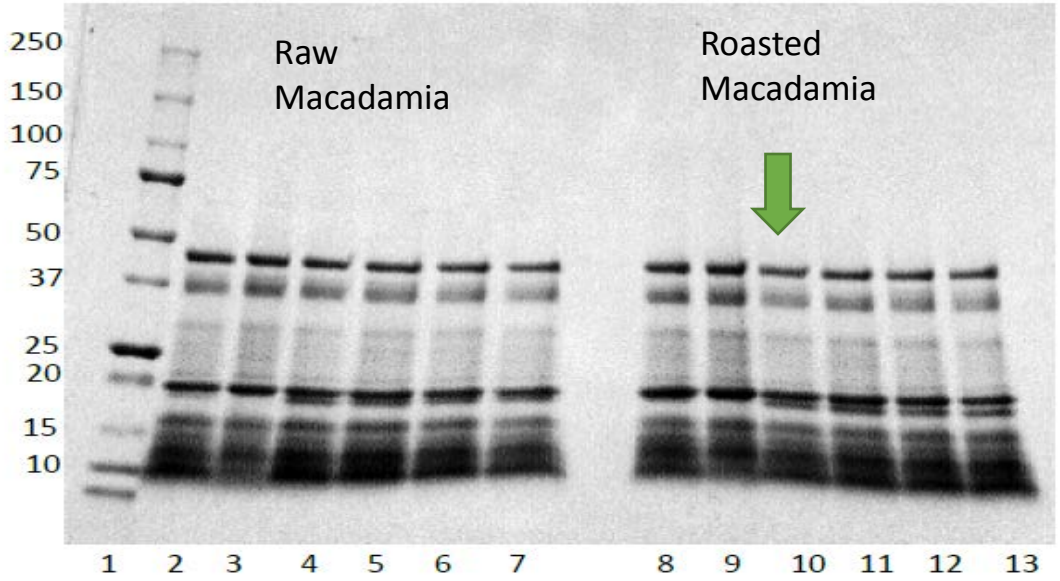

Figure 2.5 Brilliant Blue G-Colloidal stained 1-D SDS-PAGE profile of raw (lane 2-7) and roasted (lane 8-13) macadamia nut extracts under reducing condition. 15 µg of each raw and roasted macadamia protein was loaded onto each lane. Different extraction buffers were tested and the extractions were conducted at 60℃.

Lane 1 - Molecular weight marker
Lane 2 & 8 - 0.1 M Tris HCl
Lane 3 & 9 - Borate Saline Buffer (0.1 M Boric Acid + 0.025 M Sodium Borate)
Lane 4 & 10 - 0.01 PBS, 0.5 M NaCl
Lane 5 & 11 - 0.01 PBS, 1.5 M NaCl
Lane 6 & 12 - 0.025 M Tris HCl+ 1.5 M NaCl
Lane 7 & 13 - 2 M NaCl

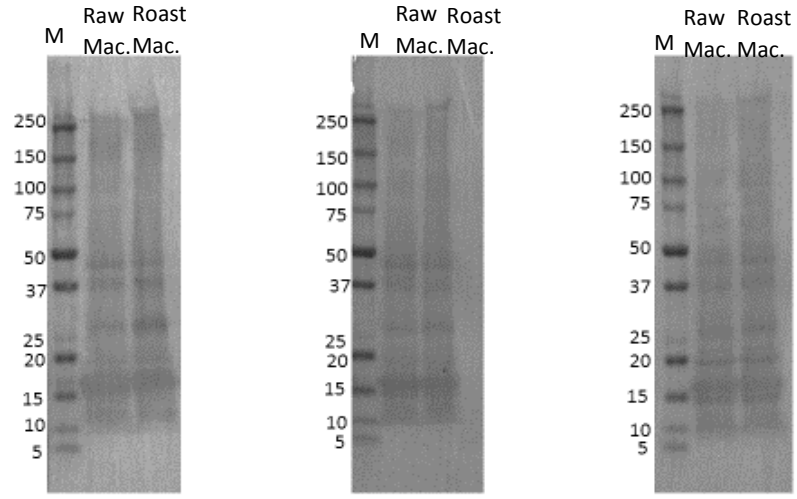

Figure 2.6 Rabbit anti-roasted macadamia nut IgG immunoblots of reduced raw and roasted macadamia extracts. [A] was probed with rabbit NE 338, [B] with rabbit NE 339, and [C] with rabbit NE 340 antisera. Each lane was loaded with 15 µg protein. M is molecular weight marker.

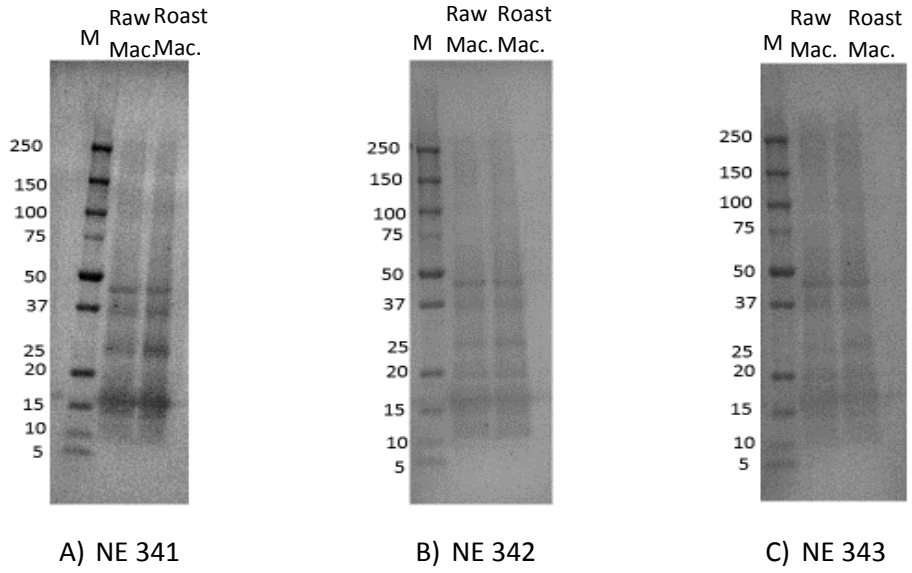

Figure 2.7 Rabbit anti-raw macadamia nut IgG immunoblots of reduced raw and roasted macadamia extracts. [A] was probed with rabbit NE 341, [B] with rabbit NE 342, and [C] with rabbit NE 343 antisera. Each lane was loaded with 15 µg of protein. M is molecular weight marker.

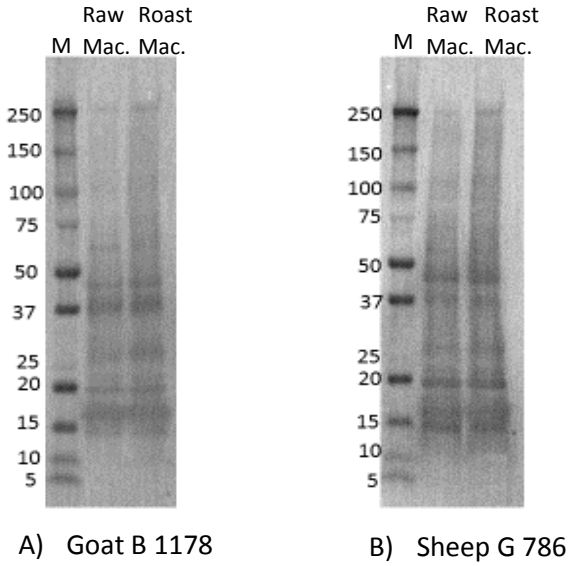

Figure 2.8 Goat and sheep anti-roasted macadamia nut IgG immunoblots of reduced raw and roasted macadamia extracts probed with [A] goat B 1178 and [B] sheep G 786 antisera. Each lane was loaded with 15 µg protein. M is molecular weight marker.

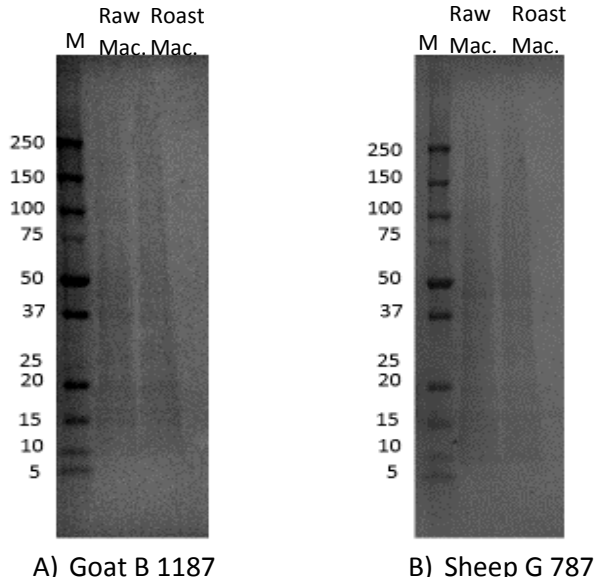

Figure 2.9 Goat and sheep anti-raw macadamia nut IgG immunoblots of reduced raw and roasted macadamia extracts probed with [A] goat B 1187 and [B] sheep G 787 antisera. Each lane was loaded with 15 µg of protein. M is molecular weight marker.

IV. CONCLUSIONS

Titer values of all the animal species were closely monitored to allow optimum selection of animals to be used in the development of a sandwich ELISA for detection of processed macadamia nut residue. 1-D SDS-PAGE analysis allowed us to determine the optimum buffer (0.01 PBS, 0.5 M NaCl, pH 7.2) for extraction of a wide molecular weight range of soluble proteins in both raw and roasted macadamia nut. IgG immunoblot analysis demonstrated that the animals mounted a robust immune response to the macadamia nut immunogen resulting in IgG antibodies with high affinity and avidity against raw and roasted macadamia nut proteins. These robust antibodies will serve as a strong foundation in development of the sandwich ELISA. The antibodies produced by the rabbits, goat, and sheep that were immunized with the roasted macadamia nut were able to recognize both raw and roasted macadamia nut residues with comparable affinity. Due to this fact, further efforts on the development of a macadamia nut ELISA discussed in detail in Chapters 3 and 4 focused on the use of the roasted macadamia antisera only.

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CHAPTER 3:DEVELOPMENT OF AN ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF MACADAMIA NUT RESIDUES IN PROCESSED FOODS

I. INTRODUCTION

The potential associated with improved quality of health and high nutritional value have contributed to the significant increase in the consumption of tree nuts in recent years (O'Neil et al., 2015). The nine commonly consumed tree nuts that are associated with a majority of tree nut-related food allergies are cashew nut, pecan, walnut, almond, hazelnut, macadamia nut, Brazil nut, pistachio, and pine nut (Teuber et al., 2003). Macadamia nut belongs to the family of *Proteaceae* of which there are nine species. There are two primary species that are consumed which are *Macadamia intergrifolia* and *Macadamia tetraphylla* (Ripperton et al., 1938). Macadamia nut seeds contain approximately 72.5% of fat, which is the highest total fat compared to all other tree nuts, 8.8% of protein, 7.1% dietary fiber and 1.5% water (Dreher et al., 1996 ; Silva et al., 2006). Of the 72.5% total fat, 57.3% is made up of monounsaturated fat (Fraser, 1999) which mostly consists of oleic acid; the remainder of the fat fraction consists of small amount of palmitoleic acid, palmitic acid, stearic acid, linoleic acid, arachidic acid, myristic and erucic acid (Venkatachalam & Sathe, 2006; Silva et al., 2006).

