Development Of An Enzyme-Linked Immunosorbent Assay (ELISA) For The Detection Of Pistachio Residues In Processed Foods

Pei Wen Lim
University of Nebraska-Lincoln, plim2@unl.edu

Follow this and additional works at: http://digitalcommons.unl.edu/foodscidiss
Part of the Other Food Science Commons

Lim, Pei Wen, "Development Of An Enzyme-Linked Immunosorbent Assay (ELISA) For The Detection Of Pistachio Residues In Processed Foods" (2010). Dissertations, Theses, & Student Research in Food Science and Technology. 10.
http://digitalcommons.unl.edu/foodscidiss/10

This Article is brought to you for free and open access by the Food Science and Technology Department at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Dissertations, Theses, & Student Research in Food Science and Technology by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.
DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF PISTACHIO RESIDUES IN PROCESSED FOODS

by

Pei Wen Lim

A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Food Science and Technology

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

Lincoln, NE

November, 2010
DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF PISTACHIO RESIDUES IN PROCESSED FOODS

Pei Wen Lim M.S.

University of Nebraska, 2010

Advisors: Stephen L. Taylor and Joseph L. Baumert

Pistachios (*Pistacia vera*) are popular snacks and consumption of pistachios is notably increasing due to their usage as ingredients for confections, ice cream, and baked goods. The increased consumption of pistachios may lead to a higher frequency of allergic sensitization and an increased prevalence of allergic reactions to pistachios. Trace amounts of undeclared pistachio allergens can pose serious health risks for food-allergic consumers, including severe anaphylactic reactions. A highly sensitive analytical method, sandwich-type enzyme-linked immunosorbent assay (ELISA) is applied as a critical tool for food manufacturers and food scientists to detect minute amounts of allergenic food residues in processed foods. The aim of the study was to develop an ELISA for the detection and quantification of pistachio residues in processed foods. California raw pistachios and a mixture of Californian, Iranian, and Turkish roasted pistachios were used as immunogens to immunize one sheep, one goat, and three rabbits. Both raw and roasted pistachio ELISAs were developed by using pooled sheep antisera as the capture reagent and pooled rabbit antisera as the detector reagent. Binding of antigen-antibody complex was visualized through a colorimetric reaction involving goat anti-rabbit IgG antibody (labeled with alkaline phosphatase) and substrate (p-nitrophenyl phosphate). A total of 102 food ingredients were evaluated using the developed pistachio ELISA for potential
cross-reactivity. Vanilla ice cream and sugar cookies with known amounts of pistachio were prepared as manufactured model foods. The sensitivity, specificity, and robustness of the assay were determined by the percent of recovery from both of the model foods.

The optimized ELISA had a limit of quantification (LOQ) of <1 ppm (1μg/g). Minor cross-reactivity was observed with cashew at a level equivalent to 4 ppm of roasted pistachio. The performance of the ELISA was not affected in the presence of the ice cream and cookie matrices. The mean percent recovery of pistachio from the vanilla ice cream, cookie dough, and baked sugar cookie are 115 ± 3.0, 131 ± 20.8, and 53.9 ± 3.0 respectively. The pistachio-ELISA developed in this study is sufficiently specific and sensitive to be used to help food manufacturers in complying with the FDA labeling guidelines and to safeguard allergic consumers from undeclared pistachio residues.
DEDICATION

To the loving memory of my late father Ah Yeang Lim, who is forever remembered. His silent inspiration, encouragement, and guiding hand on my shoulder still linger on.
ACKNOWLEDGEMENT

I would like to extend my heartfelt gratitude to my advisor Dr. Stephen Taylor for his benevolent guidance, encouragement, understanding, and unceasing assistance throughout my graduate program. He was an expert, abundant with energy and enthusiasm, which sparked my interest in research and proceed with my graduate studies. His extensive knowledge in research taught me to think wisely, gather information effectively and approach problems creatively. I would also like to express my appreciation to my co-advisor, Dr. Joseph Baumert, who is always accessible and willing to help me in research. He was knowledgeable and analytical, providing me skills to establish effective experiments. Besides, I would also like to recognize the contributions of Dr. Richard Goodman and Dr. Vicki Schlegel as my thesis committee members. Their encouragement, interest, and valuable insights for my research will always be remembered.

My sincere appreciation to Julie Nordlee for sharing her thoughtful ideas and help in developing the model foods for this study. Special thanks to Lynn Neimann and Poi-Wah Lee, for their patience and dedication in teaching me laboratory techniques and magnanimously help me with constructive comments. I am also grateful to all colleagues and graduate students in FARRP, especially Rakhi Panda, Ferdelie Gaskin, Melanie Down, and Benjamin Remington for the stimulating discussions, encouragement, and the fun we have had in the past three years.

I am deeply indebted to my late father, for his everlasting love, care and unceasing support. I would like to share my achievement with him and my mother, who both had been the most enthusiastic supporters for my graduate work and always held me
in their thoughts which gave me strength to go on. A special thanks to my sisters and
brother for their help and patient for every period I was away.

Last but not least, my sincere gratitude to all the people and the animals that have
contributed to and worked on this project; as well as friends who had been supporting me
spiritually throughout my graduate studies.
TABLE OF CONTENTS

ABSTRACT .................................................................................................................. i
DEDICATION ........................................................................................................... iv
ACKNOWLEDGEMENT ......................................................................................... v
LIST OF TABLES ................................................................................................... x
LIST OF FIGURES ................................................................................................ xi

CHAPTER 1: LITERATURE REVIEW ................................................................. 1
INTRODUCTION ..................................................................................................... 1
ADVERSE REACTIONS TO FOODS AND FOOD HYPERSENSITIVITY .......... 2
Food Allergy ........................................................................................................... 2
Mechanisms of Food Allergy .............................................................................. 4
Food Allergens ..................................................................................................... 6
Threshold Doses (minimal eliciting doses)......................................................... 8
TREE NUT ALLERGY ........................................................................................... 9
Tree Nut Allergens .............................................................................................. 12
Cross-reactivity ................................................................................................. 16
PISTACHIO NUT .................................................................................................. 17
Nutritional Values of Pistachios ......................................................................... 18
Pistachio Allergens ............................................................................................ 22

METHODS FOR ALLERGEN DETECTION IN FOODS .................................. 23
Immunosorbents ................................................................................................. 23
Enzyme-linked Immunosorbent Assays (ELISAs) .............................................. 25
Competitive ELISAs ........................................................................................... 26
Non-competitive ELISAs .................................................................................... 27
   (i) Two-stage indirect ELISA ......................................................................... 27
   (ii) Sandwich-type ELISA ............................................................................. 29
Detection of Pistachio Allergens ...................................................................... 31

ELISA DEVELOPMENT .......................................................................................... 31
Antibody Production ......................................................................................... 32
Optimizing Assay Operating Conditions of ELISA ......................................... 34
CHAPTER 2: PRODUCTION OF POLYCLONAL ANTISERA AGAINST RAW AND ROASTED PISTACHIOS

ABSTRACT ........................................................................................................................................... 53
INTRODUCTION ...................................................................................................................................... 54
MATERIALS AND METHODS .................................................................................................................. 57
  Pistachio Immunogen Preparation ......................................................................................................... 57
  Extraction of Pistachio Proteins .............................................................................................................. 57
  Polyclonal Antibody Production ............................................................................................................ 58
  Titer Determination ................................................................................................................................ 59
  Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE) ...................................... 60
  IgG-immunoblotting (Western Blotting) .................................................................................................. 61
RESULTS AND DISCUSSION .................................................................................................................... 64
  Selection of Raw and Roasted Pistachios ................................................................................................. 64
  Total and Soluble Protein Content of Raw and Roasted Pistachios ...................................................... 64
  Animal Selection Prior to Immunization ................................................................................................. 66
  Raw and Roasted Pistachio Specific Antibodies Production ................................................................. 68
    Titer Determination ................................................................................................................................ 68
    SDS-PAGE and IgG Immunoblotting ..................................................................................................... 76
CONCLUSION ........................................................................................................................................... 79
REFERENCE .............................................................................................................................................. 80

CHAPTER 3: DEVELOPMENT OF AN ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETECTION OF PISTACHIO PROTEINS IN PROCESSED FOOD

ABSTRACT .............................................................................................................................................. 83
INTRODUCTION ........................................................................................................................................ 84
MATERIALS AND METHODS .................................................................................................................... 86
  Development of Pistachio Sandwich ELISA .......................................................................................... 86
Pistachio ELISA Optimization and Validation ................................................. 89
Detection of Raw and Roasted Pistachio Using The Roasted Pistachio ELISA ..... 89
Cross- Reactivity Study .................................................................................. 89
Matrices Interferences Study ......................................................................... 91
Statistical Analysis ....................................................................................... 94
RESULTS AND DISCUSSION ....................................................................... 95
Pistachio Sandwich ELISA Standard Curves .................................................. 95
Comparison of Raw and Roasted Pistachio Standard Curves ......................... 97
Detection of Raw and Roasted Pistachio Using The Roasted Pistachio ELISA .... 97
Cross- Reactivity Studies ............................................................................... 99
Matrix Interference Studies .......................................................................... 105
CONCLUSION ............................................................................................. 110
REFERENCES ............................................................................................. 111

CHAPTER 4: PRODUCTION OF MANUFACTURED MODEL FOODS FOR DETECTION OF PISTACHIO RESIDUES .................................................. 114
ABSTRACT .................................................................................................. 114
INTRODUCTION .......................................................................................... 115
MATERIALS AND METHODS ..................................................................... 117
Preparation of Manufactured Model Foods .................................................... 117
Vanilla Ice cream ....................................................................................... 117
Sugar Cookie ............................................................................................. 120
Sample Preparation and Extraction .............................................................. 122
Vanilla Ice Cream ..................................................................................... 122
Sugar Cookies and Cookie Dough ............................................................... 123
RESULTS AND DISCUSSION ................................................................ 124
Recovery of Pistachio From Vanilla Ice Cream ........................................... 125
Recovery of Pistachio From Sugar Cookies ................................................... 129
CONCLUSION .......................................................................................... 135
REFERENCES .......................................................................................... 136
LIST OF TABLES

CHAPTER 1
Table 1. Tree nut allergens................................................................. 14
Table 2. Nutrient composition of pistachios........................................ 21

CHAPTER 2
Table 3. Protein content of raw and roasted pistachio determined by LECO (Dumas)
method (total protein content) and the Lowry method (soluble protein content)
........................................................................................................... 66

CHAPTER 3
Table 4. Cross-reactivity of different food and food ingredients in the pistachio ELISA
................................................................................................................ 101

CHAPTER 4
Table 5. Formulation of naturally incurred standards of pistachio in vanilla ice cream 119
Table 6. Formulation of naturally incurred standards of pistachio in sugar cookie ...... 121
Table 7. Mean ppm and percent recovery of pistachio from manufactured vanilla ice
cream obtained from three different locations within the ice cream freezer as
determined by the developed pistachio ELISA .................................... 127
Table 8. The mean ppm and percent recovery of pistachio from manufactured vanilla ice
cream (combination of ice cream A, B, and C) determined by the developed
pistachio ELISA .................................................................................... 128
Table 9. Mean ppm and percent recovery of pistachio from manufactured sugar cookies
determined by the developed pistachio ELISA ........................................ 133
Table 10. Mean ppm and percent recovery of pistachio from manufactured sugar cookies
(combination of sugar cookies A, B, and C) and sugar cookie dough determined
by the developed pistachio ELISA .......................................................... 134
LIST OF FIGURES

CHAPTER 1
Figure 1. Mechanism of IgE-mediated allergy reaction .................................................. 6
Figure 2. Competitive ELISA ....................................................................................... 27
Figure 3. Two-stage indirect ELISA ............................................................................ 29
Figure 4. A sandwich-type ELISA .............................................................................. 30

CHAPTER 2
Figure 5. Pre-screening of 6 rabbits, 2 sheep and 2 goats. Mean absorbance values of the sera from all animals screened by indirect ELISA. ........................................ 68
Figure 6. Titration curve for rabbit anti-roasted pistachio sera (first production bleed of rabbit NE 249) ........................................................................................................... 71
Figure 7. Immune response of individual rabbits to roasted pistachio immunogen. ....... 72
Figure 8. Immune response of individual sheep and goat to roasted pistachio immunogen. ........................................................................................................ 73
Figure 9. Immune response of individual rabbits to raw pistachio immunogen.......... 74
Figure 10. Immune response of individual sheep and goat to raw pistachio immunogen.75
Figure 11. Brilliant Blue G-Colloidal stained SDS-PAGE gel with separated raw and roasted pistachio proteins extracted using 0.01 M PBS containing 0.85% NaCl. ........................................................................................................ 77
Figure 12. Rabbit, sheep, and goat IgG-immunoblotting of raw and roasted pistachio protein extracts ........................................................................................................ 78

CHAPTER 3
Figure 13. Optimized raw and roasted pistachio sandwich ELISA standard curves. ...... 96
Figure 14. Raw and roasted pistachio sandwich ELISA standard curves using anti-roasted pistachio antibodies .................................................................................. 98
Figure 15. Evaluation of the cross-reactivity between cashew and pistachio. .......... 104
Figure 16. Standard curves of 1000 ppm pistachio spiked into 0.01 M PBS, sugar cookie and vanilla ice cream as determined by the pistachio ELISA. ................. 107
Figure 17. Sandwich ELISA standard curve of regular ground (RG) pistachio compared to the standard curve of finely ground (FG) pistachio........................................ 109
CHAPTER 4

Figure 18. Standard curve for vanilla ice cream spiked with 10,000 ppm ground pistachio (standard) as determined by the pistachio ELISA. .............................................. 126

Figure 19. Standard curve for sugar cookies spiked with 10,000 ppm ground pistachio (standard) as determined by the pistachio ELISA. .............................................. 132
INTRODUCTION

Food is essential to provide nutrients and energy for living. While typical consumed foods are safe to eat for nearly everyone, those with specific allergies to specific foods or ingredients should avoid them in order to remain symptom free. Food allergy is an adverse immunological response to a specific allergenic food. The unintended ingestion of an offending food may provoke adverse allergic reactions, ranging from mild discomfort such as rashes, hives and angioedema to life threatening reactions such as anaphylactic shock. Currently, no effective treatments are available for food allergy; the strict avoidance of the allergenic food is the only way to prevent allergic reactions. Food allergy is becoming more prevalent worldwide and is currently a serious problematic health issue in developed countries such as the United States, Canada, the European Union, Australia, and Japan (Sicherer and Sampson, 2010; van Hengel, 2007; Angus, 2000). Genetic and other factors such as changes in dietary practices, as well as an increase of exposure to allergenic foods, are believed to be responsible for the increased prevalence of food allergy (Lack, 2008; Lehrer et al., 2002).

Tree nuts including pistachios are common allergenic foods. The increased consumption of pistachio due to its beneficial health properties can result in sensitization that also increases the risk of adverse allergic reactions. As a results, food allergen labeling is mandated by the U.S. Food and Drug Administration (FDA) under the Food Allergen Labeling and Consumer Protection Act (P.L. 108-282) (FALCPA), and serves to protect pistachio-allergic consumers from ingesting pistachios because of mislabeling,
food allergen cross contact during processing, or hidden sources of pistachio. Appropriate food allergen labeling refers to a complete listing of all ingredients containing common allergenic foods, which includes a listing of the specific types of tree nuts. The development of sandwich-type enzyme-linked immunosorbent assays (ELISAs) has been a preferred approach in detecting traces of food allergens in processed foods due to its high accuracy, precision, and simple handling (Koppelman et al., 2004).

The following chapter describes the characteristics and the mechanisms of food allergy. A review of pistachio allergy and allergens, and the analytical methods for their detection in foods is also discussed in this chapter.

ADVERSE REACTIONS TO FOODS AND FOOD HYPERSENSITIVITY

Food hypersensitivities can be defined as an individualistic abnormal reaction resulting from the ingestion of foods or food additives, which include food intolerances (non-immunological reactions) and food allergies (immunological reactions) (Asero et al., 2007). Food intolerances are adverse food reactions that do not involve the immune system and are usually caused by physiologic characteristics of the host, such as lactase deficiency. Lactase deficiency is characterized by the low levels of lactase and causes difficulties in the digestion of normal amounts of ingested lactose in the body, resulting in diarrhea and bowel discomfort (Taylor and Hefle, 2001).

Food allergy

Food allergy is primarily an IgE-mediated hypersensitivity, whereby the body’s immune system responds abnormally to an ingested food antigen by producing IgE
antibodies (Burks and Ballmer-Weber, 2006; Taylor and Hefle, 2001). The prevalence of food allergy has become one of the major public health concerns worldwide, especially in developed countries such as the United States, the European Union, Canada, Japan, etc. In the United States, the population suffering from food allergy has been gradually increasing over the past decade, and estimates indicate that as many as 50,000 to 125,000 emergency room visits occur each year due to food-induced anaphylaxis in the United States (U.S.) (Ross et al., 2008; Decker et al., 2008). Studies showed an estimated 3-4% of the total population of Americans is affected by food allergies and an even higher prevalence of food allergy occurs in infants and children with prevalence levels reaching to 8% of the population (Sicherer and Sampson, 2006; Burks and Weber, 2006). Infants and children are more susceptible to food-allergic disorders due to the immature development of their immune systems and their gastrointestinal tract. Food allergy reactions presents as a rapid onset of symptoms (immediate hypersensitivity reactions) usually occurring within minutes or hours after ingestion of the offending foods. There are four main types of symptoms including dermatological (hives, angioedema, and atopic dermatitis); gastrointestinal (abdominal pain, diarrhea, nausea, and vomiting); respiratory (rhinitis and asthma); and systemic (anaphylactic shock) (Asero et al., 2007).

Food allergy not only diminishes the quality of health and life-style of the allergic individual but also their families (Munoz-Furlong, 2003). This is because no effective treatments are available for food allergy; strict avoidance of the offending food is the only way for prevention. This prevention strategy includes eliminating the offending foods and/or food ingredients in the diet by reading the food allergen labeling on every product before purchasing or consumption. For this strategy, food-allergic consumers
need to acquire more knowledge about food ingredients. However, increasing knowledge could be difficult for food-allergic consumers because of the rise of novelty food products in the market. These food products contain unfamiliar ingredients that are possibly derived from known allergenic sources. Therefore, the prevention strategy is highly dependent on food manufacturers to provide accurate labeling of ingredients and meaningful descriptive allergen labels on food products to help food-allergic consumers recognize the particular offending foods and/or ingredients. Additionally, avoidance of allergen cross-contamination from the offending foods and/or food ingredients while manufacturing, handling and preparing foods is also important for food allergy prevention. Eating at a restaurant has been challenging for food-allergic consumers because some of the cooking ingredients may not be stated in the menu, potentially leading to an allergic reaction. Moreover, sharing cooking equipment is common in restaurants and may also result in cross-contact with allergens from other foods. Therefore, consumers who at risk of a severe food-allergic reaction should carry self-injectable epinephrine that may help lower the potential risk of life-threatening anaphylactic reactions.

**Mechanisms of Food Allergy**

The body’s immune system acts as a surveillance system to protect the body from foreign substances especially infections including bacteria, viruses, and parasites. In general, the immune system does not respond to dietary proteins in a manner similar to other foreign antigens. Instead, humans are tolerant of proteins in their diet, a phenomenon known as oral tolerance. Food allergy is caused by the inherent failure in
the development or breaking down of oral tolerance in the gastrointestinal tract. This occurrence predisposes the body to produce excessive food-specific IgE antibodies (Burks and Ballmer-Weber, 2006).

The IgE-mediated allergic reaction involves antigen-presenting cells (APCs) and lymphocytes—B cells and T cells. The initial induction of food allergy requires an immunological activation called sensitization, which is believed to begin from intact fragment(s) of allergens resistant to cooking and digestive processes that penetrate into the epithelial barrier of the gut. The microfold cell (M cells), a follicle-associated epithelium which overlies the Peyer’s patches take up the allergens and transport them across M cells into the lamina propria. The Peyer’s patches are mucosa-associated lymphoid tissues that are made up of B cell follicles and an interfollicular T cell region, as well as numerous intervening APCs including macrophages, dendritic cells, and B cells. The antigens are captured by naïve B cells and also by the major histocompatibility complex (MHC) class II complex of APCs and are then presented to naïve CD4 T cells (Figure 1). The antigen-specific Th2 CD4 cells (T-helper 2 cells) and B cells become activated when antigens are presented to these cells via APCs. Subsequently, the activated antigen-specific B cells affect contact with the activated antigen-specific Th2 CD4 cells, resulting in an immune synapse formation that drives B cells to undergo proliferation and differentiation, with class switching from IgM to IgE antibodies specific for the particular allergen (McHeyzer-Williams, 2003; Lehrer et al., 2002). The antigen-specific IgE antibodies bind with high affinity to the receptors on mast cells and basophils that are then known as sensitized cells (Taylor and Hefle, 2001).
When a subsequent interaction of allergen occurs, it results in the cross-linking of two or more membrane-bound IgE molecules, which causes the mast cell and basophil to degranulate and release chemical mediators including histamine into the tissues and bloodstream. The mediators elicit the physiological effects of allergic reactions, such as vasodilatation, smooth muscle contraction, and mucus secretion (Burks and Ballmer-Weber, 2006; Lemke and Taylor, 1994; Lehrer et al., 2002).

