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DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT
ASSAY (ELISA) FOR THE DETECTION OF PECAN RESIDUES IN
PROCESSED FOODS

by

Denise Tran

A THESIS

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The Graduate College of the University of Nebraska
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Major: Food Science and Technology

Under the Supervision of Professor Joseph L. Baumert

Lincoln, Nebraska

August, 2013

**DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT
ASSAY (ELISA) FOR THE DETECTION OF PECAN RESIDUES IN
PROCESSED FOODS**

Denise Tran, M.S.

University of Nebraska, 2013

Advisor: Joseph Baumert

Pecan nuts are becoming increasingly popular due to their link to health benefits. However, the presence of undeclared pecan residues in food products can pose serious health risks for pecan-allergic consumers. Currently, analytical methods for the detection of pecan allergens are limited and have not been validated for use by the food industry to assess the effectiveness of allergen control programs. Therefore, the aim of this study was to develop a sandwich-type ELISA to detect and quantify allergenic pecan residues in processed foods. Several varieties of pecan were mixed, roasted, and ground to immunize a goat, sheep, and rabbits. The pecan-specific IgG titer and specificity of the IgG antibodies produced in the 3 species of animals were tested by direct ELISA and immunoblot, respectively. Goat and rabbit polyclonal anti-roasted pecan sera were used as capture and detector reagents, respectively, to develop the sandwich ELISA, with visualization through an alkaline phosphatase-mediated substrate reaction. The pecan ELISA had a limit of quantification of 2 ppm (2 μ g pecan/g). Because pecans are commonly used in confections and bakery products, sugar cookie, vanilla ice cream, and dark chocolate matrices were chosen to evaluate potential interference on the ELISA's performance using spike and recovery approaches. The matrices did not significantly (*p*

< 0.05) affect the sensitivity of the developed assay. The dark chocolate matrix produced a slightly higher background but still maintained an adequate dynamic range. The ELISA was highly specific except for considerable cross-reactivity to walnut. The performance of the developed assay for detection of pecan in processed (incurred) ice cream and sugar cookies was evaluated. Excellent recovery of pecan from manufactured vanilla ice cream ($103\% \pm 4.28\%$) and sugar cookie ($87.0\% \pm 5.45\%$) occurred. The developed ELISA demonstrates high specificity towards both roasted and raw pecans and thus is a potential method for food manufacturers and regulatory agencies to detect pecan residues in processed foods and facilitate the validation of allergen control programs for pecan.

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CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

Food consumption is recognized as an evolving social practice, where food no longer serves merely as sustenance but also a way to relate to other people in social, cultural and political terms (Oosterveer, 2006). Technological advancement in food manufacturing and innovative research in nutritional science has resulted in increasingly higher food quality standards and an expanding variety of foods. These processed foods cater to the fast-paced, busy lifestyle of the common consumer by delivering convenience and a wide selection of foods. Unfortunately for individuals with food allergies, foods that are typically safe for consumption by the vast majority of the population can cause adverse or even life-threatening reactions. Understandably, the joy of eating is diminished by the persistent fear of consuming a food or food ingredient that can cause unpleasant reactions. Food allergy significantly affects quality of life, and there is recognition that the prevalence of food allergy is rising. According to Sicherer et al. (2010), the prevalence of childhood tree nut allergy has increased significantly from 0.2% to 1.1% within the past ten years. Heightened awareness, particularly due to the media, has made parents and physicians more vigilant about allergies that would have otherwise been ignored. The attention on the apparent rise in food allergy has correspondingly led to implementation of safety regulation throughout the world. The Food Allergen Labeling & Consumer Protection Act (FALCPA) that was passed by the United States Congress in 2004 and officially took effect on January 1, 2006 requires labeling of packaged foods containing any of the eight “major food allergens,” which includes tree nuts such as

pecans. Pecan is venerated as the “All American Nut” because it has been used and enjoyed in the United States for many years and is the only tree nut indigenous to North America, but pecan is also currently gaining attention internationally by other countries such as India, Canada, and Dubai. Pecans have gained importance in recent years for their health benefits, especially their high antioxidant capacity and potential for reducing the risk of cardiovascular disease (Morgan and Clayshulte, 2000; Rajaram et al., 2001; USDA, 2009). The University of Georgia has recently been given a four-year, \$1.2 million grant from the U.S. Department of Agriculture to study the nutritional benefits of pecans and offer those findings to help promote the nut (USDA, 2011). With a positive spotlight focused on the pecan nut, the food industry may incorporate pecan into food products to increase nutritional value. Although a growth in pecan use is certainly a positive aspect for the industry, this also creates a situation for the increased opportunity of unintentional exposure of pecan allergens to allergic consumers. Undeclared pecan allergens in food products, often introduced unintentionally during processing, represent a major health threat. It is currently advised that the best approach to prevent allergic reactions is to abolish the offending food from one’s diet; hence, this requires that relevant analytical methodology capable of detecting traces of allergens be established to provide accurate labeling, as this is the only link to inform consumers on the composition of prepackaged foods. The food industry and food regulatory agencies currently rely on commercially available Enzyme – Linked Immunosorbent Assays (ELISA) as a tool to monitor the safety of the food products (Whitaker et al., 2005). The ELISA method is simple, quick, sensitive and specific, but there is currently no robust assay for the detection of pecan residue that has been thoroughly validated. Therefore, the

development of such a method would allow food industry and regulatory agencies to ascertain and document compliance as well support food safety investigations, respectively.

The following chapter will discuss the different adverse reactions to food, with emphasis on the characteristics and mechanisms of true food allergy. Pecan allergy and allergens, and the analytical methods for their detection in foods will also be reviewed. The last part of the chapter will focus on the critical parameters essential for reliable and robust detection of food allergens in processed foods.

ADVERSE REACTIONS TO FOOD

Adverse reactions to food are common and can be defined as any abnormal response resulting from the ingestion of a food or food ingredient, regardless of the pathophysiology (Anderson, 1986; Sampson, 2004). Nearly everyone will experience an unpleasant reaction to something eaten at some point in their lives, but only a small percentage of these reactions will truly be attributed to food allergies. It is common for the public and even some in the health profession to mistakenly cluster all abnormal reactions to food as food allergies. It is important to distinguish true food allergies from food intolerances and other non-immunological reactions because determining the specific etiology can allow correct management of these adverse reactions to food (Koppelman & Hefle, 2006).

Food Intolerances

Because adverse food reaction is a broad term indicating a link between the ingestion of a food and an abnormal response, the ability to classify these reactions into

clearer categories is a significant step towards understanding the complex issue. Adverse reactions to foods are either immune mediated or non-immune mediated reactions depending upon whether the immune system is primarily involved in causing the reaction. Reactions to foods, which are not due to immunological mechanisms, are generally regarded as food intolerances. Types of food intolerance include toxins, metabolic disorders, or other undefined reactions (Boyce et al., 2010).

Toxic food reactions are caused by the direct action of a food or food additive without the obvious involvement of immune mechanisms (Clarke et al., 1996). These reactions can occur in anyone who is exposed to the food and do not depend on host factors. The toxins may be enzymes or any agent that could cause reactions in the body. Examples of toxic reactions include nausea from bacterial food poisoning, heavy metal poisoning, and itching and flushing from histamine ingestion as seen in scombroid fish poisoning. Many commonly consumed foods contain a small amount of histamine that when ingested, is not enough to cause any harm, but histamine poisoning can result when the body's protective mechanisms are overwhelmed from large doses of histamine (Taylor, 1986).

Metabolic food reactions involve an inborn or acquired error in metabolism of nutrients such as lactase deficiency and favism (Clarke et al., 1996). In lactose intolerance, a deficiency in the enzyme, β -galactosidase, leads to an impaired ability to digest lactose. Favism is characterized by a genetic deficiency in erythrocyte glucose-6-phosphate dehydrogenase that causes an increased sensitivity to several hemolytic factors in fava beans (Taylor et al., 2000).

Another type of nonimmunological food reaction termed anaphylactoid reactions can result from substances in food that cause mast cells and basophils to spontaneously release histamine and other mediators of allergic reaction. In contrast to true food allergies that are mediated by antigen cross-link of two surface bound immunoglobulin E (IgE) antibodies which results in the release of these mediators, anaphylactoid reactions involve substances that bring about the release of these same mediators without the involvement of IgE (Taylor et al., 2000). The spontaneous release of histamine has never been identified in foods, but this mechanism is well-established with certain drugs.

Pharmacological food reactions have been defined as adverse reaction to foods or food additives that produce drug-like or pharmacologic effects in the host. These reactions tend to be dose dependent and have the potential to be elicited in a wider, more diverse group of individuals. Pharmacologic food reactions depend on metabolic differences, concurrent medication usage, food freshness, and food preparation (Keeton et al., 2008). A well-known example is caffeine, a methylxanthine derivative present in tea and coffee. Its biological action includes stimulation of the heart muscle, the central nervous system, and the production of gastrin. Another group of biologically active substances include the vasoactive amines such as histamine and tyramine. Histamine and tyramine are commonly present in food products such as cheese, fish, sauerkraut, and sausages. The effects of large doses of vasoactive amines are extremely variable, but excessive intake can cause headache, abdominal cramps, tachycardia, urticaria, and in severe cases, hypotension, bronchoconstriction, chills, and muscle pain (Bruggink, 1996).

There is a variety of individual food sensitivities thought to occur through nonimmunological, but unknown, mechanisms, and these are regarded as idiosyncratic

reactions. Food idiosyncrasies are adverse reactions to foods or food components that occur through unknown mechanisms, which can even include psychosomatic illnesses (Taylor, 1987). Sulfite-induced asthma is the best example of an idiosyncratic reaction that has been well documented to occur among certain consumers, although the mechanism remains unknown (Bush and Taylor, 1998).

Food Allergy

Adverse reactions arising from a specific immune response that occurs reproducibly on exposure to a given food depict a true food allergy (Boyce et. al., 2010). Hypersensitivity or allergy is the result of exaggerated and inappropriate immune reactions that are damaging and sometimes fatal to the host. Individuals undergoing these reactions are termed to be “hyper” sensitive to a particular antigen because their immune system is reacting in a damaging rather than a protective fashion (Kindt et al., 2007).

Reactions involving the immune system can broadly be divided into those that are IgE-mediated and non-IgE mediated. These two mechanisms can also cause mixed disorders by working together to exacerbate diseases like atopic dermatitis and eosinophilic gastrointestinal disease (Davis, 2009). Classically, IgE-mediated disorders occur when food-specific IgE antibodies on the surface of mast cells and basophils bind to circulating ingested food allergens, and activate the cells to release cytokines and other potent mediators, such as histamine. Symptoms typically manifest within one hour of exposure, and most commonly occur in the skin (urticarial, pruritus, flushing), gastrointestinal tract (vomiting, diarrhea, abdominal cramps), and respiratory tract (cough, wheezing). Cardiovascular symptoms such as hypotension are less common but

when coupled with other respiratory and other system complications, an acute life-threatening reaction known as anaphylactic shock can occur (Jarvinen-Seppo & Nowak-Wegrzyn, 2011). In contrast to IgE-mediated reactions, non-IgE-mediated reactions are caused by immunological mechanisms not involving IgE and are predominantly mediated by T-cells activation. Non-IgE-mediated reactions typically have a slower onset, developing over hours to days after food allergen exposure, which makes clinical diagnosis more difficult. The resulting symptoms are often, but not always, localized to the gastrointestinal system. Examples of non-IgE-mediated conditions include food protein enterocolitis syndrome, eosinophilic proctitis, dermatitis herpetiformis, celiac disease, and contact dermatitis (Davis, 2009).

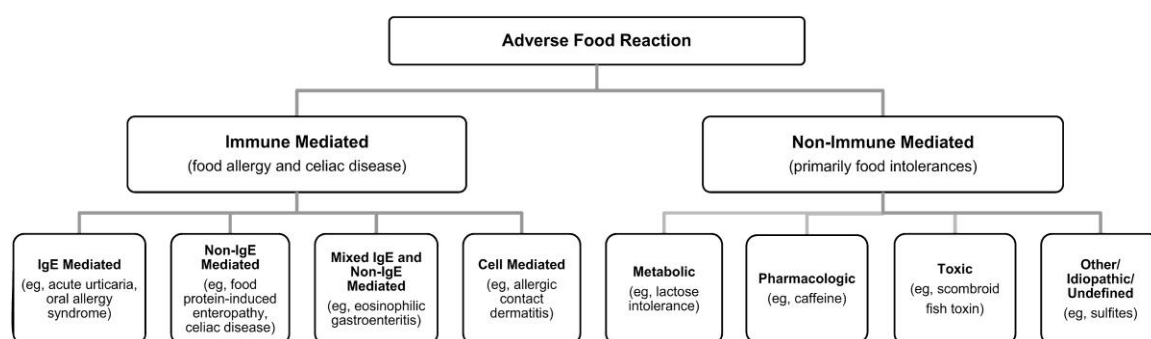


Figure 1.1 Types of adverse reactions to food (Boyce et al., 2010)

Mechanism of Food Allergy

Food allergy represents an abnormal response of the mucosal immune system to antigens delivered through the oral route (Sampson, 2004). The immune system is a complex interactive network with the capacity of protecting the host from a number of pathogens while keeping a state of tolerance to self and innocuous nonself-antigens.

Allergy is one of the immune tolerance-related diseases that arise as a direct consequence of a dysregulated immune response (Fujita et al., 2012). The resultant innate and adaptive immune responses to antigens lead to inflammatory reactions with a T-helper-2-type (Th2) cell and allergen-specific IgE predominance (Akdis, 2006).

The lumen of the gastrointestinal tract is exposed daily to an array of dietary proteins. The vast majority of proteins are tolerated through induction of T-cell anergy, deletion of reactive T cells, the generation of suppressor T cells, the formation of protective secretory IgA antibodies, and other immunological responses. This process is known as oral tolerance. However, when tolerance to a given dietary antigen is not established or breaks down, food allergy can transpire. Although food allergies may also involve other types of immunological mechanisms, the IgE-mediated mechanism is, by far, the most well-documented and understood (Burks et al., 2008; Koppleman & Hefle, 2006). The immune response in the small intestine which is responsible for the dominance of the IgE antibody generation is quite complex (Kindt et al., 2007). IgE-mediated food reactions are associated with rapid onset of symptoms and involve a two-phase process, a period of sensitization followed by the allergic reaction itself, commonly referred to the elicitation phase. The sensitization phase begins when the immune system is exposed to the immunogenic allergen and results in the induction of IgE antibody specific to that allergen. When the allergen enters the body, it is presented by an antigen-presenting cell (APC) such as dendritic cells to naïve Th0 cells via interaction of the MHC-peptide complex with the T-cell receptor, resulting in Th-cell priming and activation. Depending on key cytokines present during T-cell priming, naïve T-cells differentiate into four different (and possibly even more) “classical” effector cell subsets

comprised of Th2, Th1, Th17 cells, and induced regulatory T (Treg) cells. The presence of interleukin (IL)-4 promotes T-cell differentiation into allergen-specific Th2 cells that produce signature cytokines IL-4, IL-5, IL-9, and IL-13, but little or no interferon (IFN)- γ (Bohle, 2013). IL-5 causes an increase production of mast cells and basophils in the target organs of an atopic individual while IL-4 and IL-13 stimulate B cells to switch to IgE- and IgG4-antibody production (Pene et al., 1988; Punnonen et al., 1993). The secreted IgE antibodies then attach to high-affinity IgE receptors (Fc ϵ RI) on mast cells in the tissues or basophils in the blood (Jeurink and Savelkoul, 2006). The sensitization phase is symptomless and can occur without the development of clinical reactivity. Thus, the demonstration of IgE antibodies directed against a particular food in human blood serum is insufficient evidence for the diagnosis of a food allergy unless it is coupled to a strong history of food allergy or a positive double-blind, placebo-controlled food challenge. Once the sensitization phase occurs, the stage is set for the initiation of an allergic response. Subsequent exposure to the same food allergen can result in the cross-linking of the multivalent antigen to two IgE molecules bound to mast cells or basophils. A series of biochemical events is then initiated which causes cell membrane disruption and the release of a variety of mediators contained within granules existing in the mast cells and basophils. While several dozen substances have been identified as chemical mediators emanating from these cells, histamine is responsible for most of the immediate effects of an allergic reaction. The histamine-related effects include inflammation, pruritis, and contraction of the smooth muscles in the bloods vessels, gastrointestinal tract, and respiratory tract. Other important mediators include a variety of prostaglandins

and leukotrienes which are associated with some of the slower-developing responses observed in some food allergy cases (Koppleman & Hefle 2006).

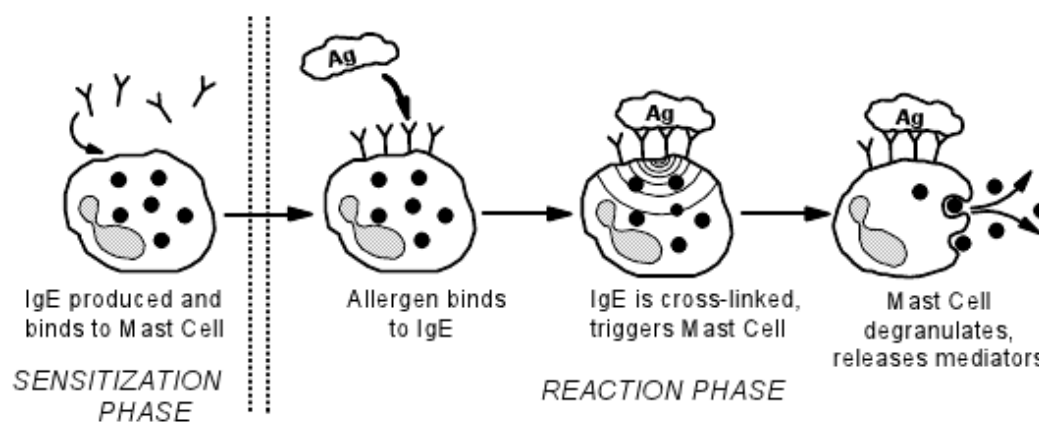


Figure 1.2 Mechanism of IgE-mediated food allergy (Gutman, 2011)

Food Allergens

Mechanisms leading to allergic responses have been well described; however, what makes a protein within a food an allergen is unknown. The contribution and interaction of genetics, environment, and protein molecular structure associated with allergic sensitization are also not well understood. Any food protein is potentially allergenic if its presentation in the appropriate context of the major histocompatibility complex (MHC) class II pathway induces an immunoglobulin switch to antigen-specific IgE and establishes sustaining memory B cells (Aalberse 2006; Aalberse & Stadler 2006; Esch 2006). In simplistic terms, a food allergen is a protein contained within a food that first elicits an IgE antibody response and on subsequent exposures elicits a clinical response. These can take the characteristics of “complete, class 1, or true food” allergens

that sensitize and elicit a clinical response or “incomplete, class 2, or nonsensitizing” food allergens that can only elicit but fail to sensitize (Jensen-Jarolim & Untersmayr 2006). Because such allergic responses require complex interactions between the protein and the immune system, they are notoriously difficult to predict. Nevertheless, it is clear that some proteins are intrinsically more allergenic than others (Huby, 2000).