Macadamia nuts are often incorporated into baked goods and confections to enhance nutritional values and flavor. It has also been widely introduced into the daily diet as part of a suggested healthy dietary plan. Intake of macadamia nut was shown to help lowered cholesterol levels in individuals with hypercholesterolemia. Elevation of plasma monounsaturated fatty acids (MUFA) was observed following consumption of macadamia nut for four weeks, with a decrease in low density lipoprotein (LDL) from

5.3% to 3% and an increase in high density lipoprotein (HDL) by 7.9% (Garg et al., 2003). Despite the nutritional benefits of macadamia nuts for the majority of consumers, macadamia nut is consider as a primary food allergen and undeclared or undetected macadamia nut residues pose a potential food safety risk to individuals with macadamia nut allergies. So far, no cure exists for macadamia nut allergy (or any other food allergies). Thus, the best preventive strategy is avoidance of the offending food by those who have macadamia nut allergies by paying close attention to food labelling and having an injectable epinephrine kit always available (Sicherer & Sampson, 2014). However, even with all of the precautions previously mentioned, some reactions may still happen if vigilance is not exercised to minimize the opportunity for cross-contact or labeling errors that could occur during processing of pre-packaged food products. The risk of cross-contact of allergenic foods can also occur at retail bakeries and delis, at foodservice establishments, and at home. The lack of vigilance can lead to tragic outcomes for allergic consumers and potential litigation of the parties involved. An example of this involves a recent law suit that was filed towards Publix supermarket by the parents of an 11 year old nut allergic child who tragically died after eating cookies that were claimed by an employee not to contain any nuts and had no nuts disclosed on the label (Green, 2015). Therefore, employee training and especially accurate labeling on food packaging are vital to make certain allergic individuals can easily determine if a product is safe for consumption.

Access to reliable tools for detection of residue from food allergens is important in assessing and mitigating the risk of undeclared allergen residue. Reliable methods used for detection and validation should be highly specific, sensitive and robust. Protein based

immunoassays and DNA based polymerase chain reaction (PCR) are the two main methods for determination of residues from the allergenic source of concern (López-Calleja et al., 2015). A recent study was carried out on the development of a real time PCR method to detect macadamia residue (DNA) in processed food products (López-Calleja et al., 2015). High accuracy with a detection limit of 0.1 mg kg^{-1} and ability to detect macadamia residues in most of the commercial food products makes this technique one of the more sensitive and reliable choices in food inspection and detection (López-Calleja et al., 2015). However, there are some limitations with the use of PCR particularly the fact that these methods quantify DNA from the source of interest but not specifically the protein fraction which has been documented to cause allergic reactions associated with foods. The detection of DNA as a surrogate marker for detection does draw into question whether or not the actual protein(s) from the allergenic source of concern are present. The absence of DNA does not guarantee the absence of proteins or vice versa.

Competitive and sandwich ELISAs are the main approaches used to quantify protein and potential allergens in foods. When involving detection of small proteins or peptides, the competitive ELISA will be the best option while the sandwich ELISA is the most commonly preferred method when it comes to detection of intact proteins from the allergenic source of interest (Poms et al., 2004). Although there are a few commercial ELISA kits for detection of macadamia nut residue available in the market (López-Calleja et al., 2015), no peer-reviewed references are available which outline the performance of these methods for detection of macadamia nut residue in processed foods. Therefore, the performance of these kits is still uncertain, especially for the detection of

macadamia nut residue in processed foods. ELISAs are quick, highly specific and also a sensitive analytical method (Taylor et al., 2009). The objective of this study is to develop a robust, sensitive, and specific ELISA for detection of macadamia nut residue in processed foods. The development includes an extensive cross-reactivity study and matrix interference studies to verify the specificity and sensitivity of the developed assay.

II. MATERIALS AND METHODS

a) Development of Sandwich ELISA

The macadamia nut standard was developed by using roasted macadamia nuts purchased from bulk.com (Boston, MA). 10 g of ground roasted macadamia nuts were added to 100 mL of extraction buffer [0.01 M Phosphate Saline Buffer (PBS) with 0.5 M NaCl, pH 7.2] to achieve a 10,000 ppm macadamia nut (mg/ml) concentration. This solution was then extracted for 1 hour in a shaking water bath at 60 °C. The heated extracts were allowed to sit for 10 minutes followed by centrifugation at 3020 x g for 30 minutes at 10 °C using a tabletop centrifuge (Sorvall® Legend ™ RT, Kendro Laboratory Products, CT). The supernatant obtained was serially diluted 3-fold (1000, 333, 111, 37, 12.3, 4.11, 1.37, 0.45, 0.15, 0.05, 0.02 ppm macadamia nut) to develop a standard curve. A 0 ppm standard curve point also included which consisted of the extraction buffer only.

The protocol used for the development of a sandwich ELISA for detection of macadamia nut residue was adapted from Hefle et al. (2001). To optimize the conditions of the ELISA for suitable sensitivity (at or below 2.5 ppm macadamia nut), different combinations of capture, detection and conjugated antibodies (i.e. checkerboard

titrations) were performed to determine the optimum pairing which included combinations of rabbit-goat (capture-detector), rabbit-sheep, goat-rabbit, and sheep-rabbit pairings. Capture and detection antibodies were tested at various dilutions for each of the combinations where the capture antisera were diluted 1:1000, 1:5000, 1:7500, and 1:10,000 in conjugate buffer while detection antibody was diluted 1:5000 and vice versa. The conjugate antibody was diluted at 1:2500. Data from the numerous preliminary assay trials are not presented in this thesis and the following discussion will focus on the optimized conditions.

The first step of the optimized assay involved a 1:10,000 dilution of rabbit antisera (capture antibody) in coating buffer (0.015 M Na_2CO_3, 0.035 M NaHCO3, and 0.02% NaN3, pH 9.6), which was then loaded onto a 96 well microtiter plate (NUNCImmuno™ MaxiSorp™ 96-MicroWell™ plates, Nagle Nunc International, NY) and incubated overnight at 4 ºC. The coated plates were washed with wash buffer (PBS containing 0.05% Tween 20 and 0.02% NaN3, pH 7.4) for a total of four cycles and each cycle with (200, 300, 200, and 300 µl) of wash buffer dispensed respectively in each well using a microplate washer (AM60, Dynex Technologies, Inc., VA) after every incubation step, except for the step after substrate incubation. After the initial washing step, 350 µl of blocking buffer containing 0.1% gelatin (300 bloom, porcine, Sigma-Aldrich Co., St. Louis, MO) in 0.01 M PBS, pH 7.4, was added to each well and incubated at 37°C for 1 hour. The addition of 100 µl of the serially diluted roasted macadamia nut extract standards to each well was followed by 1 hour incubation at 37 °C. The captured macadamia nut proteins in each standard curve dilution bound to the coated IgG antibodies. A solution containing 100 µl of goat anti-roasted macadamia nut antisera

(detector antibody) diluted 1:5,000 in conjugate buffer (0.01 M PBS containing 0.1% Bovine Serum Albumin, pH 7.4), was added to each well and again allowed to incubate for 1 hour at 37 °C. After another washing step, 100 µl/well of rabbit anti-goat IgG (conjugate antibody) labeled with alkaline phosphatase (Immunopure®, Pierce Biotechnology Inc., IL) diluted 1:5000 in conjugate buffer was added to the plates followed by a 1 hour incubation at 37 °C. The plates were then incubated for 30 minutes in a dark environment after the addition of p-nitrophenyl phosphate substrate (p-NPP, SigmaFast™, Sigma-Aldrich Co., St. Louis, MO) which allowed the formation of a yellow colored product. To stop the enzymatic reaction, 100 µl of 1 N NaOH was added into each well and the intensity of the colored product was measured immediately at 405 nm using a microplate reader (ELx808 Ultraplate, BioTek Instruments Inc., Winooski, VT). Standard curves were generated using GraphPad Prism® v.4.03 software (GraphPad Software Inc., San Diego, CA). The intensity of the colored product is directly proportionate to the concentration of macadamia nut protein present in each standard.

b) Cross-Reactivity Studies

To evaluate potential cross-reactivity in the developed assay, a total of 86 food ingredients that are commonly used in baked goods and confections were purchased from local grocery stores in Lincoln, Nebraska. Liquid samples were used as is without further processing while non-liquid samples were ground into uniform fine particles using an Oster 10-speed blender. Each sample was extracted 1:10 (w/v) with 0.01 M PBS with 0.5 M NaCl (pH 7.2) at 60 °C in a shaking water bath (Julabo SW22, Julabo USA, Inc., Allentown, PA) at 150 rpm for 1 hour followed by centrifugation at 3020 x g for 30 minutes at 10 ºC. The clarified extracts were stored at -20 °C until needed for further analysis. The soluble protein content of each sample was determined by the Lowry method (Lowry et al., 1951). The individual extracts were analyzed by the developed macadamia ELISA based on a dilution basis of the extraction buffer (1:1, 1:10, 1:100 v/v) and based upon a soluble protein content (10µg/ml, 1µg/ml, and 0.1µg/ml). Results obtained from the cross-reactivity studies were expressed in ppm apparent macadamia nut equivalents by comparing the absorbance readings obtained from sample extracts with the absorbance readings of the roasted macadamia nut standard curve dilutions.

c) Matrix Interference Studies

Three food matrices, cookies, ice-cream, and dark chocolate, were chosen as reference food matrices to assess the overall sensitivity of the developed ELISA. Edy's Grand Vanilla Ice-Cream and Keebler® Sandies® Simply Shortbread Cookies were purchased from a local grocery store in Lincoln, Nebraska. Dark chocolate samples were

obtained from a batch of Barry Callebaut dark chocolate specifically manufactured as a negative control matrix for a previous study.

Prior to extraction, the shortbread cookies were ground thoroughly with an Oster 10-speed blender, while the ice-cream and dark chocolate were melted in a shaking water bath at 60 ºC. To prepare a 10,000 ppm macadamia nut spike, 10 g of ground roasted macadamia nut was added into 100 mL of extraction buffer (0.01 M PBS with 0.5 M NaCl, pH 7.2). The mixture was then extracted in a shaking water bath at 60 °C for 1 hour followed by centrifugation at 3020 x g at 10 °C for 30 minutes. A 1000 ppm macadamia nut standard was prepared by mixing 10 mL of the 10,000 ppm spike with 90 mL of extraction buffer (0.01 M PBS with 0.5 M NaCl, pH 7.2). This 1000 ppm macadamia nut solution was then spiked into each extraction bottle containing 10 grams of cookies, ice-cream and dark chocolate. Negative controls (0 ppm) were prepared by adding 10 grams of cookies, ice-cream, and dark chocolate into individual extraction bottles. In a separate extraction bottle, 10 mL of 0.01 M PBS with 0.5 M NaCl buffer was added and used as a negative control. A total of eight samples (four 1000 ppm of each macadamia nut-spiked shortbread cookies, macadamia nut-spiked vanilla ice-cream, macadamia nut-spiked dark chocolate, macadamia nut-spiked 0.01 M PBS, and four 0 ppm of each macadamia nut-free shortbread cookies, vanilla ice-cream, dark chocolate, and 0.01 M PBS buffer) were extracted 1:10 (w/v) with extraction buffer (0.01 M PBS, 0.5 M NaCl, pH 7.2) for 1 hour in a shaking water bath at 60 ºC. This step was then followed by centrifugation at 3020 x g at 10 °C for 30 minutes and the clarified extracts were stored at -20 °C until later used for macadamia nut ELISA analysis.