Figure 1. Mechanism of IgE-mediated allergy reaction (Lehrer et al., 2002; Taylor and Hefle, 2001).
Food Allergens

Foods are generally complex, consisting of many components such as carbohydrates, lipids, proteins, and minerals. However, the allergenic food components that cause food allergy are usually naturally occurring food proteins or glycoproteins (Besler, 2001). Food allergens can be defined as food substances that can react with specific IgE antibody, inducing allergic sensitization and eliciting clinical allergic reaction. Food allergens are generally the major proteins of the food that are abundant, structurally stable and highly soluble in aqueous solution such as ovalbumin in egg, glycinin in soy, parvalbumin in fish, etc. Food proteins that are physically stable in harsh conditions have a higher potential to be allergenic; examples of these conditions are high heat during food processing or low pH and proteolysis in the digestive system of the gut (Taylor, 2001; Lehrer et al., 2002; Bush and Hefle, 1996). Moreover, food allergens are usually divalent or multivalent molecules with two or more IgE antibody-binding sites (epitopes), and each epitope will be a minimum of approximately 15 amino acid residues long (Huby et al., 2000). The IgE-binding epitopes on an allergen cross-link two mast cell- or basophil-bound specific IgE molecules and trigger degranulation and the release of inflammatory mediators such as histamine, serotonin, and leukotrienes which give the symptoms of allergy (Huby et al., 2000; Lehrer et al., 2002). Every food protein may have the potential to be an allergen but some proteins are much more commonly allergenic than others. Although, many food allergens have been characterized in a wide variety of foods, most of the food allergies (more than 90%) are generally caused by eight groups of foods that are also known as the “Big 8”. These groups include chicken eggs, cow’s milk, crustacean shellfish, fish, peanuts, soybeans, tree nuts, and wheat. The
typical allergens that affect mostly infants or young children are egg, milk, and peanut; whereas, the allergens which cause allergic reactions in older children or adults are most likely peanut, tree nuts and seafood (Sicherer and Sampson, 2010). Allergies to milk, eggs, soy and wheat in infants and young children are frequently outgrown as tolerance ultimately develops. Peanut, tree nuts (almond, Brazil nut, cashew, hazelnut, macadamia nut, pecan, pine nut, pistachio, and walnut), and seafood allergies are unlikely to be outgrown (Sampson, 2004).

**Threshold Doses (minimal eliciting doses)**

The concentration or dose of particular allergens needed to stimulate sensitization or elicit an allergic reaction is generally unknown, and varying eliciting doses of allergenic foods are observed among different individuals (Lehrer et al., 2002; Bindslev-Jensen et al., 2002). However, the amount of offending food or allergen that is needed to elicit an adverse reaction for a severe food-allergic patient may be very low in some cases. Allergic reactions have been reported in studies including contacting the lips of a person who has just eaten the offending food, skin contacting items that were contaminated with the offending food, and inhaling vapors from cooking or processing of the offending food (Bahna, 2004; Steensma, 2003).

Recently, statistical modeling approaches were used by Taylor et al. (2009) to determine the threshold dose of peanut for peanut-allergic consumers. Clinical oral challenge studies can be used to determine the lowest- and no-observed adverse effect levels (LOAELs and NOAELs) for peanut-allergic individuals. LOAEL is the lowest dose or the minimum eliciting dose to produce an adverse effect; whereas, NOAEL is the
highest dose observed not to produce any adverse effect. Statistical modeling of NOAELs and LOAELs for large groups of peanut-allergic individuals provides information on the population threshold levels. Furthermore, LOAELs and NOAELs can be used to benefit peanut-allergic consumers and physicians in order to determine the degree of care that must be employed in applying the restriction diet. Population thresholds play an important role for public health and the food industry to establish action levels to monitor the success of allergen control programs and to adjust labeling policies to protect food-allergic consumers from being exposed to doses that might elicit allergic responses.

**TREE NUT ALLERGY**

Tree nuts allergy has become increasingly common in recent years affecting approximately 0.5% of the U.S. population, ranking as the third most prevalent food allergy after crustacean shellfish and peanut allergy (Sampson, 2004; Sicherer et al., 1999; Sicherer et al., 2004). Like other food allergies, tree nut allergy is associated with an IgE-mediated allergic mechanism and often leads to itching or swelling in the mouth, rashes, eczema, and other symptoms after exposure to the particular offending nuts. In addition, tree nut allergy is one of the more severe food allergies and can involve a systemic allergic reaction that includes life-threatening anaphylactic shock (Sicherer et al., 2001; Teuber et al., 2003). Furthermore, the Food Allergy and Anaphylaxis Network (FAAN) peanut and tree nut voluntary registry in the U.S. (self-reported) showed English walnuts (34%), cashews (20%), and almonds (15%) are the most frequent causes of tree nut-allergic reactions followed by pecan (9%) and pistachio (7%), with hazelnut, Brazil nut, macadamia, and pine nut covering less than 5% respectively (Sicherer et al., 2001).
However, the data in this registry are self-reported and perhaps less reliable than comparative data involving clinical confirmation of patient histories. Clinical confirmation may involve skin prick tests with suspect foods that documents allergic sensitization but not allergic reaction or, better yet double-blind, placebo-controlled oral food challenge with the suspect food which documents the existence of an adverse reaction (Bock et al., 1988).

Thus, a comparison of the self-reported data in the registry with clinical studies of the comparative prevalence of tree nut allergies is useful. A clinical study on the prevalence for tree nut allergy was completed by Fleischer (2005). This study of 101 tree nut-allergic patients at a referral clinic confirmed that walnut and cashew were the most common allergenic tree nuts in the U.S. The percentage of these patients with allergies to specific tree nuts were walnut (30%), cashew (30%), pecan (14%), almond (8%), hazelnut (5%), macadamia nut (4%), pistachio (4%), Brazil nut (3%), and pine nut (2%). Pecan allergy shows a higher prevalence than almond in the clinical study compared to the registry but this might be expected as walnuts and pecans are closely related. In addition, more people claimed to have allergy to pistachio (7%) in the registry study compared to the clinical study (4%). Other tree nuts such as Brazil nut, hazelnut, macadamia, and pine nut show relatively low percentage (≤ 5%) in both studies. A study of young pediatric patients conducted at Mt. Sinai School of Medicine in New York showed that the comparative prevalence of tree nut allergies was walnut (26%), pecan (13%), almond (13%), cashew (11%), hazelnut (7%), pistachio (7%), pine nut (7%), and Brazil nut (4%) (Sicherer et al., 1998). This difference may be attributable to the different age range of this group of patients and the possibility that many of them had not yet
ingested cashews. Prevalence patterns are going to reflect consumption patterns to some degree. In clinical studies such as these, selection biases occur because only a certain segment of the allergic population are likely to seek assistance from these referral clinics.

Unlike egg and milk allergies, tree nut allergy is rarely outgrown. Therefore, the rate of people who are affected by tree nuts is expected to be constant and the false-positive rate of historical determination of allergy is generally low. An increase in population growth and outgrowth from peanut allergy due to the cross-reactivity between peanut and tree nuts could be the two possibilities for the outgrowing tree nut allergy (de Leon et al., 2003). The high prevalence of allergy in walnut, cashew, almond, and pecan might be due to the popular use as ingredients in foods. Tree nut allergic consumers do not always know which specific nuts they are allergic to because cross-reactivity and cross-contamination make it difficult to pinpoint the causative tree nut; therefore, further clinical tests are required to be done to verify the specific tree nut allergy. This could help consumers eliminate the unnecessary nutritional restriction in their diet. Consumers who are allergic to cashew are often allergic to pistachio as well; this is one of the examples of cross-reactivity of two proteins from a closely related family (Ahn et al., 2009; Willison et al., 2008; Garcia et al., 2000). Consumers who allergic to cashew are advised to avoid pistachio unless further clinical analysis have been done or the consumer has a history of tolerance to pistachio.
Tree Nut Allergens

Tree nut allergens are potent and often lead to severe allergic reactions such as analphylactic shock (Bock et al., 2001). Several tree nut allergens have been identified and are listed in the official allergen database of the IUIS allergen nomenclature subcommittee (International Union of Immunological Societies, IUIS; http://www.allertgen.org/List.htm) (Table 1). Most tree nut allergens are seed storage proteins (Roux et al., 2003; Teuber et al., 2003). The allergenic seed storage proteins are usually conserved proteins with homologous sequences and structures that are highly compact. The seed storage protein is important in developing plants for embryo development and other biological activities (Hoffmann-Sommergruber and Mills, 2009). Seed storage proteins are mainly soluble proteins and are classified based on their sedimentation coefficients. The 2S albumin seed storage proteins which belong to prolamin superfamily are water soluble; whereas, the 11S legumin (hexameric globulin, composed of 30-40 kDa acidic subunit and 17-20kDa basic subunit) and 7S vicilin (trimeric globulin, Mr ~50kDa) from cupin superfamily are soluble in dilute saline solution (Breiteneder and Radauer, 2004; Roux et al., 2003).

The 2S albumins are heterodimeric proteins (molecular weight, Mr, ~15kDa) consisting of two subunits that are linked by disulfide bonds. The 2S albumins are found in a variety of tree nuts, including Brazil nut (Ber e 1), cashew (Ana o 3), walnuts (Jug r 1), pecan (Car i 1), hazelnut (Cor a 14), and pistachio (Pis v 1).

The 7S vicilin-like and 11S legumin-like proteins are globular storage proteins belonging to the cupin superfamily due to their unique ‘jelly-roll’ barrel conformation. The 7S globulins are trimeric proteins that are made up of three subunits. The 11S
globulins are hexameric proteins that consist of six subunits, whereby each subunit is post-translationally cleaved into acid and basic polypeptides with intermolecular disulfide linkages. The disulfide bonds structurally stabilize the 2S albumin and 11S globulin and allow resistance to thermal denaturation and pepsin digestion. These are two common characteristics of food allergens. The 7S vicilin-like allergenic proteins have been characterized in cashew (Ana o 1), hazelnut (Cor a 11), pistachio (Pis v 3), English walnut (Jug r 2) and black walnut (Jug n 2). In addition, the 11S legumin-like allergenic proteins have been identified in almond (Pru du 6), Brazil nut (Ber e 2), cashew (Ana o 2), English walnut (Jug r 4), hazelnut (Cor a 9), pecan (Car i 4), and pistachio (Pis v 2 and Pis v 5).

In addition to seed storage proteins, other allergenic proteins such as profilin and lipid transfer proteins (LTPs) can also be found in tree nuts. These proteins are considered panallergens because they are commonly found in fruits, vegetables, nuts, seeds, and pollens. Other reasons include the proteins’ high association with IgE-mediated cross-reactivity in patients with LTP- or profilin-specific IgE production (Asero et al., 2008). A study demonstrated patients who are allergic to pollen allergens (Bet v 1) may experience allergic symptoms after the ingestion of tree nuts (Hirschwehr et al., 1992).
<table>
<thead>
<tr>
<th>Tree nuts</th>
<th>Named food allergen</th>
<th>Allergen Classification</th>
<th>Molecular weight (kDa)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Almond</td>
<td>Pru du 3</td>
<td>Lipid transfer protein</td>
<td>9</td>
<td>IUIS</td>
</tr>
<tr>
<td></td>
<td>Pru du 4</td>
<td>Profilin</td>
<td>14</td>
<td>Tawde et al., 2006</td>
</tr>
<tr>
<td></td>
<td>Pru du 5</td>
<td>60S acidic ribosomal prot P2</td>
<td>10</td>
<td>Abolhassani and Roux, 2009</td>
</tr>
<tr>
<td></td>
<td>Pru du 6</td>
<td>11S legumin-like</td>
<td>ca. 360</td>
<td>Sathe et al., 2002</td>
</tr>
<tr>
<td>Brazil nut</td>
<td>Ber e 1</td>
<td>2S albumin</td>
<td>9</td>
<td>Alcocer et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Ber e 2</td>
<td>11S globulin</td>
<td>29</td>
<td>Bartolome et al., 1997</td>
</tr>
<tr>
<td>Cashew</td>
<td>Ana o 1</td>
<td>7S vicilin-like</td>
<td>50</td>
<td>Wang et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Ana o 2</td>
<td>11S legumin-like</td>
<td>55</td>
<td>Wang et al., 2003</td>
</tr>
<tr>
<td></td>
<td>Ana o 3</td>
<td>2S albumin</td>
<td>14</td>
<td>Robotham et al., 2005</td>
</tr>
<tr>
<td>Hazelnut</td>
<td>Cor a 1</td>
<td>Pathogenesis-related protein</td>
<td>17</td>
<td>Breiteneder et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Cor a 2</td>
<td>Profilin</td>
<td>14</td>
<td>IUIS</td>
</tr>
<tr>
<td></td>
<td>Cor a 8</td>
<td>Lipid transfer protein</td>
<td>9</td>
<td>Schocker et al., 2004</td>
</tr>
<tr>
<td></td>
<td>Cor a 9</td>
<td>11S legumin-like</td>
<td>40</td>
<td>Beyer et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Cor a 10</td>
<td>Luminal binding protein</td>
<td>70</td>
<td>IUIS</td>
</tr>
<tr>
<td></td>
<td>Cor a 11</td>
<td>7S vicilin-like</td>
<td>48</td>
<td>Lauer et al., 2004</td>
</tr>
<tr>
<td></td>
<td>Cor a 12</td>
<td>oleosin</td>
<td>17</td>
<td>IUIS</td>
</tr>
<tr>
<td></td>
<td>Cor a 13</td>
<td>Oleosin</td>
<td>14-16</td>
<td>IUIS</td>
</tr>
<tr>
<td></td>
<td>Cor a 14</td>
<td>2S albumin</td>
<td>15-16</td>
<td>IUIS</td>
</tr>
</tbody>
</table>

1 Modified from Teuber et al., 2003; International International Union of Immunological Societies (IUIS); http://www.allertgen.org/List.htm.
Table 1 (continued). Tree Nut Allergens

<table>
<thead>
<tr>
<th>Tree nuts</th>
<th>Named food allergen</th>
<th>Allergen Classification</th>
<th>Molecular weight (kDa)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macadamia</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pecan</td>
<td>Car i 1</td>
<td>2S albumin</td>
<td>16</td>
<td>IUIS</td>
</tr>
<tr>
<td></td>
<td>Car i 4</td>
<td>11S legumin-like</td>
<td>55.4</td>
<td>Sharma et al., 2007</td>
</tr>
<tr>
<td>Pine nut</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pistachio</td>
<td>Pis v 1</td>
<td>2S albumin</td>
<td>7</td>
<td>Ahn et al., 2009</td>
</tr>
<tr>
<td></td>
<td>Pis v 2</td>
<td>11S globulin</td>
<td>32</td>
<td>Ahn et al., 2009</td>
</tr>
<tr>
<td></td>
<td>Pis v 3</td>
<td>7S vicilin</td>
<td>55</td>
<td>Willison et al., 2008</td>
</tr>
<tr>
<td></td>
<td>Pis v 4</td>
<td>Manganese superoxide dimutase</td>
<td>25.7</td>
<td>Ayuso et al., 2007</td>
</tr>
<tr>
<td></td>
<td>Pis v 5</td>
<td>11S globulin (acidic subunit)</td>
<td>36</td>
<td>Beyer et al., IUIS</td>
</tr>
<tr>
<td>Walnut (English)</td>
<td>Jug r 1</td>
<td>2S albumin</td>
<td>15-16</td>
<td>Teuber et al., 1998</td>
</tr>
<tr>
<td></td>
<td>Jug r 2</td>
<td>7S vicilin-like</td>
<td>44</td>
<td>Teuber et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Jug r 3</td>
<td>Lipid transfer protein</td>
<td>9</td>
<td>Pastorrello et al., 2004</td>
</tr>
<tr>
<td></td>
<td>Jug r 4</td>
<td>11S globulin</td>
<td>-</td>
<td>Teuber et al., 2003</td>
</tr>
<tr>
<td>Walnut (Black)</td>
<td>Jug n 1</td>
<td>2S albumin</td>
<td>-</td>
<td>Bannon et al., 2001</td>
</tr>
<tr>
<td></td>
<td>Jug n 2</td>
<td>7S vicilin-like</td>
<td>-</td>
<td>Bannon et al., 2001</td>
</tr>
</tbody>
</table>

- * Data not available
Cross-reactivity

Foods within a certain group or family having proteins with a high degree of similarity in amino acid sequences can cause cross-reactions in food-allergic individuals. Individuals with pollinosis can develop allergic sensitization to pollen-related food allergens. The high similarity or homologous structures between food proteins and pollens such as birch pollen, often cause IgE-mediated cross-reactivity in pollen-allergic individuals and induce oral allergy syndrome (OAS) (Vieths et al., 2002). Symptoms of OAS are elicited by cross-reaction of food proteins in fresh fruits, vegetables and nuts with pollen allergens, resulting in itching and swelling of the lips, mouth and throat. However, patients with pollen-related allergy can consume and tolerate pollen-related foods after heat treatment. Because pollen allergens are usually heat labile, the proteins such as pathogenesis related protein 10 family members (PR-10 proteins including Bet v 1, Ara h 8, Gly m 4) and profilins (Berkner et al., 2009; Mittag et al., 2004; Breiteneder and Ebner, 2000) can be easily denatured through heat processing and the IgE binding epitopes are usually destroyed in the process.

Additionally, legume plants such as soybeans, peanuts, and peas possess a common protein, 11S glycinin, which shows a positive response in serological cross-reactivity by in vitro tests (Beardslee et al., 2000; Moneret-Vaurin et al., 1999). Cashew and pistachio, belonging to the Anacardiaceae family, are another example demonstrating that the conserved seed storage proteins from the same botanical family contain highly cross-reactive allergens (Ahn et al., 2009; Willison et al., 2008; Garcia et al., 2000). However, the results demonstrated by IgE cross-reactivity from in vitro tests may be
inconsistent with the results demonstrated by in vivo tests. A common muscle protein, tropomyosin, present in crustacea and mollusks showed cross-reactivity by in vitro assays; however, many crustacea-allergic individuals are not allergic to mollusks (Leung et al., 1996).

PISTACHIO NUT

Pistachios (Pistacia vera) belong to the of Anacardiaceae family which also includes cashew, mango, and sumac. Pistachio is well known for its beige hard shells, enclosing a yellowish green edible kernel that is enveloped by purple colored skins. Pistachios grow in heavy grape-like clusters on trees, surrounded by a fleshy hull. The pistachio trees are small to medium in size, bushy and deciduous with separate male and female trees. They grow slowly to a height of about 6-9 meters with one or several trunks. In addition, the male and female flowers are apetalous, such that they spread the pollen through wind pollination and begin the growth cycle from late spring. Pistachios grow best in hot sunny areas but they can survive temperatures ranging from −10°C in winter to 40°C in summer. Throughout the summer, pistachios first grow by enlarging the nut shells until the shells harden, followed by enlargement of the nutmeats (kernels) until the nutmeats fill up the shells. In late summer, pistachio kernels become large and mature (ripen), causing the shells to split and the hulls to degrade. Thus, pistachios’ shells naturally split when they are fully ripened, a signal to harvest. Pistachio trees shed their leaves during fall and remain dormant in the winter. Moreover, pistachios have long
juvenile periods as they bear fewer drupes during the first five years and only achieve full-bearing status between 10-12 years of age (Heber and Bowerman, 2008).

Pistachios have grown natively in Middle Eastern countries such as Iran and Turkey and in western Asia countries such as India since the early ancient period (Ferguson et al., 2005). In the late 1890’s, pistachios were first introduced to the United States (U.S.) under the USDA plant exploration service and developed commercially in the southern U.S. Pistachios are primarily grown in California due to the ideal cultivation conditions, and grown sparingly in Arizona and New Mexico. The United States is the second largest pistachio producer and exporter in the world, ranking only after Iran. According to Agricultural Marketing Resource Center (AgMRC), pistachios were the third largest tree nut crop in 2008, behind almonds and walnuts. Pistachio production (in-shell) increased to 416 million pounds in 2007 compared to 238 million pounds in year 2006 (USDA, 2008). This large increase in pistachio production indicates an increased consumption of pistachios among consumers.