As with other toxicological hazards, food allergens may arise at any point in the food chain. However, they differ from most other chemical hazards as they pose a risk only to a limited and reasonably well-defined proportion of the population and are harmless to the vast majority at almost any level of intake (Krevel, 2009). The allergens in foods are almost always naturally occurring proteins. More than 160 foods have been described as causing food allergies, but allergy experts only consider a limited number of those to be of public health concern (Taylor and Hefle, 2001). Eight foods or food groups are thought to account for more than 90% of all IgE-mediated food allergies on a worldwide basis and have come to be known as the “Big Eight” (FAO, 1995). These foods or food groups are milk, eggs, fish (all species of finfish), crustacean (shrimp, crab, lobster, crayfish), peanuts, soybeans, tree nuts (almonds, walnuts, pecans, cashews, Brazil nuts, pistachios, hazelnuts, pine nuts, macadamia nuts, chestnuts, and hickory nuts), and wheat (Taylor and Hefle, 2001).

Although there is no single set of features that can discriminate between an allergenic and a non-allergenic protein, allergenic proteins tend to share certain molecular characteristics (Krevel, 2009). Common features of major food allergens are that they are typically water-soluble glycoproteins and are relatively stable to heat, acid, and proteases (Sicherer and Sampson, 2009). Food allergens, unlike inhaled or contact allergens, must

pass through the harsh environment of the digestive system, beginning immediately upon entry into the oropharynx. Following ingestion, dietary proteins undergo digestion by enzymes in the saliva and stomach as well as by gastric acid (Faria and Weiner, 2005). Stability to the proteolytic and acidic conditions of the digestive tract is considered as one of the more important characteristics of food allergens, which contributes to an increased probability of reaching the intestinal mucosa, where absorption and interaction with the immune system can occur (Masilamani et al., 2012; Moreno, 2007).

It has been established that IgE-mediated activation of effector cells requires cross-linking, and therefore, the multivalent (multiple epitopes) allergens must possess a complex structure to support the interaction with these cells. An allergen molecule can carry one or more (up to roughly 25) epitopes, complexes of amino acids constituting potential binding site(s) for allergen-specific IgE antibodies (Jeurink and Savelkoul, 2006). The IgE-binding epitopes responsible for a protein's allergenicity can either be linear or conformational. A linear epitope (also known as continuous or sequential epitope) involves a protein segment of consecutive amino acids whereas a conformational or discontinuous epitope comprises amino acids that are close in space in the folded molecule, despite being noncontiguous in the amino acid sequence. Conformational epitopes are dependent on the 3-dimensional structure of the protein and are usually displayed on the surface area of the molecule (Pomes, 2010).

Over the past 20 years, there has been an explosion in the number of allergens that have been identified and characterized. As a result, efforts to classify allergens are in progress to identify common properties and motifs that may be predictive of allergenicity. The World Health Organization (WHO) and the International Union of

Immunological Societies (IUIS) produce an official list of allergens, which is designated by the Allergen Nomenclature Sub-Committee (Chapman et al., 2007). Allergens included in this listing must induce IgE-mediated (atopic) allergy in humans with a prevalence of IgE reactivity about 5% among individuals allergic to that substance. An allergen is termed major if it is recognized by IgE from at least 50% of a cohort of allergen individuals but does not carry any connotation of allergenic potency; allergens are otherwise termed minor. The allergen designation is then based on the Latin name of the species from which it originates and is composed of the first three letters of the genus, followed by the first letter of the species finishing with an Arabic number. The numbers are determined by the order in which allergens are identified and are common to all homologous allergens (also known as isoallergens) in a given species. Isoallergens are defined on the basis of having a similar molecular mass, an identical biological function, if known, for example enzymatic action, and > 67% identity of amino acid sequences. For those species where the first three letters of a genus and the first letter of a species are identical, the second letter of the species is also used (Mills et al., 2009b).

Protein classifications have been proposed for almost as long as proteins have been studied, with the criteria used reflecting the level of knowledge that was available at the time. The first systematic attempt to impose a classification on a wide range of plant proteins was that of TB Osborne who developed a system based on the sequential extraction of proteins in water, dilute saline, alcohol-water mixtures and dilute acids or alkalis. Our increasing knowledge of protein structure and properties has allowed more systematic and scientifically valid classifications to be made, culminating in the recent availability of extensive amino-acid sequences coupled with the more limited availability

of 3D structures. The identification of conserved sequence motifs and 3D structures has allowed proteins to be classified into families and superfamilies (Shewry et al., 2006). Based on their functional and structural properties or sequence identities of 30% or greater, 65% of all plant food allergens belong to just four protein families, the prolamin, cupin, Bet v 1-like (major birch pollen allergen), and profilin families whilst animal food allergens can be classified into three main families, the tropomyosins, EF-hand proteins, and caseins (Radauer and Breiteneder, 2007; Mills, 2011).

The prolamin superfamily comprises the largest number of allergenic plant food proteins (Breiteneder & Radauer, 2004; Breiteneder, 2006). Prolamins are proline- and glutamine-rich α -helical proteins with a conserved skeleton of 8 cysteine residues that serve several biologic functions. They comprise 3 major groups of plant food allergens: the seed storage 2S albumins found in tree nuts and seeds, the defense-related nonspecific lipid transfer proteins found in soft fruits and vegetables, and cereal α -amylase/trypsin inhibitors (Breiteneder & Radauer, 2004; Kreis et al., 1985). The second major superfamily of plant food allergens, the cupins, is widely distributed among all kingdoms and shares a conserved β -barrel fold (Dunwell et al., 2000). The cupin family contains 2 groups of seed storage proteins called vicilins and legumins, which are important peanut and tree nut allergens, such as Ara h 1 from peanut and Jug r 2 from walnut. The profilin and Bet v 1 family includes tree pollinosis-associated food allergens with low stability that induce symptoms of the oral allergy syndrome. As stated previously, these 4 protein families contain approximately 65% of all plant food allergens. Of the remaining 27 allergen-containing protein families, more than 50% harbor allergenic proteins of the

plant defense system or pathogenesis-related proteins, such as the cysteine proteinases, thaumatin-like proteins, or chitinases (Breiteneder & Radauer, 2004).

The most important animal food allergens are present in milk, egg, and seafood. Mammalian milk allergens are present predominantly in 3 protein families: α -Lactalbumin is essential for milk production and is a member of glycosyl hydrolase family 22, β -Lactoglobulin is a lipocalin, and the casein family harbors the major protein constituents of milk. Ovomucoid, the most important egg allergen, is a Kazal-type serine protease. In seafood, there are 2 major groups of allergenic proteins. The tropomyosins of crustacea and mollusks play a key regulatory role in muscle contraction, and the calcium-binding parvalbumins present in fish and amphibians are important for the relaxation of muscle fibers (Chapman et al., 2007). The repertoire of allergenic proteins identified is small compared to the vast array of different proteins found in nature. The explanations for this are lacking but may in part result from conservation of surface structures in certain families, such as the Bet v 1 and parvalbumin superfamilies, which promotes IgE cross-reactivity (Jenkins *et al.*, 2005, 2007). By recognizing a significant homology across many plant and animal taxa that are sources of food, some protein families are significantly overrepresented (i.e. prolamins) and some species of plant or animal are also overrepresented as strongly polarizing allergen sources (i.e. egg, crustaceans, peanut) in many cultures (Masilamani et al., 2012).

As extensive research to identify the characteristics of non-toxic food proteins that evoke IgE-mediated allergic response in predisposed individuals continues to be carried out, a number of allergen databases have been set up to collect and curate the existing data on allergens, their physicochemical properties and their allergenic

relevance. Some of these are curated collections of allergen sequences, such as AllergenOnline (<http://www.allergenonline.org/>) and the IUIS allergen database (<http://www.allergen.org/>). The IUIS database is dedicated to providing a systematic nomenclature for allergens that allows unequivocal identification of allergens, and upon evidence of IgE binding activity of the allergenic molecules being provided, the allergen nomenclature subcommittee grants official allergen designations. Other allergen databases are linked to data on clinical reactivity of foods, such as InformAll (<http://foodallergens.ifr.ac.uk/>), whereas the Allergome database represents a rapidly updated non-peer-reviewed repository of information (<http://www.allergome.org/>). Another type of database is represented by Allfam, which groups allergens according to their protein family characteristics (<http://www.meduniwien.ac.at/allergens/allfam/>) (Hoffmann-Sommergruber et al., 2009). Our increasing understanding of the role that protein structure and properties play in predisposing certain types of proteins to becoming allergens closes the gap on our ability to predict the allergenicity of foods (Mills et al., 2007).

Impact of Processing on Food Allergens

With the growth in research and technology, a large variety of food processing methods are currently employed by the food industry, such as heating, chilling, high pressure treatment, ultra filtration, irradiation, hydrolysis, and fermentation. These processing conditions can alter immunodominant epitopes and affect protein allergenic properties (Hengel, 2007). The types of modification that the food proteins may undergo during processing include protein unfolding and aggregation, in addition to chemical

modifications (Mills et al., 2009c). For example, heat treatment can induce the loss of the tertiary protein structure and induce aggregation of allergens affecting the conformational structure. Proteolytic or hydrolytic treatments also affect the conformational structure and the linear amino acid sequence, which may destroy sequential IgE-binding epitopes. The physicochemical changes from food processing can alter the way in which food proteins are broken down during digestion and may modify the form they are taken up across the gut mucosal barrier and presented to the immune system. Along with the possibility of extensive modification during food processing, proteins are often presented within complex structures inherent to foods, which can have an impact on their ability to sensitize or elicit allergic reactions (Besler et al., 2001). Certain food matrices, such as those rich in fat, may affect the kinetics of allergen release, potentiating the severity of allergic reactions (Grimshaw et al., 2003).

Because food processing involves thermal as well as non-thermal treatments, each type of treatment may differ in its effect on epitopes. Processing has the ability to alter food in a manner that may permit masking or unmasking of allergenic epitopes which can in turn, reduce or enhance allergen recognition (Sathe et al., 2005b). The ability of patients to consume the raw form of a food but react to the processed form has been described, but rather rarely. This could be attributed to the formation of new allergenic epitopes called neoallergens as a result of change in protein conformation after processing. For example, Malanin et al. (1995) described a girl who experienced an anaphylactic reaction after ingestion of cookies containing pecan nuts, but tolerated the ingestion of raw pecans. An exclusive reactivity to a 15 kDa neoallergen from heated pecans was demonstrated in the patient.

The changes in protein structure can also affect the detection of allergenic ingredients in food products. The first factor that can be affected is the extraction efficiency. It has been reported that roasting of peanut decreases the protein extraction efficiency which impaired detection of peanut in food products (Poms et al., 2004). Secondly, because food processing can alter the integrity of both protein and DNA, the sensitivity of detection methods can subsequently be affected. Although food processing can either decrease or increase the allergenic capacity, a decrease in the sensitivity of detection methods generally occurs and quantification of the allergen content of food products is consequently hampered by food processing. Therefore, it is important to assess the reactivity of detection methods towards processed allergenic foods in order to validate the performance of such methods in relation to allergen-containing processed food products (Hangel, 2007). The complexity of food processing makes managing allergens in foods difficult but confirms the importance of understanding its impact at the molecular level if risk assessors are to move towards knowledge-based ways of managing allergen risks (Mills et al., 2009c).

TREE NUT ALLERGY

Nuts have constituted a part of mankind's diet since pre-agricultural times and tree nut consumption has recently been recognized to be a healthy dietary habit (Eaton & Konner, 1985; Crespo et al., 2006). For example, the frequency of nut intake has been associated with reduced risk of some chronic diseases, such as coronary heart diseases (Hu & Stampfer, 1999; Sabate et al., 2001), diabetes (Jiang et al., 2002) and cancers of the prostate (Mills et al., 1989; Jain et al., 1999) and colorectum (Jenab et al., 2004; Yeh

et al., 2006). The U.S. Food and Drug Administration (FDA) acknowledged the protective link between nuts against heart disease and boosted the positive image of nuts by approving the first qualified health claim for a food for immediate use on a package stating that “Scientific evidence suggests but does not prove that eating 1.5 ounces per day of most nuts, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease” (FDA, 2003). With research showing that certain nuts can benefit heart health, incorporation of nuts can add value to a product and is capable of contributing to a sustained increase in nut consumption.

Despite growing awareness and consumption, tree nuts have increasingly been found to cause adverse effects and are among the highest producers of IgE-mediated allergic reactions following food ingestion (Cochard & Eigenmann, 2011; Crespo et al., 2006). An estimated 6% of children and 3.7% of adults in the U.S. have food allergies, of which tree nut allergies affect 1.1% of the children and 0.5% of the adults (Sicherer & Sampson, 2006; Sicherer et al., 2010). Although the prevalence of tree nut allergy appears to remain steady among adults, the prevalence of childhood tree nut allergy has increased significantly (0.2% in 1997, 0.5% in 2002, and 1.1% in 2008) (Sicherer et al., 2010). In addition, tree nuts are among the most persistent food allergies with only approximately 9% of cases having the potential to outgrow or develop tolerance, making tree nut allergy a lifelong problem for the majority of sufferers (Byrne et al., 2010). Tree nut allergies are also typically associated with severe or fatal reactions and therefore, are of serious concern. For example, in a study by Bock et al. (2001), peanuts (63%) and tree nuts (31%) were responsible for more than 90% of the 32 reported fatalities due to food-induced anaphylaxis during 1994-1999.

The majority of tree nut allergies are associated with one or more of nine widely consumed tree nuts. These include almond, Brazil nut, cashew nut, hazelnut, macadamia, pecan, pine nut, pistachio, and walnut. The prevalence of each individual tree nut allergy is quite variable, but walnut has been shown to be the leading cause of food allergy in the U.S. among the nuts with cashew, almonds, and pecan following behind (Sicherer et. al., 2001; 2003; 2010). Allergic reactions to other nuts such as Brazil nuts, hazelnuts, pistachios, macadamia nuts, and pine nuts are less common triggers of food allergic reactions and have been reported anecdotally (Crespo et al., 2006). According to the Economic Research Service (ERS), a primary source of economic information and research in the U.S. Department of Agriculture (USDA), the top three nuts eaten in the United States are almonds, pecans and walnuts (ERS/USDA, 2012). Interestingly, the highest consumed tree nuts correspond to the individual tree nuts most prevalent in causing allergic reactions. It has been postulated that consumption rate is related to food allergy prevalence. For example, peanut allergy rates are higher in countries like the United States and United Kingdom, where rates of consumption are high. Shellfish, fish, and sesame allergy are much higher in areas where these foods are staples of the diet (Keet & Wood, 2007).

With today's busy lifestyle, tree nuts are convenient, tasty, nutritious, and an easy snack that are typically consumed as whole nuts (either raw, roasted, and/or salted) or used as ingredients in a variety of processed foods, especially in spreads, bakery, and confectionary products (Alasalvar & Shahidi, 2009). However, an increased use of tree nuts in processed foods generates an increase in opportunity for accidental contamination to other foodstuffs not labeled to reflect tree nut content. According to FDA Enforcement

Reports, 316 food recalls were documented in the first quarter of 2013, and analysis of FDA recall announcements by ExpertRECALL revealed that undeclared allergens or other allergen concerns remained the primary cause of recall, accounting for nearly 34% of initiated food recall (ExpertRECALL, 2013). According to the U.S. Food and Drug Administration's records of recalls for fiscal year 1999, undeclared tree nut ingredients accounted for 10% of the class I or II food product recalls (those situations in which an adverse health effect is possible) (Vierk et al., 2002).

PECAN NUT

Pecan is the only nut crop native to the North American continent that has a commercial importance. The United States produces more than 80% of the world's pecan (USDA, 2005). The pecan (*Carya illinoensis*) belongs to the family Juglandaceae, which also includes other tree nuts such as walnuts, hickory nuts, and butternuts (Rosengarten, 1984). Over 1,000 different pecan varieties have been described, although 90% of cultivated acreage is represented by only a few dozen varieties. Venkatachalam et al. (2007) compared 24 commercially important pecan cultivars and reported that all had similar biochemical composition with small but significant differences noted in certain samples. On the basis of the assessment techniques, the tested cultivars were similar, but not identical, with respect to polypeptide composition and immunoreactivity. The soluble proteins of pecans are synthesized during the cotyledon stage, while the storage protein synthesis starts from the maturation stage until post abscission. The Osborne fractionation of pecan proteins has shown the seed proteins to

consist about 60% alkali glutelins, 32% globulins, 3% prolamins, and 2% albumins (Venkatachalam et al., 2008).

Pecan Nutrition and Consumption

Pecans have a rich, buttery flavor and can be eaten fresh or used in baked goods, confectioneries, and salads. According to the U.S. Department of Agriculture Nutrient Database, pecan kernels contain 72% lipids, 14% carbohydrates, 9% protein, 3.5% water, and 1.5% ash (USDA, 2009). Pecans have gained importance in recent years for their health benefits, especially their high antioxidant capacity and potential for reducing the risk of cardiovascular disease (Morgan and Clayshulte, 2000; Rajaram et al., 2001; USDA, 2009). The University of Georgia has recently been given a four-year, \$1.2 million grant from the U.S. Department of Agriculture to study the nutritional benefits of pecans (USDA, 2011). The goal is to provide consumers with more information on the nutrient-packed nut and ultimately increase the utilization and interest of pecans. This will additionally provide support for a flourishing global market.

Pecans are already the second most widely consumed tree nuts in the United States accounting for 22% of total tree nut consumption (USDA, 2000), but with increasingly more research on the positive health benefits, this number is only anticipated to increase. Per capita consumption averaged 0.48 pound annually since 2000, slightly greater than walnut consumption but behind that of almonds (USDA, 2003). In 2010, the United States exported 40,622 metric tons (MT) of unshelled pecans valued at \$143 million. The top buyer of U.S. in-shell pecans was Hong Kong. The United States also

exported 12,948 MT of shelled pecans valued at nearly \$109 million with Canada and the European Union as the biggest customers for shelled pecans outside the United States.