During the ELISA analysis, the 1000 ppm macadamia nut-spiked food matrices that were previously extracted were serially diluted 3-fold (333, 111, 37, 12.3, 4.11, 1.37, 0.45, 0.15, 0.05, 0.02, and 0 ppm) with 0.01 M PBS buffer to obtain 12 macadamia nut concentrations. These spiked samples were analyzed in the macadamia nut ELISA and the standard curves attained from each macadamia nut-spiked matrices were compared to the developed macadamia nut ELISA standard curve prepared in PBS buffer. A one-way analysis of variance (ANOVA) test was used to analyze the difference between standard curves where the significance level was reported at $p < 0.05$.

d) Effect of Extraction Buffer Additives on Dark chocolate, Cookies, and Ice-cream Matrices

An efficient extraction buffer is very crucial in assuring proteins are well extracted from the samples. Chocolate has always been a problematic matrix in immunoassay methods, including ELISA due to its high level of tannins and other phenolic compounds (Scaravelli et al., 2008). While evaluating our representative food matrices (dark chocolate, cookies and ice-cream) in the developed macadamia nut ELISA, the dark chocolate matrix was found to interfere with macadamia nut protein extraction likely due to interfering components present in the chocolate matrix. Therefore, further evaluation was performed by adding different additives into the extraction buffer to determine the effects of additives in reducing interfering compounds present in food matrices and to increase the extraction efficiency of the macadamia nut proteins.

Additives such as fish gelatin, non-fat dry milk (NFDM), and Tween 20 have been shown to effectively reduce interfering components when added into extraction buffers. A study done by Keck-Gassenmeier and colleagues (1999) showed that recovery of peanut proteins increases from 2-3% to 60-90% when fish gelatin was added during extraction. When extraction was performed with addition of NFDM, Costa et al. (2015) found that a standard curve in a chocolate matrix had significantly improved, indicating more proteins were extracted with increasing extraction efficiency. Thus, three different additives (fish gelatin, NFDM, and Tween 20) were chosen in this study to evaluate their effects in improving extraction efficiency.

A 1000 ppm macadamia nut standard in the matrices was prepared the same way as discussed previously in the matrix interference studies with the exception that a designated amount of the selected additive was added into the buffer prior to extraction. The additives tested for each food matrix were 1% fish gelatin, 5% fish gelatin, 1% NFDM, 5% NFDM, 0.5% Tween 20, and 1% Tween 20, with each additive tested individually for all 3 of the representative food matrices.

Clarified extracts for each matrix were analyzed with the developed macadamia nut ELISA. A standard curve was prepared by serially diluting the 1000 ppm macadamia nut in matrix extract to achieve the 12 macadamia nut standard concentrations mentioned previously. The standard curves derived from spiked samples were then compared to the macadamia nut ELISA standard curve prepared in PBS buffer and to those without the addition of the extract additive. A one-way analysis of variance (ANOVA) test was used to analyze the difference between standard curves to determine if they are significantly different from one another.

e) Statistical Analysis

In a sandwich-type ELISA, the absorbance (y-axis) is directly proportional to the protein concentration bound to the plate (x-axis). The limit of detection (LOD) of the macadamia nut ELISA was determined as the mean value of the blank (wells without macadamia nut) plus three times the standard deviation of the blank. The limit of quantification (LOQ) is defined as the lowest point on the linear portion of the standard curve. Three replicates for each macadamia nut standard curve in the three food matrices were performed to ensure the reliability and consistency of the results. The statistical difference between the standard curves was determined using a one-way ANOVA test (GraphPad Prism® v.4.03 software) to determine if the performance of the developed ELISA varied significantly by potential interference of the food matrices. The raw and roasted macadamia nut standard curves were also compared and analyzed using the unpaired t-test in GraphPad Prism ® Software to determine if the developed macadamia ELISA was able to detect both raw and roasted macadamia without significance difference where $p < 0.05$ was considered significant.

III. RESULTS AND DISCUSSION

a) Macadamia Nut Sandwich ELISA Standard Curves

Different combinations of capture and detection antibodies (rabbit/goat/sheep) raised against both raw and roasted macadamia nut were analyzed in the macadamia ELISA. Only the optimized pairs and dilutions are discussed in this thesis. The best combination occurred when rabbit was used as the coated capture antisera and goat as the detector antisera at a dilution of 1:10,000 and 1:5000, respectively. Thus combination provided higher sensitivity as compared to the other combinations in which the background of the negative control or 0 ppm concentration was often found to be higher, thus limiting the sensitivity and overall utility of the assay. The remaining studies discussed in this thesis are based on these antisera combinations and respective dilutions.

Figure 3.1 shows the raw and roasted macadamia standard curves from the optimized ELISA in PBS buffer. The background values of both standard curves were low (< 0.1 absorbance unit, AU), with a limit of quantification (LOQ) of < 1 ppm (µg of macadamia nut/ml or gram of sample). A background absorbance of 0.1 AU is considered excellent for an ELISA while the LOQ of 1 ppm macadamia nut meets our desired benchmark to support a food safety risk evaluation. Based on the result of an unpaired t-test according to (GraphPad Prism® v.4.03 software), there was no significant difference between the raw and the roasted standard curves, $t(22)=0.34$, p=not significant (ns). Qualitatively, the antisera directed against roasted macadamia nut did appear to detect the proteins from the roasted macadamia nut slightly better than from the raw macadamia nut. The IgG immunoblot studies as discussed from Chapter 2 support this finding as well. The developed ELISA could reliably detect both processed and

unprocessed macadamia nut residues in a buffer matrix indicating the potential versatility of the developed assay. Thus, the roasted macadamia standard curve was used for the remaining of the study.

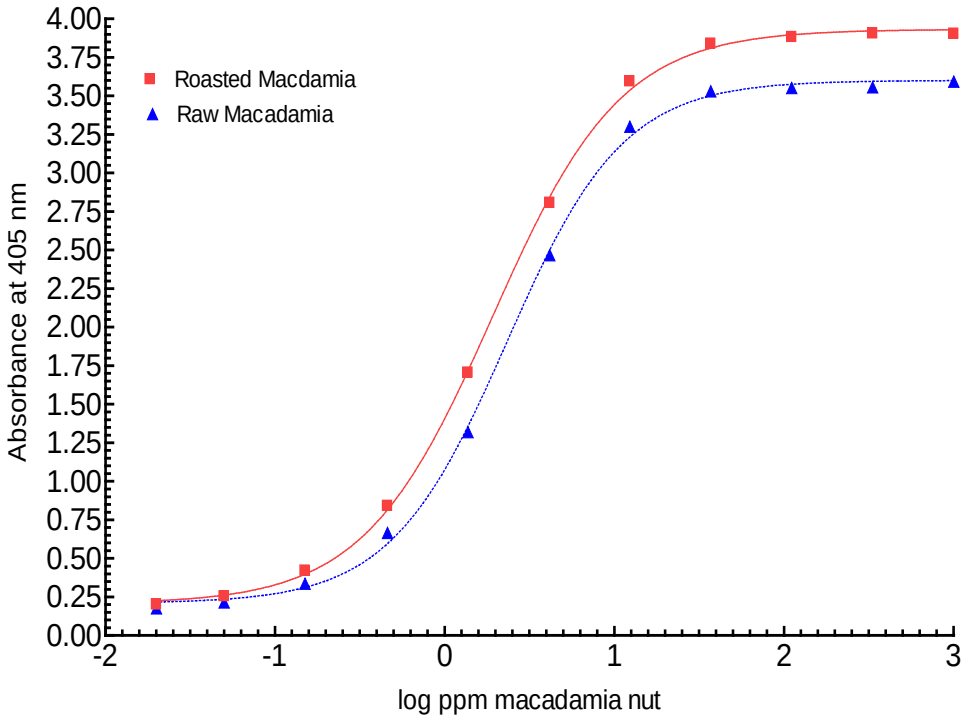

Figure 3.1 Buffer (0.01 M PBS, 0.5 M NaCl, pH 7.2) standard curves of raw and roasted macadamia nut as analyzed in the sandwich ELISA. The microtiter plates were coated with rabbit NE 338 antisera (1:10,000 dilution) followed by incubation with serially diluted raw/roasted macadamia extract. The detection antibody used was goat B 1178 antisera (1:5000 dilution). Similar standard curves were observed with rabbit NE 339 (data not shown).

b) Cross-Reactivity Studies

Individuals with multiple food allergies can react to foods within the same botanical group due to the cross-reactivity of IgE antibodies to homologous proteins. Tree nuts collectively are known to be the group of foods where cross-reactivity can be common (Immunology, 2016). Similarly, it is important to assess the potential cross-reactivity when developing an ELISA as these homologous proteins can also result in recognition of proteins from botanically similar food sources during the immunization of laboratory animals. Such cross-reactivity would limit the utility of the developed ELISA.

A total of 86 food ingredients that may be present in processed foods and those that are commonly used in baked goods and confections were evaluated in the developed macadamia nut ELISA for potential cross-reactivity (Table 3.1). Both rabbit antisera, NE 338 and NE 339 were individually used for evaluation of cross-reactivity. For the cross-reactivity evaluation based upon the soluble protein content (10µg/ml, 1µg/ml, and 0.1µg/ml) of the food extract, the only food extract that demonstrated a reading higher than the limit of quantification, (LOQ = 1.0) was nutmeg, with 1.4 and 1.5 ppm equivalent macadamia nut concentration respectively when either rabbit NE 338 and NE 339 antiserum were used as the coating antibody. This was observed at the 10µg/ml protein concentration only. However, this observation was likely due to the high color intensity of the spices rather than actual binding of nutmeg proteins to the antibodies. When the potential cross-reactivity was assessed by using a dilution series (0, 1:10, 1:100 v/v), only the pure extracts of all-spice, cinnamon, cloves, paprika, Brazil nut, poppy seeds, oregano, nutmeg, and cherries displayed minor cross-reactivity in the macadamia nut ELISA with qualitative detection ranging from 0.04 to 1.5 ppm of equivalent

macadamia nut (Table 3.2). The undiluted extracts may cause higher signal due to the high concentration of interfering substances in these ingredients, thus leading to an elevated background response when analyzing these pure ingredients that could be misinterpreted as a false positive result. Similar findings have been reported for ELISAs by others (Fæste et al., 2006). These minor cross-reactive or interfering foods are unlikely to cause a false positive problem when formulated into a processed food product as the amount of these ingredients used in food formulations are typically in very low concentrations as compared to the full strength extracts used in this study.