**Nutritional Values of Pistachios**

Pistachios are one of the most nutrient-dense foods (Table 2). A one ounce serving of pistachios, approximately 49 kernels, provides about 160 calories and 6 g of protein; together with 13 g of fat, 3 g of dietary fiber, and 7 g of monounsaturated fat. Additionally, pistachios are also excellent sources of minerals, vitamins, and powerful antioxidant compounds such as luteins, zeaxanthins, and anthocyanins. Luteins are the compounds that make pistachios appear yellowish-green (Giuffrida et al., 2006).
Moreover, the purple skin of pistachio is caused by the presence of anthocyanins, compounds that provide strong antioxidant effects on human health. Pistachio nuts were listed as one of the heart-healthy snacks according to Gebauer (2008). Studies demonstrated consuming a one ounce serving size of pistachios daily can constrain weight gain and improve the blood cholesterol profile (Edwards et al., 1999; Gebauer et al., 2008).

The high nutritional value of pistachio makes it stand out among tree nuts, especially its high amount of monounsaturated fatty acids that replace the saturated fatty acids. This profile reduces the level of LDL (low density lipoprotein) cholesterol and elevates the level of good cholesterol, HDL (high density lipoprotein) in the body. The replacement of saturated fatty acids with monounsaturated fatty acids, coupled with an abundant intake of antioxidants, helps reduce the risk of cardiovascular disease (Sheridan et al., 2007; Tokusoglu et al., 2005; Dietary Guidelines for Americans, 2005).

Pistachio nuts are primarily sold in the grocery stores as a roasted and salted snack food. Due to the high nutritional value and unique green color, pistachios are widely used in the catering industry as ingredients for ice cream, cakes, chocolates, cookies, pasta, pudding, salads, and flavorings. However, the increased consumption is leading to an increase in the frequency of allergic sensitization. Tree nuts (including pistachios) are recognized as commonly allergenic foods. Several proteins in pistachio are now recognized as pistachio allergens (Pis v 1, 2S albumin; Pis v 2, 11S globulin; Pis v 3, 7S vicilin) in the literature (Ahn et al. 2009; Willison et al., 2008). Therefore, the
significant increase in consumption of pistachios may lead to sensitization to pistachio nut, which may provoke food allergy symptoms
Table 2. Nutrient composition of pistachios

<table>
<thead>
<tr>
<th>Calories</th>
<th>160</th>
<th>Protein</th>
<th>6 (g)</th>
<th>Total fat</th>
<th>13 (g)</th>
<th>Carbohydrate</th>
<th>8 (g)</th>
<th>Dietary Fiber</th>
<th>3 (g)</th>
</tr>
</thead>
</table>

**Minerals (% DV)**

<table>
<thead>
<tr>
<th>Mineral</th>
<th>3</th>
<th>7</th>
<th>9</th>
<th>14</th>
<th>8</th>
<th>0</th>
<th>4</th>
<th>18</th>
<th>17</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnesium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphorus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copper</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Manganese</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selenium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Vitamins (% DV)**

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>2</th>
<th>17</th>
<th>3</th>
<th>2</th>
<th>1</th>
<th>25</th>
<th>4</th>
<th>0</th>
<th>3</th>
<th>0</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiamin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Riboflavin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Niacin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pantothenic Acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin B6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin B12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin E-α-tocopherol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin K</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Lipids (g)**

<table>
<thead>
<tr>
<th>Lipid Type</th>
<th>1.5</th>
<th>7</th>
<th>4</th>
<th>61</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated Fat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monounsaturated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Phytosterols (mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Carotenoids (mcg)**

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>94</th>
<th>342</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta-carotene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lutein &amp; zeaxanthin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Pistachio Allergens

Until recently, little was known about specific pistachio allergens. However, the allergenic proteins of pistachios are primarily seed storage proteins. A few scientists have been investigating the cross-reactivity of the *Anacardiaceae* family allergens in the past decade. Pistachio nuts are known to cross-react with cashew nuts because they belong to the same family, which means cashew-allergic patients may show positive food allergy reactions when they ingest pistachio nuts (Hasegawa et al., 2009). According to studies conducted by Fernández (1995) and Parra et al. (1993), IgE antibodies obtained from the sera of patients with pistachio nut allergy cross-reacted with other *Anacardiaceae* members, including cashew nuts and mango seeds. In addition, Funes et al. (1999) conducted studies utilizing both *in vivo* and *in vitro* techniques to identify *Anacardiaceae* allergens. The protein profiles of cashew nuts, mango seeds, and pistachio nuts were assessed by SDS-PAGE followed by immunoblotting. The presence of specific IgE against *Anacardiaceae* was also determined by skin prick test and radioallergosorbent test (RAST) test. In these studies, the common allergenic pistachio nut proteins, separated by SDS-PAGE, had estimated molecular weights of approximately 14, 30, 40, and 55kDa.

To date, five pistachio allergens have been identified and listed on the IUIS allergen nomenclature subcommittee and *NCBI* databases. The pistachio allergens are Pis v 1 (2S albumin, 7kD), Pis v 2 (11S globulin, 32kD) (Ahn et al. 2009), Pis v 3 (vicilin-like protein, 55kD) (Willison et al., 2008), Pis v 4 (Manganese Superoxide Dimutase, 25.7kD) (Ayuso et al., 2007), and Pis v 5 (11S globulin, 36kD).
Pis v 1 and Pis v 2 were identified as major allergens of pistachio because Ahn et al. (2009) demonstrated that the serum of 19 out of 28 (68%) pistachio-allergic patients showed IgE binding to the 7kDa protein, while 14 out of 28 (50%) of patients bound to the 32 kDa faction. Based on a comparison of peptide sequences with cashew nuts, results showed that Pis v 1 and Pis v 2 share 64% sequence identity to Ana o 3 (cashew allergen) and 48% to Ana o 2 (cashew allergen), respectively. This could explain the high potential of cross reactivity between pistachio and cashew.

Pis v 3 (vicilin-like protein, 55kD) is perhaps the most thoroughly studied pistachio allergen, and sera from pistachio-allergic subjects has been shown to cross-react with the cashew allergen, Ana o 1. In studies of IgE binding using the sera of pistachio-allergic patients, 37% of the sera reacted to Pis v 3 and cross-reacted with Ana o 1 (Willison et al., 2008). The work conducted by Willson et al. (2008) on the identification of Pis v 3 (55kD) complemented the results previously reported by Funes et al. (1999), which indicated that this 55kD protein is a common allergen of pistachio nuts. Little published research exists on the other two possible pistachio allergens - Pis v 4 and Pis v 5. However, they are listed on the Allergome allergens database.

METHODS FOR ALLERGEN DETECTION IN FOODS

**Immunoassays**

To avoid undeclared allergens in foods, reliable detection and quantitation methods for food allergens are essential for both the food industry and food-allergic consumers. Immunoassays are often used for the detection of soluble allergenic proteins. The detection of proteins is relevant because allergens are proteins. Immunoassays are highly
sensitive and specific, depending on the binding specificity of affinity of the antibody to the antigen. Antibodies do not recognize the whole antigenic molecules. In fact, antibodies non-covalently bind to antigen epitopes (also known as antigenic determinants) throughout the protein molecule. The non-covalent bonding includes hydrogen bonds, electrostatic bonds, Van der Waals forces, and hydrophobic interactions, which are reversible and affect the strength of the interactions between antibodies and antigens. In immunoassays, antibodies that bind directly to the specific antigen are known as primary antibodies. As proteins, the primary antibodies raised from one animal species are usually structurally distinct and can be used as the antigen to give rise for the secondary antibodies in another animal species. The secondary antibodies are more often the anti-immunoglobulin antibodies, directly bound to the constant region of specific immunizing antibodies. For example, injecting rabbit immunoglobulin into goat will then give rise to goat anti-rabbit immunoglobulin antibodies. The secondary antibodies are useful in immunoassays because they are usually conjugated or labeled with enzymes and are used as detection antibodies to determine the extent of binding of antigen to primary antibodies (Deshpande, 1996).

Various immunoassay formats can be considered and include the radioallergosorbent test (RAST), enzyme allergosorbent test (EAST), enzyme-linked immunosorbent assay (ELISA), SDS-polyacrylamide gel electrophoresis (PAGE) with immunoblotting, and rocket immunoelectrophoresis (RIE) (Besler, 2001).

RAST and EAST rely on the binding of specific IgE antibodies in human sera to food allergens bound to the solid phase. However, they are not suitable for the routine determination of food allergens by the food industry. This is because the specificity of
human serum IgE varies among sensitized individuals, it is unusual to find appropriately allergic serum donor. Also, the limited amount of serum and the use of radioactive materials in the RAST make it less preferable. The sandwich-type ELISA is the most popular method used by the food industry to detect allergenic food residues due to its high sensitivity, specificity, speed, and simplicity.

SDS-PAGE with immunoblotting is a powerful immunochemical technique applied to identify allergenic proteins in food by the immunoreaction between antibody and allergen (Poms et al., 2004). Immunoblotting also characterizes protein antigens by determining the relative molecular weight of the polypeptide chains. Immunoblotting is often used with the SDS-PAGE under reducing conditions. The antigen sample is first denatured and separated by SDS-PAGE on the basis of its molecular mass and size. The separated antigen is subsequently transferred out of the gel to a nitrocellulose membrane or polyvinylidene difluoride (PVDF) membrane. The transferred antigen located on the membrane is then identified using specific antibodies followed by labeled secondary antibodies. Although immunoblotting provides a reliable qualitative method to detect the presence of a protein antigen, the denaturation process in immunoblotting might result in disruption of the epitope of an antigen which affects the extent of binding between antigen and antibody. SDS-PAGE and immunoblotting are immensely useful for screening for the presence of antigen in a sample and may be used to select the right antibody for the use in another immunoassay system, such as ELISA.

**Enzyme-linked Immunosorbent Assays (ELISAs)**

ELISAs typically employ IgG antibodies that are raised in animals directed against allergens or proteins from specific foods (Besler, 2001; Goodwin, 2004). The
antibodies can either be monoclonal or polyclonal antibodies, which bind to a very specific binding site and multiple binding sites of antigens, respectively. The binding of the antigen and antibody is detected using an enzyme linked to a secondary antibody, which turns the antigen-antibody complex into a colored product when enzyme substrate is added. ELISAs are useful methods in detecting and quantifying allergenic proteins in foods, providing relatively fast, highly specific, sensitive, and very robust allergens detection.

Basically, ELISA holds four principle steps, consisting of coating, blocking, reacting of antigen and antibody, and developing color. ELISA can be in either a competitive or non-competitive format (Yeung, 2006). These two assays are used depending on the purpose of experiments and also the types of antibodies and antigens.

**Competitive ELISAs**

The competitive ELISAs are also known as competitive inhibition ELISAs. The competitive assays apply two antigens, whereby one is coated on the microtiter plate and another is added in a sample solution containing a limited amount of primary antigen-specific antibody which competes for binding to this antibody (Yeung, 2006) (Figure 2). Technically, the antigens in the sample solution are added to the plates and bind to the primary antibody, before competing for the binding of the antigen coating on the wells. The excess or unbound antibodies remaining in the well are then washed off by the washing step, followed by adding secondary enzyme-conjugate antibody to the plate to detect the bound antigen-antibody complexes in the wells. The color is developed when the substrate of the enzyme is added. The intensity of the color is inversely proportional to the concentration of the antigens present in the solution sample. For example, a high
concentration of antigens in the solution demonstrates more binding between antigens and antibodies that gradually result in fewer antibodies that are bound to the antigens in the wells. Thus, less color will be developed. Competitive ELISAs are often used for antigen quantification and cross-reactivity testing, which help in justifying the affinity and avidity of the antibody and antigen interactions.

![Diagram of Competitive ELISA]

Figure 2. Competitive ELISA

**Non-competitive ELISAs**

There are two types of non-competitive ELISAs: (i) two-stage indirect ELISA and (ii) sandwich-type ELISA.

(i) **Two-stage indirect ELISA**

This format is also called an antigen-coated ELISA. This is the easiest approach to ELISA, and is normally used for antibody quantification or titer determination. Basically,
a serial dilution of antigens is first coated on the solid phase of the microtiter plate, followed by incubation with primary antibodies (Figure 3). The bound antigen-antibody complexes are subsequently detected by addition of secondary enzyme-conjugate antibodies which specifically attach to the primary antibodies. Levels of color are then developed depending on the bound antigen-antibody complexes when enzyme substrates are added to the plate. The colors are proportional to the concentration of the antigens in the sample. The level of color can be measured by an ELISA-plate reader at an appropriate wavelength. The concentration or the results of the analyte-containing sample can be interpolated from the standard curve based on the absorbance values. This process can be done by using an ELISA reader operating computer software, such as the Graphpad Prism® to generate a typical sigmoidal-shaped standard curves, which is plotted as the standard protein concentration versus the corresponding mean absorbance value of replicates.
Figure 3. Two-stage indirect ELISA

(ii) **Sandwich-type ELISA**

The sandwich ELISA is the most commonly used analytical method for the detection and quantification of specific allergenic proteins (Yeung, 2006; Goodwin, 2004; Besler, 2001). The sandwich ELISAs use a pair of antibodies, as capture and detector, directed against two or more distinct epitopes on antigens, which makes the detection more specific against particular antigens.

The capture antibodies specific for the antigens are first coated on the microtiter plate. After coating, a series of dilutions of the antigens in the sample solution and antigen standard are added and captured by the antibodies on the plate. The bound antigens are subsequently detected by adding a specific amount of detector antibodies whereby the
antigens get trapped and “sandwiched” in between the capture and detector antibodies.

Multiple washing steps are performed in between each step in order to remove the excess or unbound proteins. As with other ELISAs, the bound antigen-antibodies complexes are detected by the addition of the enzyme-conjugated secondary antibodies (second antibody which will bind specifically to the detector antibody), followed by incubation of the enzyme substrate. As a result, the colorimetric signal produced during the enzymatic reaction is proportional to the amount of enzyme-conjugate bound to the plate as measured with the ELISA plate reader. A direct relationship exists between the concentration of the antigen-antibody and the intensity of the signal (or color). As the concentration of antigen in the sample increases, the color becomes more intense (Figure 4).

Figure 4. A sandwich-type ELISA
Several ELISAs have been developed for the detection and quantitation of allergenic residues in various food products, including peanut (Hefle et al., 1994), almond (Hlywka et al., 2000), egg (Hefle et al., 2001), walnut (Niemann et al., 2009), soybean (Koppelman et al., 2004), casein (Hefle and Lambrecht, 2004), and mustard (Lee et al., 2008).

Detection of Pistachio Allergens

ELISA method has yet to be developed for the quantitative determination of pistachio residues in foods. To date, a conventional PCR (Barbieri and Frigeri, 2006) and a novel real-time PCR (Brezna et al., 2008) are the only published methods for the qualitative detection of pistachio in foods. PCR methods have the advantage of being highly sensitive. PCR methods detect the presence of DNA from a given source but not the presence of specific proteins in food samples. Proteins and DNA may not share the same fate in food processing making PCR less reliable than ELISA for the detection of allergen residues. Since proteins elicit allergic reaction rather than DNA, ELISA is more appropriate for the detection and quantitation of pistachio residues in foods.

ELISA DEVELOPMENT

ELISA provides relatively rapid, sensitive, specific, accurate, robust, simple, and cost effective analytical methods to the food industry for food allergen detection in supporting allergen control within HACCP and prerequisite programs. ELISA can detect allergenic residues even at a very low level including micrograms per gram (µg/g) or parts per million (ppm). The effectiveness of ELISA depends on many critical components and factors including the selection of the appropriate antigen to raise good antisera and as an
antigen standard for the assay, the use of reagents and buffers to optimize the assay system, and the examination of antibody production and performance in the immunoassay system, in terms of assessing potential non-specific binding through cross-reactivity and matrix interference studies (Goodwin, 2004; Immer, 2006; Yeung, 2006). Various quality elements in ELISA development are described below.

(i) **Antibody Production**

In ELISA, the detection of food allergen depends on the animal antisera that bind specifically to the antigen used for immunization. The most critical point to develop an effective ELISA is the right selection of food allergen used as target protein. Decisions can be made about alternative strategies to develop antisera; for example, choosing a protein antigen from raw or roasted/processed material and choosing a crude protein extract from foods or specific food allergens for producing antibodies (Besler, 2001). Generally, the allergenic protein in the processed form is favored for immunoassay development. This form will be beneficial to be used as an antibody for detecting the allergen in most processed foods. In most ELISAs for food allergen detection, crude protein extracts from allergenic foods are used to make antibodies. These antibodies are capable of recognizing most of the allergenic proteins in the allergenic foods. However, they can lead to other issues by cross-reactivating with related allergenic proteins, usually with the same or a closely related family (Vieths et al., 2002). Alternatively, using specific or isolated allergenic protein to make antibodies is relatively specific and sensitive. However, the availability and concentration of the specific protein may be varied due to various species or cultivation of the allergenic foods present in the food products. Moreover, processing may result in protein denaturation that may reduce the
immunoreactivity of the specific allergen and eventually diminish its detection. In fact, food companies are generally concerned about whether whole allergenic foods (i.e. peanut) are present in the food product, not just the presence of one allergenic protein (Hefle et al., 2006). In addition, the absence of other molecules or contaminant proteins of a target protein from an appropriate food source is essential for antibody production in order to produce highly precise, specific and sensitive antibody against particular antigen. This absence can also avoid the potential false positive results due to cross-reactivity of antibody against other contaminant proteins for later use in the immunoassay.

Antibody production requires a process called immunization, whereby a selected antigen is injected into a laboratory animal through subcutaneous and/or intramuscular routes with an appropriate adjuvant. The purpose of using adjuvant is to enhance the immune response of the animal by increasing the immunogenicity and the efficiency of antigen presentation to increase the number of antibody-secreting B-cells. Complete Freund’s adjuvant (CFA) and Incomplete Freund’s adjuvant (IFA) are the most popular adjuvants used for immunization. A good quality antiserum, characterized by its high affinity and avidity, is the key to success for an immunoassay. Starting from a low dose of immunogen for primary injection is necessary to produce antibodies with high affinity and avidity (Harlow and Lane, 1998). In addition, to maintain the production of antibody with high titer, booster injections are applied at approximately three to four week intervals after the first injection (Harlow and Lane, 1998; Hefle et al., 2006). The booster injection usually uses 10-50% of the primary dose. This low dose of antigens promotes an immune response favorable to class switching from IgM to IgG antibodies. IgG antibody has less avidity but more affinity than IgM antibody. As the immunization
undergoes several booster injections within a period, these injections cause antibody of the IgG class to dominate, eventually resulting in high titer and higher affinity.

During immunization, the quality of producing antibody production should be monitored. The titer of antibodies can be quantified using indirect ELISA through the mid-linear point of the titration curve. In addition, the specificity and affinity of the antibodies can be evaluated using immunoblotting. A good quality antigen-specific antibody, used as capture and detection antibody, is crucial for a sensitive and specific ELISA.

(ii) Optimizing Assay Operating Conditions of ELISA

Assay optimization is an essential step to achieve optimum test performance of ELISA for allergen detection. Once the assay format has been selected along with good quality antibody production, a number of required reagents and conditions are needed to perform the ELISA analysis. They include the (a) solid phase support and coating reagents, (b) sample preparation, and (c) various buffers used in the assay system, as well as time and temperature.

(a) Solid phase support and coating reagents

A solid phase support as coating carrier is one of the essential elements to begin the ELISA analysis. A 96-well polystyrene microtiter plate (in a 12 x 8 format) is commonly used in ELISA. The microtiter plate is made from highly hydrophobic material composed of a long carbon chain with benzene rings attached to every alternate carbon. This material gives the microtiter plate a greater capacity to bind proteins (antigen and antibody) through hydrophobic interactions between the non-polar structure of the protein and the solid matrix (Crowther, 1998). Antigen or antibody can be coated directly or
indirectly on the surface of the plate. However, the effectiveness of coating still depends on the nature of binding capability of the proteins. Some proteins may bind firmly to the plate, whereas, some may detach from the plate after binding for a short period of time. Generally, a high titer antiserum is favorable for coating because more antibodies will bind to the plate. However, the concentration of antiserum used to coat the plate is also a concern. Neither a high nor low concentration of antiserum is ideal for use because at a high concentration, proteins will over fill the space on the plate and cause protein molecules to bind with each other through protein-protein interactions. These protein-protein interactions are weaker than the hydrophobic interactions between proteins and the plate, so they will have a high tendency to dissociate from each other during the assay, which eventually affects the efficacy of the actual proteins bound. On the other hand, at a low concentration of the coating material, insufficient antibody or antigen is present for use in detecting the antigen. In most cases, 1 µg/ml or 10 µg/ml of protein concentration is sufficient for the use of coating (Kemeny, 1991). Some other components along with the coating process, such as coating buffer, time, and temperature may also affect the coating efficiency.