Pecan Allergy and Allergens

Pecans, while having economic and nutritional benefits, also underlie an important food safety issue. Pecans are included in the group of the 8 most common foods capable of inducing an allergic reaction, often referred to as the “Big 8” allergens. Nine percent of self-reporting tree nut-allergic patients listed pecan as their allergen (Sicherer et al., 2001). Pecan is considered as a type of nut allergy that can manifest itself quite early in life and persist through adulthood, resulting in lifelong afflictions (Fleischer et al., 2005; Roux et al., 2003).

Two allergenic proteins have been described to date in pecan and include a 2S albumin, Car i 1 (16 kDa), and an 11 S legumin, Car i 4 (55.4 kDa) (Sharma et al., 2011a, Sharma et al., 2011b). Pecan proteins were demonstrated to be thermally stable by Venkatachalam et al. (2006), and they also showed that pecan protein solubility and antigenic reactivity were not directly correlated; suggesting that loss in protein solubility during the extraction and testing phase alone may not be always reliably and predictably related to the loss in antigenicity. Most recently, the identification of 2S albumin as the most digestion resistant protein from pecan and its ability to retain IgE binding activity despite extensive digestion has been described (Spiric, 2011).

MANAGING ALLERGENS IN THE FOOD INDUSTRY

The rising awareness of food allergies has become a public health concern. Because there is currently no effective cure for food allergies and its potential life-

threatening consequences, individuals suffering from these conditions have to avoid consuming problematic foods, typically for the rest of their lives. Therefore, allergic consumers need to be provided with relevant information about allergens in manufactured foods to make an informed choice about what is safe to eat (Kerbach et al., 2009). The ability to keep food allergic individuals safe includes the assistance of friends and family, school authorities, and medical professionals, but the cooperation with the food industry and enforcement authorities is especially vital. With the average consumer already balancing a number of considerations when deciding what to eat, such as the cost, taste, and nutrition, food allergic consumers have the additional life-saving need to avoid allergens (Connors et al., 2001; Barnett et al., 2011a, 2011b). Because accurate and unambiguous labeling of food products is vital, the food industry and regulatory agencies have been put under greater pressure to ensure the food they provide is safe (Cevdek, 2010).

As with other hazards, an increase in regulation has occurred in an attempt to better manage food allergens. The Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA) passed by the U.S. Congress, effective January 1, 2006, amended the Federal Food, Drug, and Cosmetic Act (FFDCA) and requires manufacturers to clearly identify in plain English on their food labels if a food product has any ingredients that contains protein derived from any of the eight major allergenic foods and food groups. If allergenic foods are present, food labels must have either the word “contains” followed by the name of the originating food source, or the common name of the allergen followed in parentheses by the name of the food source, in the list of ingredients (FDA, 2004). The intended goal of FALCPA was to improve food labeling information for the millions

of consumers who suffer from food allergies. Changes in food labeling legislation have led to significant improvement; however, unintended allergenic constituents can be present in foods as a result of manufacturing and other operations. The passing of the Food Safety Modernization Act, which focuses more on preventing food safety problems including food allergens rather than relying primarily on reacting to problems, emphasizes the importance of validation and verification of allergen control approaches (FDA, 2011).

Allergen management has evolved with the growing knowledge and understanding of the issue, but application of allergen management principles is still inconsistent. Individual manufacturers are currently interpreting risk in the supply chain differently, as there are no agreed approaches to perform risk assessment to a common standard (Ward et al., 2010). Initially, little was known about the key determinants of risk; namely how sensitivity and reactivity to allergens varied across the susceptible population and in response to the dose consumed. Industry's approach to date has been based around existing Good Manufacturing Practices (GMPs) assuring segregation of allergenic ingredients and systematic declaration of allergens on labels where mandated. In the absence of threshold levels and insufficient knowledge about the levels of allergens required to provoke adverse reactions, many manufacturers have seemingly adopted a "fail-safe" approach using conservative precautionary labeling practices that relay potential risk of allergen residue that may be present in the food product despite their best efforts to remove allergens on shared equipment. This has consequently led to the proliferation of precautionary labeling and a reduction in the effectiveness of this measure in limiting risk as allergic individuals take risks in the face of reduced food

choices. If precautionary labels are ignored, risk to consumers actually increase (Sampson et al., 2006).

Current allergen management focuses largely on the hazard. This has driven conservative industry standards around control of unintentional allergen cross-contact during food manufacture, where allergen management and cleaning approaches can sometimes “chase molecules around the supply chain” (Ward et al., 2010). Complete elimination of allergens from food plants or dedicating lines to specific allergens is not practical. Therefore, the proposition of moving from a hazard based approach for allergen management to one based on risk seems ideal. With the risk-based approach, the possibility that all industrially manufactured food could eventually carry a precautionary label transpires, unless agreement can be reached on a consistent approach to decision making for the use of precautionary warning statements, such as quantitative management action levels. Risk management does not seek to eliminate the risk, which is generally regarded as impossible unless there is no exposure, but to reduce the probability of harm to a level considered tolerable. What is tolerable generally reflects the balance of different stakeholder interests (Ward et al., 2010). With the effort towards a risk-based approach in allergen management, reliable quantification methods for allergenic residues in processed foods become crucial.

Detection Methods for Allergens in Foods

The manufacturing of foods is a multifaceted process, and with each step involved, creates various opportunities for the presence of undeclared allergens to inadvertently appear in a product. Some of the reasons for the occurrence of hidden

allergens in processed foods include cross-contact through shared equipment, carry-over from rework material, unknown ingredients in raw material, or contamination from maintenance or cleaning tools (Besler et al., 2002). Reliable food allergen detection methods are vital for many food manufacturers to avoid expensive food product recalls, as well as for food protection agencies all over the world to assure the safety of food to consumers. In terms of allergen quantification, the scientific community has somewhat reached a dead end until agreed thresholds are established. Nevertheless, some attempts have been taken to indicate how much is too much by means of risk assessment methodologies (Albillos, 2012).

Until harmonization is achieved in regards to the ultimate system for allergen management, compliance with current regulations and the prevention of unintentional allergens into food products must remain the principal goal to support the safety of food-allergic consumers. Thus, analytical methods become an essential tool for validation of sanitation practices by food processors, compliance verification against established ingredient declaration requirements, confirmation of Good Manufacturing Practices (GMP), and enforcement by food regulators. These analytical systems should be cost-effective, specific, and sensitive enough to reliably detect traces of food allergens in a diverse range of food matrices (Kerbach et al., 2009). However, establishing robust analytical methods can be challenging due to the lack of universally recognized reference materials and scientifically sound threshold levels. Threshold levels for specific allergic reactions determined until now by double-blind placebo-controlled food challenges (DBPCFC) range between less than 1 mg and more than 1 g of allergenic protein depending on the food concerned and the selected individuals (Taylor & Hourihane,

2008). There seems to be a general assumption that the detection limits for different food products need to be around 10 ppm [mg allergen (protein)/kg food] or lower, depending on the particular food (Koppelman *et al.*, 1996, Poms and Anklam, 2004).

Currently, several analytical approaches have been developed for the detection and quantification of allergen traces in food products. The methods employed typically focus on targeting the allergenic protein(s) for the source of concern or a marker that indicates the presence of the allergenic commodity (Cucu *et al.*, 2013; Poms *et al.*, 2006). The various allergen detection techniques include immunochemical methods such as enzyme-linked immunosorbent assays (ELISAs) and lateral flow devices (dipsticks), DNA-based methods such as polymerase chain reaction (PCR), mass spectrometry, and ATP tests. Immunochemical methods depend on antibodies to reliably detect the allergenic protein. They can give qualitative or quantitative results and are fairly rapid and sensitive. ELISAs, especially lateral flow devices, can be used in the food processing facility for rapid assessment of the removal of allergenic residue from equipment surfaces. The PCR method detects DNA sequences indicative of the allergenic foods. This technique relies on heat stable DNA polymerase to amplify the DNA fragment and PCR's high specificity allows it to be a good method for verifying ELISA or immunochemical assay results. The need for a separated clean room for PCR analysis and the expense of needed equipment are limitations for using PCR in the food processing facility. Mass spectrometry, which detects proteins and peptides, is also an excellent confirmatory method due to its high sensitivity, but the high cost of equipment and laborious, time-consuming process renders it not useful for routine analyses. Nonspecific methods, such as ATP and total protein tests, are useful to check routine cleaning and sanitation effectiveness, but lack the

specificity necessary for allergen validation and verification. The choice of allergen detection method depends on various factors such as the ultimate purpose, type of sample, food matrix, processing effect, turn-around time, availability of equipment, and cost (Jackson, 2010).

Enzyme-linked Immunosorbent Assays (ELISAs)

Although technical approaches designed to detect the presence of food allergens have been available for a number of years, ELISA based methods remain the most commonly used by the food industry and official food control agencies (Abbot et al., 2012; Hengel, 2007). Because proteins are the causative agent in food allergy, ELISAs have an advantage over other methods by detecting the actual allergen protein molecule instead of a surrogate marker such as DNA or ATP. The ELISA is an immunochemistry format that is based on specific binding between an antigen and an antibody, commonly allergen specific IgG antibody raised in animals such as rabbits, sheep and goats. An epitope, also called an immunodeterminant region, on the antigen surface is recognized by the antibody's binding site. The type of antibody and its affinity and avidity for the antigen determines the assay's sensitivity and specificity (Koivunen & Krogsrud, 2006). In the ELISA method, an enzyme is covalently linked to a specific antibody and when the antibody recognizes a target antigen, the complex will bind to it. The enzyme component of the antibody-enzyme complex catalyzes a reaction with the addition of a suitable substrate that ultimately produces a colored product. Thus, the presence of the colored product indicates the presence of the antigen and the extent of reaction permits the measurement of small quantities of antigen (Berg et al., 2002). The basic ELISA is

distinguished from other antibody-based assays because separation of specific and non-specific interactions occurs via serial binding to a solid surface, usually a polystyrene multi-well plate, and because the test allows for easy visualization of results without the additional concern of radioactive materials use. ELISAs are relatively quick and simple to carry out and can handle a large numbers of samples in parallel.

All ELISA protocols include five common steps beginning with the coating of antibody or antigen on a solid phase followed by the addition of a blocking buffer containing a nonspecific protein which occupies the remaining uncoated surface on the solid phase. The blocking step minimizes nonspecific reactions and also protects the adsorbed antigen or antibody from surface denaturation (Nielsen, 2010). Different immunoassay reagents are then incubated at a specified temperature and time followed by washing. Thorough incubation and washing steps are critical between the addition of reagents to ensure sufficient binding of antibody-antigen complexes and separation of bound and free substances (Nielsen, 2010). The last step involves a color reaction that can be visualized and measured rapidly using specially designed multichannel spectrophotometers. This allows data to be stored and analyzed statistically.

A key feature of the flexibility of ELISA is that more than one system or format can be used to measure the same analyte. The variety of formats allows for a certain amount of flexibility, which can be adjusted based on the antibodies available, the results required, or the complexity of the samples (Crowther, 2001). For example, immunoassay signals can be detected directly or indirectly. In the direct ELISA, antigens are immobilized and enzyme-conjugated primary antibodies are used to detect or quantify antigen concentration. It is more important for the antibodies in this ELISA format to be

more purified for the enzyme conjugation procedure. In the indirect ELISA, primary antibodies are not labeled, but detected instead with enzyme-conjugated secondary antibodies that recognize the primary antibodies.

Both direct and indirect ELISAs can be further configured into two types of formats, competitive and noncompetitive ELISAs (Nielsen, 2010). The competitive ELISA is the preferred format for the detection of relatively small proteins. In competitive formats, unlabeled analyte (usually antigen) in the test sample is measured by its ability to compete with labeled antigen in the immunoassay. The unlabeled antigen blocks the ability of the labeled antigen to bind because that binding site on the antibody is already occupied. Thus, in a competitive immunoassay, less signal measured in the assay means more of the unlabeled (test sample) antigen is present. The amount of antigen in the test sample is inversely related to the amount of colorimetric product produced as a result of the specific enzyme/substrate reaction measured in the competitive format (Abbot Diagnostics, 2008). Competitive ELISA methods have been described for some food allergens (Mariager et al.1994, Yeung and Collins 1996, Holzhauser and Vieths 1999a, Koppelman et al.1999, Roux et al. 2001) with sensitivities down to 0.4 mg/kg.

Noncompetitive ELISAs involve the detection and quantification of primary antibody-antigen complexes immobilized on the solid-phase by the amount of enzyme linked to the detection antigen or antibody molecules to produce a colored product in the assay solution. In contrast to competitive ELISAs, the color intensity that results at the end of the assay is positively related to the amount of the target molecules. One of the most popular variations for a noncompetitive ELISA is the sandwich format. This is also

the most commonly used type of immunoassay for the detection of potential food allergens (Nielsen, 2010). Sandwich immunoassays involve immobilization of a capture antibody on a solid phase support, such as on the wells of microplates. The solution containing the target antigen is introduced and antibody-antigen binding occurs. A second antigen-specific, labeled antibody is added and it also binds to the analyte, forming a sandwich. Since both capture and detecting antibodies need to bind, the antigens must have at least two antigenic sites or epitopes. A suitable substrate is then added and reacts with the enzyme, producing a colored product that is directly proportional to the concentration of the analyte in the sample solution (Lipton et al., 2000). The direct conjugate binding with the antigenic targets on the captured antigen is accordingly called the direct sandwich ELISA. The indirect sandwich ELISA is quite similar to the direct sandwich ELISA, but differ in that the detecting antibodies are not labeled with enzyme and an additional antibody, an antispecies enzyme-conjugated one, is applied to bind to the unlabeled detecting antibodies. The bound conjugate is then processed as in direct sandwich ELISAs. The advantage of the indirect sandwich ELISA is that any number of different sources of antibodies can be added to the captured antigen, provided that the species in which it was produced is not the same as the capture antibody. More specifically, the enzyme conjugated antispecies antibody does not react with the antibodies used to capture the antigen (Crowther, 2001). Sandwich ELISA methods have been developed for several food allergens (Hefle et al., 1994, 2001, Tsuji et al., 1995; Holzhauser and Vieths, 1999b, Koppelman et al., 1999, Hlywka et al., 2000; Wei et al., 2003; Kaw et al., 2008; Lee et al., 2008; Panda et al., 2010; Gaskin & Taylor, 2011) and

numerous test kits have become commercially available in this format during the last decade.

Detection Methods for Pecan

Unlike other tree nuts, such as walnut and hazelnut, studies on pecan from a food allergy stance are limited. In spite of its increasing importance for the food industry, only a few analytical methods to detect pecan in food have been published. Venkatachalam et al. (2006) reported the development of an enzyme linked immunosorbent assay (ELISA) method capable of detecting pecan proteins in the range of 32–800 ng pecan protein/ml. The main function of this developed ELISA was to assess the stability of pecan antigens subjected to thermal processing treatments and to in vitro simulated intestinal fluid (SIF) and simulated gastric fluid (SGF) digestion conditions. More recently, Polenta et al. (2010) developed a competitive ELISA to detect traces of pecan that is more relevant for the food industry. The ELISA method developed in the research allowed the detection of pecan proteins in complex matrices, such as milk chocolate, at levels as low as 1 ng pecan protein/ml which is an improvement on the ELISA developed by Venkatachalam et al., (2006). This study also analyzed cross-reactivity, with walnut showing a level of interference lower than 10%. Although the study carried out by Polenta et al. (2010) assessed specificity and selectivity of the polyclonal antibodies produced for the ELISA method, the food commodities used in the challenge studies to test for cross-reactivity with the competitive ELISA were not reflective of ingredients or foods that pecan is commonly present in, such as baked goods and confections. In addition, the assessment of the method's performance in food matrices was not sufficient because the procedure

involved spiking milk chocolate with protein extracts instead of naturally incurred standards, which is the ultimate evaluation of an ELISA according to Taylor et al. (2009). Naturally incurred standards involve incorporation of the allergenic food residues into the food formulation which is then processed in a manner that mimics industrial food processing. This method evaluates the effects of processing on the allergenic food residues in a food matrix (Taylor et al., 2009). Although Polenta et al. (2012) examined the ELISA's ability to detect pecan traces after processing treatments in a later study, the study focused on direct treatment to the pecans and did not consider food matrices, which can have a considerable effect on allergen ELISAs. The quantitative extraction and recovery of allergenic food residues from food matrices is perhaps the most important concern because the food matrix may contain components that interfere with the ELISA by inhibiting antigen-antibody binding, reacting with epitopes, or having interfering enzymatic activity (Taylor et al., 2009).

Two DNA-based methods have also been published for pecan. Brezna and Kuchta (2008) developed a polymerase chain reaction (PCR) method for the detection of pecan DNA in food, with a practical detection limit of 0.01% (w/w). Hubalkova and Rencova (2011) recently developed a one-step PCR method for the simultaneous detection of the major allergens of pecan and Brazil nuts, but resulted in lower sensitivity [0.1% (w/w) or 1 g kg⁻¹] system. Although the PCR technique can improve detection limits, there are some concerns regarding the use of DNA-based methods as the offending molecules that cause allergic reactions are proteins, and processing can affect protein and DNA differently (Poms et al. 2004). The absence of DNA does not necessarily indicate absence of protein. In addition, immunological-based methods are advantageous due to better

suitability for on-line analysis of production lines, easier handling, and requirement of less sophisticated equipment (Jackson, 2010). Overall, the developed methods for the detection of pecan are certainly sensitive, but they all lack the validation necessary for use with processed foods which is an essential facet of the food industry.

Pecan Cross-reactivity

Cross-reactivity, defined as a positive response to a sample that does not contain any of the target antigens, can be a major problem for detection methods (Abbott et al., 2010). Cross-reactions arise because the cross-reacting antigen shares or has common epitopes which is structurally similar to ones on the immunizing antigen (Mayer, 2006). Using human specific IgE serologic techniques, Goetz et al. (2005) determine that walnut, pecan, and hazelnut represent a group of strong cross-reacting allergens. A similar result was reported in another study in which allergosorbent tests were used (Gillespie et al., 1976). Because both walnuts and pecan belong to the Juglandaceae family, cross reactive proteins are expected. Correspondingly, the cross reactivity between the two species of nut has been described. For instance, the interference of pecan was reported in the development of an ELISA method for the detection of walnut traces in food (Niemann et al., 2009). In vitro cross-reactivity between pecan and walnut is not unexpected (Teuber et al, 2000). Sharma et al. (2011a, 2011b) demonstrated in vitro cross-reactivity of pecan allergens, Car i 1 and Car i 4, with walnut major allergens, Jug r 1 and Jug r 4, respectively. In addition, high degree of amino acid identity between pecan and walnut epitopes were also evaluated by utilizing the BLAST program accessible at the Web site of the National Center for Biotechnology Information

(www.ncbi.nlm.nih.gov/BLAST/), which revealed an 88% sequence identity between Car i 1 and Jug r 1 along with a 95% sequence identity between Car i 4 and Jug r 4.