Table 3.1 Cross-reactivity analysis of various food and food ingredients in the macadamia nut ELISA

Ingredient	Soluble Protein Content (mg/ml)	Equivalent macadamia nut (ppm)	Ingredient	Soluble Protein Content (mg/ml)	Equivalent macadamia nut (ppm)
Tree nuts			**Legumes**		
Almond	21.8	<1.0	Carob powder	7	<1.0
Hazelnut	24.9	<1.0	Chickpea	22.8	<1.0
Walnut	7.3	<1.0	Green pea	9.8	<1.0
Cashew	22.4	<1.0	Lima beans	9.7	<1.0
Pecan	4.3	<1.0	Peanut	16.6	<1.0
Brazil nut	17.9	<1.0	Soy milk	4.5	<1.0
Pistachio	37.2	<1.0	**Cereals**		
Pine nut	18.2	<1.0	Buckwheat flour	19.7	<1.0
Coconut flakes	4	<1.0	Yellow cornmeal	9.7	<1.0
Coconut milk	0.6	<1.0	Oats	3.3	<1.0
Almond milk	0.4	<1.0	Potato flour	5.1	<1.0
Seeds			Rice flour	15.7	<1.0
Anise	7.1	<1.0	Tapioca flour	ND	<1.0
Caraway	16.1	<1.0	Whole wheat flour	8	<1.0
Celery	6.4	<1.0	Sorghum flour	16.1	<1..0
Fennel	10.8	<1.0	Quinoa flour	11.8	<1.0
Mustard seeds	44.1	<1.0			
Sesame	12.5	<1.0			
Flax	16.1	<1.0			
Poppy	16.6	<1.0			
Sunflower	12.8	<1.0			

Soluble protein content of extracts determined by Lowry method
ppm = part per million (µg/ml)
ND = soluble protein content not determined

Table 3.1 (continued) Cross-reactivity analysis of various food and food ingredients in the macadamia nut ELISA

Ingredient	Soluble Protein Content (mg/ml)	Equivalent macadamia nut (ppm)	Ingredient	Soluble Protein Content (mg/ml)	Equivalent macadamia nut (ppm)
Baking			**Spices/flavoring**		
Brown sugar	0.6	<1.0	All spice	19.3	<1.0
Butter	0.1	<1.0	Almond extract	0.1	<1.0
Cocoa powder	14.6	<1.0	Basil	ND	<1.0
Cream cheese	3.5	<1.0	Cinnamon	ND	<1.0
Cream of tartar	ND	<1.0	Cloves	ND	<1.0
Dark chocolate	7.3	<1.0	Cumin	13.9	<1.0
Dried egg white	397.4	<1.0	Garlic powder	15.6	<1.0
Whole egg	26.9	<1.0	Ginger power	13.5	<1.0
Egg yolk	30.1	<1.0	Nutmeg	2.4	1.38
Light corn syrup	0.1	<1.0	Onion powder	8	<1.0
Non-fat dry milk	36	<1.0	Oregano	33.8	<1.0
Honey	1	<1.0	Paprika	15.9	<1.0
Lemon juice	0.2	<1.0	Vanilla extract	1.8	<1.0
Liquid malt extract	9.7	<1.0			
Maltodextrin	0.1	<1.0			
Refined sugar cane	0.1	<1.0			

Soluble protein content of extracts determined by Lowry method
ppm = part per million (µg/ml),
ND = soluble protein content not determined

Table 3.1 (continued) Cross-reactivity analysis of various food and food ingredients in the macadamia nut ELISA

Ingredient	Soluble Protein Content (mg/ml)	Equivalent macadamia nut (ppm)	Ingredient	Soluble Protein Content (mg/ml)	Equivalent macadamia nut (ppm)
Fruits/veg			**Oil**		
Apple	0.5	<1.0	Canola	ND	<1.0
Blueberry	1.1	<1.0	Olive	0.2	<1.0
Broccoli	1.8	<1.0	palm	ND	<1.0
Cherries	4.3	<1.0	Peanut	ND	<1.0
Cranberry	3.9	<1.0	Sunflower	0.1	<1.0
Dates	4.7	<1.0	Macadamia nut	ND	<1.0
Kiwi	1.4	<1.0	**Food coloring**		
Banana chips	0.5	<1.0	Caramel color		<1.0
Fresh banana	0.1	<1.0	**Functional ingredients**		
Orange	0.8	<1.0	Guar gum	0.9	<1.0
Pineapple	1.2	<1.0	Xanthan gum	1	<1.0
Raisins	5.9	<1.0			
Mango	ND	<1.0			

Soluble protein content of extracts determined by Lowry method
ppm = part per million (µg/ml),
ND = soluble protein content not determined

Table 3.2 Ingredients with minor cross-reactivity or matrix inference detected in the macadamia nut ELISA

Ingredients	Cross-reactivity, equivalent macadamia nut (ppm)	
	Dilution (pure extract)	Dilution (pure extract)
	Rabbit 338	Rabbit 339
All spice	0.34	0.42
Cinnamon	0.34	0.49
Cloves	0.11	0.14
Paprika	0.04	0.11
Brazil nut	0.11	0.13
Poppy seeds	0.31	0.45
Nutmeg	1.30	1.47
Oregano	0.52	0.34
Cherries	0.32	0.18

*Three different dilutions were carried out (0, 1:10, 1:100 v/v), but cross-reactivity was only detected in pure extracts of the above ingredients. No detection was observed in diluted samples.
ppm = part per million (µg/ml)

	Protein Concentration, µg/ml	
	Rabbit 338	Rabbit 339
Nutmeg	1.38	1.47

*Three different protein concentrations were carried out (0.1, 1, 10 µg/ml), but cross-reactivity was only detected in nutmeg at 10 µg/ml. No detection was observed in lower protein concentration samples.
ppm = part per million (µg/ml)

c) Matrix Interference Studies

Matrix interferences can result in false positive responses but more likely cause false negative results in immunoassays due to the decrease in sensitivity that the matrix can cause on occasion. Interfering compounds present in samples and subsequent extracts can hinder the extractability of the proteins of interest, interfere with antibody-antigen binding, and interfere with the enzyme activity of the assay which in turn decreases the sensitivity of the assay (Lee et al., 2004; Khuda et al., 2012). Spiked food matrices (cookies, ice cream, and dark chocolate) were tested in the developed macadamia nut ELISA to evaluate potential matrix interferences in common food products where macadamia nut residue may need to be assessed and to determine the overall sensitivity of the developed ELISA. Figures 3.2 and 3.3 show the standard curves of all three food matrices with antiserum from rabbits NE 338 and NE 339 used as the capture antibody, respectively. Standard curves developed in the negative control extract of the various matrices were also compared to standard curve of the buffer control which was generated by extracting macadamia nut directly in 0.01 M PBS with 0.5 M NaCl, pH 7.4. Statistical analysis based on one-way ANOVA test indicated that there were no significant differences ($p < 0.05$) between the standard curves developed by spiking the cookie and ice-cream matrices with the macadamia nut. Both of these standard curves were very similar to the buffer control standard curve indicating that the matrix did not affect extraction of the spiked macadamia nut and did not interfere with the ELISA. However, it was notable that the dark chocolate standard curve was significantly less sensitive than the other two standard curves and the buffer control standard curve. These trends were observed when rabbit NE338 and NE339 antisera was used as the capture antibody

source. Interfering components such as tannins, polyphenols and fat content present in chocolate can greatly affect the detection of an analyte using antibody-based methods thereby reducing the sensitivity and efficacy of the assay (Poms et al., 2005).

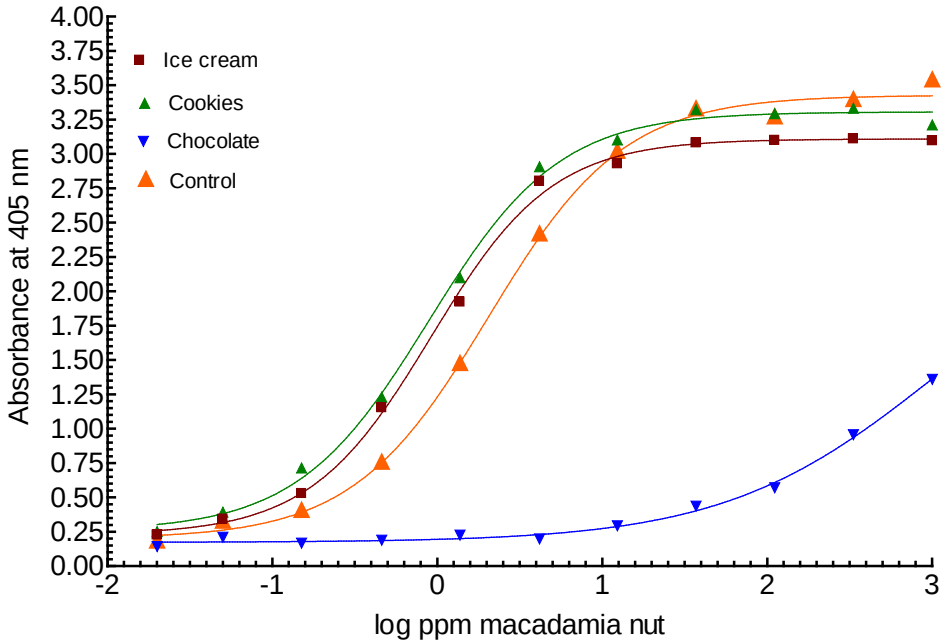

Figure 3.2 Roasted macadamia nut spiked vanilla ice-cream, sugar cookies, and dark chocolate standard curves as determined by the macadamia nut ELISA. Antisera of rabbit NE 338 was used as capture antibody and goat B 1178 antisera was used as detecting antibody. Ground roasted macadamia nut was spiked into ice cream (red), cookies (green), dark chocolate (blue), and 0.01 M PBS, 0.5 M NaCl (orange; buffer control) to achieve a 1000 ppm macadamia nut standard and serially diluted 1:3 (v/v) to develop the standard curves.

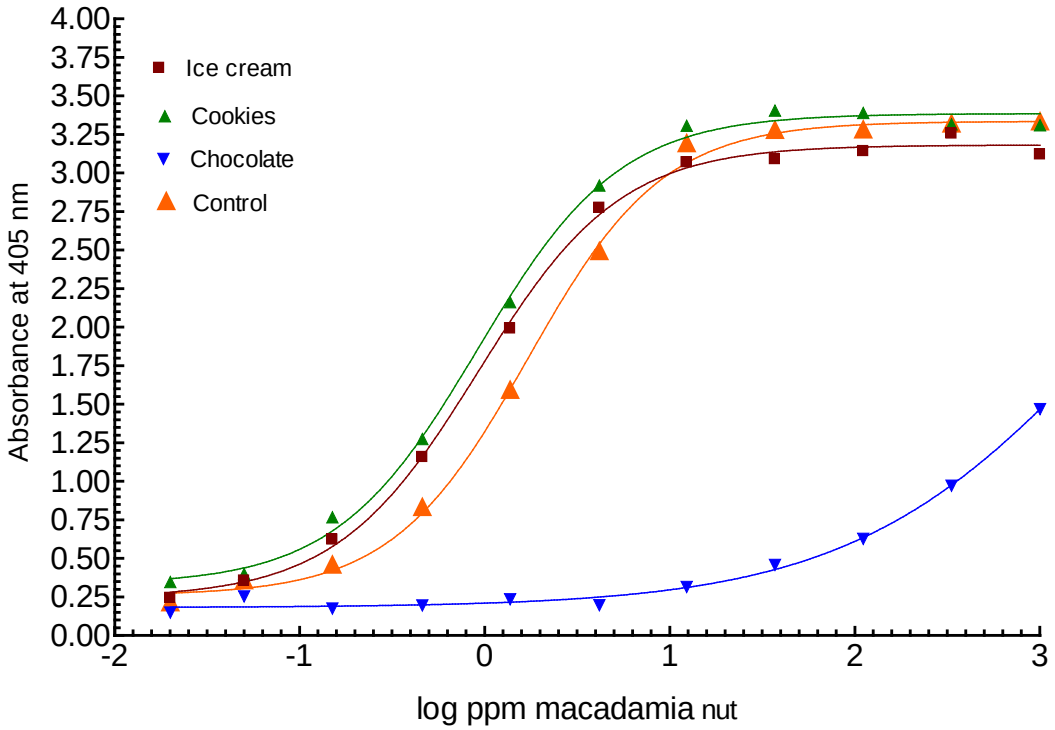

Figure 3.3 Roasted macadamia nut spiked vanilla ice-cream, sugar cookies, and dark chocolate standard curves as determined by the macadamia nut ELISA. Antisera of rabbit NE 339 was used as capture antibody, and goat B 1178 antisera was used as detecting antibody. Ground roasted macadamia nut was spiked into ice cream (red), cookies (green), dark chocolate (blue), and 0.01 M PBS, 0.5 M NaCl (orange; buffer control) to achieve a 1000 ppm macadamia nut standard and serially diluted 1:3 (v/v) to develop the standard curves.

d) Effect of Extraction Buffer Additives on Dark chocolate, Cookies, and Ice cream Matrices

A few different approaches have been found to be effective in reducing these matrix interferences in previous studies. Adding a nonionic detergent such as Tween 20 or including a protein source such as fish gelatin or non-fat dry milk (NFDM) in the extraction buffer have shown to help increase extraction efficiency of the target proteins of interest by binding tannins and other phenolic compounds (Lee et al., 2004). Furthermore, diluting sample extracts has also been found to be convincing in eliminating some interfering components (Ahmed, 2001).