(b) Sample preparation

The purpose of ELISA is to determine the antigen or allergen residue in a food sample. The results of allergen detection can be less meaningful even with high sensitivity of the antibody if sampling and sample preparation are poor. In many cases, allergenic proteins are not distributed homogenously in foods. Additional mixing and grinding of foods to smaller particle sizes and homogenized mixture are required during the sampling process to obtain a representative sample for testing. In addition, dissolving
the food sample in an appropriate extraction buffer is critical to obtain a complete extraction of the antigens from all foods samples. Although phosphate buffer usually works well in simple extraction for most food samples, foods like chocolate containing tannin or food with high fat content may need additional additives in the buffer such as fish gelatin or non-fat dry milk, to improve the extraction efficiency of the assay. The use of fish gelatin and non-fat dry milk in the extraction buffer has shown a tendency to improve the binding properties and to minimize the background due to non-specific binding (Immer, 2006; Besler, 2001, Keck-Gassenheimer et al., 1999).

(c) Buffers, time, and temperature

The choice of buffers for coating, extracting, blocking, and washing; in terms of composition, concentration, and pH plays an important role in providing optimum conditions in each processing step. The most common buffers used in ELISA are a pH 9.6 carbonate or bicarbonate buffer for coating, pH 7.4 phosphate buffered saline solution for regular extraction and washing buffers, and pH 7.4 phosphate buffer for blocking (Kemeny, 1991; Crowther, 2001). The blocking reagents consist of fish gelatin, non-fat dry milk, casein, and casein hydrolysate, as well as rabbit, horse, bovine and calf serum (Kemeny, 1991; Crowther, 2001). The blocking step is performed after the coating and washing steps to fill up any space or gap between coated proteins on the plate; hence, preventing non-specific binding for any protein to the plate.

Furthermore, time and temperature can be other factors affecting detection in the assay. In each step, adequate time is required for the interactions of proteins with the plate (for coating) and antibody to the antigen, as well as the enzymatic reactions for color development. The optimum binding of antibody to antigen can be achieved within 1
or 2 hours (Kemeny 1991). Temperature can also affect the binding and dissociation rate of the proteins. A plate is typically coated at 4°C overnight or at 37°C for one hour depending on the desired working schedule. However, the subsequent process of the assay including blocking, washing, sample or antigen adding, and primary and secondary antibodies incubations are usually conducted at 37°C for an hour (Kemeny, 1991).

(iii) Assay validation

After a successful assay optimization, the quality and the performance of the assay are assessed by a series of analytical test procedures for validation. Validation is crucial in ELISA development to ensure that the assay complies with established specifications for ELISA’s use in achieving proper standards of accuracy and reliability. The validation of an assay requires large scale intra- and inter-laboratory trials with the replicated procedure to obtain sufficient data to support and document the validity. The tests or procedures conducted for a validation study are necessary to characterize the performance of the assay. The characteristics that require consideration during validation include accuracy, precision, specificity, detection limit, quantitation limit, and robustness of the assay (Lipp et al., 2005).

- Accuracy is defined as the closeness of the test results obtained by the method to the true value derived from the reference standard. In practice, the known amount of analyte (e.g. allergenic food protein extract) is usually added within the range of the method standard curve and determined as the percentage of recovery by the assay of the known added amount of analyte in the sample or can be determined the difference between the means of added analyte and reference standard.
- **Precision** is defined as the degree of consistency for repeated test results under the same operating condition to measure the reproducibility or repeatability of the assay. Precision is often statistically expressed as the mean and standard deviation and is used for statistical comparisons for intra- and inter-laboratory collaborations for the validation of ELISAs.

- **Specificity** is the ability to identify the analyte between the compounds that may be structurally closely related or matrix components that are present in the test sample.

- **Detection limit** is characterized as the limit of the assay that enables detection of the lowest amount of analyte in a sample. However, the amount is not required to be quantified.

- **Quantitative limit** is the limit that can determine the lowest amount of analyte in a sample with an acceptable level precision and accuracy.

- **Robustness** is a measure of the ability of assay to resist small changes or susceptibility to variation conditions such as pH, temperature, or minimal time change during normal usage, in which it provides substantially reliable results.

A cross-reactivity study is often conducted by evaluating the potential cross-reactivity of food commodities to assess their response and the specificity of the assay. In addition, a matrix interference study or a spike-and-recovery study are used for analyzing the effectiveness of the assay in detecting the allergenic residues in different complex food matrices. This study is useful to classify the potential occurrence of non-specific binding towards the antigen-specific antibody that will diminish the sensitivity and selectivity of the assay.
SUMMARY

Food allergy is an IgE-mediated, adverse immune response to food. Food allergies are known to be caused by major food proteins that are highly resistant from heat and proteolysis, most commonly the Big 8 allergenic food groups such as cow’s milk, crustacean shellfish, egg, fish, peanut, tree nuts, soy, and wheat. Tree nut allergy is one of the food allergies that has increased in prevalence and often leads to severe anaphylactic reaction. Pistachio is growing in popularity owing to its nutritional benefits. Indeed, pistachio allergy has gained attention lately due to the drastic increase of food products using pistachio to meet consumer demands. Five pistachio allergens (Pis v 1, 2, 3, 4 and 5) have been identified as allergenic proteins in pistachio. Food-allergic individuals are advised to avoid offending foods to prevent suffering from severe or sometimes fatal allergic reactions. Proper food allergen labeling on food products is essential to help food-allergic individuals to easily identify the offending food or ingredients that they should avoid. Undeclared food allergen in foods can pose serious health risks to food-allergic consumers. Under the Food Allergen Labeling and Consumer Protection Act (FALCPA) of 2004, all food products containing ingredients derived from the Big 8 allergenic food groups must be listed on the label of the products. A fast, sensitive, specific, and reliable allergen detection method, such as ELISA plays an important role in detecting allergen residues thereby assuring the health of food-allergic consumers. Additionally, ELISA can be used to investigate food allergy incidents and the compliance of food industries with allergen labeling regulations. The development of a pistachio ELISA can also help to prevent unnecessary food allergy reactions to pistachio in susceptible individuals. Because an ELISA may lead to reduced use of precautionary
labeling (“may contain”), this advancement may also prevent unnecessary dietary restrictions for pistachio-allergic individuals that may adversely affect their nutritional status and quality of life. At the same time, the use of this ELISA will assist companies in complying with the FDA labeling guidelines.
REFERENCES


Ling, M., Ye, J., Cockrell, G., Sampson, H., Bannon, G.A., Stanley, J.S., Burks, A.W.

Characterization of Two Major Allergens in English and Black Walnut.

Unpublished.


CHAPTER 2: PRODUCTION OF POLYCLONAL ANTISERA AGAINST RAW AND ROASTED PISTACHIOS

ABSTRACT

Development of a qualitative and quantitative method for the detection of allergenic food residues in foods is essential to assist food manufacturer and food regulatory agency to safeguard food allergic consumer from undeclared allergenic protein in foods. The aim of the study is to develop of an enzyme-linked immunosorbent assay (ELISA) for the detection of pistachio residues in foods. ELISA uses the immunized antibody to measure the presence of specific allergen. Polyclonal anti-sera raised against a mixture of roasted pistachio and raw pistachios were developed separately in three rabbits, one goat, and one sheep. The immune responses of each animal and antibody production were evaluated and quantitated using non-competitive ELISA. Throughout the immunization process, all animals had a good immune response with high antibody production, titer \( \geq 10,000 \). The high titer anti-sera were selectively pooled for the later use for ELISA development. The raw and roasted pistachio soluble proteins profiles were successfully demonstrated using SDS-PAGE. The soluble proteins of raw and roasted pistachio were approximately ranged from 9 – 75 kD. The reactivity of the animal antibodies against raw and roasted pistachio was qualitatively performed by immunoblots. All anti-sera showed ability to bind to wide variety of pistachio proteins, which indicated the antibodies are capable for the use in the pistachio-ELISA development.
INTRODUCTION

Food allergies continue to increase in prevalence throughout the world, especially in industrialized nations. Food allergies can occasionally be severe and even life-threatening so avoiding specific allergenic foods in the diet is the only way to protect food allergic consumers from food–induced allergic reactions. As a result of the increased awareness of this public health issue, food industry and regulators have paid more attention to food safety and other preventive measures such as the implementation of allergen control plans or HACCP plans as a means to decrease the chance of undeclared allergens in packaged food products. In addition, regulation on food allergen labeling has become more rigorous; the Food Allergen Labeling and Consumer Protection Act (FALCPA) of 2004 amended the Food, Drug and Cosmetic Act and now requires food manufacturers to clearly identify and label the food ingredients derived from the Big 8 (crustacean shellfish, egg, fish, milk, peanuts, tree nuts, soybeans, and wheat) allergenic foods.

As a result, food allergen detection is increasingly needed that are sufficiently specific, sensitive, fast and reliable for routine use by food industry. Enzyme-linked Immunosorbent Assay (ELISA) is proven to be a very effective analytical method to detect and quantify allergenic residue in various food products. In the past decade, several ELISAs including peanut (Hefle et al., 1994), hazelnut (Holzhauser and Vieths, 1999), almond (Hlywka et al., 2000), egg (Hefle et al., 2001), walnut (Niemann et al., 2009), soybean (Koppelman et al., 2004), casein (Hefle and Lambrecht, 2004), and mustard (Lee et al., 2008), lupine (Kaw et al., 2008) have been successfully developed. These ELISAs assist food manufacturers with the assessment of the allergen control plan and provide regulatory agencies with the ability to monitor labeling compliance.
FALCPA also requires a description of the specific food allergen in the food labeling. For example, if the ingredient list includes tree nuts, the specific type of tree nuts must be defined if the tree nut is derived from one of the 19 tree nuts considered by FDA as a major allergenic tree nut. Thus, the need for ELISAs to detect individual tree nut allergen has risen, especially for pistachio due to the increased availability of pistachio products in the market.

The sandwich-type ELISA is an immunochemical method for measuring the presence of a specific allergenic protein (antigen). It commonly uses polyclonal antisera containing IgG antibodies typically from two different species, where by the antigen is bound in between two IgG antibodies. Polyclonal antibodies are useful to detect food allergens due to their ability to detect multiple proteins in a given food or several antibody binding epitopes on specific allergenic proteins. Polyclonal antibodies contain antibodies that bind to multiple sites on the antigen (each antibody identifies a different epitope on an antigen) and often create stable multivalent interactions between antigens and antibodies; thus, giving a much higher avidity for the antigen. However, polyclonal antibodies may also increase nonspecific interactions with other substances that lead to nonspecific background problems. As a result, selecting the right sources for polyclonal antibody production is very important. In addition, analyzing the animal pre-bleed before immunizations are initiated is also a crucial step in order to determine whether the animal is sensitized to food proteins and would thereby contain IgG antibodies in the sera that will cross-react with other antigens or carry more nonspecific interaction. These animals will be ineligible for immunization. ELISA development requires a sufficient amount of specific IgG antibodies that have high affinity and avidity to the antigen of interest. The
titer of the polyclonal antibodies can be very decisive for choosing the right antibody. In addition, immunostaining such as immunoblotting can demonstrate the specificity of antibodies binding (Harlow and Lane, 1988).

The objective of the study is to produce high titer polyclonal IgG antibodies against raw and roasted pistachio proteins that were later used for the development of a sensitive pistachio-ELISA. Titer determination and immunoblotting were used to examine the production of polyclonal antibodies including the specificity and affinity interaction of the antibodies to the pistachio antigens.
MATERIALS AND METHODS

Pistachio Immunogen Preparation

Three sources of roasted pistachio (California, Iranian, and Turkish pistachio) and raw pistachio from California were selected. Pistachios were shelled manually by hand. All shelled pistachios were washed three times with deionized water, dried with multiple layers of paper towel, then thoroughly air dried in the fume hood for 24 hours. All varieties of pistachio were tested for allergenic residue using other tree nut assays including almonds (Neogen Veratox®), hazelnuts (Neogen Veratox®), pecan (FARRP in-house ELISA), and walnut (FARRP in-house ELISA) to ensure that no residual contamination of these tree nuts remained in the pistachios before preparing the immunogen. Equal parts of roasted Californian, Iranian, and Turkish pistachios were then ground to a fine particle size and mixed using a 16 speed Oster blender (Niles, IL) to obtain a homogenized, ground pistachio sample. Raw Californian pistachio was also ground in this manner.

Extraction of pistachio proteins

The soluble protein from the ground pistachio was extracted 1:10 (w/v) in 0.01 M phosphate buffered saline (PBS) (pH 7.4; 0.002 M NaH₂PO₄, 0.008 M Na₂HPO₄, 0.85% NaCl) using gentle rocking (Labquake™ Shake, Barnstead|Thermolyne Corp., Dubuque, IA), overnight at room temperature. The next day, the extracts were centrifuged at 3612 × g (4,100 rpm) in a tabletop centrifuge (Sorvall® Legend™ RT, Kendro Laboratory Products, Newton, CT) at 10°C for 30 minutes. The soluble protein of the pistachio extract and the total protein content of the ground pistachio were determined by the
Lowry method (Lowry et al., 1951) and the Dumas Method with a LECO instrument (AOAC, 2000), respectively. The ground pistachio was divided into aliquots and stored at -20°C until used for immunization of animals for the development of polyclonal antibodies. The pistachio extract was also divided into aliquots and stored at -20°C until used for titer determination.

**Polyclonal Antibody Production**

Polyclonal antibodies were developed by Covance Research Products (Denver, PA). California raw pistachios and a mixture of Californian, Iranian, and Turkish roasted pistachios were selected to immunize animals for antibody production. Three New Zealand rabbits, one goat, and one sheep were immunized with each pistachio immunogen according to the protocol as described below.

*Rabbits:*

Three rabbits were immunized with the mixture of the roasted pistachio immunogen and three rabbits were immunized with the raw Californian pistachio immunogen. For initial immunization, each rabbit was injected subcutaneously at multiple sites with a total of 250 μg of protein emulsified in Freund’s Complete Adjuvant (FCA). Subsequent booster injections were administered at 21-day intervals by using 125 μg of protein in Freund’s Incomplete Adjuvant (FIA) through the subcutaneous route. Test bleeds were collected at 10 and 24 days post-booster injection to monitor the development of pistachio-specific antibodies.

*Goat and Sheep:*

One goat and one sheep were immunized with the mixture of the roasted pistachio immunogen and one goat and one sheep were immunized with the raw Californian
pistachio immunogen. For initial immunization, both goat and sheep were injected intramuscularly with a total of 1000 μg of protein emulsified with Freund’s Complete Adjuvant (FCA). Subsequent booster immunizations were administered at 21-day intervals with a total of 500 μg in Freund’s Incomplete Adjuvant (FIA). Test bleeds were collected 10 days after each boost to monitor the antibody production.

**Titer Determination**

The pistachio-specific IgG antibody titer levels produced by the 6 rabbits, 2 goats, and 2 sheep were determined by a non-competitive or indirect ELISA as described by Hefle et al. (2001) with little modification. Roasted or raw pistachio extract (soluble protein) was coated on the microtiter plate (NUNC-Immuno™ MaxiSorp™ plates, Nagle Nunc International) with a 10-fold serial dilution (10 μg/ml, 1 μg/ml, and 0.1 μg/ml) in coating buffer (0.5 M carbonate bicarbonate buffer, pH 9.6) and incubated overnight at 4°C. The following day, the plate was washed 4 times with wash buffer (0.025 M PBS (0.005 M NaH₂PO₄, 0.02 M Na₂HPO₄, 0.85% NaCl, pH 7.4 containing 0.5% bovine serum albumin (BSA) (RIA grade, Sigma Chemical Co. # 10868) and 0.2% Tween 20 (Polyoxyethylene Sorbitan Monolaurate, Bio-Rad Laboratory Inc. # 170-6531) and blocked using 350 μl/well of 1% BSA in 0.025 M PBS for 1 hour at 37°C. After an hour of incubation with the blocking solution, the plate was again washed as described above and then a 10-fold serial dilution of anti-sera (diluted with conjugate buffer, same reagents as in the wash buffer) was added to the plate and incubated for two hours at 37°C. Following a third wash step, 100 μl/well of a 1:5,000 dilution of alkaline phosphatase-conjugated anti-species IgG antibodies (Immunopure®, Pierce Biotechnology, Inc., rabbit anti-goat IgG # 31300, goat anti-rabbit IgG # 31342, and
rabbit anti-sheep IgG # 31360) diluted with conjugate buffer was added, followed by incubation at 37°C for 2 hours. The plates were again washed and then incubated with 100 µl/well p-nitrophenyl phosphate substrate (Sigma Fast™, Sigma-Aldrich # N 2770) at room temperature for 30 minutes in a dark environment. The enzymatic reaction was stopped by addition of 1 N NaOH. The absorbance values were measured at 405 nm using a microplate reader (ELx808 Ultraplate, Bio-teck Instruments, Inc.). Titration curves were constructed by using GraphPad Prism® software (GraphPad Prism® software, Inc., San Diego, CA). Titer is defined in the Food Allergy Research and Resource Program laboratory as the log reciprocal of the mid-linear portion of the titration curve when 1µg/ml of pistachio immunogen is coated onto the microtiter plates. Antisera from each animal with titers >10,000 were selected, pooled and later used for the development of the pistachio ELISA.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)**

The soluble protein profiles of raw and roasted pistachio were demonstrated by SDS-PAGE using a Bio-Rad Mini Protean® II cell (Bio-Rad Laboratories, Hercules, CA) with Ready Gel® precast gels (15% Tri-HCl; Bio-Rad Laboratories, Hercules, CA).

Sample buffer was prepared by combining 0.50 ml of Laemmli sample buffer [62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue (Bio-Rad Laboratories, Hercules, CA)] with 27 mg of dithiothreitol (DTT) Cleland’s Reagent. Subsequently, previously extracted raw and roasted pistachio proteins (1:10 w/v in 0.01 M PBS) were individually mixed with the sample buffer at a 1:1 ratio. The samples were then heated at 100°C and centrifuged at 13,060 x g (Microcentrifuge Model 235 V, Fisher Scientific, Pittsburg, PA) for five minutes in each step. The Ready Gel® and running
buffer [25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3 (Bio-Rad Laboratories, Hercules, CA)] were prepared and assembled according to the manufacturer’s instruction. Five µL of the molecular weight markers (Precision Plus Protein™ standards; Bio-Rad Laboratories, Hercules, CA) and 10 µg of each pistachio (raw and roasted) protein were loaded in wells of the gels. The electrophoresis was completed within 35 minutes, under a constant voltage of 200 Volts. The gels were then removed from the electrophoresis cell and placed into a disposable Petri dish with extra careful handling. The separated protein bands in the gels were fixed for 30 minutes using Fixing Solution 5X Concentrate [60% (w/v) trichloroacetic acid and 17.5% (w/v) 5-sulfosalicylic acid (Sigma-Aldrich Company, Inc., St. Louis, MO)] diluted 1:5 with deionized water. The gels were then stained with Brilliant Blue G-Colloidal Concentrate [0.1% (w/v) Brilliant Blue G, 0.29 phosphoric acid and 16% saturated ammonium sulfate (Sigma-Aldrich Company, Inc., St. Louis, MO)] for at least two hours. After staining, the gels were destained with a solution of 10% acetic acid in 25% (v/v) methanol for one minute and then rinsed with 25% methanol (methanol, analytical grade, Fisher Scientific, Pittsburg, PA). The destaining process was continued by placing the gels in 25% methanol for approximate 24 hours prior captured the gel images using a Kodak Gel Logic 440 Imaging System (Eastman Kodak Company) coupled with the Kodak 1D v. 3.6.5 software (Kodak Scientific Imaging Systems, New Haven, CT).