ELISA DEVELOPMENT

Immunoassay techniques provide complementary and alternate approaches in reducing the use of costly, sophisticated equipment and analysis time while still maintaining reliability and remarkable sensitivity. Immunoassay techniques in their most simple forms provide excellent screening tools to detect adulteration and contaminations qualitatively. The immunoassay provides an invaluable tool for the food industry to use in quality control, safety assurance and allergen monitoring (Bonwick & Smith, 2004). Although the tool can be utilized in various different applications, all ELISAs have the same goal, which is to be highly optimized for sensitivity, specificity, precision, and robustness. To achieve optimal performance with an ELISA, there are several critical components to consider in the development process, which will be described in the following section.

Antibody Production

The first step in the development of an immunoassay is the preparation of suitable antibodies (Bonwick & Smith, 2004). ELISAs, like other immunoassays, rely on the ability of antibodies to interact and bind antigen as a means of generating a measureable result. Therefore, the choice of antibodies is of prime importance and lays the foundation for a successful assay. Antibody reagents are developed from either polyclonal or monoclonal antibodies. Polyclonal IgG antiserum is generated in animals, most commonly sheep, rabbits, or goats. The animals produce the antiserum, just as a human

would, as a defense mechanism when exposed to an antigen. Antiserum usually contains a mixture of antibodies that recognize and bind to the same antigen, but they may attach to different epitopes. Monoclonal antibodies differ from polyclonal antibodies in that they are highly specific for a single epitope on a multivalent antigen. They are produced from a single cell line using hybridoma technology and mouse myeloma cell lines.

Hybridomas are antibody-producing tumor cells that produce many copies of the same antibody and grow easily in laboratory cell culture. An advantage of monoclonal antibodies is that the hybridoma cell line that produces them is potentially “immortal” and can produce the same antibodies consistently and indefinitely (Abbott Diagnostics, 2008). Monoclonal antibodies (MAb), because they recognize a single epitope, provide high specificity at the expense of sensitivity, as only one antibody molecule can bind to the antigen. Polyclonal antibodies (PAb) provide higher sensitivity due to the possibility of multiple antibodies binding to a single antigen molecule, but have a higher risk of cross-reactivity as the epitope is less precisely defined (Karaszkiwicz, 2008). The selection of a polyclonal versus a monoclonal antibody depends on the amount of antibody required, the application, and whether an antibody is needed that detects multiple proteins or epitopes of a single protein (polyclonal) or one that recognizes a single epitope (monoclonal). Regarding the food industry, polyclonal antibodies are commonly utilized to circumvent the previously mentioned encumbering effects of food processing on the detection of allergenic foods. Polyclonal antibodies are more tolerant of small changes in the nature of the antigen and provide a detection system that is less likely to fail completely to identify the presence of denatured or altered proteins, which

can occur in food processing, because of their ability to recognize multiple epitopes (Besler et al., 2002; Hefle et al., 2006; Lipman et al., 2005).

Because of their common use for the detection of allergenic foods, the production of polyclonal antibodies will be described further. The common approach for immunization of animals begins by an initial injection of the antigen of interest. The choice of antigen, also known as the immunogen due to its ability to stimulate an immune response, can be as general as a protein extract of the whole food, or as specific as a highly purified protein (Crevel, 2006). In particular, for the detection of allergenic residues, the production of successful antibodies can be attributed to the form of the immunogen. Various aspects should be considered such as the use of raw or heat-processed immunogen, the choice of variety, and the immunogen preparation process. Simple preparation steps such as the amount of processing and washing of the starting materials to remove agricultural contaminants is often overlooked, but are underestimated factors in increasing sensitivity and reducing cross-reactivity in polyclonal antibodies, respectively. Because the food industry is generally concerned whether any protein for the allergenic source is present as opposed to just one allergen protein, using a crude extract of allergenic food to make antibodies is appropriate (Hefle et al., 2006). There are no characterized, standardized food extracts for use in immunoassay development. Each laboratory uses its own food extracts for production of antibodies, for use as control samples, and for development of serial calibrators for quantitative assays. The raw material used to develop food extracts, as well as the extraction procedure, can lead to considerable lab-to-lab variability in extract composition (Williams et al., 2012). Therefore, each developed method must be appropriately validated for its specific

purpose. Subsequent boosting injections follow the initial injection of the antigen following a strict schedule. Seven to ten days after the first boosting injection, the first bleed is usually taken. The subsequent bleeds are then taken at regular intervals, usually monthly. The immunogen is commonly given as an emulsified solution with either complete or incomplete adjuvant. The immunogen for the first injection is prepared with complete adjuvant and the immunogen for the subsequent injections is prepared with incomplete adjuvant. Freund's complete adjuvant is an oil emulsion containing nonionic detergent with killed mycobacteria. The incomplete adjuvant is the same oil emulsion but without killed mycobacteria. Addition of killed mycobacteria in the first immunization aims to attract macrophages and other appropriate cells to the injection site. Injection of immunogen prepared in this manner will provide low-level stimulations of the animal immune system and minimize breakdown of the immunogen by metabolic enzymes, to ensure maximal exposure of the immune system to a foreign protein, thus generating a strong response. Animals are generally immunized either by subcutaneous, intraperitoneal, intradermal, or intramuscular routes or a combination of these. The choice of route can vary according to the volume being injected, the physical nature of the immunogen, the buffers, the species, and the stage of immunization (Harlow and Lane, 1988; Deshpande, 1996). Larger volumes are usually administered to animals via the subcutaneous route, but intraperitoneal injection is frequently used in rodents. Intramuscular or intradermal injections are used for slower immunogen release. Freund's adjuvant is most often injected subcutaneously and intramuscularly due to the proficiency in forming depot sites and slow immunogen release. Subcutaneous injection releases immunogen slower than other routes, and is the preferred route for booster injections due

to a decreased chance of anaphylaxis. For larger animals, subcutaneous and intramuscular routes are normally used (Hefle et al., 2006).

The dose of immunogen is also another factor to consider when immunizing animals for the production of antibodies. The desired result is an antibody with high avidity and affinity. Because large doses may induce tolerance, using the lowest amount of immunogen necessary to achieve antibody production is usually the best approach. The primary or initial injection of animals is usually the largest dose with a typical dose for rabbits being 100 µg of antigen and for larger animals, 500-1000 µg of antigen is common. Primary injections are followed by smaller booster injections in the range of 10-50% of the primary dose to promote clonal selection for high-affinity antibody. After the initial injection, high avidity but low affinity IgM antibodies are produced but soon switch to IgG antibodies with increasing affinity as immunization continues. The interval between booster injections should allow adequate time for the circulating antibody level to drop low enough to prevent prompt clearance of the injected immunogen. A three to four week interval is recommended between the primary injection and a booster injection and four to six week intervals is sufficient for subsequent booster injections (Deshpande, 1996; Hefle et al., 2006).

During the immunization period, blood is routinely collected from the animal and serum is isolated from the blood. This serum is usually rich in antibodies that recognize the injected antigen, and is called the antiserum. (Abbott Diagnostics, 2008). High quality antiserum is crucial in immunoassays. The immunized animals' antibody production should be consistently monitored. The quality of antibody is often reported as antibody titers, which measure the presence and amount of specific, functional antibody present in

the serum. Titers are carried out on serum samples using an indirect ELISA, in which the antigen is coated onto microtiter plates and the antiserum is diluted serially and added to plates. The mid-linear point of the resulting titration curve is then used to define the quantity of antibodies specific for the antigen. This is often referred to as the titer value. The serum samples utilized for evaluation are collected when IgG production rate peaks, which is about 7-14 days post booster injection. In the beginning of the immunization process, small test bleeds are taken until sufficient titer is reached and then larger volume samples, often referred to as production bleeds, are acquired thereafter (Hefle et al., 2006). Once a good titer and affinity have been developed, individual bleeds can then be pooled to form a larger quantity of homogenous antiserum. In different animals, it is not generally possible to reproduce antibodies with the exact titer and specificity for the same immunogen, as the immune response of individual animals differ even though they may be physiologically identical. However, once useful antibody is attained, up to millions of assays can be prepared from one animal. Assays can be prepared even after the animal is no longer available, as antisera can be stored frozen for many years without loss of activity (Lee & Kennedy, 2007).

Optimizing Assay Operating Condition of ELISA

Once acceptable antibodies have been developed, the next step includes optimizing the assay to meet performance requirements. This optimization is an imperative process whereby the assay is fine tuned to give the required sensitivity, specificity, speed, and other critical factors essential to the assay's application (Law, 1996). Although the ELISA is a powerful and well-characterized application, attempting

to develop and optimize a specific assay can be difficult. Because the method involves the assembly of a large immune complex with multiple components, failure to capture signal can potentially be caused by various factors. There are several crucial parameters to consider for the optimization process such as the initial immobilization of the biomolecule to the solid surface, the detection system, sample preparation, reagent concentration and various buffers used in the assay system along with incubation time and temperature conditions.

Once high quality antibodies are established, the optimization of the surface to which the antigens and antibodies are immobilized is critical. The surface is an integral component of any assay due to its effect on biomolecules as they attach or do not attach to the solid support matrix. Just like the quality of antibodies lays the foundation for a robust ELISA, the performance of an assay will only be successful if the antibodies and antigen are effectively immobilized. The attachment phenomenon is controlled by the chemical properties of the surface, and can be influenced by other factors such as pH and temperature. The utilization of polystyrene microplates, especially the 96-well plates, is common because of their versatility and high throughput. Correspondingly, new developments in automated plate washing and readers have supported the appeal and ease of microplates (Gibbs, 2001b). The attachment of proteins or peptides to a plate is by passive adsorption that is mediated primarily by hydrophobic interactions, but some electrostatic forces may also contribute (ThermoScientific, 2010). Once the solid phase format is decided, various elements such as the composition of the coating buffer, incubation time and temperature, and the concentration of reagent must be optimized for effective binding to the surface.

The next critical step in creating a robust immunoassay is the blocking of the plate. The blocking step stabilizes proteins bound to the surface and prevents non-specific binding (NSB) of other proteins or biomolecules to unoccupied spaces on the surface, which can be detrimental to the specificity and sensitivity of the assay results. Various blocking reagents can be used to saturate these unoccupied binding sites without taking an active part in specific assay reactions. The blocking method can depend on the type of surface, the type of biomolecule immobilized to the surface, and the type of detection system employed. Generally, there are two major classes of blocking reagent, proteins and detergents. Non-ionic detergents such as Tween 20 are considered temporary blockers because they do not provide a permanent barrier to biomolecule attachment to the surface and can be washed away. Instead, non-ionic detergents can be beneficial when included in the wash buffer to further decrease nonspecific binding. Unlike non-ionic detergents, proteins are permanent blockers and only need to be added once after the surface is coated with the capture molecule. Some of the most commonly used protein blockers include bovine serum albumin, non-fat dry milk or casein, and fish gelatin (Gibbs, 2001a).

ELISAs require the use of an appropriate enzyme label and a matching substrate that is suitable for the detection system being employed to produce a measurable signal. Typically an enzyme is attached to the secondary antibody which must be generated in a different species than primary antibodies (i.e. if the primary antibody is a rabbit IgG antibody then the secondary IgG antibody would be an anti-rabbit IgG antibody from goat, chicken, etc., but not rabbit). For colorimetric assays, horseradish peroxidase and alkaline phosphatase are common enzymes used as labels (Gibbs, 2001d). These enzymes

are typically used because they each meet most, if not all, of the criteria necessary to produce a sensitive, inexpensive, and easily performed assay. These criteria include stability at typical temperatures (4, 25, and 37°C), greater than six months shelf life when stored at 4°C, commercially available, capable of being conjugated to an antigen or antibody, inexpensive, easily measurable activity, high substrate turnover number, and unaffected by biological components of the assay (Raskhit, 2006). For all enzyme-linked immunoassays, the final stage is the addition of the enzyme substrate. The substrate is chosen for its quantitative yield of a colored reaction product. The rate of color development is proportional, over a certain range, to the amount of enzyme conjugate present. Choosing the best substrate for any type of assay depends on the sensitivity desire, the timing requirements, and the detection device to be used. The most commonly used substrates for peroxidase are TMB (dual function substrate), ABTS (2,2'-azino-di [3-ethylbenzthiazoline] sulfonate), and OPD (o-phenylenediamine), and the most widely used substrate for alkaline phosphatase is *p*-NPP (*p*-nitro-phenylphosphate). During enzyme-substrate reaction, temperature and light can cause an "edge effect," where the optical density in edge wells is higher or lower than center wells. Therefore, it is recommended that incubation be completed in the dark at room temperature to ensure the assay's outcome is as controlled as possible (Gibbs, 2001d). Development conditions and timing associated with the enzyme-substrate reaction are several requirements that need to be optimized to develop a precise, accurate and reproducible assay.

Once all reagents are decided, the ideal concentrations of each reagent must be established empirically. Utilizing microtiter plates, checkerboard titrations can be performed to assist in the assessment. The checkerboard titration involves the dilution of

two reagents against each other to examine the activities at all the resulting combinations (Luttmann et al., 2006). The optimum combination of reagents and their concentrations should generate a standard curve that displays the correct sensitivity, range, and linearity for a robust ELISA (Thermo Scientific, 2010). The operating range of an assay is the interval of analyte concentrations (amounts) over which the method provides suitable accuracy and precision (OIE, 2012). Time and temperature conditions for all reagent incubations can also be analyzed and optimized at this stage. It should be kept in mind that very few immunoassay variables can be changed independently. For example, increasing the incubation temperature to effectively speed up the assay can alter the specificity and change precision (Law, 1996).

With respect to processed foods, the manufacturing process and complex matrices can affect the successful preparation of samples prior to analysis. Next to the efficiency with which the antibody or antibodies are able to detect the antigen of interest, the efficiency with which these antigens are extracted from sample is the most important parameter that can affect the overall performance of an ELISA-based method (Abbott et al., 2010). Any analytical method will only detect what is extracted, and therefore, this important step can cause erroneous results if not optimized (Cucu et al., 2013). Food matrices are complex systems with various compounds such as fatty acids, phenolic compounds, surfactants, and endogenous enzymes, which can affect the sample extraction and lead to suboptimal assay performance. Dark chocolate is an example of a problematic matrix due to its high content of protein-binding tannins. To improve recovery, various additives, such as dry-powdered milk or gelatin, can be included in the extraction buffer (Williams et al., 2012). Before extraction can even be carried out, the

sample must be homogenous, and there must be a sufficient number of samples to be representative of the whole. Proper grinding and mixing procedures can ensure homogeneity and improved extraction of target analytes. Accurate sampling is critical in order to obtain meaningful results from any type of analytical assay and avoid possible uncertainty and error of measurement. When considering an appropriate sampling scheme, it is important to first consider the objective of the test (Lipton et al., 2000). Even the most sensitive methods can result in poor allergen detection if sample preparation is poor.

Assay Validation

Method validation is the process of demonstrating that the combined procedures of sample preparation and analysis will yield acceptably accurate, precise, and reproducible results for a given analyte in a specified matrix (Lipton, 2000). Validation is necessary to demonstrate the performance and reliability of a method and to determine the confidence that can be placed in the results it generates. The parameters that need to be assessed to fully characterize the performance of an analytical method are: accuracy, precision, sensitivity, specificity, and ruggedness (Law, 1996).

Accuracy is defined as the closeness of the results obtained by the procedure to the true value and can be demonstrated by measuring the recovery of analyte from spiked or incurred samples. The impact of sample matrix effects should be considered during this process. Results are commonly reported as the mean recovery at several levels across the quantitative range. Ideal percent recovery range from 80 to 120% (Abbot et al., 2012; Lipton et al., 2000; Mihaliak & Berberich, 1995).

Precision is the degree of dispersion among a series of measurements of the same sample tested under specified conditions (OIE, 2012). Precision can be evaluated in several ways by testing the same replicated sample, and the results are typically cited as the standard deviation or coefficient of variation (CV). Oftentimes, precision can be evaluated by determining variability between replicates assayed at various concentrations on the standard curve (intra-assay precision) and by how much variability occurs between multiple assays performed on different days (inter-assay precision) (Lathey, 2003; Lipton et al., 2000). The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the lower limit of quantification (LLOQ), where it should not exceed 20% of the CV (Huber, 2010). Interlaboratory tests can be carried out later in the validation study as part of a larger collaborative trial depending on the intent of the experiment (Thompson et al., 2002). This second phase of validation will obtain a more definitive assessment of precision in terms of repeatability and reproducibility for the assay (Judson et al., 2013).

The sensitivity of the assay is commonly expressed in terms of the detection limit of the system. For quantitative assays, there is not only an indicator of the minimum detectable analyte concentration, but a quantification limit is also applied to describe the sensitivity of the assay. In broad terms, the limit of detection (LOD) is the smallest amount or concentration of analyte in the test sample that can be reliably distinguished from zero while the limit of quantification (LOQ) is the lowest amount of an analyte that can be quantitated with suitable precision and accuracy (Huber, 2010; Thompson et al., 2002). When estimating the LOD, it is common to assume that it is the signal strength of a blank increased by three times the standard deviation of the blank (Abbott et al., 2010).

This method provides at best an estimate, and relies on normal Gaussian distribution of the blank measurements around zero. This can generally be assumed for methods such as ELISA, but the LOQ is best determined experimentally (CCMAS, 2010).