For our study, different additives (1% & 5% NFDM, 1% & 5% fish gelatin, 0.5% & 1% Tween 20) were tested for their effects on the standard curves developed in all three matrices (dark chocolate, cookies, and ice cream). The inclusion of the extraction additives did not create high background absorbances for the standard curves developed in all three food matrices (Figures 3.4, 3.5 and 3.6). It was clearly shown that all of the additives significantly increased the extraction efficiency of macadamia nut proteins in the dark chocolate matrix (Figure 3.4). The extraction additives likely help to eliminate non-specific binding of the tannins that are found in dark chocolate that can limit the solubility of the proteins of interest. While no significant difference was observed in the standard curves developed in the cookie and ice cream matrices in the presence of additives, the addition of the additives did not have a detrimental effect on the sensitivity of the developed ELISA either so there is no harm in standardizing the extraction buffer to include any of these additives (Figures 3.5 and 3.6, respectively). Similar trends were observed for the standard curves developed using antiserum from rabbit NE339 (data not

shown). Overall, slightly better sensitivity was observed in the standard curves developed in all three matrices when the extraction buffer with 5% NFDM was utilized.

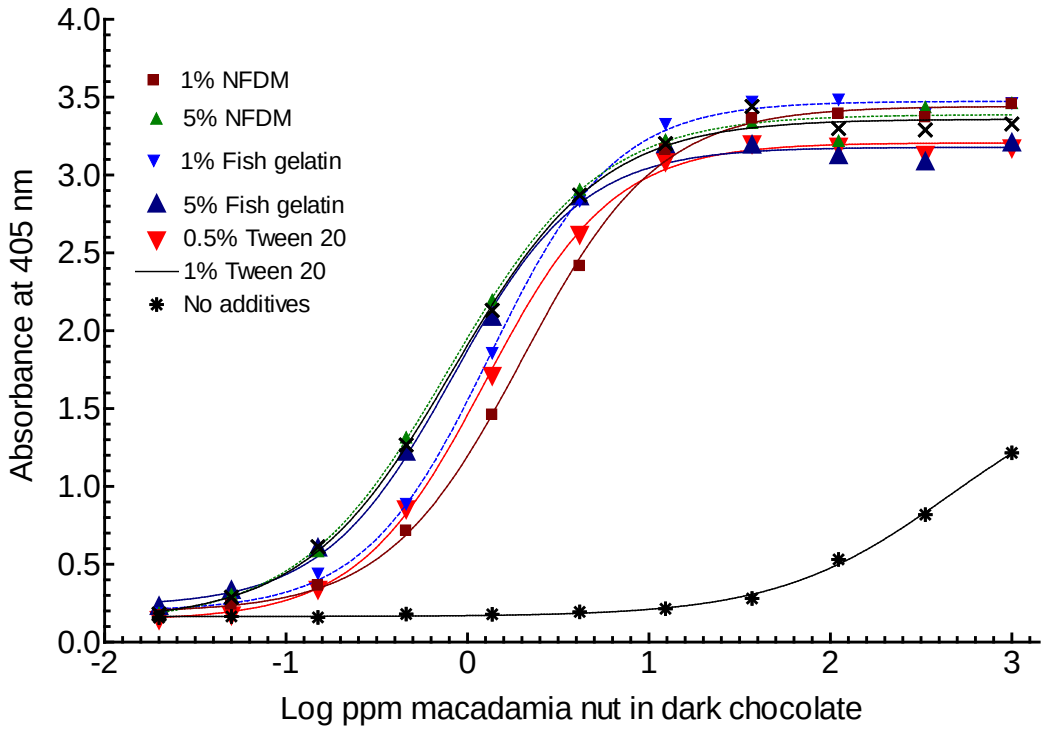

Figure 3.4 The effect of extraction additives on the optimization of roasted macadamia nut-spiked dark chocolate standard curves were compared as determined by the macadamia nut ELISA. Antisera of rabbit NE 338 was used as capture antibody and goat B 1178 antisera was used as detecting antibody.

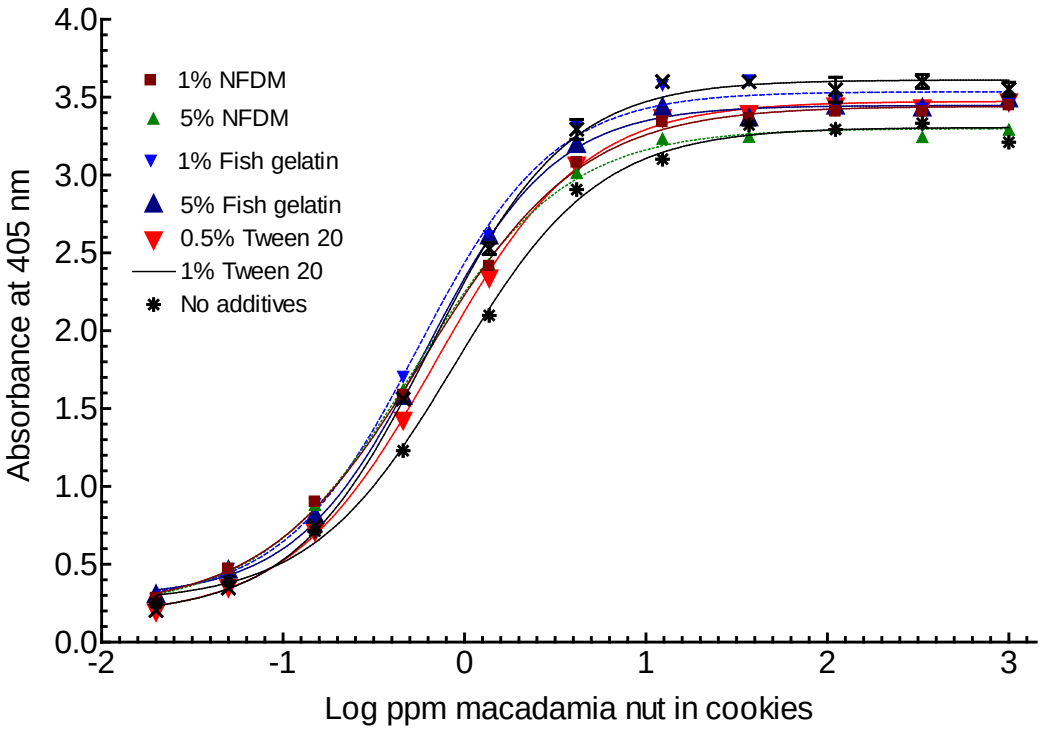

Figure 3.5 The effect of extraction additives on the optimization of roasted macadamia nut-spiked cookie standard curves were compared as determined by the macadamia nut ELISA. Antisera of rabbit NE 338 was used as capture antibody and goat B 1178 antisera was used as detecting antibody.

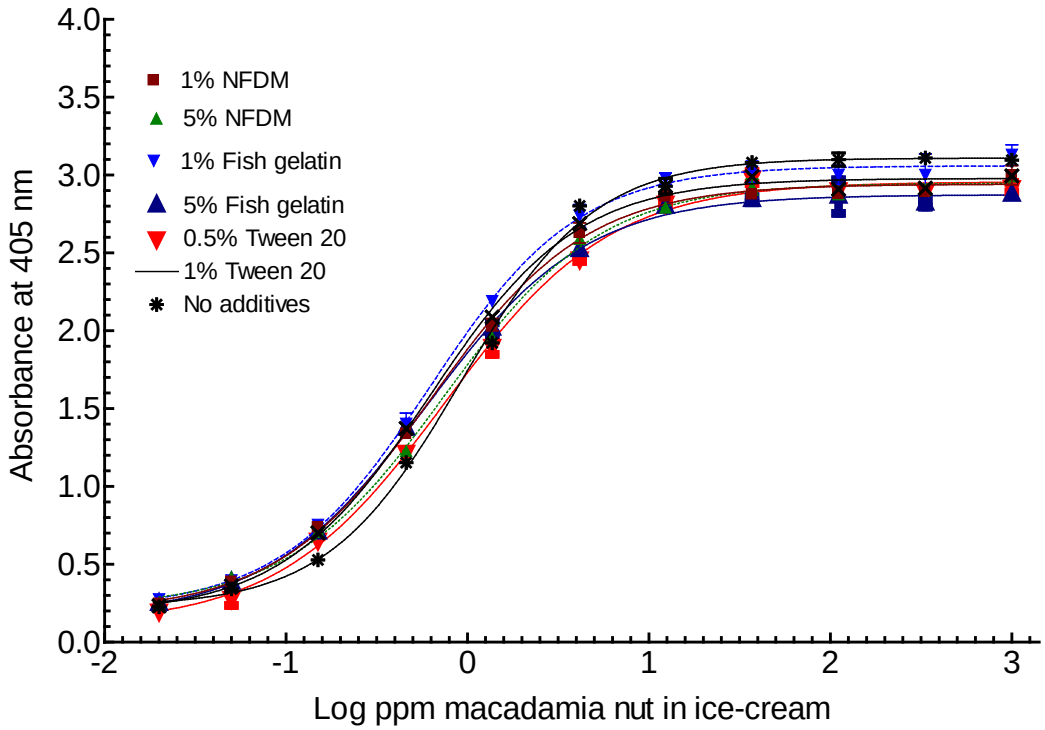

Figure 3.6 The effect of extraction additives on the optimization of roasted macadamia nut-spiked ice cream standard curves were compared as determined by the macadamia nut ELISA. Antisera of rabbit NE 338 was used as capture antibody and goat B 1178 antisera was used as detecting antibody.

IV. CONCLUSIONS

A sensitive and specific macadamia nut sandwich ELISA was developed utilizing rabbit antisera as the capture antibody and goat antisera as detector antibody, where antisera of both species were raised against roasted macadamia. These IgG antibodies were able to detect both raw and roasted macadamia nut residues equally well. Thus, the roasted macadamia ELISA was selected and used in this study and the remaining manufactured model food studies described in Chapter 4. No significant cross-reactivity was observed across 86 ingredients commonly used in baked goods and confections that would limit the utility of the developed ELISA. Matrix interferences present in dark chocolate greatly impacted the extraction efficiency of macadamia nut proteins. However, the use of extraction additives aided in reducing the effects of the interfering components and increased the extraction efficiency of the macadamia nuts without increasing the background signal. No significant difference was observed in cookie and ice cream matrix with and without having additives during extraction. So, the extraction additives did not have a detrimental effect on the sensitivity of the ELISA when used for analysis in these matrices. The extraction buffer with 5% NFDM was found to be the best additive in all three matrices to maintain the needed sensitivity of the assay of approximately 1 ppm macadamia nut.