**IgG-immunoblotting (Western Blotting)**

The binding affinity and specificity of animal (rabbits, goat, and sheep) IgG antibodies directed against both raw and roasted pistachio proteins were demonstrated by IgG-immunoblotting. Raw and roasted pistachio proteins were separated by SDS-PAGE
as described as above. The separated pistachio proteins on unstained gels were then transferred onto polyvinylidene difluoride (PVDF) membranes (Immoblin-P PVDF membrane, 0.45 μm, Millipore Corporation, Billerica, MA) by electrophoresis with a constant voltage of 65 Volts for 1 hour and 20 minutes. Subsequently, the membranes were placed in Petri dishes containing adequate amount of Ponceau S staining solution (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid) for a quick and reversible staining. This process allowed protein bands to be located on the membranes. Three separate lanes of each raw and roasted pistachio protein were cut into strips in order to access the animal (rabbit, goat, and sheep) IgG antibodies independently in the following steps. The membranes were rinsed several times with distilled water to remove the excess Ponceau S solution prior to blocking (0.01 M PBS, 0.2% BSA, 0.05% Tween 20) at room temperature for two hours with gentle rotation. Animal IgG antibodies worked as primary antibodies which specifically attached to pistachio proteins bound to the PVDF membrane. Rabbit antibodies were diluted 1:8000 with the blocking buffer; whereas, goat and sheep antibodies were both diluted 1:5000 in blocking buffer. Each strip of raw and roasted pistachio protein membranes was incubated individually with three animals’ antibodies at room temperature for one hour. The membranes were washed four times for five minutes each with wash buffer (0.01 M PBS with 0.05% Tween 20) in every washing step. Later, each membrane was incubated for an hour with the corresponding secondary antibodies, which were goat anti-rabbit IgG antisera, rabbit anti-sheep antisera, and rabbit anti-sheep antisera, each conjugated with horseradish peroxidase (Immunopure®, Pierce Biotechnology, Inc., Rockford, IL) diluted 1:40,000 in blocking buffer. All membranes were then washed as described previously, followed by a final
incubation with the DAB (3’3 – diaminobenzidine) substrate solution (Pierce Technology, Inc., Rockford, IL) until the bands were clearly demonstrated. The membranes were washed to remove residual substrate and thoroughly air dried before capturing the images with a Kodak Gel Logic 440 Imaging System (Eastman Kodak Company) coupled with the Kodak Gel Logic ID v. 3.6.5 software (Kodak Scientific Imaging Systems, New Haven, CT).
RESULTS AND DISCUSSION

Selection of Raw and Roasted Pistachios

The development of a specific and sensitive sandwich ELISA for detection of pistachio residue required the use of antisera (IgG antibodies) from two animal species (used as capture and detector antibodies). Therefore, it was vital to choose the right samples for immunization at the very beginning of the study. California, Iran, and Turkey are the main pistachios producers in the world. Different sources of pistachios were included to develop a robust ELISA that is capable of detecting pistachio proteins in foods regardless of the varying sources and varieties of pistachio. In addition, in-shell pistachios from dedicated pistachio farms or producers practicing good manufacturing practices (GMP) were strongly preferred for sample selection because these pistachios have less potential for cross-contamination with other tree nuts. Careful selection and minimizing cross-contamination are vital to prevent production of antibodies that might also bind to other tree nut proteins which in turn decrease the specificity of the assays.

Thus, three sources of roasted pistachios from California, Iran, and Turkey were selected; a mixture of roasted pistachios from the three sources was used for immunization to produce antibodies against roasted pistachios. Due to the difficulties of finding the raw Iranian and Turkish pistachios in the United States, only raw Californian pistachios were selected for the raw pistachio immunizations.

Total and Soluble Protein Content of Raw and Roasted Pistachios

The total and soluble protein contents of raw and roasted pistachios are demonstrated in Table 3. Raw and roasted pistachios consist of an approximate total protein concentration of 25.6% and 24.1% respectively. In addition, raw pistachios
consist 11 mg/ml of soluble protein content; whereas, roasted pistachios consist only 8 mg/ml.

Based on the results, the total protein contents of both pistachio samples were much higher than the soluble protein contents. These results were reasonable because the LECO (Dumas) method quantified the total nitrogen content in the particular samples, which did not only include the nitrogen within proteins but also the nitrogen of the nucleic acid to measure the crude protein concentration of foods. On the other hand, the Lowry method is a colorimetric technique to determine the soluble protein levels in foods by measuring only tyrosine and tryptophan contents in proteins. Knowing the total and soluble protein contents were beneficial in estimating the protein content of the raw and roasted pistachios. Roasted pistachios have less protein content compared to raw pistachios. Under the roasting process, pistachio proteins were exposed to high temperature (vary from 100-180°C) (Sharma, 1985) that may have caused the unfolding (denaturing) of secondary and tertiary structure of the proteins. Denaturation of the proteins may have lowered the solubility of some of the proteins thereby decreasing their extractability as compared to the raw pistachio samples.
Table 3. Protein content of raw and roasted pistachio determined by LECO (Dumas) method (total protein content) and the Lowry method (soluble protein content)

<table>
<thead>
<tr>
<th>Methods</th>
<th>Total Protein Content</th>
<th>Soluble Protein Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw Pistachio(^a)</td>
<td>25.6%</td>
<td>11 mg/ml</td>
</tr>
<tr>
<td>Roasted Pistachio(^b)</td>
<td>24.1%</td>
<td>8 mg/ml</td>
</tr>
</tbody>
</table>

\(^a\)Raw pistachio is only includes the Californian variety

\(^b\)Roasted pistachio is a composite sample consisting of roasted Californian, Iranian, and Turkish pistachios.

**Animal Selection Prior to Immunization**

Good quality antibodies should be specific, sensitive, and have high affinity to the antigens of raw and roasted pistachio in order to develop a specific and sensitive sandwich-ELISA.

Before the immunization process, sera from 10 animals including 6 rabbits (NE 249-NE 254), 2 goats (B 859 and B 867), and 2 sheep (G 510 and G 511) were pre-screened using a non-competitive ELISA to determine if the animals were sensitized to raw and roasted pistachios, raw cashew, roasted cashew, soy, and oat proteins prior to the initiation of the immunization protocol. The pre-screening process was critical to select animals that would produce good quality antisera that did not contain antibodies directed against other food proteins that would thereby interfere with sensitivity of the developed ELISA method. Prior sensitization to cashew, soy and oats were not recommended in this
study to avoid false-positive results or non-specific binding that can cause high background absorbance reading value.

Based on the results demonstrated by the non-competitive ELISA (Figure 5), all rabbits demonstrated low absorbance values ($\leq 0.1$ OD) against raw and roasted pistachio, and raw and roasted cashew; however, all rabbits demonstrated higher absorbance values against soy and oat proteins than the goats and sheep. These results are possibly caused by the natural diet of rabbits consisting of soy and oat resulting in the production of IgG antibodies directed against these food proteins. Even though the absorbance values against soy and oats from rabbits were high compared to other ingredients, the absorbance values were still low enough ($< 1$ OD) that the use of this antisera would not significantly increase the background in the developed ELISA. Both the sheep and the goats had low levels of pre-existing IgG antibodies against the food ingredients tested (Figure 5). Overall, the 10 animals showed low pre-existing antibodies against all the ingredients. Rabbits NE 249, NE 250, NE 251, sheep G 510, and goat B 859 were chosen to be immunized with roasted pistachio; whereas, rabbits NE 252, NE 253, NE 254, sheep G 511, and goat B 867 were selected for raw pistachio immunizations.
Figure 5. Pre-screening of 6 rabbits (NE), 2 sheep (B) and 2 goats (G). Mean absorbance values of the sera from all animals screened by indirect ELISA. One µg/ml of soluble raw pistachio, roasted pistachio, raw cashew, roasted cashew, soybean, and oat proteins were coated onto microtiter plates. A 1:10 serial dilution of individual sera and 1:5000 dilution of enzyme-labeled secondary antibody were used. Each absorbance value represents the mean of 3 readings.

**Raw and Roasted Pistachio Specific Antibodies Production**

**Titer Determination**

IgG antibodies directed against raw and roasted pistachio proteins were produced by 10 animals over a 90-week immunization period. Antibody production was monitored during the immunization process using a non-competitive or indirect ELISA for the titer determination. The IgG antibody titer was determined by monitoring the binding of antibody (1:10 (v/v) serial dilution of sera in buffer) that bound to the previously coated
antigens (1 µg/ml pistachio proteins) on the microtiter wells. The alkaline phosphatase-conjugated anti-species IgG antibodies detected the bound antibodies. Addition of the p-NPP substrate produced a yellow-colored product that could be measured on a microplate reader. The intensity of the color developed indicated the amount of specific antibodies in the antisera. The absorbance value for each serum sample was measured at a wavelength of 405 nm wavelength. A titration curve was generated using GraphPad Prism software by plotting the absorbance values on the Y-axis and log antibody dilution on the X-axis. The titer value was determined as the log reciprocal of the mid-linear portion of the titration curve. The Figure 6 provides an example of the titer titration curve from the first production bleed of rabbit NE 249. Based on the titration curve, the mid-linear portion of the curve corresponded to the log antibody dilution of -4.839; in which the titer was determined as log reciprocal or antilog [(4.839) = 6.9 x 10^4] of the mid-linear portion of the dilution curve using a microtiter plate coated with 1.0 µg roasted pistachio protein/ml. The titer value of each animal varies after each booster (immunization). This variability was expected due to the different responses of individual animals to the antigens and environmental factors that may affect the immune response at different time points. However, animals that produced an antibody titer ≥ 10,000 were considered as optimum and placed into production bleed to obtain higher levels of sera at each collection point. Production bleeds with a titer level of ≥ 10,000 were then selected and pooled for the use for the development of the pistachio ELISA (see Chapter 3).

The progress of the antisera of rabbits (NE 249, 250, 251) to roasted pistachio in the 91-week immunization process is demonstrated in Figure 7. Titer values of all rabbit antisera > 10,000 occurred 10 weeks after the first immunization. The rabbits were placed
on production bleeds beginning at the 11th week and throughout the duration of the immunization protocol resulting in a total of 39 production bleeds per rabbit. Due to the availability of the production bleeds at the start of the ELISA development, a total of 17 production bleeds from rabbits NE 249 (weeks 17-31), NE 250 (weeks 17-31), and NE 251 (weeks 17-31) were selected. Equal amounts of each production bleed were obtained and mixed into a pooled rabbit antiserum for the development of ELISA.

Figure 8 shows the immunization of the sheep G 510 and B 859 over 91 weeks in response to roasted pistachio. The sheep and goats were also placed on production bleeds after the 10th week when both animals’ antisera achieved titer values of > 10,000. A total of 28 and 27 production bleed were collected from the individual sheep and goats, respectively. Pooled sheep and goat antisera were taken accordingly from the production bleeds taken on weeks 13-31 after the initial immunization and were used for the development of the pistachio ELISA.

Similarly, Figure 9 and Figure 10 show the immunization response to raw pistachio of rabbits (NE 252, NE 253, and NE 254) over 91 weeks and both sheep (G 511) and goat (B 867) over 91 weeks, respectively. The three rabbits were placed on production bleeds 13 weeks after the first immunization; whereas, the first production bleed of the sheep and goat occurred 10 weeks after the first immunization. A pool of rabbit anti-raw pistachio antiserum was compiled from 22 production bleeds from three rabbits: NE 252 (weeks 17-31), NE 253 (weeks 15-31), and NE 254 (weeks 17-31). Pooled sheep and pooled goat antisera were compiled from the production bleeds collected on weeks 13-31.
Overall, an excellent immune response to raw and roasted pistachio protein was achieved in all of the animals resulting in high titer IgG antibody values in a relatively short period of time.

Figure 6. Titration curve for rabbit anti-roasted pistachio sera (first production bleed of rabbit NE 249). The vertical line indicates the mid-linear portion of the titration curve. Titer was defined as the log reciprocal of the mid-linear portion of the dilution curve using microtiter plate coated with 1.0 μg roasted pistachio protein/mL. Each data point represents the mean of triplicate readings and the standard deviation for each point is < 0.1.
Figure 7. Immune response of individual rabbits to roasted pistachio immunogen. Data points are the mean of triplicate readings.
Figure 8. Immune response of individual sheep and goat to roasted pistachio immunogen. Data points are the mean of triplicate readings.
Figure 9. Immune response of individual rabbits to raw pistachio immunogen. Data points are the mean of triplicate readings.
Figure 10. Immune response of individual sheep and goat to raw pistachio immunogen. Data points are the mean of triplicate readings.
**SDS-PAGE and IgG Immunoblotting**

SDS-PAGE and IgG immunoblotting were used as supplementary tools to verify the quality of the antisera in terms of validating the specificity and affinity of the specific IgG antibodies against raw and roasted pistachio proteins. Through SDS-PAGE, the soluble proteins of raw and roasted pistachio were separated on the gels by their molecule mass. The Brilliant Blue G-colloidal staining was used to visualize separated proteins on the gels. The estimated molecular weights of soluble proteins of raw and roasted pistachio ranged from 9 – 75 kD (Figure 11). Based on the SDS-PAGE gel images, no significant difference between raw and roasted pistachio proteins were detected. These results indicate that pistachio proteins were heat stable proteins, where by all the major proteins were still highly intact after roasting and the solubility of the proteins was not significantly affected; hence, each protein of the roasted pistachio was clearly demonstrated on the gel. Five major pistachio allergens, Pis v 1 (7 kD, 2S albumin), Pis v 2 (32 kD, 11S globulin), Pis v 3 (55 kD, 7S vicilin), Pis v 4 (25.7 kD, manganese superoxide dismutase-like protein), and Pis v 5 (36 kD, 11S globulin) have been previously characterized (Ahn et al., 2009; Willison et al., 2008; Ayuso et al., 2007; Beyer, Accession number: EU410073.1 [accessed October 20, 2010; http://www.ncbi.nlm.nih.gov/nucleotide/171853009]).

The reactivity of the pooled rabbit, sheep, and goat antisera analyzed by IgG immunoblotting is shown in Figure 12. All the antisera bound to a variety of soluble pistachio polypeptides with molecular weights ranging from 10 to 75 kD. However, two bands of approximately 17 and 18 kD present in the SDS-PAGE for raw and roasted pistachio were faded on the immunoblots. These two bands were faintly detected by
sheep and goat antisera, but were not detected on the rabbit antibodies. These results could be due to the low or limited affinity of antibodies to the particular proteins. Nevertheless, the immunoblots qualitatively indicated that the antibodies were able to bind to a wide variety of pistachio proteins, which allowed for their use in developing ELISA that is capable for detecting pistachio residue.

Figure 11. Brilliant Blue G-Colloidal stained SDS-PAGE gel with separated raw and roasted pistachio proteins extracted using 0.01 M PBS containing 0.85% NaCl. Approximately 10 μg of each of raw and roasted pistachio proteins were loaded onto each lane. MW is the molecular weight standards.
Figure 12. Rabbit, sheep, and goat IgG-immunoblotting of raw and roasted pistachio protein extracts. Approximately 10 µg of pistachio proteins were loaded onto each lane. Raw pistachio protein extracts (lane 1, Brilliant Blue G-colloidal stain of a 15% SDS-PAGE resolving gel); raw pistachio proteins bound to rabbit (lane 2), sheep (lane 3), and goat (lane 3) IgG antibody; roasted pistachio protein extracts (lane 5, Brilliant Blue G-colloidal stain of a 15% SDS-PAGE resolving gel); roasted pistachio proteins bound to rabbit (lane 6), sheep (lane 7), and goat (lane 8) IgG antibody. MW is the molecular weight standards (Brilliant Blue G-colloidal staining for comparison only).
CONCLUSION

Stringent selection of animals prior to immunization is important to obtain good quality antibodies that serve as effective reagents for the later development of the pistachio-ELISA (discussed in detail in Chapter 3). Consequently, the polyclonal antisera directed against raw and roasted pistachios were successfully developed with high titer values of $\geq 10,000$ in six rabbits, two sheep, and two goats. The high specificity and affinity of the polyclonal antibodies directed against raw and roasted pistachio as demonstrated by IgG immunoblotting have provided a sufficiently sensitive and robust response to pistachio proteins that can be utilized in the development of an ELISA for the detection of pistachio residue.
REFERENCES


CHAPTER 3: DEVELOPMENT OF AN ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETECTION OF PISTACHIO PROTEINS IN PROCESSED FOOD

ABSTRACT

Pistachio is one of the potent food allergens that often leads to severe allergic reactions and even life-threatening anaphylactic shock in pistachio-allergic individuals. Pistachio-allergic individuals are accessible sensitized to other tree nuts such as cashew due to the similar homology protein structure from the same family. Products that contain tree nuts other than pistachio are most often avoided by pistachio allergic individuals, because usually multiple types of tree nuts will be handled and processed under same equipment or facility, results in frequent allergens cross-contamination. The undeclared of pistachio in food products or the unintentional ingestion of pistachio can pose a serious health risk to pistachio-allergic individuals. Therefore, specific methods with sensitivity in low part-per-million (ppm) are necessary to detect and quantify trace amount of pistachio residues in foods.

The aim of the study is to develop a sandwich-type ELISA to detect the presence of pistachio residues in foods. Both raw and roasted pistachio ELISAs were developed by utilizing the pooled sheep antisera as capture reagent and pooled rabbit antisera as detector reagent. Binding of antigen-antibody complex was visualized through a colorimetric reaction involving goat anti-rabbit IgG antibody (labeled with alkaline phosphatase) and substrate (p-nitrophenyl phosphate). A total of 102 food ingredients were tested by using the developed roasted pistachio ELISA assay to evaluate the
specificity and potential cross-reactivity of the assay. A known amount of pistachio was added into ice cream and cookie matrices to evaluate assay performance.

The optimized ELISA had a limit of quantification (LOQ) of <1 ppm (1 μg/g). Minor cross-reactivity was occurred with cashew at a level equivalent to 4 of roasted pistachio. The performance of the ELISA was not affected in the presence of ice cream and cookie matrices. Pistachio can be qualitatively detected by pistachio-ELISA and quantitatively measured to the very low range of 1 ppm.

**INTRODUCTION**

Pistachio (*Pistacia vera*), a tree nut belonging to the same Anacardiaceae family as cashew, mango, and sumac, is well known for its high nutritional values (good source of fiber, vitamins and minerals), heart health effects, and antioxidant properties (Ryan et al., 2006). Pistachios are commonly eaten fresh or as a roasted and salted snack, or are used as ingredients in confectionary, ice cream, baked products, pudding, and some gourmet products. However, the presence of pistachio in foods may pose a serious health threat to pistachio-allergic consumers. The severity of reported allergic reactions to pistachio range from mild and transitory reactions to severe anaphylactic allergic reactions (Liccardi, 1997; Liccardi et al., 1996; Fernandez et al., 1995; Parra et al., 1993). There is no treatment for food allergy thus strict avoidance of the specific allergenic food in the diet is the only way for prevention of a reaction. Therefore, food allergic consumers need to be vigilant in carefully reading labels on packaged foods and be aware of potential cross-contact concerns that may introduce the offending food ingredients into a product before consuming. Indeed, food manufacturers and food regulators have the
responsibility to ensure a safe and wholesome product is produced. For example, food manufacturers must be diligent with food handling, including handling of ingredients through all the stages of processing to minimize the possibility of food allergen cross-contamination and also provide accurate and adequate labeling information on the finished products. In addition, food regulators should supervise food manufacturers and ensure they provide competent food allergen labeling statements on the food products complying with the labeling law—Food Allergen Labeling and Consumer Protection Act (FALCPA) of 2004. A quantitative detection of pistachio residue can be very useful in detecting the unintentional contamination of pistachio residues in foods and verifying proper labeling with concomitant reductions in risk to pistachio allergic consumers.

Currently, two analytical methods for the detection of pistachio in foods have been demonstrated, i.e., conventional PCR and the real-time PCR methods that are based on the specific DNA (Barbieri and Frigeri, 2006; Brežňa et al., 2008). Although both PCR methods are effective and sensitive approaches, it is not a preferable method to be used by the food industry for detection of allergenic proteins in foods. In addition to the high cost of technical equipment and training, DNA and protein do not share the same fate in foods; PCR only detects the presence of DNA from a given source but not the specific allergenic protein in food samples which make PCR less desirable for monitoring the removal of allergenic proteins in food processing facilities. The Enzyme-linked Immunosorbent Assay (ELISA) is an analytical method which is highly sensitive, specific, and fast, as well as a reliable allergen detection method extensively used by food industry for the detection of allergenic residues in foods. The antibodies that are used in the ELISA system are usually very specific for a particular epitope or protein. However,
ELISAs are limited when used with closely related foods that have proteins with a high degree of amino acid homology and may contain common epitopes. The antibodies may not be able to distinguish the cross-reactive epitope, resulting in false positive results. In addition, the food matrix may be a concern in ELISA detection as complexity may hinder the actual antigen/antibody interaction thereby affecting the ELISA detection efficiency (Taylor et al., 2009). Establishing cross-reactivity and matrix interference studies is thus essential to evaluate the ELISA performance thoroughly. The objective of this study is to develop a sensitive, specific, and robust pistachio-ELISA, as well as to validate the assay for the detection of pistachio residues in two food matrices, sugar cookies and vanilla ice cream.