Specificity is the ability to unequivocally assess the analyte in the presence of components which may be expected to be present. The presence of interferents such as matrix components, non-specific binding of reactants to the solid phase, and biomolecules with cross-reactive epitopes can affect the extent that a method can accurately quantify the target analyte (Vessman, 1996). Such interferences may cause falsely reduced or elevated responses in the assay that negatively affect its analytical specificity (OIE, 2012). For example, a positive response to a sample that does not contain any of the target analyte can occur and cause a false positive result. Therefore, the possibility of cross-reactions should be evaluated with a wide selection of substances, especially those that are genetically similar or likely to be analyzed for the presence of the target analyte (Abbott et al., 2010). In addition, a non-specific response can occur from a substance or substances in the final extract other than the specific protein analyte and is referred to as a matrix effect (CCMAS, 2010). A test for general matrix effect can be made by applying the method of analyte additions (also called “standard additions”) to a test solution derived from a typical test material. The test should be completed using the same final dilution as the normal procedure produces, and the range of additions should encompass the same range as the procedure-defined calibration validation. If the calibration is linear, the slopes of the usual calibration function and the analyte additions plot can be compared for significant difference. A lack of significance confirms that there is no detectable general matrix effect. If the calibration is not linear, a more complex

method is needed for a significance test, but a visual comparison at equal concentrations will usually suffice (Thompson et al., 2002). The entire range of matrix types for which the method will be applied must be available for the method validation. Moreover, several examples of each type must be used to estimate normal range of recoveries for that matrix type. If it is likely that the history of the material will affect the recovery of the analyte such as the technical processing or cooking of foodstuffs, then examples at different stages of the processing must be obtained (Thompson et al., 1999).

SUMMARY

Adverse reactions to food is a growing issue, but true food allergies, the IgE-mediated adverse responses involving the immune system, are of most concern due to the debilitating and life-threatening effects that can result. Modern research and technology has increased our knowledge of food allergens and their resilient characteristics. Of the eight major allergenic foods, tree nut allergy has increased in prevalence, especially in children, and is attributed to more severe reactions. The pecan is an American nut that is showing great prospects in the global market due to the growing attention on the nut's nutritional health benefits.

Because the sensitivity of food allergy sufferers to specific food allergens varies widely between individuals with very small amounts of the allergenic component able to trigger an allergic reaction in some cases, it is essential to clearly communicate to consumers the safety of their food through accurate labeling (Scaravelli, 2008). With state-of-the-art technology, the processed food industry has experienced steady growth, which has also led to the concern of increased opportunity for unintentional incorporation

of allergens into processed, packaged foods. Therefore, reliable detection methods for allergenic residues in processed foods are essential for the food industry and regulatory agencies to determine and document compliance as well as support food safety investigations, respectively. Although detection methods have become more available within the last few years, limitations exist and care must be taken when choosing an approach for detecting the presence of allergenic food residues. Because manufactured foods are very complex and the processes involved can change the nature of food allergens, it is essential to choose methods that are well validated. The terms “valid” or “validity” refer to whether estimates of test performance characteristics are unbiased with respect to the true parameter values (OIE, 2012). In general, validation should check that the method performs adequately for the purpose throughout the range of analyte concentrations and test materials to which it is applied (Thompson et al., 2002). The ELISA method is currently the most commonly used and preferred method for the detection of food allergens because it is relatively simple, quick, sensitive and specific, but there is currently a lack of such an assay for the detection of pecan residues that is thoroughly suited for the food industry. Therefore, the objective of this study was to develop a robust and validated ELISA for detecting pecan residues in processed foods. It is expected that such a test will assist food companies comply with current FDA labeling guidelines and other legislation focused on preventing hazards, such as food allergens, from hindering the safety of food products. This in turn will help prevent unnecessary food allergic reactions to pecan in susceptible individuals and increase their food choice and quality of life.

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CHAPTER 2: EVALUATION OF PECAN PROTEIN EXTRACTION EFFICIENCY AND PRODUCTION OF POLYCLONAL ANTISERA AGAINST ROASTED PECANS

INTRODUCTION

The prevalence and awareness of food allergy is rising. Considering the array of food choices and frequent social gatherings centered around food, this condition impacts the sensitive individual and also schools, camps, sports teams, restaurants, airlines, and undoubtedly family and friends. Heightened awareness and an increase in media coverage have made parents and physicians more vigilant about allergies they would have otherwise ignored. The attention has correspondingly led to the implementation of safety regulation throughout the world. For example, the Food Allergen Labeling & Consumer Protection Act of 2004 (FALCPA) in the United States requires labeling of packaged foods containing any of the eight “major food allergens.” This includes milk, eggs, wheat, soybean, peanuts, tree nuts, fish and crustacean shellfish (FDA, 2004). Of the “Big 8” allergens, allergic reactions to tree nuts are among the leading causes of fatal and near-fatal reactions to foods (Jarvinen-Seppo & Nowak-Wegrzyn, 2011). Similar to peanut allergies, most individuals who are diagnosed with an allergy to tree nuts tend to have a lifelong allergy (Byrne et al., 2010). To further exacerbate the situation, tree nuts are ingredients in many unexpected foods due to their nutritional and functional properties (Rajamohamed & Boye, 2010).

The pecan is a particularly important tree nut in the United States as it is the only nut crop native to the North American continent that has a commercial importance. The United States produces more than 80 percent of the world’s pecan (USDA, 2005).

Although pecans have been enjoyed in the United States for many years, the nut is also gaining interest internationally by other countries such as India, Canada, and Dubai. Moreover, consumption of pecan is increasing popularity due to its link to multiple health benefits (Carter, 2012). Pecans have been praised for their high antioxidant capacity and LDL cholesterol lowering effects (Hudthagosol et al., 2010; Rajaram et al., 2001).

With a positive spotlight on the pecan nut and the potential for a growing global market, incorporation of pecan nut into more food products is expected. However, increased usage also parlays into unintentional exposure of pecan allergens to allergic consumers. Undeclared pecan allergens in food products represent a major health threat. It is currently advised that the best way to prevent allergic reactions is to abolish the offending food from one's diet. Hence, relevant analytical methodology are needed for detection of traces of allergens to provide accurate food labeling, since this is the only link for consumers to know the composition of prepackaged foods.

The food industry and food regulatory agencies currently rely on commercially available Enzyme – Linked Immunosorbent Assays (ELISAs) as an analytical method to monitor the safety of food products (Whitaker et al., 2005). The ELISA method is simple, quick, sensitive and specific, but there is currently no robust assay for the detection of pecan residue that has been thoroughly validated. Therefore, the development of such a method would allow the food industry and regulatory agencies to ascertain and document compliance as well support food safety investigations, respectively.

Immunochemical methods use antibodies as reagents to detect and quantify target antigens. These immunological reagents are therefore the backbone of every

immunoassay system. Antibodies are a large family of glycoproteins that share key structural and functional properties. Functionally, they can be characterized by their ability to bind both antigens and specialized cells or proteins of the immune system. Structurally, antibodies are often depicted as Y-shaped molecules, each containing four polypeptides with two identical polypeptide units called heavy chains and another two called light chains. The region of an antigen that interacts with an antibody is called an epitope or an immunodeterminant region. The binding of an antibody to the antigen is dependent on reversible, noncovalent interactions. The binding site of an antibody can accommodate from 6 to 10 amino acids. Small changes in the antigen structure (such as a single amino acid) can affect the strength of the antibody-antigen interaction (Koivunen & Krogsrud, 2006). The measure of the binding strength between a single epitope and a single combining site on the antibody is called antibody affinity. Avidity is another parameter used to characterize antibody-antigen binding and refers to the overall strength of binding between multivalent antigens and antibodies (Mayer, 2006). High affinity and avidity antibodies are required in immunoassays because they can bind more antigen in a shorter period of time and form more stable complexes than their low-affinity counterparts (Koivunen & Krogsrud, 2006).

The sandwich ELISA commonly utilizes polyclonal antisera containing IgG antibodies typically from two different animal species, to bind the antigen. Polyclonal antibodies are particularly useful to detect food allergens in processed foods. Due to their ability to recognize multiple epitopes, they are more tolerant to small changes in the nature of the antigen and provide a detection system that is less likely to fail completely to identify the presence of denatured or altered proteins, which can occur in food

processing (Besler et al., 2002; Hefle et al., 2006; Lipman et al., 2005). Therefore, it is important to ensure that the antibodies in an ELISA for specific use in the food industry are able to efficiently detect the target antigen at all steps during the manufacturing process.

Although the production of high quality antibodies is essential for the development of a successful ELISA, the efficiency at which the target protein is extracted is the most important parameter that can affect the overall performance of an ELISA-based method (Abbott et al., 2010). The results of allergen detection can be less meaningful, even with highly sensitive antibodies, if sampling and sample preparation are poor. Any analytical method will only detect what is extracted, and therefore, this important step can cause erroneous results if not optimized (Cucu et al., 2013). The target protein must be optimally solubilized in the extraction solvent, and that can be considerably influenced by the type of solvent, the pH and ionic strength of the extraction solvent along with the extraction time and temperature. In a study by Sathe and colleagues (2009), pecan proteins were found to be the least soluble seed proteins among those tested with aqueous extraction buffers. The difference in solubility may be due to the presence of nonprotein components such as the high phenolic content in pecans that may interact with the proteins and thereby alter protein solubility (Venkatachalam et al., 2008). Hence, the pecan nut requires additional attention to optimize the parameters for high extraction efficiency. For example, several studies have assessed the extraction efficiency of pecan proteins using various buffers, and they all invariably indicated that 0.1 M NaOH (pH 12.9) followed by buffered saline borate (BSB, pH 8.45) were the most effective solutions for extracting the highest concentration of pecan proteins (Sathe et al.,

2009; Spiric, 2011; Venkatachalam et al., 2008). Despite the high extraction efficiency, the molarity and high pH of these buffers are not compatible in immunoassays because they may affect the antibody-antigen interaction. Alternatively, saline extraction buffers of neutral pH provide suitable conditions for ELISA systems (Spiric, 2011). Studies have shown that the amount of total soluble pecan protein content increases with increasing concentrations of salt present in the extraction buffer (Spiric, 2011; Venkatachalam et al., 2008). Spiric (2011) noted that the ionic strength of the extraction solution has a greater impact on pecan protein extractability than pH. In this study, the solubility of high molecular weight proteins began to increase at 1 M NaCl concentration although pecan proteins started to precipitate out of solution at high levels of salt (>2 M NaCl). A solution of 0.01 M PBS, 1 M NaCl (pH 7.2) was able to extract acceptable pecan protein levels compared to high pH solvents. It is thus expected that this buffer would be compatible to detection of pecan allergens via an ELISA method.

The objective of this study was to produce specific polyclonal IgG antibodies against pecan proteins that could be used as potential reagents for the development of a robust sandwich type ELISA to detect pecan residues in processed foods.

MATERIALS AND METHODS

Pecan Immunogen Preparation

Multiple varieties of soft and hard shell pecans were obtained from various sources in Mississippi, Georgia, and Texas. Six hundred grams of pecans with intact shells (no cracks or separations) from each lot were washed in deionized distilled water and dried with multiple changes of paper towels to remove any adhering foreign proteins.

Washing and drying were carried out for a total of six times. The washed, in-shell pecans were then thoroughly air dried under a fume hood. The pecans were shelled by hand and any inner debris was removed. After shelling, the pecans were washed three times, dried with multiple changes of paper towels and thoroughly air dried in a fume hood. Roasting was accomplished by spreading pecans into a single layer on a baking sheet and roasting for 10 minutes at 270°F. Each lot was ground to a small particle size and bagged separately; 50 grams of each lot was pooled for a composite. The composite was frozen and ground three times by freezing at -70 °C followed by grinding while still frozen until a fine particle size was achieved (16-speed Oster blender, Niles, IL). The ground, roasted pecans were then defatted by washing 1:5 (w/v) with ethyl ether (five times), followed by a 1:5 (w/v) wash with acetone (two times). The defatted material was filtered after each washing step, and the retained powdered pecans were thoroughly air-dried. The final defatted ground protein immunogen was reserved for subsequent immunization of the animals, and the soluble protein content of the pecan immunogen was measured using the Lowry method (Lowry et al., 1951).

Polyclonal IgG Antibody Production

Polyclonal antibodies were developed at Covance Research Products (Denver, PA) using standard immunization protocols described by Harlow and Lane (1988). One sheep, one goat, and three New Zealand white rabbits were immunized with the defatted, ground roasted pecan immunogen. For initial immunization, each rabbit was injected subcutaneously at multiple sites with the defatted ground pecan containing a total of 250 µg pecan protein suspended in Freund's Complete Adjuvant (FCA) to form a slurry.

Subsequent booster injections were administered at 21-day intervals by using defatted ground pecan containing 125 µg pecean protein in either Freund's Incomplete Adjuvant (FIA) or TiterMax Classic through the subcutaneous route. A rotation of adjuvant was used for these subsequent boosts (two months with FIA then the following month TiterMax Classic). Test bleeds were collected at 10 and 24 days post-booster injection to monitor the antibody production. The goat and sheep were immunized using the same protocol as the rabbits except defatted ground pecan containing a total of 1,000 µg pecan protein was used for the initial immunization with FCA and the booster immunizations included defatted ground pecan containing 500 µg pecan protein with FIA or TiterMax Classic. Test bleeds from the goat and sheep were collected 10 days after each boost to monitor the production of the antibodies.

Extraction of soluble pecan

Before titer values could be determined, optimum extraction of soluble pecan proteins must precede in order to obtain the most reliable results. If insufficient protein is extracted, that can generate low titer values and cause inaccurate assessment of the produced antisera. Because the total amount of soluble pecan protein content has been found to increase with increasing concentrations of salt in the extraction buffer (Spiric, 2011; Venkatachalam et al., 2008), this study aspired to evaluate if increasing the salt concentration in the extraction buffer (0.01 M PBS, 0.15 M NaCl, pH 7.2) currently utilized in our lab could improve the extraction efficiency of pecan proteins in our samples. The high salt buffer (0.01 M PBS, 1 M NaCl, pH 7.2) described in Spiric's study (2011) demonstrated an improvement in the sensitivity of allergen detection by a

non-competitive ELISA format. Hence, our study applied this buffer in the evaluation of the anti-roasted pecan antisera produced from the five animals. The extraction efficiency of pecan by both a low salt extraction buffer and high salt buffer were compared to determine the best solution for all further analysis of pecan proteins and use for the development of the pecan ELISA. Before the extraction, raw and roasted pecans were prepared as previously described for the immunogen (Pecan Immunogen Preparation). The ground raw and roasted pecan were then individually extracted 1:10 (w/v) in the low salt buffer (0.01 M PBS, 0.15 M NaCl, pH 7.2) as well as the high salt buffer (0.01 M PBS, 1 M NaCl, pH 7.2) for one hour in a 60°C shaking water bath. The samples were then centrifuged at 3,612 x g (4,100 rpm) for 30 minutes in a tabletop centrifuge (Sorvall® Legend™ RT, Kendro Laboratory Products, Newton, CT) at 10°C. The supernatant was collected and filtered through a 0.45 µm membrane. The soluble protein content of each sample extract was estimated using the Lowry method (Lowry et al., 1951), and then divided into aliquots before being stored at -20°C until used for titer determination, SDS-PAGE analysis, and IgG immunoblotting.

Titer Determination

The polyclonal IgG antibody production from each animal was monitored by determining the titer values using a non-competitive ELISA as described by Hefle and others (2001), with appropriate modifications. Microtiter plates (NUNC-Immuno™ MaxiSorp™ 96-MicroWell™ plates, Nunc Nunc Intl., Rockster, NY, USA) were coated with three different soluble protein concentrations (10 µg/ml, 1 µg/ml, and 0.1 µg/ml) from the roasted pecan extract in coating buffer (0.5 M carbonate bicarbonate

buffer, pH 9.6). After an overnight incubation, the plates were washed four times with conjugate buffer [0.025 M PBS (0.005 M NaH_2PO_4 , 0.02 M Na_2HPO_4 , 0.85% NaCl, pH 7.4 containing Tween 20 (Polyoxyethylene Sorbitan Monolaurate, Bio-Rad Laboratoty Inc.))] using an automated microplate washer (AM60, Dynex Technologies, Inc., VA), and then 350 μl /well of blocking buffer [0.025 M PBS, 0.1% gelatin (300 bloom, porcine, Sigma-Aldrich Co., St. Louis, MO) pH 7.4] was added and incubated for one hour at 37°C. The plates were washed again as previously described, and then a 10-fold serial dilution of pecan-specific goat, sheep, or rabbit antisera diluted in conjugate buffer was added to the plate and incubated for two hours at 37°C. After another wash step, 100 μl /well of the corresponding alkaline phosphatase-conjugated anti-species IgG antibodies [rabbit anti-goat IgG, goat anti-rabbit IgG, and rabbit-anti-sheep IgG (Immunopure®, Pierce Biotechnology, Inc., Rockford, IL)] diluted 1:5,000 with conjugate buffer was added and incubated at 37°C for two hours. The plates were washed one last time followed by the addition of 100 μl /well of p-nitrophenyl phosphate (p-NPP) substrate solution (p-NPP SigmaFast™ Tablets, Sigma Chemical Co., St. Louis, MO). The plates were placed in a dark environment for 30 minutes, and then the enzymatic reaction was stopped by adding 1 N NaOH (100 μl /well). The product absorbance was measured at 405 nm using a microplate reader (ELx808 Ultraplate, BioTek Instruments, Inc., Winooski, VT).

Titration curves from the pecan-specific IgG antibodies were constructed by using GraphPad Prism® v.4.03 software (GraphPad Prism® software, Inc., San Diego, CA). In this study, titer was defined as the log reciprocal of the mid-linear portion of the resulting titration curve when 1 $\mu\text{g}/\text{ml}$ of pecan protein was coated onto the microtiter plate

(Maxisorp, Nalge Nunc International, Rochester, NY) (Hefle et al., 2001). Antibodies with the highest titers (>10,000) from the most recent animal bleeds were pooled accordingly (as described in the results sections) and used for the development of the pecan ELISA.

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

The soluble protein profiles of raw and roasted pecan were examined by SDS-PAGE under reducing conditions using a Bio-Rad Mini Protean® Tetracell (Bio-Rad Laboratories, Hercules, CA). Polyacrylamide precast resolving gels (15% Tris-HCl; 8.6cm x 6.8cm x 1.0mm; Bio-Rad Laboratories, Hercules, CA) were used to separate the proteins. Protein samples were prepared by mixing the extract in a 1:1 ratio with Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue; Bio-Rad Laboratories) containing 350 mM dithiothreitol (DTT Cleland's Reagent; Bio-Rad Laboratories). The mixture was heated for five minutes in a boiling water bath, cooled to room temperature, and centrifuged at 13,000 x g for five minutes immediately before loading samples in the SDS-PAGE gel. Stock 10X Tris/Glycine/SDS Buffer (Bio-Rad Laboratories) was diluted 1:10 with reverse osmosis (RO) water to achieve a final 1X running buffer. Five µl of standard molecular weight markers (Precision Plus Protein Dual Color Standards, Bio-Rad Laboratories) and 10 µg of protein from each sample were loaded in separate wells on the gel for each run. The electrophoresis run time was approximately 35 minutes at a constant voltage of 200 V. The run was stopped when the leading bromophenol blue front line reached the end of the gel. Proteins 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, Inc.) diluted 1:5 with RO water and stained with Coomassie Brilliant Blue R-250 Staining Solution (Bio-Rad Laboratories) overnight. The following day, the gels were destained with Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad Laboratories) to remove residual dye from the gel. The final gel images were captured using a Kodak Gel Logic 440 imaging system (Eastman Kodak Company) and the corresponding Kodak 1D v.3.6.5 software (Kodak Scientific Imaging System, New Haven, CT).