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CHAPTER 4:PRODUCTION OF MANUFACTURED MODEL FOODS FOR DETECTION OF MACADAMIA NUT RESIDUES BY ELISA

I. INTRODUCTION

Although allergic reactions caused by macadamia nuts are less common as compared to other potent allergens derived from peanut and other tree nuts such as walnut and cashew nut, sensitized individuals may still experience anaphylactic reactions (Fleischer et al., 2005; Pallares, 2000). Complete avoidance from offending foods is the best approach in preventing possible allergic reactions. However, there is still a risk in coming in contact with allergic components due to unintended cross-contact during food processing on occasion. This is especially true when tree nuts are used in different product formulations that share the same production line and equipment. Therefore, it is crucial for manufacturing facilities to adhere to good manufacturing practices, ensure that proper cleaning protocols are in place and that these cleaning protocols are validated using sensitive and specific methods to monitor the removal of residue (FDA, 2015).

Sandwich ELISAs and ELISA-based lateral flow methods are the most commonly used analytical platforms by food manufacturers and health agencies for tracking proteins residues from allergenic sources due to their high specificity and sensitivity in detecting proteins across different food matrices (Taylor et al., 2009). Nonetheless, processing effects from both thermal and non-thermal techniques that are used during food production could possibly alter the structure and conformational epitopes of food allergens, thereby affecting antibody binding to epitopes and limiting the sensitivity of ELISA methods (Alvarez & Boye, 2012). As a result, the percent recovery of allergenic proteins might be subject to inaccuracies.

To assess the robustness and overall performance of the developed ELISA, using incurred standards in model foods mimicking the actual manufacturing process is the best approach (Koppelman & Hefle, 2006). Sugar cookies and vanilla ice cream were chosen to be the model foods in our incurred macadamia nut standard study. Eight different incurred levels were prepared by incorporating a known amount of powdered macadamia nut-sugar mix into other ingredients needed to prepare each food matrix. The extraction of successive incurred standards for each final food product was then evaluated using the developed macadamia nut sandwich ELISA.

II. MATERIALS AND METHODS

a) Antisera Selection for the Incurred Model Food Study

Antisera of both rabbit NE 338 and NE 339 were individually used as the capture antibody (diluted in 1:10,000 in coating buffer) while goat B 1178 antisera, diluted in 1:5000, was used as detecting antibody of all studies as discussed in Chapter 3. While analyzing the homogeneity of mixing for macadamia nut-sugar mix which was used as the spike material in model foods tested, three different antisera were tested separately as coating antibody: pooled NE 338, pooled NE 339, and a pool of (NE 338 + NE 339) diluted 1:10,000 in 0.01 M PBS buffer to evaluate any possible variation. Antisera of rabbit NE 338 + NE 339 were pooled by combining equal volumes of pooled NE 338 antisera and pooled NE 339 antisera. As discussed in the Results section, no significant differences were observed between the standard curves that utilized the individual rabbit antisera or the pool of both rabbit antisera, therefore the pool of NE338+339 was used for the incurred model foods study described in detail in the following section.

b) Preparation of Incurred Model Foods

i) Sugar Cookies

Ingredients needed for baked sugar cookies were purchased from local grocery stores in Lincoln, NE, which included Betty Crocker™ sugar cookie mix, butter, and eggs. Prior to baking the cookies, a concentrated spike material of ground macadamia nut and sugar was prepared by grinding 1 g of ground roasted macadamia nut with 99 g of granulated sugar to generate a 10,000 ppm macadamia nut standard. Five subsamples of the 10,000 ppm powdered macadamia nut-sugar mix were randomly drawn and extracted 1:10 (w/v) with 0.01 phosphate buffered saline (0.01 M PBS with 0.5 M NaCl, pH 7.2) containing 5% non-fat dry milk (NFDM). Samples were extracted in a shaking water bath (Julabo SW22, Julabo USA, Inc., Allentown, PA) at 60 ºC for 1 hour followed by centrifugation (Sorvall® Legend™ RT, Kendro Laboratory Products, Newton, CT) of the extracts at 3020 x g for 30 minutes at 10 ºC. The clarified supernatant was analyzed using the developed macadamia nut ELISA to ensure the homogeneity of mixing. Following the homogeneity analysis, a calculated spike of 1526 ppm macadamia nut-sugar mix was prepared by mixing 36.4 g of the 10,000 ppm macadamia nut and powdered sugar mixture from the previous step into 463.6 g of sugar using a Kitchen AidTM 5 Quart Artisan Mixer, KSM150OPS (Kitchen Aid, St. Joseph, MI). The same extraction procedure and homogeneity analysis as previously mentioned were carried out to verify the uniformity of mixing. Eight different incurred macadamia nut levels in cookie dough (0, 1, 2.5, 5, 15, 25, 50, 100 ppm macadamia nut) were then individually prepared by adding a specific amount of the 1526 ppm macadamia nut-sugar mix into the rest of the

ingredients needed for sugar cookies. Table 4.1 shows the designated amount of each ingredient needed for all eight batches of sugar cookies.

Each incurred level of cookies dough was prepared individually starting with 0 ppm followed by the next lowest macadamia nut level. The specific amount of powdered macadamia nut-sugar mix was added into the Betty Crocker™ sugar cookie mix and these dry ingredients were mixed for 30 minutes using the Kitchen Aid™ mixer at the lowest speed prior adding the rest of the ingredients. After adding butter and eggs, the batter was mixed for 15 minutes with a stop every 5 minutes to manually fold the dough from bottom of the mixing bowl to the top using a spatula. This allowed the spiked macadamia nut to be well distributed in the cookie dough. To prevent any carryover of macadamia nut proteins in between batches, all utensils and the mixing bowl were thoroughly cleaned with hot soapy water and rinsed off with distilled water. Each level of cookie dough was kept in labeled Ziploc bags and stored at -20 °C until needed for further baking and analysis.

Prior to baking, all batches of cookie dough were allowed to come to room temperature and each dough ball was weighed out to approximately 30 g and placed on lined baking sheets. Each level of incurred cookie dough was baked separately for 15 minutes at 375 °F then allowed to cool to room temperature. Cookies were weighed and recorded before and after baking in order to account for moisture loss during the baking process. The remaining raw cookie dough and the baked cookies from each incurred macadamia nut level were evaluated using the developed macadamia nut ELISA in three independent trials to assess the effects of heat processing on the recovery of macadamia nut residue.

Table 4.1 Formulation for naturally incurred macadamia nut in sugar cookies.

Naturally Incurred Macadamia Nut Standards in Sugar Cookies (grams, g)								
Ingredient	**100 ppm***	**50 ppm**	**25 ppm**	**15 ppm**	**5 ppm**	**2.5 ppm**	**1 ppm**	**0 ppm**
Macadamia-free sugar cookie mix (negative control)	335.2	351.6	359.8	363.1	366.4	367.2	367.7	368
Macadamia nut-sugar mix (1526 ppm)	32.8	16.4	8.2	4.9	1.6	0.8	0.3	0
Butter	87	87	87	87	87	87	87	87
Egg	45	45	45	45	45	45	45	45
Total weight	***500***	***500***	***500***	***500***	***500***	***500***	***500***	***500***

*****ppm = parts per million, µg/g

ii) Vanilla Ice cream

The ingredients needed to prepare vanilla ice-cream include heavy whipping cream, half & half cream, vanilla extract and sugar. These ingredients were purchased from a local grocery store in Lincoln, NE. The same batch of 1526 ppm macadamia nut-sugar mix prepared earlier for the sugar cookies was used to make the macadamia nut incurred vanilla ice cream. Eight different incurred macadamia nut levels of vanilla ice cream (0, 1, 2.5, 5, 15, 25, 50, 100 ppm) were individually prepared by adding a specific amount of 1526 ppm macadamia nut-sugar mix into the rest of the ingredients needed for vanilla ice cream. Table 4.2 gives the designated quantities of each of the ingredients needed for all eight batches of vanilla ice cream.

Each batch of ice cream was prepared independently in ascending order starting with 0 ppm and proceeding to the next to the highest macadamia nut level. The canister of the ice cream maker (Conair Cuisinart 1.5 Quart Frozen Yogurt Ice Cream Maker, Model ICE-21, North American Electrical Standards) was kept in the freezer overnight before each use. The ice cream maker was set up the following day by placing a rotator inside the double insulated frozen canister. The specific amount of chilled heavy whipping cream, half & half cream, vanilla extract and sugar were poured into the rotating frozen canister. A soft custard-like texture was formed after 10 minutes of rotation and the designated amount of macadamia nut-sugar mix was then added to obtain the desired level of macadamia nut in each batch of ice cream. The canister was rotated for an additional 15 minutes to obtain a homogeneous mixture with well distributed macadamia nut incorporated in the churned ice cream. To prevent any carryover of macadamia nut proteins between batches, the ice cream maker was thoroughly cleaned with hot soapy water, rinsed with distilled water, and allowed to air dry. The finished ice

cream was kept in labeled Ziploc bags and stored at -20 °C until needed for further analysis. Each incurred batch was evaluated using the developed macadamia nut ELISA in 3 independent trials to assess the effects of processing on the recovery of macadamia nut residue.

Table 4.2 Formulation of naturally incurred macadamia in vanilla ice-cream

Naturally Incurred Standards in Vanilla Ice-cream (grams, g)								
Ingredients	**100 ppm ***	**50 ppm**	**25 ppm**	**15 ppm**	**5 ppm**	**2.5 ppm**	**1 ppm**	**0 ppm**
Heavy whipping cream	211	211	211	211	211	211	211	211
Half & half cream	211	211	211	211	211	211	211	211
Vanilla extract	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5
Sugar	38.7	55.1	63.3	66.6	69.9	70.7	71.2	71.5
Macadamia nut-sugar mix (1526 ppm)	32.8	16.4	8.2	4.9	1.6	0.8	0.3	0
Total Weight	***500***	***500***	***500***	***500***	***500***	***500***	***500***	***500***

*****ppm = parts per million, µg/g

c) Extraction and Evaluation of Incurred Model Foods

i) Baked Sugar Cookies and Unprocessed Cookie Dough Analysis

Baked cookies from all eight incurred macadamia nut levels were ground separately into fine particle sizes using an Osterizer® blender (Sunbeam Corporation, Delray Beach FL) prior to extraction. Three subsamples from each batch of ground cookies and dough were extracted 1:20 (w/v) in 0.01 M phosphate buffered saline (PBS in 0.5 M NaCl, pH 7) in a shaking water bath (Julabo SW22, Julabo USA, Inc., Allentown, PA) at 60 °C for 1 hour followed by centrifugation at 10 °C for 30 minutes at 3020 x g on a tabletop centrifuge (Sorvall® Legend™ RT, Kendro Laboratory Products, Newton, CT). The clarified supernatants were analyzed for recovery of macadamia nut using the developed macadamia nut ELISA.