MATERIALS AND METHODS

Development of Pistachio Sandwich ELISA

Standard Curve Development

Ground raw pistachios from California and a mixture of ground, roasted Californian, Turkish, and Iranian pistachios were used for the development of raw and roasted pistachios standards, respectively. A 10,000 part per million (ppm; µg/g) concentration of raw and roasted pistachios were independently extracted 1:10 (w/v) with 0.01 M phosphate buffered saline [(PBS), pH 7.4, 0.85% saline and 0.02% NaN₃] with additional 1% of nonfat dry milk (NFDM) at 60°C in a shaking water bath for two hours. The extracts were then centrifuged for 30 minutes at 4200 rpm at 10°C. The supernatants (soluble protein extracts) were saved and stored overnight at 4°C. These 10,000 ppm
extracts were serially diluted 3-fold (1000, 333, 111, 37, 12.3, 4.11, 1.37, 0.45, 0.15, 0.05, 0.02, and 0 ppm) to develop a standard curve.

**Primary Antibodies**

The pooled animal anti-raw and anti-roasted pistachio antisera (described in detail in Chapter 2) that had a titer level >10,000 were used as capture and detection antibodies (primary antibodies) for the ELISA development. Preliminary trial assays were completed with different combinations of capture and detector antisera (rabbit-goat, rabbit-sheep, goat-rabbit, and sheep-rabbit) and varying dilutions of antisera (1:1000 to 1:50,000 dilutions of antisera in PBS buffer). A 1:25,000 dilution of sheep anti-pistachio antisera and a 1:15,000 dilution of rabbit anti-pistachio antisera were selected as the capture and detection antibodies, respectively.

**Raw and Roasted Pistachio sandwich ELISAs**

The raw and roasted pistachio–sandwich ELISA were developed based on the protocol described by Hefle et al. (2001). Both raw and roasted pistachio–ELISAs were developed using the same extraction and incubation conditions, and the same concentration of diluted antisera and antigen in buffers. The initial step included a 1:25,000 dilution of sheep antisera in coating buffer (0.015 M Na$_2$CO$_3$, 0.035 M NaHCO$_3$, and 0.02% NaN$_3$, pH 9.6) that was applied to microtiter plates (NUNC-Immuno™ MaxiSorp™ 96-MicroWell™ plates, Nagle Nunc International, NY) and incubated overnight at 4°C. The following day, the plates were washed four times with wash buffer (PBS containing 0.05% Tween 20 and 0.02% NaN$_3$, pH 7.4) using a microplate washer (AM60, Dynex Technologies, Inc., VA) after every incubation step, excluding the step after the incubation of the substrate. After the initial washing step, the
plates were filled with 350 µl/well of blocking buffer consisting of 0.1% gelatin (300 bloom, porcine, Sigma – Aldrich Co., St. Louis, MO) in 0.01 M PBS and incubated for one hour at 37°C. Later, 100 µl of the serially dilutioned pistachio standards (3-fold dilutions; standard curve points include 1000, 333, 111, 37, 12.3, 4.11, 1.37, 0.45, 0.15, 0.05, 0.02, and 0 ppm) were loaded to the plates, followed by a one hour incubation at 37°C. The pistachio proteins bound to the capture antibodies were detected by adding 100 µl/well of rabbit antisera (1:15,000 dilution in conjugate buffer (0.01 M PBS containing of 0.1% bovine serum albumin [RIA grade, USB Corporation, Cleveland, OH], pH 7.4]) to the plates and incubated for one hour at 37°C. The subsequent binding of antigen-antibody complexes were visualized by the addition 100 µl/well of goat anti-rabbit IgG labeled with alkaline phosphatase [Immunopure®, Pierce Biotechnology, Inc., Rockford, IL] at a 1:5,000 dilution in conjugate buffer and incubated for one hour at 37°C prior to the final addition of substrate, p-nitrophenyl phosphate (SigmaFast™, Sigma – Aldrich Co., St. Louis, MO). The plates were placed under a dark environment at room temperature for 30 minutes for color development. After the 30 minute incubation, 1 N NaOH was added to the plates to stop the enzymatic reaction. The intensity of colored product was measured using a ELx 808 Ultra Micro Plate Reader (Bio-Tek Instruments, Inc. Winooski, Vermont) at 410 nm. Standard curves were constructed using GraphPad Prism® Version 4 Software (GraphPad Software Inc., San Diego, Calif, U.S.A.). The variability between the raw and roasted pistachio ELISA standard curves was evaluated using the unpaired t-test statistical function in the GraphPad Prism ® Software.
Pistachio ELISA Optimization and Validation

Detection of raw and roasted pistachio using the roasted pistachio ELISA

The roasted pistachio ELISA was selected to use in the following validation studies. The sheep antisera and rabbit antisera directed against roasted pistachio were used to determine if similar binding to the raw pistachio proteins resulted when using the anti-roasted pistachio antisera. This study was performed as previously described with the development of the roasted pistachio ELISA, which utilized a 1: 25,000 dilution of sheep antisera and a 1: 15,000 dilution of rabbit antisera as capture and detection antibodies, respectively. A 10,000 ppm concentration each of raw and roasted pistachio extracts were prepared (see the extraction described in the standard curve development section above) and serially diluted 3-fold to construct two standard curves. Subsequently, the two serially diluted pistachio extracts were added to the pre-coated microtiter plates and carried out the following steps of the roasted pistachio ELISA as described above were completed. Two standard curves were then generated by GraphPad Prism ® Software and evaluated with the unpaired t-test.

Cross-Reactivity Study

A total of 102 foods and food ingredients that are commonly used in cooking or baking were selected for the cross-reactivity study in order to investigate any of the proteins that have potential to form false-positive on pistachio ELISA. All the samples were purchased from local grocery stores in Lincoln, NE. Oil samples and non-liquid samples of large particle size such as tree nuts and fruits were ground into smaller, uniform size using an Osterizer® blender (Sunbeam Corporation, Delray Beach FL).
Samples extractions

With the exception of oil sample extracts, all sample extracts were prepared under the same extraction methods and conditions outlined according to the ELISA extraction described previously at the standard curve development section. All samples were extracted 1:10 (w/v) with 0.01 M PBS (pH 7.4, 0.85% saline and 0.02% NaN₃) containing 1% of nonfat dry milk (NFDM) at 60°C in a shaking water bath for two hours. The soluble proteins were then centrifuged for 30 minutes at 4200 rpm at 10°C. The clarified extracts were stored at 4°C until used. The soluble protein content of the samples was determination by the Lowry method (Lowry et al., 1951).

Oil samples such as vegetable oil, olive oil, etc. were handled differently as they required concentration of the extract due to the low levels of soluble protein found in the oil. The oil samples and extraction buffer (0.01 M PBS consisting of 1% NFDM) were blended at a ratio of 1:5 (w/v) using Osterizer® blender. The oil mixtures were then heated at 60°C in a shaking water bath for two hours. Subsequently, the oil mixtures were concentrated by dialysis. The tubing (Spectra/Por® 3 membrane, molecular weight cutoff of 3,500 daltons, Spectrum® Laboratories, Inc., Rancho Dominquez, CA) was coated with polyethylene glycol (PEG-2000, Fluka BioChemika, Ronkonkoma, NY). Concentration of the oil extracts was considered complete when the final volumes were reduced by two-thirds of their original volume. The extracts were refrigerated at 4°C until used for protein content determination by the Lowry method and cross-reactivity analysis.

Using the soluble protein content determined by the Lowry method, all extracts were analyzed by the developed pistachio ELISA based on soluble protein content.
(10µg/ml, 1µg/ml, and 0.1µg/ml) and on an extract concentration basis (1:1, 1:10, 1:100).

The results of the cross-reactivity were expressed in the equivalent pistachio levels by comparing the absorbance readings obtained from the samples with the absorbance readings of the standard curve points.

Matrices Interferences Study

Spike and Recovery Experiments using Ground Pistachio in Ice Cream and Sugar Cookies

Two food matrices (vanilla ice cream and sugar cookies) were selected to evaluate the potential matrix interference on the sensitivity and performance of the developed pistachio ELISA. Vanilla ice cream and sugar cookies were purchased from a local grocery store in Lincoln, NE. Both vanilla ice cream and sugar cookie were pre-screened for undeclared pistachio proteins or relevant proteins in the products using the developed pistachio ELISA.

Prior to the extraction, melted ice cream and cookies were grounded and mixed thoroughly using the Osterizer® blender (Sunbeam Corporation, Delray Beach FL). Subsequently, 0.1 g of ground roasted pistachio was added to the extraction bottle containing 10 g of the vanilla ice cream and sugar cookies separately in order to prepare a level of 10,000 ppm pistachio for the standard. Separately, 10 g of each pistachio-free sample (0.01 M PBS with 1% of NFDM, vanilla ice cream and sugar cookie) were prepared as negative controls (0 ppm). A total of six samples (three 10,000 ppm of each pistachio-spiked 0.01M PBS with 1% NFDM, pistachio-spiked sugar cookies, pistachio-spiked vanilla ice cream; and three 0 ppm of each pistachio-free 0.01 M PBS with 1% NFDM, vanilla ice cream, and sugar cookies) were extracted at a 1:10 (w/v) dilution
with the extraction buffer (100 ml of 0.01 M PBS with 1% NFDM) at 60°C in a shaking water bath for 2 hours, followed by the 30 minutes centrifugation at 4100 rpm (3612 × g) at 10°C. The supernatants (extracts) were stored at 4°C until used for the pistachio ELISA analysis on the following day.

On the next day, the 10,000 ppm sample extracts were serially diluted 1:3 with the corresponding negative control extracts to achieve a standard curve with 12 pistachio concentration levels (1000, 333, 111, 37, 12.3, 4.11, 1.37, 0.45, 0.15, 0.05, 0.02, and 0 ppm). These standards were then evaluated by the sandwich ELISA using sheep capture and rabbit detector antibodies as described above (pistachio-ELISA). A total of three standard curves were generated and compared in order to assess the possible matrix interference. The differences between each standard curve were analyzed using a one-way ANOVA test (significant differences were reported at the p < 0.05 level).

**Spike and Recovery Experiments Comparing Regular Ground Pistachio and Finely Ground Pistachio in Ice Cream**

The objective of this study was to evaluate the differences between the standard curves of regular ground pistachios and finely ground pistachios. Vanilla ice cream was purchased from a local grocery store in Lincoln, NE. The vanilla ice cream was pre-screened for undeclared pistachio proteins or relevant proteins that would affect the outcome of the analysis using the developed pistachio ELISA. Each treatment of ground pistachios was prepared independently. Powdered sugar was prepared by grinding white granulated sugar using a coffee grinder (Krups®).
Regular ground pistachios were prepared using the Osterizer® blender (Sunbeam Corporation, Delray Beach FL). Ground pistachios (0.015 g) and 5 g of powdered sugar were added to 10 g of pistachio-free vanilla ice cream in an extraction bottle to achieve a 10,000 ppm of spiked standard.

Finely ground pistachios required sugar to be used as a carrier to help grind the pistachios into a very small particle size (powder-like). In order to prepare a 10,000 ppm spiked standard, 1.5 g of ground pistachios were blended with 50 g of white granulated sugar using coffee grinder (Krups®). Five grams of the finely ground pistachio-sugar mix were taken from the bulk mixture and added to the extraction bottle with 10 g of pistachio-free vanilla ice cream. In addition, a negative control was prepared by adding 10 g of pistachio-free ice cream with 5 g of powdered sugar in extraction bottle.

All samples, including the two 10,000 ppm spiked standards of regular ground pistachios and finely ground pistachios, and the negative control, were extracted 1:10 (w/v) in 150 ml of 0.01 M PBS with additional 1% NFDM for 2 hours at 60°C in a shaking (150 rpm) water bath, followed by centrifugation (3612 x g) at 10°C for 30 minutes. The clarified solutions were divided into aliquots and stored at 4°C until used for the pistachio ELISA analysis on the following day. The 10,000 ppm sample extracts were serially diluted 1:3 with the corresponding negative controls to achieve standard curve points with 12 pistachio concentration levels (1000, 333, 111, 37, 12.3, 4.11, 1.37, 0.45, 0.15, 0.05, 0.02, and 0 ppm). These standards were then evaluated by sandwich ELISA using sheep capture and rabbit detector antibodies as described at the above pistachio-ELISA. The differences between each standard curve were analyzed using a one-way ANOVA test (significant differences were reported at the p < 0.05 level).
Statistical Analysis

The quantitative sandwich-type ELISA uses a direct relationship between the signal variable (y-axis, in units of absorbance) and the analyte (protein) concentration variable (x-axis), which means the greater the signal, the greater the concentration of bound analyte. Based on the standard curve, the limit of detection (LOD) of pistachio ELISA was mathematically represented as the mean value of the blank (wells without pistachio extract added) plus three times the standard deviation of the blank. The limit of quantification (LOQ) was determined as the lowest point on the linear portion of the standard curve. In order to evaluate the consistency and reliability of the results, three replicates were completed for each pistachio extraction and each extract was analyzed in triplicate on three different days.

Raw and roasted pistachio standard curves were compared and analyzed using the unpaired t-test in GraphPad Prism ® Software in order to determine the differences between two standard curves. The performance and responses of the matrix interference study were analyzed by determining the difference between the standard curves in various food matrices (sugar cookie and vanilla ice cream) and the standard curve in 0.01 M PBS (with 1% NFDM) using the one-way ANOVA test in GraphPad Prism ® Software (comparing the means of three groups).
RESULTS AND DISCUSSION

Pistachio sandwich ELISA standard curves

Raw and roasted pistachio ELISAs were successfully developed by using a combination of two different animals anti-raw or anti-roasted antisera. Both raw and roasted pistachio ELISAs utilized the optimized concentration of a 1:25,000 dilution of sheep antisera as capture antibody and a 1:15,000 dilution of rabbit antisera as detection antibody. Raw and roasted standard curves from the optimized pistachio ELISAs are shown in Figure 13. Both ELISAs had very low background value (< 1.0 absorbance unit, AU), with a limit of quantification (LOQ) of < 1 ppm (µg of whole raw/roasted pistachio/ml). The low background value from the standard curves was used as a tool indicating the level of existing non-specific binding in the assays. Additionally, the highly specific and sensitive antibodies against the raw/roasted pistachio proteins can be used to develop a sensitive ELISA with a LOQ of < 1 ppm. The specificity of the antibodies in this study were consistent with the results demonstrated by IgG immunoblotting experiments outlined in Chapter 2. Both of these results indicate that the developed pistachio ELISA will be very responsive and specific in detecting pistachio residue in foods.
Figure 13. Pistachio sandwich ELISA standard curves. The microtiter plates were coated with raw/roasted pistachio-specific sheep antisera (1:25,000 dilution), followed by incubation with various concentrations of ground raw/roasted pistachio extract. Raw/roasted pistachio-specific rabbit antisera (1:15,000 dilution) were used as detection antibody. Each data point represents the mean of three replicates with three analyses per replicate. (Each standard curve was derived from 12 readings with an average standard error of <0.13 AU).
Comparison of raw and roasted pistachio standard curves

Both raw and roasted standards were very similar when they were visually compared. However, this assumption then required statistical judgment and support. The differences between raw and roasted pistachio ELISA standard curves were analyzed using the unpaired t-test in GraphPad Prism ® Software. There were no significant differences between the raw and roasted pistachio standards curves (p > 0.05). Therefore, roasted pistachio standards were selected as pistachio ELISA and carried on to the following cross-reactivity and matrices interference studies for assay validation.

Detection of raw and roasted pistachio using the roasted pistachio ELISA

The raw and roasted pistachio standard curves generated by using antisera of roasted pistachio were demonstrated on Figure 14. This comparison was conducted to ascertain if the anti-roasted pistachio antibody was able to recognize the unheated proteins from raw pistachio similarly as the roasted form. After the comparison by t-test, there were no significant differences (p > 0.05) between raw and roasted pistachio standards. Therefore, it was demonstrated that the antisera directed against roasted pistachio was able to bind to raw pistachios equivalently. As a result of this analysis, it was determined that only the anti-roasted pistachio antisera needed to be used in the subsequent development of the pistachio ELISA rather than a mixture of raw and roasted pistachio antibodies.
Figure 14. Pistachio sandwich ELISA standard curves. The microtiter plates were coated with roasted pistachio-specific sheep antisera (1:25,000 dilution), followed by incubation with various concentrations of ground raw (dotted curve) and roasted (solid curve) pistachio extract. Roasted pistachio specific rabbit antisera (1:15,000 dilution) were used as detection antibody. Each data point represents mean of four replicates with three analyses per replicate. Each standard curve was derived from 6 measurements with an average standard error of <0.10 AU.
Cross-Reactivity Studies

Cross-reactivity studies are needed when developing ELISA, especially when using polyclonal antisera. This is because polyclonal antisera contains abundant mixtures of antibodies with variable binding affinities toward many antigens. The specificity of the developed polyclonal antibodies to pistachio proteins was determined by cross-reactivity studies. Cross-reactivity tests can also be used to evaluate whether other ingredients from relevant food matrices might have interaction with the antibodies that would result in false-positive readings or increased background in the developed ELISA. Neither cross-reactivity nor false positive results can be neglected. The impact of unintentional cross-reactivity or false positive results could be enormous, causing unnecessary product recall or rejection by a company and eventually monetary loss.

Cross-reactivity was assessed by evaluating the soluble proteins extracted from a wide range of foods that were botanically related to pistachios (such as cashew, mango, and sumac derived from Anacardiaceae family) and food ingredients that are often incorporated in recipes containing pistachio with the developed pistachio ELISA (Table 4). Cashew was the only food that showed cross-reactivity with pistachio with an equivalent pistachio level of 4 ppm. Cross-reactivity was not detected with any of the other foods and ingredients. The cross-reactivity of cashew with pistachios was expected due to the similar botanical family relationship (Hasegawa et al., 2009). Many studies have demonstrated the cross-reactivity of the homologous seed storage proteins between cashew and pistachio, such as the 2S albumin, 7S vicillin, and 11S globulin (Anh et al., 2009; Willison et al., 2008, Goetz et al., 2005; Garcia et al., 2000; Fernandez et al., 1995).
A further cross-reactivity assessment was completed on cashew in order to verify the reactivity manner of cashew on pistachio ELISA. This assessment was carried out using the pistachio-specific sheep and rabbit antisera toward a 3-fold serially diluted cashew and pistachio extract standard curve. A linear reactivity with pistachio antibodies occurred on the cashew standard, which indicates the presence of common antigenic determinants (epitopes) between cashew and pistachio (Figure 15). The cross-reactivity between cashew and pistachio affects accuracy and reliability of the results if using the pistachio ELISA to detect undeclared pistachio residue in the food products that contain cashew as ingredients.
Table 4. Cross-reactivity of different food and food ingredients in the pistachio ELISA

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Protein Contents (mg/ml)</th>
<th>Equivalent pistachio level (ppm)</th>
<th>Ingredients</th>
<th>Protein Contents (mg/ml)</th>
<th>Equivalent pistachio level (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anacardiaceae</strong></td>
<td></td>
<td></td>
<td><strong>Egg</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cashew</td>
<td>4.51</td>
<td>4.0</td>
<td>Spray-dried whole egg</td>
<td>48.34</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Mango (dried)</td>
<td>0.13</td>
<td>&lt;1.0</td>
<td><strong>Flours and starches</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mango (flesh and skin)</td>
<td>1.4</td>
<td>&lt;1.0</td>
<td>Buckwheat flour</td>
<td>7.62</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Mango (seed)</td>
<td>10.82</td>
<td>&lt;1.0</td>
<td>Corn flour</td>
<td>1.73</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Sumac</td>
<td>2.86</td>
<td>&lt;1.0</td>
<td>Corn starch</td>
<td>ND</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td><strong>Tree nuts</strong></td>
<td></td>
<td></td>
<td>Oats</td>
<td>.52</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Almond</td>
<td>9.7</td>
<td>&lt;1.0</td>
<td>Potato flour</td>
<td>2.87</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Brazil nut</td>
<td>6.41</td>
<td>&lt;1.0</td>
<td>Rice flour</td>
<td>.76</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Chestnut</td>
<td>ND</td>
<td>&lt;1.0</td>
<td>Tapioca flour</td>
<td>.79</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Coconut flakes</td>
<td>0.52</td>
<td>&lt;1.0</td>
<td>Wheat flour</td>
<td>2.25</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Coconut milk</td>
<td>0.17</td>
<td>&lt;1.0</td>
<td>Wheat gluten</td>
<td>4.26</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Hazelnut</td>
<td>9.17</td>
<td>&lt;1.0</td>
<td>Yellow corn meal</td>
<td>1.29</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Macadamia nut</td>
<td>2.85</td>
<td>&lt;1.0</td>
<td><strong>Food coloring</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pecan</td>
<td>2.57</td>
<td>&lt;1.0</td>
<td>Annatto</td>
<td>3.47</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Pine nut</td>
<td>4.98</td>
<td>&lt;1.0</td>
<td>Caramel color</td>
<td>51.38</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Walnut</td>
<td>3.97</td>
<td>&lt;1.0</td>
<td>Citrus Red 2</td>
<td>ND</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td><strong>Dairy</strong></td>
<td></td>
<td></td>
<td>FD&amp;C Red 3 and 40</td>
<td>ND</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Butter</td>
<td>ND</td>
<td>&lt;1.0</td>
<td>FD&amp;C Yellow 5</td>
<td>1.64</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Cream cheese</td>
<td>2.37</td>
<td>&lt;1.0</td>
<td>Yellow 6</td>
<td>11.19</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Non-fat dry milk</td>
<td>22.16</td>
<td>&lt;1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whey</td>
<td>6.72</td>
<td>&lt;1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole milk</td>
<td>2.37</td>
<td>&lt;1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Protein content of extracts determined by the Lowry method, ppm = parts per million (µg/g), ND = not detected
Table 4 (continued). Cross-reactivity of different food and food ingredients in the pistachio ELISA