IgG immunoblotting (Western Blotting)

Because allergic consumers may be exposed to nut allergens that are raw or processed, both forms of proteins in the seed could be responsible for eliciting allergic reactions. For this reason, it is important to assure that the antibodies used for allergen detection are able to detect both forms (Sathe et al., 2009). The binding affinity and specificity of the polyclonal goat, sheep, and rabbit IgG antibodies for both raw and roasted pecan proteins were demonstrated by IgG immunoblotting. Pecan extracts were separated by molecular weight by SDS-PAGE under reducing conditions as previously described. Two gels were run simultaneously, one for protein stain and the other for immunoblotting. After electrophoresis, the gels for immunoblotting were equilibrated in transfer buffer (1X Tris/ Glycine Buffer, Bio-Rad Laboratories; 20% Methanol, Analytical Grade, Fisher Scientific) for 15 minutes and transferred onto a polyvinylidene difluoride (PVDF) membrane (Immunoblin-P PVDF membrane, 0.45 μ m, Millipore Corporation, Billerica, MA) using a modified method of Towbin et al. (1979) and Harlow and Lane (1988). The transfer was carried out for 80 minutes at a constant

voltage of 65 V. The membrane was subsequently washed three times for five minutes in RO water with gentle rotation to remove residual transfer buffer. To verify the successful transfer of soluble proteins, the membrane was soaked in Ponceau S staining solution [0.1% (w/v) Ponceau S in 5% (v/v) acetic acid] for a few minutes. Multiple washes in RO water was used to remove the Ponceau S stain from the membrane before blocking with 0.01 M PBS containing 0.05% Tween 20 (PBS-T, pH 7.4) with 0.2% bovine serum albumin (BSA, RIA grade, USB Corp., Cleveland, OH) for two hours at room temperature with gentle rotation. After washing the membrane four times for five minutes each in PBS-T, the pooled animal anti-pecan IgG antibodies were added to the membrane. Rabbit antibodies were diluted 1:8,000 (v/v) in blocking buffer while sheep and goat antibodies were both diluted 1:10,000 (v/v) in blocking buffer. The membranes were washed as previously described to remove unbound IgG antibodies. Each membrane was incubated for an hour with the corresponding horseradish peroxidase conjugated secondary antibodies (polyclonal) which included goat anti-rabbit IgG antisera, rabbit anti-sheep antisera, and rabbit anti-goat antisera (Product # 31340, 31480, 31402; Immunopure®, Pierce Biotechnology, Inc., Rockford, IL). All the secondary antibodies were diluted 1:25,000 (v/v) in blocking buffer. Another wash was performed before finally incubating the membranes with the DAB (3,3'-diaminobenzidine) substrate solution (Pierce Technology, Inc., Rockford, IL) for 15 minutes at room temperature to visualize the bound secondary antibodies. The membranes were finally washed in several changes of PBS-T to remove residual substrate and thoroughly air dried before capturing the images with a Kodak Gel Logic 440 Imaging System (Eastman Kodak Company)

utilizing the Kodak Gel Logic ID v.3.6.5 software (Kodak Scientific Imaging Systems, New Haven, CT).

RESULTS AND DISCUSSION

Titer Determination

The titers of pecan-specific IgG antibodies in sera of the five immunized animals (goat B 895, sheep G 538, rabbit NE 269, rabbit NE 270, and rabbit NE 271) were monitored throughout the immunization process to verify successful immunization and determine the bleeds that would be useful for development of the pecan ELISA. Titers were performed on the collected bleeds from each animal using an indirect, non-competitive ELISA with soluble pecan proteins coated onto microtiter plates. The final intensity of the yellow-colored product measured at 405 nm directly corresponded to the amount of specific antibodies in the antisera for each animal. Titration curves were generated using GraphPad Prism Software v.4.03 with 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. Figure 2.1 provides an example of a titration curve generated from the anti-roasted pecan antiserum of goat B 895 (production bleed 1/19/12) using a microtiter plate coated with 1.0 µg roasted pecan protein/ml. By taking the log reciprocal of the log antibody dilution at the mid-linear portion of the curve, which corresponded to -5.061, the titer value was determined to be 115,080. Titer values were obtained for all bleeds collected from the five animals throughout the immunization period. For polyclonal antisera, the level and quality of the antibodies produced will vary from animal to animal and from a single animal over time

(Boenisch, 2009). Therefore, to help ensure the successful development of a sensitive sandwich ELISA, an arbitrary titer value of 10,000 was established as the minimum acceptable value. As a result, production bleeds from each individual animal with a titer level of 10,000 or more were selected and pooled for the use in the development of the pecan ELISA.

The comparison of titer values generated with plates coated with pecan extracted from either the low salt buffer (0.01 M PBS, 0.15 M PBS, pH 7.2) or high salt buffer (0.01 M PBS, 1 M NaCl, pH 7.2) are displayed in Figure 2.2 (rabbits) and 2.3 (goat and sheep). The utilization of the low salt buffer generated values that trended over or below the 10,000 arbitrary baseline value set for acceptable antisera while the high salt buffer showed a 1 to 2 log increase in titer values. These results highlight the importance of obtaining appropriate protein extraction for accurate analysis of antisera because inadequate extraction of antigen can lead to low values which would deem the antisera to be poor or mediocre when in fact they are quite superior as demonstrated by the titer values established after a more robust extraction buffer was utilized for pecan protein extraction. The low salt extraction buffer failed to sufficiently extract high molecular weight proteins which are typically the proteins of highest abundance in pecan kernel and the proteins the rabbit, goat, and sheep antisera primarily recognized (discussed further in the next section). Titers carried out with the pecan antigen extracted with the low salt buffer ceased after the 18th month for the rabbits and after the 15th month for the goat and sheep due to insufficient interaction by the antibodies.

The production progress of antisera by rabbits (NE 269, 270, 271) to roasted pecan during the immunization process is presented in Figure 2.2. All three rabbits

produced antisera against pecan with titer values greater than 10,000 by the third month post-initial injection, and production bleeds began in the fourth month (16 weeks after the initial injection). Due to the abundant amount of suitable antisera already available at the beginning of this study, the most recent bleeds at that point in time with the highest titers were selected and pooled. For each individual rabbit, equal amounts of chosen production bleeds between months 16-24 of immunization were mixed together, aliquoted, and stored at -20°C for further use in the development of the pecan ELISA.

The goat (B 895) and sheep (G 538) immune response to the roasted pecan immunogen throughout the immunization process is shown in Figure 2.3. Both animals displayed a similar response to the rabbits with titer values greater than 10,000 obtained by the third month post-initial injection, and equal amounts of selected production bleeds between months 16-24 of immunization were mixed together for each individual animal. The pooled goat and sheep antisera were divided into aliquots prior to storage at -20°C to minimize freeze/thaw cycles which can damage the antibodies. Overall, the high titer values attained from all five animals demonstrated their excellent immune responses to roasted pecan, and provided a good foundation for the development of an ELISA for the detection of pecan.

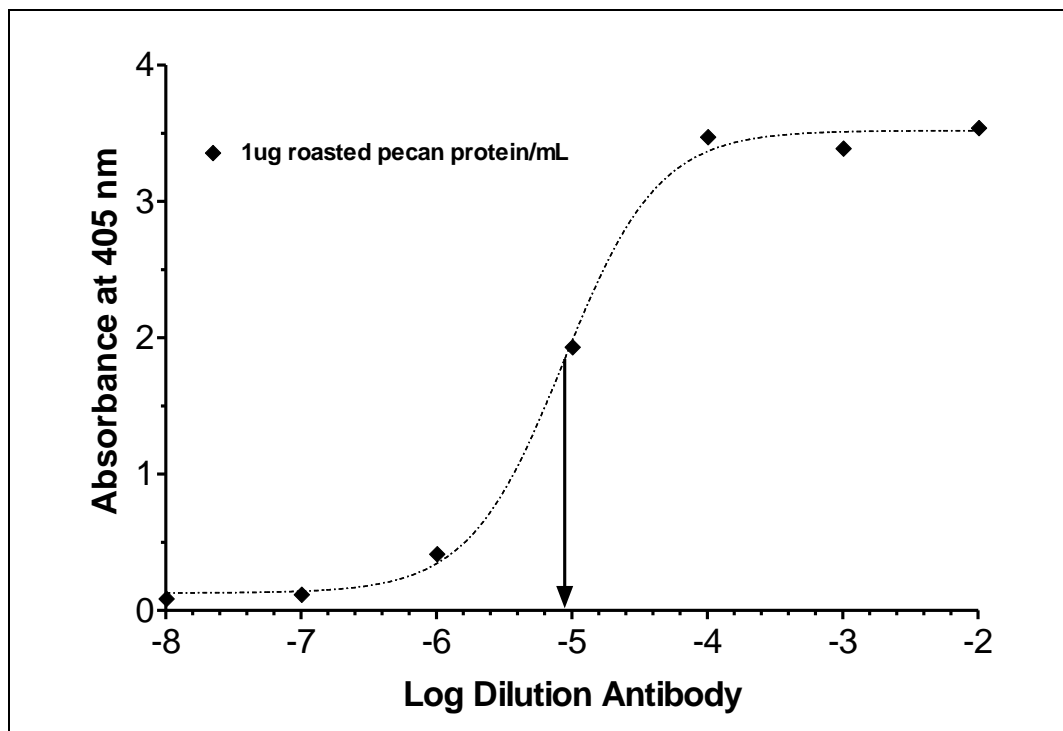


Figure 2.1. Titration curve obtained with the anti-roasted pecan antiserum from goat (B895) using a microtiter plate coated with 1.0 μg roasted pecan protein/ml. The vertical line indicates the mid-linear portion of the titration curve. Titer was determined by determining the log reciprocal of the mid-linear portion of the titration curve. Each data point represents the mean of triplicate readings and the standard deviation for each point is <0.1 absorbance unit (AU).

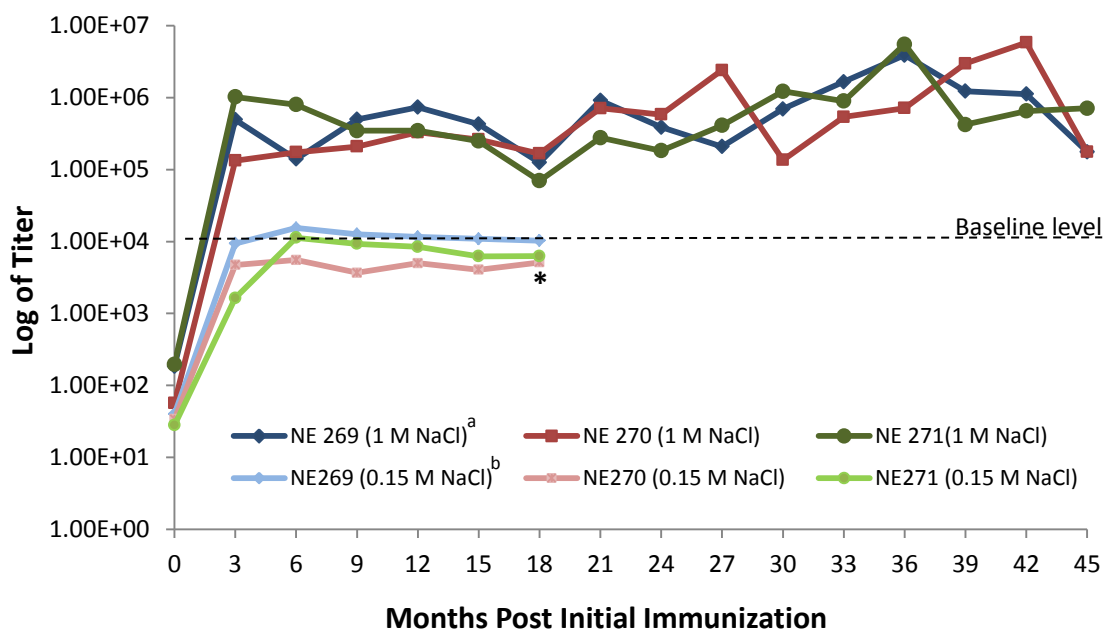


Figure 2.2. Immune response of individual rabbits (NE 269, 270, 271) to roasted pecan immunogen. Data points are the mean of triplicate readings. Titer analyses from plates coated with pecan extracted by either a high or low salt buffer are compared.

*Titers using the low salt buffer ceased after the 18th month for the rabbits due to insufficient interaction by the antibodies.

^a Pecan antigen coated on plates for titer analysis were extracted using a high salt buffer (0.01 M PBS, 1 M NaCl, pH 7.2)

^b Pecan antigen coated on plates for titer analysis were extracted using a low salt buffer (0.01 M PBS, 0.15 M NaCl, pH 7.2)

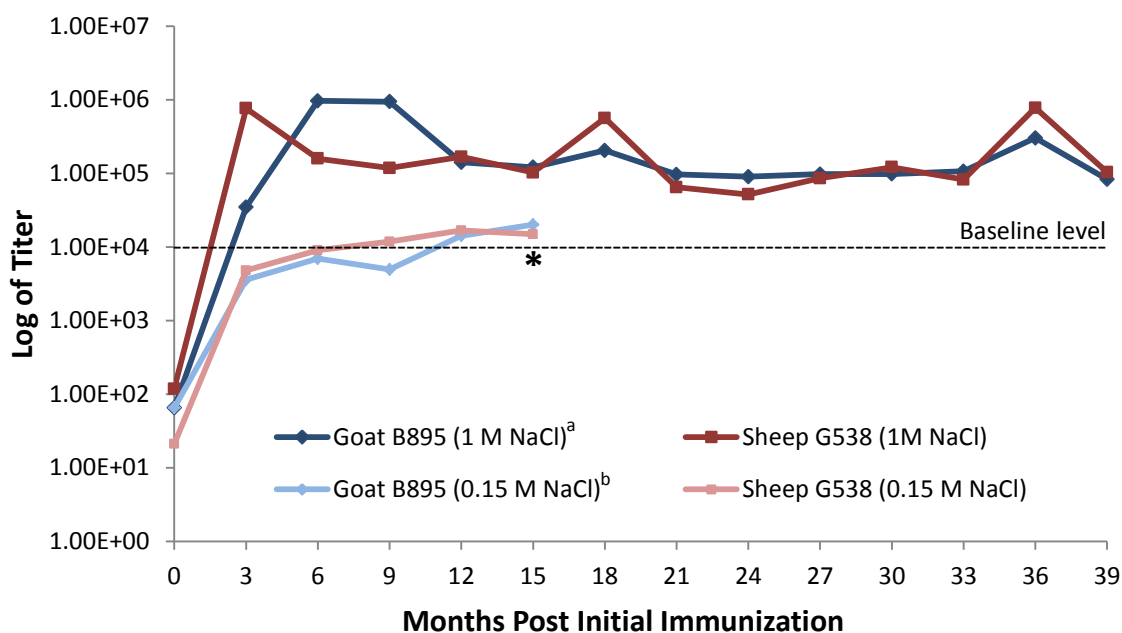


Figure 2.3. Immune response of goat (B 895) and sheep (G 538) to roasted pecan immunogen. Data points are the mean of triplicate readings. Titer analyses from plates coated with pecan extracted by either a high or low salt buffer are compared.

*Titers using the low salt buffer ceased after the 15th month for the goat and sheep due to insufficient interaction by the antibodies.

^a Pecan antigen coated on plates for titer analysis were extracted using a high salt buffer (0.01 M PBS, 1 M NaCl, pH 7.2)

^b Pecan antigen coated on plates for titer anylsis were extracted using a low salt buffer (0.01 M PBS, 0.15 M NaCl, pH 7.2)

SDS-PAGE and IgG Immunoblotting

The effectiveness with which the antibodies used in the ELISA to detect the antigen of interest, along with the efficiency with which these antigens are extracted from sample, are the most important parameters that can affect the overall performance of an ELISA-based method (Abbott et al., 2010). Electrophoresis and IgG immunoblotting were performed to verify the specificity and affinity of the IgG antibodies against raw and roasted pecan. Because both raw and processed forms of pecan may be used in food products, it is essential to validate that the antibodies are robust enough to detect both forms. The role of salt content on pecan protein solubilization was also examined by comparing the SDS-PAGE protein profiles of raw and roasted pecan proteins extracted with 0.01 M PBS containing either 0.15 M or 1 M NaCl followed by evaluating the antibodies' ability to bind these proteins. One-dimensional SDS-PAGE separated the pecan proteins by their molecular mass, regardless of their original charge, and the proteins were further visualized by Coomassie Brilliant Blue stain after electrophoresis. The protein content of the pecan extracts prepared using PBS with low (0.15 M NaCl) and high (1 M NaCl) salt content are shown in Table 2.1, and the resulting SDS-PAGE gel profiles of the pecan extracts are shown in Figure 2.4. Although the general patterns for each protein extract were similar, the staining intensity for the raw pecan extracts was slightly higher than the roasted pecan. Nevertheless, the pecan proteins were still clearly presented after roasting, indicating that the solubility of the proteins was not significantly affected by the heating process. Also judged qualitatively by the staining intensity of the protein bands, the increase of salt content in the PBS extraction buffer effectively enhanced the solubility of pecan proteins for both raw and roasted pecan, especially the

higher molecular weight proteins. The increase of extracted pecan protein by the high salt buffer correspondingly supports the superior titer values described earlier in the titer determination section. The major polypeptide bands from the pecan extracts prepared with the high salt buffer were located at 12, 16, 18, 20, 22, 34, 50, 55, and 75 kDa which are relatively consistent with the electrophoretic profile of pecan proteins in other studies (Spiric, 2011; Venkatachalam et al., 2007). The bands at the estimated molecular weights of 12 kDa and 55 kDa likely represent the characterized pecan allergens of Car i 1 and Car i 4, respectively.