The 0 ppm baked sugar cookies and cookie dough were used as negative controls. Standard curves were prepared by serially diluting the 1000 ppm positive control 1:3 (v/v) in negative control ground cookie and dough extracts, respectively, to obtain12 macadamia nut standards (1000, 333, 111, 37.0, 12.3, 4.12, 1.37, 0.457, 0.152, 0.051, 0.017, and 0 ppm). The 1000 ppm macadamia nut standard was achieved by adding 1 ml of 10,000 ppm macadamia extract prepared beforehand as described in Chapter 3 into 9 ml of 0 ppm baked cookies and dough extract respectively. Standard curves were constructed using GraphPad Prism v.4.03 software (GraphPad Software Inc., San Diego, CA). Percentage recovery of macadamia nut in baked cookies and cookie dough were calculated as the recovered ppm level over the expected ppm level of macadamia nut added. The moisture after baking was also taken into account to determine the actual ppm

level of macadamia remaining after water loss. The result was based on triplicate extractions of each incurred level analyzed in 3 independent trials.

ii) Vanilla Ice cream

The 1000 pm positive control was prepared by adding 1 ml of 10,000 ppm macadamia nut extract prepared beforehand as described in Chapter 3 into 9 ml of 0 ppm ice cream extract, and the 0 ppm vanilla ice cream was used as negative control. All batches of frozen ice-cream were allowed to thaw before extraction. Three subsamples from each incurred macadamia nut level along with samples of negative control were extracted 1:20 (w/v) in 0.01 M PBS with 0.5 M NaCl (pH 7.4) containing 1 % Tween 20 in a shaking water bath (Julabo SW22, Julabo USA, Inc., Allentown, PA) at 60 °C for 1 hour followed by centrifugation at 10 °C for 30 minutes at 3020 x g on a tabletop centrifuge (Sorvall® Legend™ RT, Kendro Laboratory Products, Newton, CT). The clarified supernatants were analyzed for recovery of macadamia nut using the developed macadamia nut ELISA.

A standard curve was developed by serially diluting the 1000 ppm positive control 1:3 (v/v) in negative control ice cream to generate 12 macadamia standards (1000, 333, 111, 37.0, 12.3, 4.12, 1.37, 0.457, 0.152, 0.051, 0.017, and 0 ppm) and the GraphPad Prism v.4.03 software (GraphPad Software Inc., San Diego, CA) was used to generate the standard curve. The percentage recovery of macadamia nut in the incurred ice cream was calculated as the recovered ppm level over the expected ppm level of macadamia nut added. The result was based on triplicate extractions of each incurred level analyzed in three independent trials.

III. RESULTS AND DISCUSSION

a) Antisera Selection for Incurred Model Food Study

Figure 4.1 compared the standard curves that were generated in the 0.01 M PBS buffer using different combinations of pooled rabbit antisera. Since all curves were comparable, we selected a combined pool of NE 338 & 339 at equal volumes for the rest of the study.

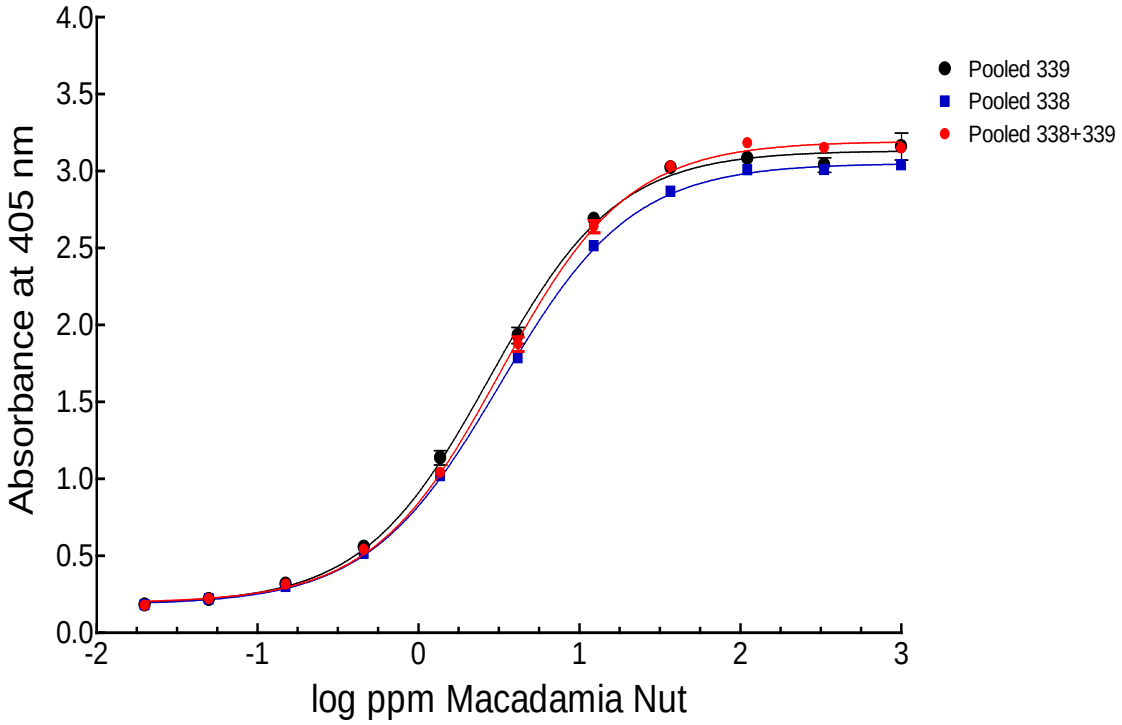

Figure 4.1 Comparison of standard curves developed using antisera from pooled NE 338, NE 339 and NE 338 + NE 339 as capture antibody respectively at a 1:10,000 dilution of antisera in 0.01 M PBS buffer. The detecting antibody used was goat B 1178 antisera at a 1:5000 dilution. Each data point represents 8 readings (n=4, each with data analyzed in duplicate), with an average standard error of < 0.03 AU.

b) Recovery of Incurred Macadamia Nut from Baked Sugar Cookies and Cookie Dough

The relative amount of recovered macadamia nut from baked sugar cookies and cookie dough were estimated by generating a standard curve with ground macadamia nut spiked into a negative control extract of either baked cookies or cookie dough and serially diluting the 1000 ppm macadamia nut spiked standard 1:3 (v/v). Figure 4.2 shows the standard curve developed in the negative control baked cookies and cookie dough as compared to the standard curve produced in 0.01 M PBS with 0.5 M NaCl. The results of one-way ANOVA test at the $p < 0.05$ significance level (GraphPad Prism® v.4.03 software) showed that there was no significance between baked cookies, cookie dough, and the PBS curves. While no statistical differences were observed between the standard curves, qualitatively the standard curved spiked into the baked cookie or cookie dough extracts shifted the standard curve slightly to the left compared to the standard curve prepared in the PBS buffer. This shift generated a slightly longer dynamic range of the developed standard curves in the cookie matrices which thereby provided slightly more sensitivity of the developed assay. Table 4.3 shows the recovery of macadamia nut from both baked cookies and cookie dough at each incurred levels obtained from the 3 trials. The percentage recovery of macadamia nut in baked cookies was much lower in comparison to the unprocessed cookie dough. Processing such as baking under high heat can significantly affect the physiochemical and immunological properties of proteins within a food matrix. Maillard browning reactions can result in changes of protein conformation, loss of solubility and interfere with the antigen-antibody reactions. Dilution has been implemented as one of the direct and efficient way in reducing

interfering components. Overall, the recovery of macadamia nut by the developed ELISA was quite sufficient for the cookies before and after the baking process with recoveries generally falling in the ±30% of expected range that is accepted for food allergen detection methods by that analytical community. We did observe lower recoveries at the 1, 2.5 and 5 ppm incurred levels in the baked cookies which is lower than we would prefer. This could be due to a number of factors including the homogeneity of the incurred material or dilution errors that may have occurred. Overall, the developed macadamia nut ELISA is considered to be a sensitive analytical method for detecting macadamia nut residues in food products and would serve as a reliable monitoring tool.

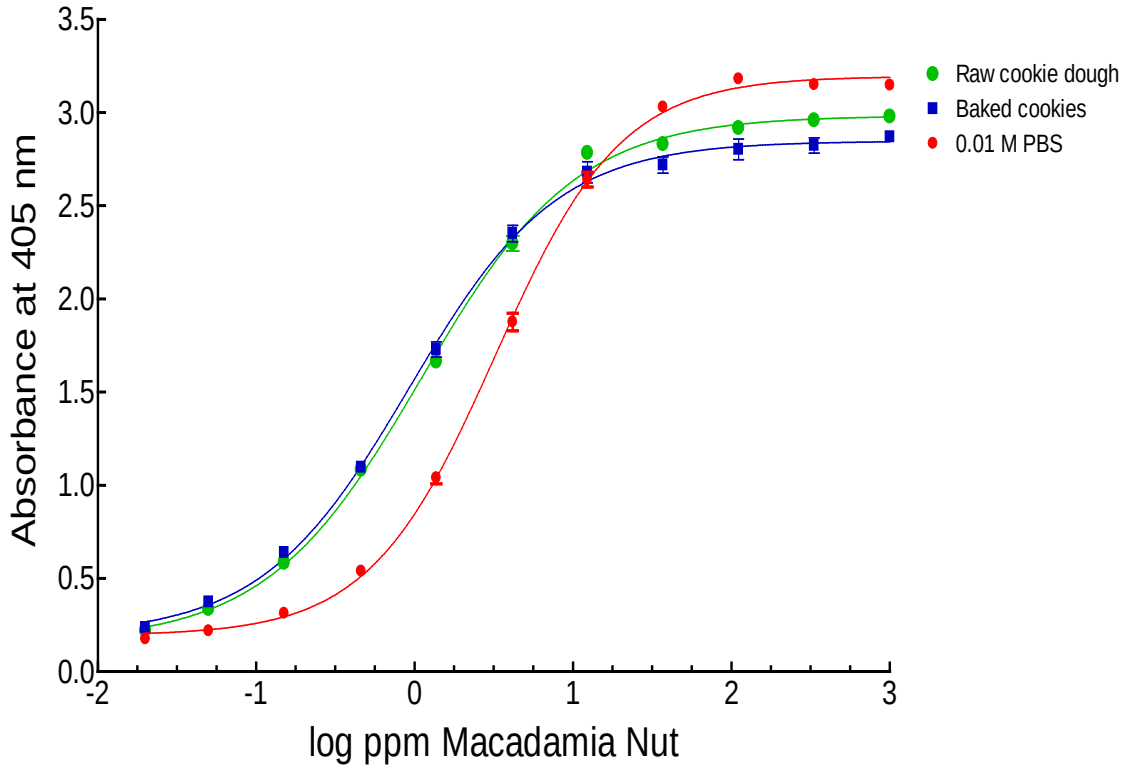

Figure 4.2 Comparison of standard curves between baked cookies, cookie dough, and 0.01 M PBS with 0.5 M NaCl. A 1000 ppm macadamia nut spike was serially diluted 1:3 (v/v) with a negative control extract of baked cookies, cookie dough, or 0.01 M PBS with 0.5 M NaCl, respectively. Each data point represents 8 readings (n=4, each with data analyzed in duplicate), with an average standard error of < 0.05 AU.

Table 4.3 Recovery of incurred macadamia nut from baked cookies and cookie dough model foods as determined by the macadamia nut ELISA. Sample were extracted 1:20 (w/v).