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Protein Contents (mg/ml)</th>
<th>Equivalent pistachio level (ppm)</th>
<th>Ingredients</th>
<th>Protein Contents (mg/ml)</th>
<th>Equivalent pistachio level (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fruits</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blueberry (dried)</td>
<td>ND</td>
<td>&lt;1.0</td>
<td>Xanthan gum</td>
<td>ND</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Cherries</td>
<td>0.47</td>
<td>&lt;1.0</td>
<td>Yeast-active dry</td>
<td>8.37</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Cocoa powder</td>
<td>10.75</td>
<td>&lt;1.0</td>
<td>Anise seed</td>
<td>6.15</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Cranberry (dried)</td>
<td>0.99</td>
<td>&lt;1.0</td>
<td>Basil</td>
<td>7.68</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Dates</td>
<td>1.96</td>
<td>&lt;1.0</td>
<td>Caraway</td>
<td>2.59</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Orange (flesh and skin)</td>
<td>0.07</td>
<td>&lt;1.0</td>
<td>Cinnamon</td>
<td>7.86</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Pineapple</td>
<td>0.76</td>
<td>&lt;1.0</td>
<td>Clove</td>
<td>33.42</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Raisins (dried)</td>
<td>1.30</td>
<td>&lt;1.0</td>
<td>Coriander</td>
<td>6.39</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Tomato (sun-dried)</td>
<td>3.38</td>
<td>&lt;1.0</td>
<td>Cumin</td>
<td>9.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td><strong>Herbs and Spices</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cocoa powder</td>
<td>10.75</td>
<td>&lt;1.0</td>
<td>Mace ground</td>
<td>3.87</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Dates</td>
<td>1.96</td>
<td>&lt;1.0</td>
<td>Mustard ground</td>
<td>23.53</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Orange (flesh and skin)</td>
<td>0.07</td>
<td>&lt;1.0</td>
<td>Nutmeg</td>
<td>2.02</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Pineapple</td>
<td>0.76</td>
<td>&lt;1.0</td>
<td>Onion powder</td>
<td>1.58</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Raisins (dried)</td>
<td>1.30</td>
<td>&lt;1.0</td>
<td>Oregano</td>
<td>12.97</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Tomato (sun-dried)</td>
<td>3.38</td>
<td>&lt;1.0</td>
<td>Paprika</td>
<td>7.82</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td><strong>Functional Ingredients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barley malt</td>
<td>4.83</td>
<td>&lt;1.0</td>
<td>Garlic powder</td>
<td>6.58</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Calcium sulfate</td>
<td>ND</td>
<td>&lt;1.0</td>
<td>Ginger powder</td>
<td>7.09</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Carrageenan</td>
<td>ND</td>
<td>&lt;1.0</td>
<td>Mace ground</td>
<td>3.87</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Corn syrup</td>
<td>ND</td>
<td>&lt;1.0</td>
<td>Mustard ground</td>
<td>23.53</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Guar gum</td>
<td>ND</td>
<td>&lt;1.0</td>
<td>Nutmeg</td>
<td>2.02</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Lemon juice</td>
<td>ND</td>
<td>&lt;1.0</td>
<td>Onion powder</td>
<td>1.58</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Orange juice</td>
<td>0.76</td>
<td>&lt;1.0</td>
<td>Oregano</td>
<td>12.97</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Pectin</td>
<td>ND</td>
<td>&lt;1.0</td>
<td>Paprika</td>
<td>7.82</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Potassium acid tartrate</td>
<td>ND</td>
<td>&lt;1.0</td>
<td>Pepper black</td>
<td>2.16</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Potassium sorbate</td>
<td>ND</td>
<td>&lt;1.0</td>
<td>Pepper white</td>
<td>ND</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Salt</td>
<td>ND</td>
<td>&lt;1.0</td>
<td>Thyme</td>
<td>11.38</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>ND</td>
<td>&lt;1.0</td>
<td>Wasabi (paste)</td>
<td>0.01</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Vanilla extract</td>
<td>0.52</td>
<td>&lt;1.0</td>
<td>Fresh onion</td>
<td>0.14</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

Protein content of extracts determined by the Lowry method, ppm = parts per million (µg/g), ND = not detected
Table 4 (continued). Cross-reactivity of different food and food ingredients in the pistachio ELISA

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Protein Contents (mg/ml)</th>
<th>Equivalent pistachio level (ppm)</th>
<th>Ingredients</th>
<th>Protein Contents (mg/ml)</th>
<th>Equivalent pistachio level (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Legumes</strong></td>
<td></td>
<td></td>
<td><strong>Seeds</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Green peas</td>
<td>7.68</td>
<td>&lt;1.0</td>
<td>Flax seeds</td>
<td>9.07</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Peanuts</td>
<td>2.31</td>
<td>&lt;1.0</td>
<td>Poppy seeds</td>
<td>6.94</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Peanut flour</td>
<td>4.55</td>
<td>&lt;1.0</td>
<td>Sesame seeds</td>
<td>4.65</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Soy nut</td>
<td>1.86</td>
<td>&lt;1.0</td>
<td>Sunflower seeds</td>
<td>3.13</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Soy lecithin</td>
<td>ND</td>
<td>&lt;1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soy flour</td>
<td>25.58</td>
<td>&lt;1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soy sauce (acid- hydrolyzed)</td>
<td>2.89</td>
<td>&lt;1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soy sauce (naturally brewed)</td>
<td>3.47</td>
<td>&lt;1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Oils</strong></td>
<td></td>
<td></td>
<td>Sugar and sweeteners</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canola oil</td>
<td>14.49</td>
<td>&lt;1.0</td>
<td>Beet sugar</td>
<td>0.13</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Hydrogenated vegetable oil</td>
<td>3.50</td>
<td>&lt;1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Olive oil</td>
<td>2.76</td>
<td>&lt;1.0</td>
<td>Cane sugar</td>
<td>ND</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Palm oil</td>
<td>0.06</td>
<td>&lt;1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partially hydrogenated oil</td>
<td>0.15</td>
<td>&lt;1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean oil</td>
<td>2.85</td>
<td>&lt;1.0</td>
<td>Dextrose</td>
<td>0.16</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>3.56</td>
<td>&lt;1.0</td>
<td>Honey</td>
<td>0.55</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Molasses</td>
<td>4.29</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

Protein content of extracts determined by the Lowry method, ppm = parts per million (µg/g), ND = not detected
Figure 15. Evaluation of the cross-reactivity between cashew (solid line) and pistachio (dotted line). Standard curves were developed using of various concentrations of cashew and pistachio and analyzed using the roasted pistachio ELISA. Standard curves were developed by diluting each corresponding food extract level 1:3 (v/v) in PBS + 1% NFDM. Each standard curve was derived from 8 measurements with an average standard error of <0.07 AU.
**Matrix Interference Studies**

The developed pistachio assay is highly specific and sensitive for the detection of allergenic pistachio residues in a simple buffer system (0.01 M PBS + 1% NFDM); however, the detection of pistachio residues can be challenging when present in a food matrix due to interference substances such as fatty acids and phenolic compounds (Taylor et al., 2009). Fatty acids have been demonstrated to cause antibody denaturation; whereas, phenolic compounds have been shown to non-specifically bind to antibodies, which then impede the interaction between antigen and antibodies in the assay system (Hefle, 1995; Keck-Gassenmeier et al., 1999). In addition, the food matrix may negatively affect the efficiency of extraction needed prior to the ELISA analysis. Extraction of allergenic proteins from the food matrix may also be difficult due to the effects of pH, which can decrease of protein solubility, chemical modification, enzymatic reaction, and protein aggregation.

**Spike and Recovery Experiments using Ground Pistachio in Ice Cream and Sugar Cookies**

The matrix interference study (also known as spike-and-recovery) experiment plays an important role in assay validation. The main concept is to evaluate the effectiveness of the pistachio assay in different food matrices. Baked products and confections are most at risk due to the common use of pistachios in these products and potential for cross-contact with non-pistachio containing products. Therefore, sugar cookies and vanilla ice cream were selected for the spike and recovery experiment. The effect of these food matrices on the pistachio ELISA were interpreted by comparing the standard curves in the two food matrices to the standard curved developed in buffer. The
red, green, and blue standard curves as shown in Figure 16 indicate the standard curves generated by pistachio spiked in PBS + NFDM, sugar cookies, and vanilla ice cream, respectively. Based on the statistical results (ANOVA, \( p > 0.05 \)), there were no significant differences in the slopes among the three standard curves indicating that the sugar cookie and vanilla ice cream matrices did not affect the sensitivity and effectiveness of pistachio assay.
Standards were prepared by spiking 1000 ppm of roasted pistachio into the food matrices and diluting 1:3 (v/v) in each of the corresponding negative control food extracts. Each standard curve was derived from 9 measurements with average standard error of <0.04 AU.
Spike and Recovery Experiments Comparing Regular Ground Pistachio and Finely Ground Pistachio in Ice Cream

This study was conducted to investigate if finer particle-size pistachio ground with sugar had higher extraction efficiency or extraction yield (more soluble pistachio protein extracted) as compared to regular ground pistachio. Material preparation could be used for one of the parameters to assess the material integrity and homogeneity, or even the manner of the extracted protein on the ELISA method (Zeleny and Schimmel, 2010). Figure 17 shows the standard curves of the two types of pistachio preparations – regular grind (RG) and fine grind (FG). Based on the results obtained from the pistachio ELISA and evaluated by the one-way ANOVA ($p>0.05$), no significant differences in the slopes between the two curves were detected. This investigation showed both preparations containing different particle sizes did not affect the extraction efficiency of the extracted proteins and that the proteins were sufficiently homogeneous in both preparations.
Figure 17. Sandwich ELISA standard curve of regular ground (RG) pistachio compared to the standard curve of finely ground (FG) pistachio. Both standards curves were developed by serially diluting extract 3-fold from 1000 ppm to 0 ppm in the vanilla ice cream matrix containing 0.01 M PBS and 1% NFDM. Each data point represents the mean of 9 measurements with average standard error of <0.03 AU.
CONCLUSION

Raw and roasted pistachio ELISAs were developed utilizing raw and roasted sheep antisera as capture antibody and rabbit antisera as detector antibody, respectively. Both ELISAs are capable of quantitatively detecting pistachio proteins as low as 1 ppm (LOQ). The standard curves developed using raw and roasted pistachio specific antibodies were statistically similar. Additionally, roasted pistachio antibodies were also able to detect unheated pistachio, thus eliminating the need to use both raw and roasted antibodies in the developed pistachio ELISA. Thus, the roasted pistachio ELISA was selected for further ELISA validation. However, cross-reactivity occurred between cashew and pistachio at the equivalent pistachio level of 4 ppm but no additional cross-reactivity or non-specific binding resulted with the any of the other tested food or food ingredients. Based on these cited results, it is expected that accuracy and reliability of the results will be affected when using the pistachio ELISA to detect undeclared pistachio residue in the food products that contained cashew as ingredients. The sensitivity of the pistachio ELISA was not affected by the presence of sugar cookie and vanilla ice cream matrices.
REFERENCES


CHAPTER 4: PRODUCTION OF MANUFACTURED MODEL FOODS FOR DETECTION OF PISTACHIO RESIDUES

ABSTRACT

The positive health effects of pistachio have lead to an increase usage of pistachio in recipes as ingredients. However, the increase pistachio exposure in foods increases the risk of allergic reactions in pistachio-allergic individuals. Sandwich-ELISA has been a reliable allergen detection method to help food manufacturers in food allergen control plan in order to reduce the risk of allergen cross-contact and to assure the safety of the susceptible individuals.

The validation of the developed pistachio-ELISA was demonstrated by naturally-incurred standards in vanilla ice cream, sugar cookie dough, and sugar cookies by adding known amount of pistachios into the foods formulations and processed under the stimulated conditions similar to food industry practices. Excellent recoveries of pistachio were obtained in vanilla ice cream and sugar cookie dough (before baking) with mean of percent recovery of 115% ± 3.0%, 131% ± 20.8%, respectively. An acceptable of pistachio were recovered from the baked sugar cookies with the mean of percent recovery of 53.9% ± 3.0%. The comparatively lower recovery of pistachio from sugar cookies is likely due to protein aggregation or cross-linking due to the loss of solubility of protein during baking. The complex food matrix and food processing with heat treatment affect the allergenicity of allergens and also interrupts the reactivity between allergen and antibody for detection often leading to false negative or false positive results.
INTRODUCTION

Pistachio is a popular tree nut that is often eaten as a roasted snack. In recent years, positive health effects have been reported for pistachio nuts (Gebauer et al., 2008; Sheridan et al., 2007; Kocyigit et al., 2006; Edwards et al., 1999). Researchers discovered that pistachio contains many types of beneficial fatty acids (predominantly monounsaturated fatty acids), minerals, and antioxidants such as γ-tocopherol, β-carotene, lutein, selenium, flavonoids, and phytoestrogens (USDA, 2009), which have been linked to multiple health benefits. However, increased consumption and also their incorporation into a variety of food recipes, such as salads, pesto, baked products, and ice cream has led to an increase in the prevalence of allergic sensitization to pistachio, resulting in adverse reactions.

Although pistachio is a less common cause of allergic reactions among sensitized individuals compared to several other tree nut allergies such as walnut and almond, pistachio allergy is as potent as other tree nut allergies and can cause severe anaphylactic reactions (Porcel et al., 2006; Sicherer et al., 1998). Because pistachio-allergic individuals can only prevent pistachio-related food allergy by avoidance; undeclared pistachio residues in foods can cause serious health risks including the risk of an anaphylactic reaction (Sicherer and Sampson, 2006; Crevel, 2005). Food manufacturers and regulatory bodies must work together to minimize the risk for food-allergic individuals from ingesting undeclared offending food residues (Huggett and Hischenhuber, 1998). Each production step must be strictly examined: from raw material handling through food processing to food packaging and labeling. Food manufacturers and public health agencies use sandwich-ELISA to detect allergenic food residues in
other foods. Due to its high sensitivity and specificity, sandwich-ELISA can distinguish specific allergenic food residues and provide reliable detection of allergens in various food matrices (Taylor et al., 2009). ELISA is sensitive and can have a low limits of quantification for allergenic residues in the simple buffer systems or when extracted from food matrices. However, food processing can disrupt the quantitative and qualitative features of ELISA by causing interactions between the food matrix components and the target protein, such as protein aggregation or chemical cross-linking (Mills and Mackie, 2008). These interactions can change the conformational epitopes of a target protein or make the target protein difficult to detect with specific antibodies in ELISA. The solubility of the target protein can also be influence how easily it can be extracted from the matrix.

In order to assess the overall ELISA performance in terms of the efficiency and robustness, the accepted approach is to validate uses the detection of naturally incurred standards in the model food studies (Taylor et al., 2009; Hefle and Koppelman, 2006). In this approach, naturally incurred standards are prepared by incorporating known amounts of allergenic food residues to into relevant food matrices followed by processes commonly used in food processing. The ability to extract and detect the target allergenic food residue is then evaluated in the processed food product. Thus, the developed pistachio-ELISA was used to examine and evaluate naturally incurred standards of pistachio in vanilla ice cream, sugar cookie dough and sugar cookies.
MATERIALS AND METHODS

Preparation of manufactured model foods

Vanilla Ice cream

The formulation of vanilla ice cream was obtained from the website of allrecipes.com – vanilla ice cream II. The ingredients used for preparing vanilla ice cream were heavy whipping cream, half and half cream, white granulated sugar, and vanilla extract; the ingredients were purchased from local grocery stores in Lincoln, NE. Several containers of heavy whipping cream and half and half cream of the same manufactured lot numbers were obtained so that every level of the spiked-ice cream were prepared from the same raw ingredients. Before making ice cream, the pistachio ELISA was used to pre-screen every ingredient for the possible presence of pistachio to prevent pistachio contamination and high background absorbance. Each ingredient was added according to the unit of mass (gram, g) to ensure that all levels of naturally incurred standards had a precise spike amount (µg/g) of pistachio. Eight batches of vanilla ice cream (1121 g each) were prepared with different levels of pistachio: 0, 1, 5, 10, 25, 50, 100, and 1000 ppm (Table 5). Initially, a more highly concentrated spike material of powdered pistachio-sugar mix was used at 112,100 ppm (11.21 g ground pistachio in 1121 g of white sugar) by grinding more coarsely ground pistachio and sugar with the Krup™ coffee grinder. Four sub-samples were collected from the powdered pistachio-sugar mix to test for homogeneity of mixing using the pistachio ELISA. The eight different levels of naturally incurred pistachio were each prepared independently by proceeding from the lowest level (0 ppm) to the highest level. After completing one batch of ice cream, the ice cream
maker was cleaned thoroughly with soap and hot water followed by rinsing with distilled water to avoid carryover of pistachio protein from batch-to-batch.

The Speed Freeze canister of the ice cream maker (Deni Scoop Factory Compact Automatic Ice Cream & Frozen Dessert maker, Model 5000, Keystone Manufacturing Company, Buffalo, NY) was kept frozen in the freezer overnight every time before use. A designated amount of the ice cream mixture components (combination of heavy whipping cream, half and half, and sugar) was prepared and stored in the refrigerator to chill until use. In the following day, the ice cream maker was set up by placing a rotator inside the frozen canister. Before pouring the chilled ice cream mixture into the rotating frozen canister, a specific amount of vanilla extract was added to the chilled ice cream mixture. A soft custard-like texture of ice cream was formed after 15 minutes of rotation. Subsequently, specific amounts of powdered sugar and the powdered pistachio-sugar mix were added to the soft ice cream to achieve the desired level of pistachio in each batch of ice cream. The canister was rotated for an additional 10 minutes to homogenize the distribution of pistachio throughout the ice cream. The finished ice cream was kept in Ziploc bags and stored in the freezer at -20°C.
Table 5. Formulation of naturally incurred standards of pistachio in vanilla ice cream

<table>
<thead>
<tr>
<th>Naturally incurred standards in vanilla ice cream (grams, g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredients</td>
</tr>
<tr>
<td>-----------------------------------------------------------</td>
</tr>
<tr>
<td>Heavy whipping cream</td>
</tr>
<tr>
<td>Half &amp; half cream</td>
</tr>
<tr>
<td>White sugar</td>
</tr>
<tr>
<td>Vanilla extract</td>
</tr>
<tr>
<td>Powdered sugar</td>
</tr>
<tr>
<td>Pistachio-powdered sugar</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

<sup>a</sup>ppm - parts per million, µg/g
Sugar cookie

Seven levels (0, 1, 5, 10, 25, 50, 100 ppm) of pistachio in were added to regular sugar cookie formulation (Table 6). The ingredients included commercial Betty Crocker™ sugar cookie mix, butter, and egg. Before proceeding, all ingredients were pre-screened for the possible presence of undeclared pistachio or interfering substances using the pistachio ELISA. Due to the high fat content of pistachios, sugar was added to assist in grinding the pistachio nuts into relatively smaller particles without forming into a pasty pistachio butter. A Krups™ coffee grinder was used to finely grind 0.6803 g of pistachio and 50 g of white sugar, giving a mix of fine powdered pistachio-sugar mix (13,606 ppm). The fine particle size of pistachio allowed for easier incorporation into other ingredients and even distribution in the sugar cookies. A calculated working spike of 135.8 ppm powdered pistachio-sugar cookie mix was prepared beforehand by mixing 10 g of the powdered pistachio-sugar mix into 992 g of sugar cookie mix through a Kitchen Aid™ 5 Quart Artisan Mixer, KSM150OPS (Kitchen Aid, St. Joseph, MI). Subsequently, four sub-samples of the pistachio-sugar cookie mix from different areas of the mixing bowl were taken to test for homogeneity using the pistachio ELISA.