Table 2.1. Soluble protein content of raw and roasted pecan estimated by the Lowry method.

EXTRACTION BUFFER	RAW PECAN (mg/ml)	ROASTED PECAN (mg/ml)
0.01 M PBS, 0.15 M NaCl (pH 7.2)	1.58	1.40
0.01 M PBS, 1 M NaCl (pH 7.2)	2.43	2.01

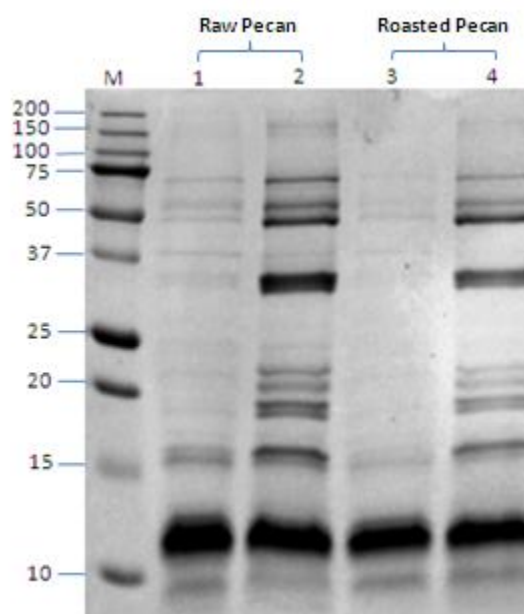


Figure 2.4. SDS-PAGE analysis of pecan protein extracts under reducing conditions. Separated proteins were stained with Brilliant Blue G-Colloidal stain following electrophoresis. Total protein load on each lane was approximately 10 μ g.

M = Molecular weight markers

Lane 1- Raw pecan protein extracted in 0.01 M PBS, 0.15 M NaCl

Lane 2- Raw pecan protein extracted in 0.01 M PBS, 1 M NaCl

Lane 3- Roasted pecan protein extracted in 0.01 M PBS, 0.15 M NaCl

Lane 4- Roasted pecan protein extracted in 0.01 M PBS, 1 M NaCl

Immunoassay analytical sensitivity depends on how strongly the antibody binds to the antigen. IgG immunoblot analyses showed that all five animal's antisera were reactive against pecan protein extracts and recognized a similar soluble protein pattern. There was no significant difference in the immunoblots from both raw and roasted pecan extracts, confirming that processing did not affect the antigenicity of the pecan proteins used for immunization of the animals. This is consistent with the results presented by Venkatachalam et al. (2006). As observed previously with the SDS-PAGE results, the extraction of proteins from pecan was influenced by the concentration of salt in the extraction buffer. This in turn, had an impact on the detection of pecan by the animal antisera demonstrated by immunoblotting. Figures 2.5 and 2.6 show a significant reduction of antibody binding in both the raw and roasted pecan samples extracted by the low salt buffer, which remained consistent for all five animals.

The anti-pecan protein IgG profiles of the sheep and goat were comparable with goat antisera showing a slightly more intense binding, particularly at the 15 kDa band (Figure 2.5). All three rabbits also displayed similar binding profiles with only minor differences (Figures 2.6 and 2.7). The protein bands detected by rabbit NE271 appeared fainter compared to the other two rabbits, which may indicate low affinity interaction. Rabbit NE270 exhibited a slight increase in band intensity at the higher molecular weight proteins (≥ 20 kDa) while rabbit NE269, showed more intense binding at the lower molecular weight proteins (≤ 15 kDa). Nevertheless, the immunoblots qualitatively indicated that the antibodies were able to bind to a wide variety of pecan proteins and supported the high titer values of all five animals. Thus, the pooled antisera from each

individual animal were deemed suitable for further use in the development of an ELISA to detect pecan residues.

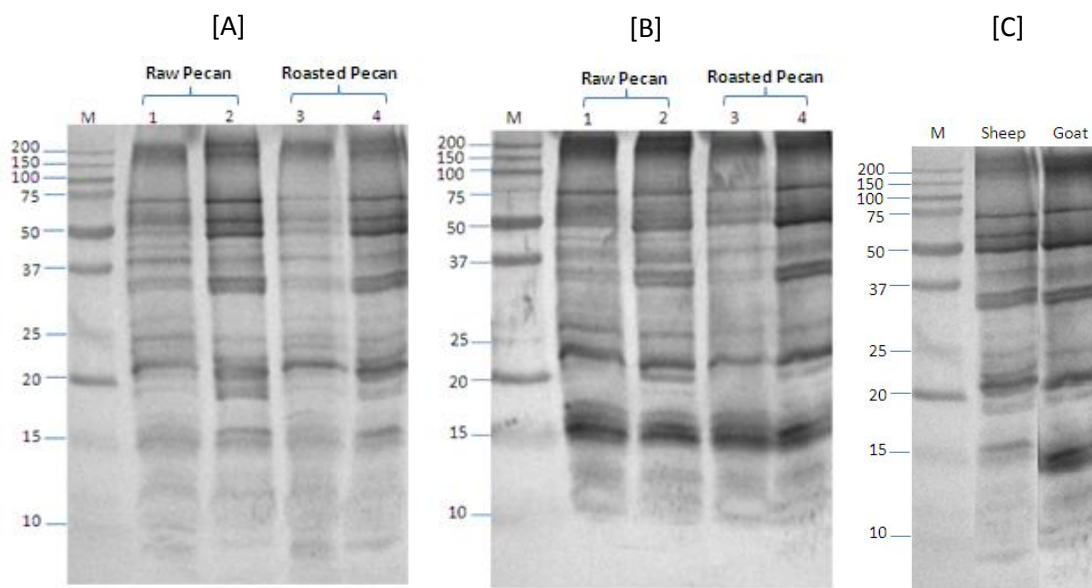


Figure 2.5. Immunoblots of raw and roasted pecan extracts probed to [A]-sheep G538 and [B]-goat B895 antisera. [C]- Comparison of sheep G538 and goat B895 immunoblots against roasted pecan protein extracted in 0.01 M PBS, 1 M NaCl

M = Molecular weight markers

Lane 1- Raw pecan protein extracted in 0.01 M PBS, 0.15 M NaCl

Lane 2- Raw pecan protein extracted in 0.01 M PBS, 1 M NaCl

Lane 3- Roasted pecan protein extracted in 0.01 M PBS, 0.15 M NaCl

Lane 4- Roasted pecan protein extracted in 0.01 M PBS, 1 M NaCl

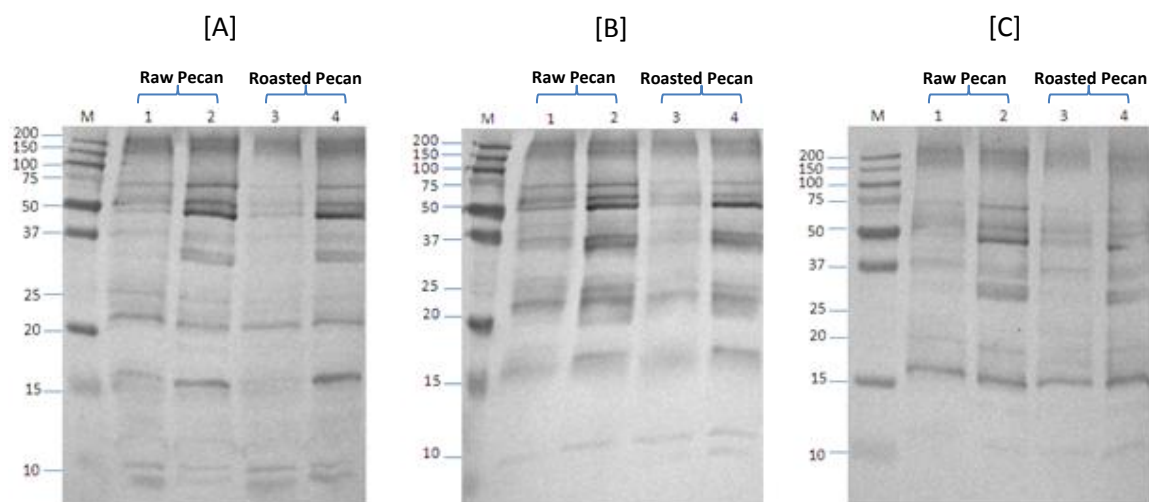


Figure 2.6. Immunoblots of raw and roasted pecan extracts probed to rabbits [A]-NE269, [B]-NE270, and [C]-NE271 antisera.

M = Molecular weight markers

Lane 1- Raw pecan protein extracted in 0.01 M PBS, 0.15 M NaCl

Lane 2- Raw pecan protein extracted in 0.01 M PBS, 1 M NaCl

Lane 3- Roasted pecan protein extracted in 0.01 M PBS, 0.15 M NaCl

Lane 4- Roasted pecan protein extracted in 0.01 M PBS, 1 M NaCl

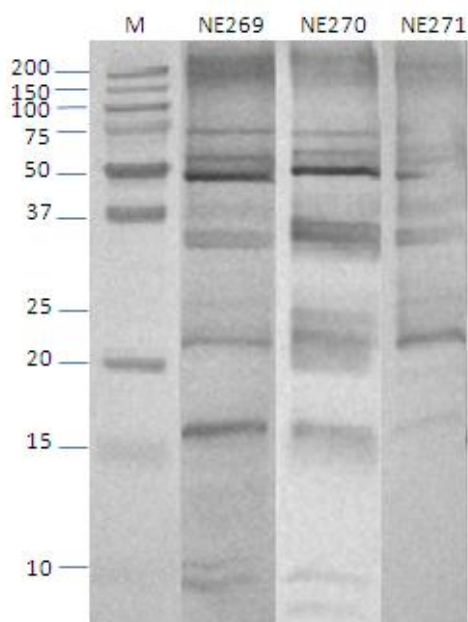


Figure 2.7. Comparison of rabbit immunoblots against roasted pecan protein extracted in 0.01 M PBS, 1 M NaCl. M = Molecular weight marker

CONCLUSIONS

The careful selection of antibodies and optimum extraction of the proteins of interest provide the framework for developing a successful ELISA. It is also imperative to consider the processing conditions for various food products which can affect the detection of food allergens. In this study, the high titer values attained from goat, sheep, and three rabbits demonstrated their excellent immune responses to roasted pecan. All five animals were also able to recognize both raw and roasted pecan proteins separated by SDS-PAGE, with only minor differences in binding patterns. SDS-PAGE analysis indicated that the solubility of pecan proteins was not significantly affected by the heating process while the increase of salt content in the PBS extraction buffer effectively enhanced the solubility of pecan proteins for both raw and roasted pecan. The results of the low titer values and insufficient SDS-PAGE protein profiles of the pecan proteins extracted with the low salt buffer suggest that inadequate protein was extracted for interaction with the anti-pecan antibodies. The optimization of protein extraction is necessary because the quality of valuable antisera can easily be misjudged. Because of the favorable results, the high salt concentration (1 M NaCl) in PBS was used for all further extractions in this study. The pooled antisera from each individual animal displayed sufficient specificity and affinity towards the soluble pecan proteins, supporting their suitability for potential use in the development of an ELISA to detect pecan residues.

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

INTRODUCTION

Pecans are one of the few commercial agricultural crops indigenous to North America. The pecan (*Carya illinoensis*) belongs to the Juglandaceae family, which also includes other tree nuts such as walnuts, hickory nuts, and butternuts (Rosengarten, 1984). Over 1000 different pecan varieties have been described, although 90% of cultivated acreage is represented by only a few dozen varieties. Venkatachalam et al. (2007) compared 24 commercially important pecan cultivars and determined that they all had similar, but not identical biochemical characteristics, polypeptide composition, and immunoreactivity. The soluble proteins of pecans are synthesized during the cotyledon stage while the storage protein synthesis starts from the maturation stage until post abscission. The Osborne fractionation of pecan proteins has revealed the seed proteins to consist about 60% alkali glutelins, 32% globulins, 3% prolamins, and 2% albumins (Venkatachalam et al., 2008).

Pecans have a rich, buttery flavor and are widely used in confectioneries and desserts. They are a high calorie food (690 kcal/100 g) comprising of about 9% protein, 72% fat, 14% carbohydrate, and 10% fiber (Venkatachalam and Sathe, 2006; USDA, 2005). Pecans have gained importance in recent years for their health benefits, especially their high antioxidant capacity and potential for reducing the risk of cardiovascular disease (Morgan and Clayshulte, 2000; Rajaram et al., 2001; USDA, 2009).

While pecans have economic and nutritional benefits, they have been linked to food safety issues, specifically as containing potent allergens. Pecans are included in the group of the eight most common foods capable of inducing an allergic reaction, often referred to as the “Big 8” allergens. Milk, eggs, wheat, peanuts, fish, crustacean shellfish, soybean, and other tree nuts also belong to this group that are thought to be responsible for 90% of all IgE-mediated allergies worldwide (FAO, 1995). Nine percent of self-reporting tree nut-allergic patients list pecan as an allergen (Sicherer et al., 2001). Pecan is considered as a type of nut allergy that can manifest itself quite early in life and persist throughout adulthood, resulting in lifelong afflictions (Fleischer et al., 2005; Roux et al., 2003).

Two allergenic proteins have been described to date in pecan and include a 2S albumin, Car i 1 (~16 kDa), and an 11 S legumin, Car i 4 (~55 kDa) (Sharma et al., 2011a, Sharma et al., 2011b). Pecan proteins were demonstrated to be thermally stable by Venkatachalam et al. (2006), who also showed that pecan protein solubility and antigenic reactivity were not directly correlated; suggesting that loss in protein solubility during the extraction and testing phase may not always be reliably and predictably related to the loss in antigenicity. Most recently, pecan allergen Car i 1 was determined to be the most digestion resistant protein from pecan with the resultant peptides maintaining IgE binding activity (Spiric, 2011).

Unlike other tree nuts such as walnut and hazelnut, studies on the allergenicity of pecan are limited. In spite of its increasing importance for the food industry, only a few analytical methods to detect pecan in food have been published. Moreover, there are currently no commercially available methods for the food industry and regulatory

agencies to use for monitoring pecan residue in finished food product or on shared equipment surfaces. Venkatachalam et al. (2006) reported the development of an enzyme linked immunosorbent assay (ELISA) method capable of detecting pecan proteins in the range of 32–800 ng pecan protein/ml. The main function of the this developed ELISA was to assess the stability of pecan antigens subjected to thermal processing treatments and to in vitro simulated intestinal fluid (SIF) and simulated gastric fluid (SGF) digestion conditions. The potential usefulness of this ELISA to detect pecan residues in a variety of food matrices was not explored. More recently, Polenta et al. (2010) has developed a competitive ELISA to detect traces of pecan in several food matrices. This particular ELISA method was able to detect pecan proteins in milk chocolate at levels as low as 1 ng pecan protein/ml which is an improvement on the ELISA developed by Venkatachalam et al., (2006). This study also analyzed cross-reactivity, with walnut showing a level of interference lower than 10%. Although the study carried out by Polenta et al. (2010) assessed specificity and selectivity of the polyclonal antibodies produced for the ELISA method, the food commodities used in the challenge studies to test for cross-reactivity with the competitive ELISA were not reflective of ingredients or foods that pecan is commonly used in such as baked goods and confections. In addition, the assessment of the method's performance in food matrices was not sufficient because the procedure involved spiking milk chocolate with protein extracts instead of naturally incurred standards, which is the ultimate evaluation of an ELISA according to Taylor et al. (2009). Naturally incurred standards involve incorporation of the allergenic food residues into the food formulation which is then processed in a manner that mimics industrial food processing. This method evaluates the effects of processing on the

allergenic food residues in a food matrix (Taylor et al., 2009). Although Polenta et al. examined the ELISA's ability to detect pecan traces after processing treatments in a subsequent study (2012), the study focused on direct treatment to the pecans and did not consider food matrices, which can have a considerable effect on allergen detection.

Two DNA-based methods have also been published for detection of pecan residue. Brezna and Kuchta (2008) developed a polymerase chain reaction (PCR) method for the detection of pecan DNA in food, with a practical detection limit of 0.01% (w/w). Hubalkova and Rencova (2011) recently developed a one-step PCR method for the simultaneous detection of the major allergens of pecan and Brazil nuts, but resulted in a less sensitive [0.1% (w/w) or 1 g/kg] system. Although the PCR technique can be a beneficial method, there are some concerns regarding the use of DNA-based instead of protein-based methods for allergen analysis. For example, the offending molecules that cause allergic reactions are proteins, and processing can affect protein and DNA differently (Poms et al. 2004). The absence of DNA does not necessarily indicate absence of protein. In addition, immunological-based methods present certain advantages such as greater suitability for analysis on production lines, easier handling, and requirement of less sophisticated equipment (Jackson, 2010). Overall, the methods that have been developed for the detection of pecan are certainly sensitive, but they all lack the necessary validation to be applicable with processed foods which is an essential facet of the food industry.

When validating an analytical method for food allergens, it is important to recognize that food processing can deeply affect the extractability and detection of target analytes (Cucu, 2011; Platteau, 2011). A robust ELISA method for the detection of pecan

would provide the industry with a convenient analytical tool to ensure product compliance with the requirements enforced by regulatory agencies, but the effective monitoring of unintended pecan contamination in complex food matrices constitutes a formidable challenge (Poms et al. 2004). Nonetheless, an analytical method such as the ELISA can be valuable if it is developed correctly and well validated for its intended use.

As described previously in Chapter 2, the development of an ELISA begins with high quality antibodies. Once all immunoreagents are acquired, the ideal concentrations of each reagent must be established empirically. Utilizing microtiter plates, checkerboard titrations can be performed to assist in the assessment. The checkerboard titration involves the dilution of two reagents against each other to examine the activities at all the resulting combinations (Luttmann et al., 2006). The optimum combination of reagents and their concentrations should generate a standard curve that displays the correct sensitivity, range, and linearity for a robust ELISA (Thermo Scientific, 2010). The operating range of an assay is the interval of analyte concentrations over which the method provides suitable accuracy and precision (OIE, 2012). For quantitative assays, sensitivity is often described through the limit of detection (LOD) and limit of quantitation (LOQ). LOD is defined as the lowest concentration or mass of analyte in a test sample that can be distinguished from a true blank sample at a specified probability level while LOQ is the lowest level of analyte in a test sample that can be reasonably quantified at a specified level of precision. When estimating the LOD, it is common to assume that it is the signal strength of a blank increased by three times the standard deviation of the blank (Abbott et al., 2010). This method gives at best an estimate and relies on normal Gaussian distribution of the blank measurements around zero. This can

generally be assumed for methods such as ELISA, but the LOQ is best determined experimentally (CCMAS, 2010).