	Cookie Dough (Before baking)		Sugar Cookie (After baking)[a]	
Incurred Macadamia Nut Level (ppm)[b]	Mean ppm Recovery[c]	% Recovery[d]	Mean ppm Recovery[c]	% Recovery[d]
0	ND[e]	NA[f]	ND[e]	NA[f]
1	ND	NA	ND	NA
2.5	5.0 ± 0.2	201 ± 8	0.46 ± 0.01	18 ± 0.1
5	4.96 ± 0.07	99 ± 1	2.5 ± 0.1	50 ± 2
15	22.2 ± 0.8	147 ± 5	11.2 ± 0.7	74 ± 5
25	36.0 ± 0.8	144 ± 3	20.1 ± 0.5	80 ± 2
50	57 ± 2	115 ± 4	46 ± 1	94 ± 2
100	102 ± 2	102 ± 2	81.5 ± 0.9	81.5 ± 0.9

[a]Amount of macadamia nut available after baking was calculated to account for moisture loss of cookie samples after baking (mean moisture loss from cookie samples = 5.3 % ± 0.2 %)
[b]ppm - parts per million (mg of ground macadamia nut per kg of cookie)
[c]Data are mean ± standard error (n=3 trails)
[d]Percent recovery calculated as the ratio of the average ppm macadamia nut recovered to the expected macadamia nut in the finished product
[e]ND – not detected
[f]NA – not applicable

c) Recovery of Incurred Macadamia Nut from Vanilla Ice Cream

The relative amount of recovered macadamia nut from vanilla ice cream was estimated by generating a standard curve with ground macadamia nut spiked into negative control ice cream extract and serially diluting the 1000 ppm macadamia nut spiked standard 1:3 (v/v) as previously discussed. Figure 4.3 shows the standard curve developed in the negative control vanilla ice cream extract as compared to the standard curve produced in 0.01 M PBS with 0.5 M NaCl. An unpaired t-test at the $p < 0.05$ significance level (GraphPad Prism® v.4.03 software) indicates that there was no significant between the standard curves that were developed in the ice cream and PBS extracts. Table 4.4 shows the recovery of macadamia nut from vanilla ice cream at each incurred level obtained from 3 trials where samples were extracted at 1:20 (w/v) without the addition of Tween 20 whereas Table 4.5 shows percentage of recovery when samples were extracted at 1:20 with the addition of 1% Tween 20. The latter extraction protocol results in an improved and excellent rate of macadamia nut recovery in the incurred vanilla ice cream model with a mean percent recovery increase from 58 % ± 2 % to 89 % ± 2 %. Additives such as Tween 20 have been widely used as an agent to help improve extraction efficiency. Douwes et al. (1995) demonstrated that the extraction efficiency was seven times higher in the presence of Tween 20 as compared to extraction carried out without the additive. The addition of the non-ionic detergent coupled with the 1:20 extraction dilution increased the extraction efficiency of macadamia nut significantly in our study. The relatively high rate of recovery indicates that the process of mixing and freezing during the ice cream making process does not affect the efficacy of developed ELISA in reliably detect macadamia residues.

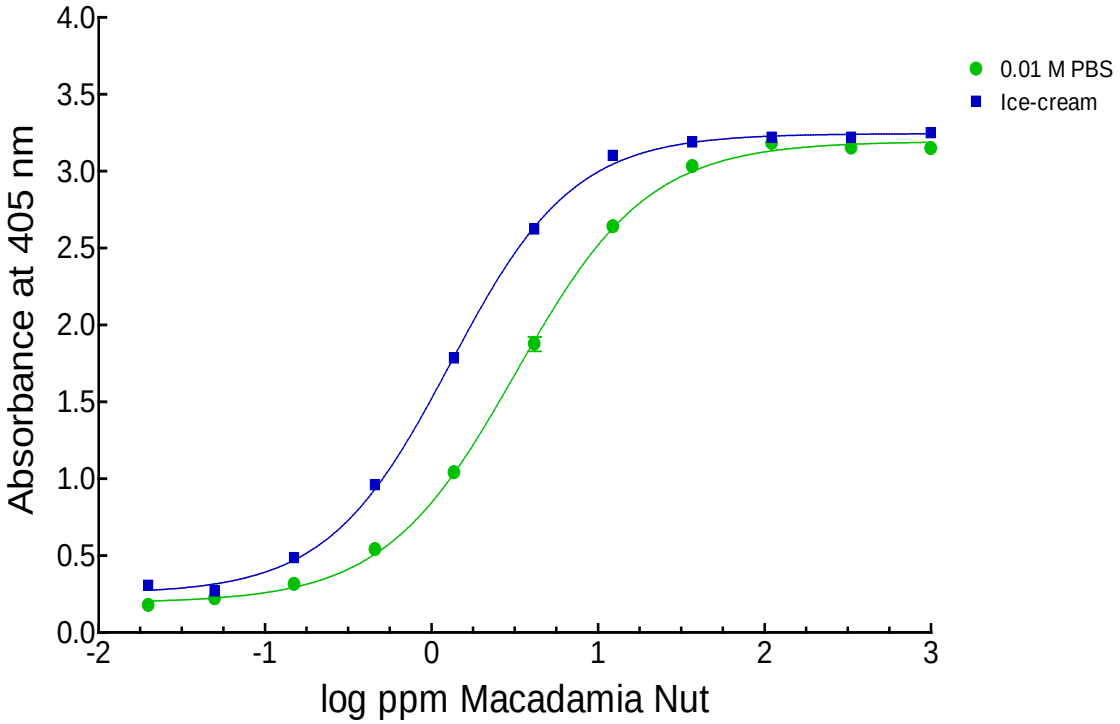

Figure 4.3 Comparison of macadamia nut standard curves developed in vanilla ice cream extract or 0.01 M PBS with 0.5 M NaCl containing 1% Tween 20. A 1000 ppm macadamia nut spike was serially diluted 1:3 (v/v) with a negative control extract of vanilla ice cream or 0.01 M PBS with 0.5 M NaCl, respectively. Each data point represents 8 readings (n=4. Each data point analyzed in duplicate), with an average of standard error of < 0.02 AU.

Table 4.4 Recovery of macadamia nut from the incurred vanilla ice cream model food as determined by the macadamia nut ELISA. Samples were extracted at 1:20 without Tween 20.

VANILLA ICE CREAM		
Incurred Macadamia Nut Level (ppm[a])	Mean ppm Recovery[b]	% Recovery[c]
0	ND[d]	NA[e]
1	ND	NA
2.5	1.39 ± 0.06	53 ± 3
5	2.76 ± 0.03	55.2 ± 0.6
15	8.95 ± 0.08	59.5 ± 0.5
25	14.9 ± 0.1	59.8 ± 0.5
50	30.9 ± 0.4	61.7 ± 0.8
100	60 ± 7	60 ± 7

[a]ppm – parts per million (mg of ground macadamia nut per kg of vanilla ice cream)
[b]Data are mean ± standard error (n=3 trails)
[c]Percent recovery calculated as ratio of the average ppm macadamia nut recovered to the expected macadamia nut in the incurred finished product
[d]ND – not detected
[e]NA – not applicable

Table 4.5 Recovery of macadamia nut from the incurred vanilla ice cream model food as determined by the macadamia nut ELISA. Samples were extracted at 1:20 with 1% Tween 20.

VANILLA ICE CREAM		
Incurred Macadamia Nut Level (ppm[a])	Mean ppm Recovery[b]	% Recovery[c]
0	ND[d]	NA[e]
1	ND	NA
2.5	2.08 ± 0.05	83 ± 2
5	4.70 ± 0.06	94 ± 1
15	14.5 ± 0.3	97 ± 2
25	24 ± 1	97 ± 4
50	40.0 ± 0.9	80 ± 2
100	83 ± 2	83 ± 2

[a]ppm – parts per million (mg of ground macadamia nut per kg of vanilla ice cream)
[b]Data are mean ± standard error (n=3 trails)
[c]Percent recovery calculated as ratio of the average ppm macadamia nut recovered to the expected macadamia nut in the finished product
[d]ND – not detected
[e]NA – not applicable

IV. CONCLUSIONS

Macadamia nut incurred sugar cookies and vanilla ice cream were used as model foods in accessing the sensitivity and efficiency of the developed macadamia nut ELISA in detecting macadamia residues in processed foods. Ice cream and unprocessed cookie dough had higher rates of recovery in comparison to the baked cookies. This could be due to the fact that processing under high heat tends to aggregate most proteins, including the macadamia nut proteins, which potentially lowers their solubility. The lower quantitative extraction recovery of heat processed/baked macadamia proteins may therefore be attributed to a decreased solubility and subsequent slight structural alterations of the target proteins that may limit the immunochemical detection. While the recovery of macadamia nut residue was slightly lower in the baked cookie model food, the recovery did range from 50 to 94% over most of the incurred levels which is typical of ELISA methods for detection of heat processed residues. Overall, the recovery of macadamia nut in both model foods shows that the developed ELISA can sufficiently detect macadamia nut proteins and can reliably serve as a tool in quantifying and monitoring macadamia nut residues in processed foods.

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SUMMARY

Tree nuts are listed as one of the allergenic food sources included in the Big Eight food group that are commonly found to be associated with adverse food allergic reactions. Clinical reports on macadamia nut allergy are less common compared to other priority food allergens, but hidden macadamia nut poses potential health and safety risks in individuals that have heightened sensitivity to macadamia nut. The food industry relies upon good manufacturing practices, robust cleaning protocols and sensitive detection methods to mitigate the risk of allergen cross-contact in food processing facilities. These analytical methods must be capable of detecting protein residue from the allergenic source of interest that have undergone thermal and non-thermal processing. The aim of our study was to develop a robust, sensitive and specific macadamia nut sandwich ELISA capable of detecting processed macadamia nut residues in food products with a limit of quantification of approximately 1 ppm. Rabbit and goat antisera were found to be the optimum pairing in the developed ELISA as the capture and detector antibodies, respectively. Cross-reactivity is more commonly found among foods in the same botanical family, so assessing potential cross-reactivity as well as potential matrix interferences becomes important for the overall validation of the developed ELISA. In this study, nutmeg was the only ingredient among all 86 food ingredients that were analyzed that demonstrated a matrix interference that was higher than the limit of quantification (LOQ=1.0) of the ELISA. However, the high color intensity from pigments or other components of the spices are likely to be the contributing factor in the higher reading. Interfering components can often contribute to false negative results which can affect the efficiency and sensitivity of the ELISA when testing pure spices or highly pigmented ingredients. The addition of extract additives helped to improve the

extraction efficiency by reducing interfering compounds. The addition of 5% NFDM was found to be the optimum additive among several that were tested in this study. Thermal and non-thermal processing such as baking and freeze-thaw cycles may result in conformational or linear changes in epitopes of food allergen as well as affect the overall solubility of target proteins. Thus, it is important to validate the robustness of an ELISA on the recovery of target proteins that have gone through typical processing steps that are used to produce a finished food product that consumers would eat to assure that the method provides the user with a reliable tool to validate the mitigation of allergen cross-contact. Therefore, macadamia nut incurred model foods (cookie and ice cream) were produced with known levels of macadamia nut to evaluate the robustness of the developed ELISA by evaluating the rate of recovery of macadamia nut in the incurred processed foods. Overall, excellent recovery of macadamia nut from both processed and unprocessed foods was observed. Based on our study, the developed ELISA is sufficiently sensitive and will serve as a tool for the food industry, regulators or others to reliably detect macadamia nut residues in processed foods.