The seven levels of pistachio in sugar cookie were made individually by combining designated amounts of pistachio-free sugar cookie mix and the powdered pistachio-sugar mix, butter, beaten egg, and powdered sugar. All of the batters were mixed with the mixer for an equal length of time and speed (kitchen aid, 6-10 speed for 15 minutes). At five minute intervals, the mixer was stopped and the dough was mixed by folding manually from the bottom to top of the mixing bowl using a spatula to attain a
well distributed sample within every level of the mixing bowl. Between batches, the mixing bowl and utensils were thoroughly cleaned with hot soapy water to wash off the fat and dough residues and rinsed with a cycle of distilled water. All levels of cookie dough were kept in Ziploc bags and stored at 4°C until use. The sugar cookies containing different levels of pistachio were baked at 375°F for seven minutes. All cookies were weighed before and after baking to determine the moisture loss of the cookies after baking. The remaining sugar cookie dough and three baked cookies from each level were taken and tested using the pistachio ELISA.

Table 6. Formulation of naturally incurred standards of pistachio in sugar cookie

<table>
<thead>
<tr>
<th>Naturally incurred standards in sugar cookie (grams, g)</th>
<th>Ingredients</th>
<th>100 ppm(^a)</th>
<th>50 ppm</th>
<th>25 ppm</th>
<th>10 ppm</th>
<th>5 ppm</th>
<th>1 ppm</th>
<th>0 ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pistachio-free sugar cookie mix (negative control)</td>
<td>0</td>
<td>248</td>
<td>372</td>
<td>446.4</td>
<td>471.2</td>
<td>491.04</td>
<td>496</td>
<td></td>
</tr>
<tr>
<td>Pistachio-sugar cookie mix (135.8 ppm)</td>
<td>501</td>
<td>250.5</td>
<td>125.25</td>
<td>50.1</td>
<td>25.05</td>
<td>5.01</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Powdered sugar</td>
<td>0</td>
<td>2.5</td>
<td>3.75</td>
<td>4.5</td>
<td>4.75</td>
<td>4.95</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Butter</td>
<td>118.3</td>
<td>118.3</td>
<td>118.3</td>
<td>118.3</td>
<td>118.3</td>
<td>118.3</td>
<td>118.3</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>61</td>
<td>61</td>
<td>61</td>
<td>61</td>
<td>61</td>
<td>61</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Total weight</td>
<td>680.3</td>
<td>680.3</td>
<td>680.3</td>
<td>680.3</td>
<td>680.3</td>
<td>680.3</td>
<td>680.3</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) ppm - parts per million, µg/g
Sample Preparation and Extraction

Vanilla Ice Cream

Three sub-samples of each level (from 0 ppm to 1000 ppm) were collected from three different areas of the Ziploc bag; top (A), middle (B), and bottom (C). The level of 0 ppm ice cream was used as the negative control. A 10,000 ppm pistachio standard or positive control was prepared by adding 0.1 g of ground pistachio to 10 g of the 0 ppm vanilla ice cream. All samples including the positive and negative samples, and the three sub-sample of every level were softened and extracted 1:10 (w/v) in 0.01 M phosphate buffered saline (pH 7.4, 0.85% saline + 0.02% NaN$_3$) + 1% nonfat dry milk (NFDM) in a shaking water bath (Julabo SW22, Julabo USA, Inc., Allentown, PA) at 150 rpm at 60$^\circ$C for two hours. Sample extracts were centrifuged at 3,162 x g (4,100 rpm) for 30 minutes at 10$^\circ$C in a tabletop centrifuge (Sorvall® Legend™ RT, Kendro Laboratory Products, Newton, CT). The clarified supernatants were analyzed for recovery of pistachio using the pistachio ELISA.

A standard curve was developed with serially diluted (1:3 v/v - 1000, 333, 111, 37.0, 12.3, 4.12, 1.37, 0.457, 0.152, 0.051, 0.017, and 0 ppm) positive control in the negative control vanilla ice cream extract. The corresponding ELISA results of the extracted samples were analyzed and compared with the standard curve. The recovery results were obtained as a ratio (in percent) of the recovered ppm level to the ppm level of added pistachio.
Sugar Cookies and Cookie Dough

Three subsamples of sugar cookies were ground into fine particle sizes using an Osterizer® blender (Sunbeam Corporation, Delray Beach FL). The 0 ppm cookie dough and sugar cookies were used as negative controls. Additionally, a pistachio cookies standard or positive control was prepared by adding 0.05g of roasted pistachio to 5g of the 0 ppm cookie dough and sugar cookie respectively to achieve a 10,000 ppm standard for pistachio-cookie dough and pistachio–sugar cookies. All samples of the naturally incurred cookie dough and sugar cookies, positive, and negative controls were extracted as described above for ice cream. The extracts were kept at -4°C for the evaluation of pistachio levels using the pistachio ELISA on the following day. Two individual serially diluted standard curves (0 – 1000 ppm) developed in the negative control (0 ppm) sugar cookies and cookie dough, respectively, were used to determine the amount of pistachio recovered from the baked sugar cookies. The percent recovery of pistachio from the baked sugar cookies was calculated as a ratio (in percent) of the recovered ppm level to the ppm level of added pistachio. The moisture loss during baking was taken into account to calculate the actual ppm level of added pistachio available in the sugar cookies after baking.
RESULTS AND DISCUSSION

The spike and recovery experiment in Chapter 3 demonstrated the efficiency and specificity of the pistachio ELISA in the presence of food matrices. However, the spiking of allergenic food particles into food matrices did not truly represent the actual effect of food processing on the allergenic food residues. Various combinations of food matrices and food processing conditions could affect the allergenic source proteins in the final product, especially the extraction efficiency. One possible impact is the enhancement of protein-carbohydrate interactions from heat-accelerated chemical reactions, such as the Maillard browning reaction. In addition, the detection of allergenic food residues could be affected by protein aggregation with loss of solubility, proteins denaturation due to pH effects, emulsion formation, protein hydrolysis, and other factors dependent upon the nature of the food processing operation (Taylor et al., 2009).

Therefore, the ultimate evaluation of the developed pistachio ELISA must be evaluated to determine the recovery of pistachio from processed model foods. In this study, known levels of ground pistachio were incorporated into vanilla ice cream and sugar cookies and processed under simulated conditions similar to food industry practices. Vanilla ice cream and sugar cookies are ideal selections for this study, because both products are common applications for pistachios. Furthermore, ice cream and cookies are examples of products frequently made with shared equipment used for formulations that do not include pistachio. Thus, the pistachio ELISA could have value in assessing the cleaning of any shared equipment but necessitates the ability to detect the residues effectively in processed food matrices.
**Recovery of pistachio from vanilla ice cream**

The standard curve generated with ground pistachio in the vanilla ice cream negative control was used (shown in Figure 18) for estimating the relative amount of pistachio in each sample. The mean ppm and percent recovery for each level of pistachio in vanilla ice cream are shown in Tables 7 and 8. The mean percent recovery of pistachio from the vanilla ice cream was 115% ± 5.0%. Comparable recoveries were obtained from the samples obtained from different locations with the ice cream freezer (A, B, and C) as shown in Table 7 indicating the consistent distribution of pistachio within each batch of ice cream in the freezer. Ice cream freezing had a minimal effect on the extraction and detection of pistachio proteins using the pistachio ELISA. The high percent recovery from the naturally incurred ice cream demonstrated the high extraction efficiency of the pistachio in ice cream and the high specificity and efficiency of the pistachio ELISA in detecting and quantifying pistachio residues from this matrix.
Figure 18. Standard curve for vanilla ice cream spiked with 10,000 ppm ground pistachio (standard) as determined by the pistachio ELISA. The 1,000 ppm standard was prepared by adding 0.01 g of ground pistachio into 10 g of vanilla ice cream negative control, followed by extraction 1:10 (w/v) in 0.01 M PBS + 1% NFDM in a shaking water bath for 2 hours. The extracted standard was centrifuged. Three-fold serial dilutions of the standard extract were made to generate the standard curve. Each data point represents the average of twelve measurements, with a standard deviation of <0.04 absorbance unit.
Table 7. Mean ppm and percent recovery of pistachio from manufactured vanilla ice cream obtained from three different locations within the ice cream freezer as determined by the developed pistachio ELISA

<table>
<thead>
<tr>
<th>Added pistachio level (ppm)</th>
<th>Ice cream A = “top”</th>
<th>Ice cream B = “middle”</th>
<th>Ice cream C = “bottom”</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ppm recovery&lt;sup&gt;a&lt;/sup&gt;</td>
<td>% recovery&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Mean ppm recovery</td>
</tr>
<tr>
<td>0</td>
<td>BLQ&lt;sup&gt;d&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;e&lt;/sup&gt;</td>
<td>BLQ</td>
</tr>
<tr>
<td>1</td>
<td>1.2 ± 0.0</td>
<td>123 ± 4.8</td>
<td>1.3 ± 0.0</td>
</tr>
<tr>
<td>5</td>
<td>5.9 ± 0.2</td>
<td>117 ± 4.9</td>
<td>6.3 ± 0.4</td>
</tr>
<tr>
<td>10</td>
<td>11.3 ± 0.7</td>
<td>113 ± 3.2</td>
<td>12.3 ± 0.4</td>
</tr>
<tr>
<td>25</td>
<td>26.9 ± 1.4</td>
<td>108 ± 5.7</td>
<td>26.3 ± 0.7</td>
</tr>
<tr>
<td>50</td>
<td>46.0 ± 0.3</td>
<td>92.1 ± 0.6</td>
<td>47.1 ± 1.1</td>
</tr>
<tr>
<td>100</td>
<td>108 ± 3.5</td>
<td>108 ± 3.5</td>
<td>119 ± 8.6</td>
</tr>
<tr>
<td>1000</td>
<td>1200 ± 45.1</td>
<td>120 ± 4.5</td>
<td>1150 ± 52.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> ppm-parts per million (mg of ground pistachio per kg)
<sup>b</sup> Data are mean ± standard error (n=4)
<sup>c</sup> Percent recovery calculated as ratio (in percent) of average ppm pistachio recovered to the available (ppm) pistachio in the finished product
<sup>d</sup> BLQ – below limit of quantification (<1 ppm)
<sup>e</sup> NA – not applicable
Table 8. The mean ppm and percent recovery of pistachio from manufactured vanilla ice cream (combination of ice cream A, B, and C) determined by the developed pistachio ELISA

<table>
<thead>
<tr>
<th>Added pistachio level (ppm&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>Mean ppm recovery&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% recovery&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>BLQ&lt;sup&gt;d&lt;/sup&gt;</td>
<td>NA</td>
</tr>
<tr>
<td>1</td>
<td>1.3 ± 0.0</td>
<td>127 ± 2.9</td>
</tr>
<tr>
<td>5</td>
<td>6.1 ± 0.2</td>
<td>122 ± 3.2</td>
</tr>
<tr>
<td>10</td>
<td>12.3 ± 0.4</td>
<td>123 ± 3.6</td>
</tr>
<tr>
<td>25</td>
<td>26.5 ± 0.6</td>
<td>106 ± 2.4</td>
</tr>
<tr>
<td>50</td>
<td>47.2 ± 0.7</td>
<td>94.4 ± 1.4</td>
</tr>
<tr>
<td>100</td>
<td>114 ± 3.7</td>
<td>114 ± 3.7</td>
</tr>
<tr>
<td>1000</td>
<td>1170 ± 35</td>
<td>117 ± 3.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> ppm-parts per million (mg of ground pistachio per kg)

<sup>b</sup> Data are mean ± standard error (n=12)

<sup>c</sup> Percent recovery calculated as ratio (in percent) of average ppm pistachio recovered to the available (ppm) pistachio in the finished product

<sup>d</sup> BLQ – below limit of quantification (<1 ppm)

<sup>e</sup> NA – not applicable
Recovery of pistachio from sugar cookies

The preparation of a naturally incurred standard of pistachio in sugar cookies is more challenging than experienced with ice cream. The challenges for a baked product matrix consist of: assurance of thorough mixing of raw ingredients (negative control sugar cookie mix, eggs, and butter) and the concentrated spike (135.8 ppm powdered pistachio-cookie mix) to obtain a homogenous naturally incurred standard, ensuring intensive cleaning of mixing equipment between batches, and accounting for moisture loss during baking. All of the wet ingredients including eggs and butter should be added according to their weight (g) as with all dry ingredients to standardize the total final weight for each batch. A large batch of liquid eggs should be prepared and mixed to eliminate variations in egg weight as a variable (Taylor et al., 2009). In addition, using the same length of time to mix the ingredients and scraping the bowl frequently enhance the incorporation of wet ingredients into dry ingredients, achieving a more homogenous cookie dough.

Baking causes moisture loss, which affects the final concentration (ppm levels) of pistachio in the cookies. The final concentration for the batch was calculated by dividing the amount of pistachio added by the final total weight of the cookie. Moisture loss was accounted for by weighing each cookie before and after baking. The average percent moisture loss after baking was 7.0% ± 0.8% (data details not shown).

The standard curve generated by adding ground pistachio to the sugar cookies negative control was used (shown in Figure 19) for estimating the relative amount of pistachio in each sample. The percent recovery of pistachio from the baked sugar cookies
is summarized in Tables 9 and 10. Comparable recoveries of each level were obtained from the samples obtained from different locations with the oven (A= left, B= center, and C = right) as shown in Table 9. The average of percent recovery of pistachio from sugar cookie A, B, and C were 50.1% ± 5.4%, 58.6% ± 3.7%, and 53.1% ± 3.8%, respectively. The highest amount of pistachio was recovered from sugar cookie B. This result may be due to the uneven heat distribution in the oven resulting non-uniformity of baking of the finished product in different zones or locations of the oven. As sugar cookie B positioned at the center of the oven received the least heat transferred from out to inside compared to sugar cookies A and C which were positioned at the side of the oven. Hence, proteins of cookie B would be less affected. Overall, the average percent recovery of pistachio from the sugar cookies was 53.9% ± 4.3%. One of the possible reasons for the minimal recovery of the pistachio from the baked sugar cookies is the effect of the high heat application on the immunological and physicochemical properties of the pistachio proteins within the food matrix. Pistachio protein might lose its solubility in the baked food matrix due to aggregation or protein-carbohydrate interactions such as the Maillard browning reaction. The loss of solubility would result in a decrease of the quantitative extraction of pistachio from the cookie matrix for ELISA detection. In addition, the high temperature treatment and the Maillard reaction could result in changes to the conformation of pistachio proteins, thus interfering with epitope binding (reduction of antigen-antibody interaction). Finally, the possibility exists of interference associated with some of the food matrix components following the baking process, which could also interfere with antigen-antibody interactions.
The lower recovery of pistachio proteins was quite evidently associated with the high heat treatment of baking as shown by a comparison of pistachio recovered from the sugar cookie dough before baking (Table 10). More than 100% recoveries of pistachio were obtained from the cookie dough. The such high recoveries (up to 158%) of pistachio from the cookie dough is not clear. However, a significantly lower recovery was obtained after baking. Thus, baking either lowers the ability to extract pistachio proteins from the matrix or affects the ability to detect the pistachio proteins using the pistachio ELISA. Although the quantitative accuracy of the pistachio ELISA is not optimal for the sugar cookie and perhaps other food matrices subjected to heat processing, the presence of pistachio residue in heat-processed foods can still be detected qualitatively by the pistachio ELISA.
Figure 19. Standard curve for sugar cookies spiked with 10,000 ppm ground pistachio (standard) as determined by the pistachio ELISA. The 1,000 ppm standard was prepared by adding 0.01 g of ground pistachio into 10 g of sugar cookies negative control, followed by extraction 1:10 (w/v) in 0.01 M PBS + 1% NFDM in a shaking water bath for 2 hours. The extracted standard was centrifuged. Three-fold serial dilutions of the standard extract were made to generate the standard curve. Each data point represents the average of nine measurements, with a standard deviation of <0.07 absorbance unit.
Table 9. Mean ppm and percent recovery of pistachio from manufactured sugar cookies determined by the developed pistachio ELISA

<table>
<thead>
<tr>
<th>Added pistachio level (ppm)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Before baking</th>
<th>After baking</th>
<th>After baking</th>
<th>After baking</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cookie A</td>
<td>Cookie B</td>
<td>Cookie C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Final pistachio Concentration (ppm)</td>
<td>Mean ppm recovery&lt;sup&gt;b&lt;/sup&gt;</td>
<td>% recovery&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Final pistachio Concentration (ppm)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>BLQ&lt;sup&gt;d&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>1.1</td>
<td>0.6 ± 0.1</td>
<td>50.6 ± 8.3</td>
<td>1.1</td>
</tr>
<tr>
<td>5</td>
<td>5.5</td>
<td>2.5 ± 0.2</td>
<td>45.8 ± 3.1</td>
<td>5.5</td>
</tr>
<tr>
<td>10</td>
<td>10.6</td>
<td>6.6 ± 0.8</td>
<td>62.4 ± 7.1</td>
<td>10.7</td>
</tr>
<tr>
<td>25</td>
<td>26.9</td>
<td>13.5 ± 1.8</td>
<td>50.1 ± 6.6</td>
<td>26.9</td>
</tr>
<tr>
<td>50</td>
<td>53.4</td>
<td>26.6 ± 1.8</td>
<td>49.8 ± 3.4</td>
<td>53.6</td>
</tr>
<tr>
<td>100</td>
<td>109</td>
<td>45.7 ± 4.1</td>
<td>42.1 ± 3.8</td>
<td>107</td>
</tr>
</tbody>
</table>

<sup>a</sup> ppm-parts per million (mg of ground pistachio per kg)

<sup>b</sup> Data are mean ± standard error (n=3)

<sup>c</sup> Percent recovery calculated as ratio (in percent) of average ppm pistachio recovered to the available (ppm) pistachio in the finished product

<sup>d</sup> BLQ – below limit of quantification (<1 ppm)

<sup>e</sup> NA – not applicable
Table 10. Mean ppm and percent recovery of pistachio from manufactured sugar cookies (combination of sugar cookies A, B, and C) and sugar cookie dough determined by the developed pistachio ELISA

| Added pistachio level (ppm) | Before baking | | After baking | | |
|-----------------------------|---------------|-----------------|-----------------|-----------------|
|                             | Dough         | Sugar cookie    | Dough           | Sugar cookie    |
|                             | Mean ppm recovery | % recovery^c | Mean ppm recovery | % recovery^c |
| 0                           | BLQ^e          | NA^f           | BLQ             | NA             |
| 1                           | 1.1 ± 0.0      | 111 ± 4.0      | 0.6 ± 0.0       | 56.2 ± 4.6     |
| 5                           | 5.6 ± 0.3      | 112 ± 5.2      | 2.9 ± 0.2       | 54.3 ± 3.2     |
| 10                          | 12.9 ± 0.8     | 129 ± 7.9      | 6.6 ± 0.3       | 61.3 ± 3.1     |
| 25                          | 39.1 ± 13.6    | 157 ± 54.4     | 13.9 ± 0.7      | 51.4 ± 2.6     |
| 50                          | 61.0 ± 5.9     | 122 ± 11.8     | 26.7 ± 0.9      | 50.0 ± 1.7     |
| 100                         | 158 ± 41.2     | 158 ± 41.2     | 54.1 ± 2.8      | 50.2 ± 2.6     |

^a ppm-parts per million (mg of ground pistachio per kg)
^b Data are mean ± standard error (n=3)
^c Percent recovery calculated as ratio (in percent) of average ppm pistachio recovered to the available (ppm) pistachio in the finished product
^d Data are mean±SE (n=9)
^e BLQ – below limit of quantification (<1 ppm)
^f NA – not applicable
CONCLUSION

The capability of the pistachio ELISA to detect pistachio residues in other foods has been assessed by the model foods (vanilla ice cream and sugar cookies) used in this study. Excellent recoveries of pistachio were obtained in vanilla ice cream and sugar cookie dough (before baking). However, a lower recovery of pistachio was obtained in baked sugar cookies, which is believed to be caused by the impact of heat processing on pistachio proteins, possibly owing to the aggregation of the proteins which lowers their solubility. If the pistachio proteins are less soluble after baking, the insolubility would affect the sensitivity of the pistachio ELISA for the detection of pistachio residues in baked foods. Insoluble aggregates of pistachio proteins may still pose a risk to pistachio-allergic individuals. However, even with the lower recovery from baked sugar cookies, this study demonstrates that the food industry can use the pistachio ELISA to monitor and quantify pistachio residues in processed foods. Additional research should be directed at optimizing the extraction efficiency from baked goods or the ability of the pistachio ELISA in detecting pistachio residues in heat-processed foods such as baked products.
REFERENCES