Specificity, the ability to unequivocally assess the analyte in the presence of components that may be expected to be present, is another aspect that should be evaluated for a reliable detection method. The presence of interferents such as 1) matrix components, 2) non-specific binding of reactants to the solid phase, and 3) biomolecules with cross-reactive epitopes can affect the extent that a method can accurately quantify the target analyte (Vessman, 1996). Such interferences may cause falsely reduced or elevated responses in the assay that negatively affects its analytical specificity (OIE, 2012). For example, a positive response to a sample that does not contain any of the target analyte can occur and cause a false positive result. Therefore, the possibility of cross-reactions should be evaluated with a wide selection of substances, especially those that are genetically similar or likely to be analyzed for the presence of the target analyte (Abbott et al., 2010). In addition, a non-specific response can occur from a substance or substances in the final extract other than the specific protein analyte and is referred to as a matrix effect (CCMAS, 2010). A test for general matrix effect can be made by applying the method of analyte additions (also referred to as spiking) to a test solution derived from a typical test material. The test should be completed in a way that provides the same final dilution as the normal procedure produces, and the range of additions should encompass the same range as the procedure-defined calibration validation.

The objective of this study was to develop a sensitive and specific sandwich ELISA capable to detecting pecan in various food matrices. Cross-reactivity studies and matrix interference studies were carried out to verify the developed method's reliability.

MATERIALS AND METHODS

Development of Pecan Sandwich ELISA

Ground, roasted pecan from various sources in Mississippi, Georgia, and Texas were used for the development of pecan standards. A 1,000 part per million (ppm; $\mu\text{g/ml}$) concentration of roasted pecans was prepared by mixing 0.05 mg of the ground, roasted pecan into 50 ml of simple buffer (PBS). This 1,000 ppm pecan standard was extracted 1:10 (w/v) with 0.01 M phosphate buffered saline [(PBS), 1 M NaCl, pH 7.2] at 60°C in a shaking water bath. The extract was then centrifuged at $3,612 \times g$ (4,100 rpm) for 30 minutes at 10°C using a tabletop centrifuge (Sorvall® Legend™ RT, Kendro Laboratory Products, CT). The supernatant was collected and filtered through a 0.45 μm membrane. The final clarified supernatant containing the soluble pecan proteins was 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 pecan) with extraction buffer to develop a standard curve.

Preliminary assay trials using checkerboard titrations were performed to generate an ELISA standard curve with the optimal combinations of capture, detection, and conjugate antibody concentrations. For the capture and detection antibodies, the pooled animal anti-roasted pecan antisera (described in Chapter 2) that had a titer value $> 10,000$ were tested with different combinations of coating antibody-secondary antibody of each animal (goat-rabbit, sheep-rabbit, rabbit-goat, and rabbit-sheep) at various dilutions ranging from 1:3,000 to 1:40,000 in PBS buffer. The appropriate conjugate antibody was also tested at dilutions ranging from 1:3,000 to 1:5,000.

The roasted pecan sandwich ELISA was developed based on the protocol described by Hefle et al. (2001). To begin, the pooled goat antisera diluted 1:5,000 in coating buffer (0.015 M Na₂CO₃, 0.035 M NaHCO₃, and 0.02% NaN₂, pH 9.6) was applied to the wells of the microtiter plates (NUNC-Immuno™ MaxiSorp™ 96-MicroWell™ plates, Nunc International, NY). The coated plates were incubated overnight at 4°C. The following day, the plates were washed 4 times with wash buffer (PBS containing 0.05% Tween 20 and 0.02% NaN₃, pH 7.4) using a microplate washer (AM60, Dynex Technologies, Inc., VA). This washing procedure was carried out after every incubation step with the exception of the substrate incubation. After the coated plates were washed, blocking buffer containing 0.1% gelatin (300 bloom, porcine, Sigma-Aldrich Co., St. Louis, MO) in 0.01 M PBS, pH 7.4, were added to the wells of the plates (350 µl/well) and incubated for 1 hour at 37°C. After the subsequent washing step, the serially diluted pecan extract standards were loaded onto the plate (100 µl/well) and allowed to incubate at 37°C for 1 hour. The captured pecan proteins bound to the antibodies coated on the plate were detected by adding 100 µl/well of the pooled rabbit (NE 270) antisera, which was diluted 1:4,000 with conjugate buffer (PBS containing 0.1% BSA, pH 7.4). Another 1 hour incubation at 37°C followed. The plates were washed and 100 µl/well of goat anti-rabbit IgG labeled with alkaline phosphatase (Immunopure®, Pierce Biotechnology Inc., IL) diluted 1:5,000 in conjugate buffer was added to the plates and incubated for 1 hour at 37°C. The bound antigen-antibody complexes were visualized by the addition of *p*-nitrophenyl phosphate substrate (*p*-NPP, SigmaFast™, Sigma-Aldrich Co., St. Louis, MO). The plates were then placed in a dark environment and incubated for 30 minutes to develop color. The enzymatic reaction was

stopped by adding 100 μ l/well of 1 N NaOH and the intensity of the colored product was immediately measured at 405 nm using a microplate reader (ELx808 Ultraplate, BioTek Instruments Inc., Winooski, VT). Standard curves were generated using GraphPad Prism® v.4.03 software (GraphPad Software Inc., San Diego, CA).

Cross-Reactivity Studies

To assess the specificity of the antibodies in the developed ELISA, 116 foods and food ingredients commonly used in baked goods and confections, as well as foods derived from the Juglandaceae family, were evaluated for potential cross-reactivity. All the foods were purchased from local grocery stores in Lincoln, Nebraska. All solid samples were ground into a small, uniform size using an Osterizer® Blender (Sunbeam Corporation, Delray Beach, FL). Liquid samples including oils were used “as is” without further processing. All samples were extracted 1:10 (w/v) with 0.01 M PBS (1 M NaCl, pH 7.2) at 60°C in a shaking water bath (Julabo SW22, Julabo USA, Inc., Allentown, PA) at 150 rpm for 1 hour. The samples were then centrifuged for 30 minutes at 3,612 x g (4,100 rpm) at 10°C. The supernatant was collected and then filtered through a 0.45 μ m membrane. The clarified extracts were stored at 4°C until used. The soluble protein content for each sample extract was estimated using the Lowry method (Lowry et al., 1951). All food samples were diluted in the extraction buffer (0.01 M PBS, 1 M NaCl, pH 7.2) and then analyzed in the developed pecan ELISA based on soluble protein content (10 μ g/ml, 1.0 μ g/ml, and 0.1 μ g/ml) and on extraction concentration basis (1:1, 1:10, and 1:100). Results of the cross-reactivity were expressed as ppm apparent pecan equivalents by comparing the absorbance readings obtained from the sample extracts to

the standard curves that were constructed based on ground roasted pecan standards prepared in extraction buffer.

Matrix Interference Studies

Because pecan is commonly incorporated into bakery products and confections, vanilla ice cream, sugar cookies, and dark chocolate were chosen as reference food standards to evaluate the potential matrix interference on the sensitivity and performance of the developed pecan ELISA. Vanilla ice cream and ingredients to prepare sugar cookies (Betty Crocker™ sugar cookie mix, butter, and egg) were purchased from a local grocery store in Lincoln, Nebraska. Dark chocolate samples were obtained from a batch of Barry Callebaut dark chocolate specifically manufactured as a negative control standard for a previous study. All foods products and ingredients were pre-screened for undeclared pecan proteins or relevant proteins using the pecan ELISA.

The sugar cookies were made according to the directions on the Betty Crocker™ sugar cookie mix package. All appropriate ingredients (Betty Crocker™ sugar cookie mix, butter, and egg) were combined and mixed for 15 minutes using a Kitchen Aid™ 5 Quart Mixer, KSM150OPS (Kitchen Aid, St. Joseph, MI). At 5 minute intervals, the mixer was stopped and the dough was folded manually from the bottom to the top using a spatula. The cookie dough was scooped with a stainless-steel spring-loaded scoop and placed 2 inches apart on an aluminum covered baking sheet. The sugar cookies were then baked in an oven at 375°F for 15 minutes.

Prior to spiking and extraction, the dark chocolate and baked cookies were ground using an Osterizer® blender (Sunbeam Corporation, Delray Beach, FL). Ten grams of

dark chocolate, ice cream, and baked cookie were placed into individual extraction bottles, and the dark chocolate and ice cream were allowed to melt in a heated water bath. To prepare a 1,000 ppm pecan standard for the matrices, 0.01 grams of ground roasted pecan were added to each extraction bottle containing 10 grams of dark chocolate, ice cream, or baked cookie. A 1,000 ppm pecan standard in 0.01 M PBS was also prepared by adding 0.01 grams of ground roasted pecan to 10 ml of the simple buffer. Negative controls (0 ppm) were prepared by putting 10 grams of pecan-free dark chocolate, vanilla ice cream, and sugar cookies into separate extraction bottles. Ten milliliters of 0.01 M PBS buffer was also added to its own extraction bottle to use as a negative control. A total of eight samples (four 1,000 ppm of each pecan-spiked 0.01M PBS, pecan-spiked sugar cookies, pecan-spiked dark chocolate, pecan-spiked vanilla ice cream; and four zero ppm of each pecan-free 0.01 M PBS buffer, dark chocolate, vanilla ice cream, and sugar cookies) were extracted 1:10 (w/v) with the extraction buffer (100 ml of 0.01 M PBS, 1 M NaCl, pH 7.2) containing 1% non-fat dry milk (NFDM) at 60°C in a shaking water bath for 1 hour, followed by centrifugation at 3,612 x g (4,100 rpm) for 30 minutes at 10°C. The supernatants were filtered through a 0.45 µm membrane and the clarified extracts were stored at 4°C until used for the pecan ELISA analysis.

A series of standards were prepared by serially diluting the 1,000 ppm pecan spiked extracts 1:3 (v/v) with the corresponding negative control extracts to achieve 12 pecan concentration levels ranging from 0 to 1,000 ppm pecan (1000, 333, 111, 37, 12.3, 4.11, 1.37, 0.45, 0.15, 0.05, 0.02, and 0 ppm pecan). The samples spiked with pecan were then analyzed in the pecan ELISA. The standard curves attained from pecan-spiked food matrices were compared with the pecan ELISA standard curve prepared in PBS.

Statistical analysis of the difference between the standard curves was performed using a one-way analysis of variance (ANOVA) test at the $p < 0.05$ significance level.

Statistical Analysis

The limit of detection (LOD) of the pecan standard curves was determined as the mean absorbance value of the blank (wells without pecan) plus 3 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. The results were based on the mean of 6 replicates with 3 analyses per replicate. To determine if any of the food matrices interfered with the performance of the developed ELISA, the difference between the standard curves was determined using a one-way ANOVA test (GraphPad Prism® v.4.03 software). The raw and roasted pecan standard curves generated by the pecan ELISA were also compared using an unpaired t-test (GraphPad Prism® v.4.03 software) in order to determine if the developed ELISA was able to detect both raw and processed pecan without any significant differences. A p -value < 0.05 was considered significant.

RESULTS AND DISCUSSION

Pecan Sandwich ELISA Standard Curves

Several combinations of goat/sheep/rabbit coating and detection antibody as well as several dilutions were investigated during the optimization of the pecan ELISA. Only the optimized coating and detection antibody as well as the antisera dilutions are presented in this thesis. The developed ELISA used a combination of goat (B 895) antisera for the IgG capture antibody and rabbit (NE 270) antisera for the IgG detection

antibody with an optimized dilution of 1:5,000 and 1:4,000, respectively. The optimum concentration for the conjugate antibody (anti-rabbit alkaline phosphatase conjugated IgG) was determined to be a 1:5,000 dilution. Because unprocessed and processed forms of pecan are incorporated into food products and both can be responsible for eliciting an allergic reaction, it was essential to verify that the developed ELISA could reliably detect both forms. Figure 3.1 compares the raw and roasted pecan standard curves produced by the optimized ELISA. Both standard curves are distinctly similar and exhibit low background values (< 1.0 absorbance unit, AU) at the zero ppm pecan standard curve point. The limit of quantification (LOQ) for both curves was 1.5 ppm (μg of pecan/ml) with a corresponding dynamic range of 1.5 ppm to 50 ppm. The raw and roasted pecan curves had a lower limit of detection (LOD) of 0.017 ppm and 0.020 ppm (μg of pecan/ml), respectively based on calculations outlined by Abbot et al. (2010). Results from the unpaired t-test (GraphPad Prism® v.4.03 software) verified there was no significant difference (p -value of < 0.05 was considered significant) between the raw and roasted standard curves, $t(22) = 0.168$, $p = \text{not significant (ns)}$. The specificity of the antibodies in this study was consistent with the previous results reported for the IgG immunoblot experiments in Chapter 2. Concisely, the animal antibodies utilized in this ELISA are robust enough to detect both forms of pecan, and the roasted pecan standard curve can be applied when detecting unprocessed and processed forms of pecan. Therefore, a standard curve prepared with roasted pecan was used for the remaining study.

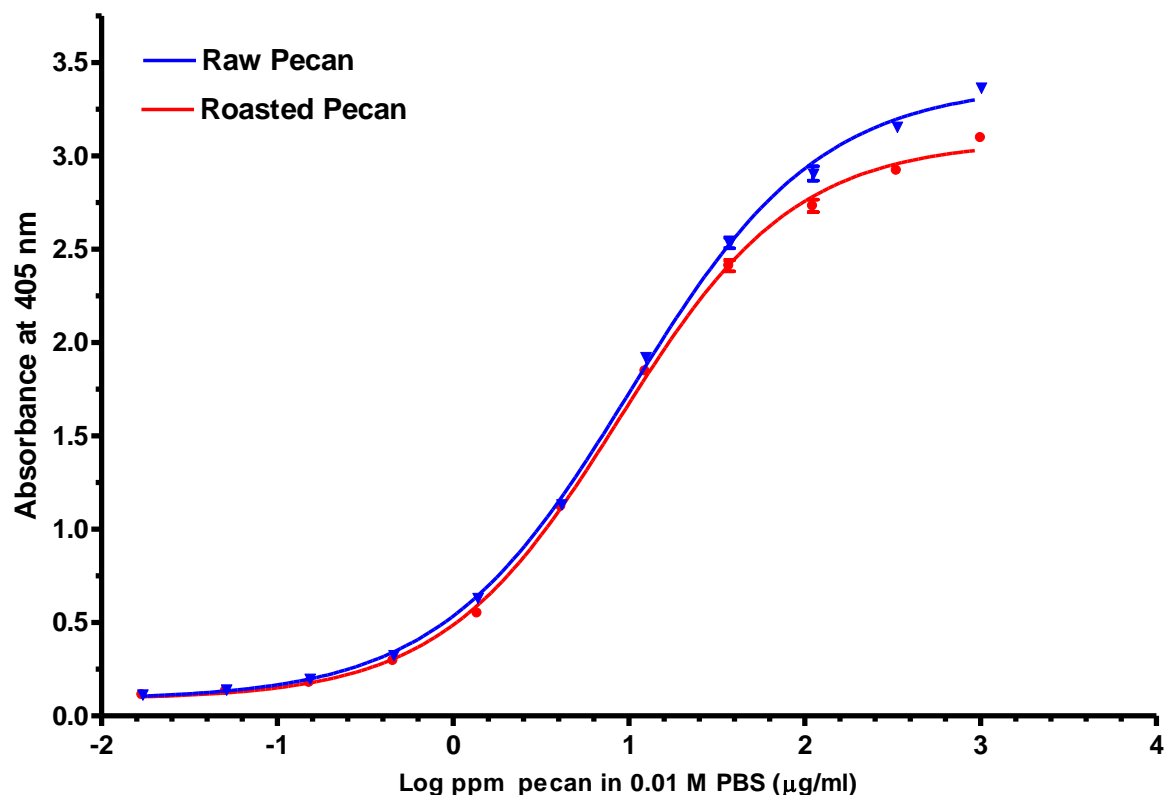


Figure 3.1. Raw and roasted pecan sandwich ELISA standard curves in 0.01 M PBS (1 M NaCl). The microtiter plates were coated with goat antisera (1:5,000) followed by incubation with various concentrations of raw or roasted pecan extract. Rabbit NE 270 antisera (1:4,000) was used as detection antibody and the conjugate antibody (anti-rabbit IgG produced in goat) was applied at a dilution of 1:5,000. Each data point represents the mean of six replicates with three analyses per replicate. (Each standard curve was derived from 18 readings with a mean standard error of < 0.02 AU).

Cross-Reactivity Studies

Cross-reactivity, defined as a positive response to a sample that does not contain any of the target antigens, can be a major problem for detection methods (Abbott et al., 2010). Cross-reactions arise because the cross-reacting antigen shares or has common epitopes which is structurally similar to the ones on the immunizing antigen (Mayer, 2006). The detection of cross-reacting antigens is more common with polyclonal antisera due to the heterogenous mixture of antibodies and their affinity to multiple epitopes. Determining cross-reactions is imperative in developing an ELISA to detect food allergens because false positive results can cause unnecessary product rejection or recall which are both time-consuming and costly to food companies. The specificity of the polyclonal antibodies utilized in the developed pecan ELISA was evaluated with 116 different foods (Table 3.1) including ingredients that may be present in processed foods containing pecan and foods botanically related to pecan such as walnut. Walnut was the only food that showed cross-reactivity in the pecan ELISA at an equivalent pecan level greater than 1 ppm. As previous studies have shown similar outcomes, this was not surprising because pecan and walnuts are part of the same family (Juglandaceae) and have been shown to contain proteins that share a high degree of amino acid sequence homology. Extracts of mustard seed, hazelnut, apple, and allspice displayed only minor (0.9, 0.4, 0.3, 0.3 ppm equivalent pecan level, respectively) cross-reactivity in the pecan ELISA. To further investigate the observed cross-reactivities, 1,000 ppm walnut and mustard seed extracts were serially diluted 3-fold and assessed in the pecan ELISA. The standard curves of both extracts as compared to pecan are shown in Figure 3.2. The 1,000 ppm diluted mustard seed extract produced a flat line which signified the cross reactivity

previously detected was attributed to a matrix issue. Mustard seed and the other minor cross-reactive foods are unlikely to cause a problem because they are incorporated into typical food formulations at much lower concentrations than the full-strength extracts tested in this study. On the other hand, walnut displayed a very prominent cross-reactive curve that would certainly affect the outcome of pecan detection in a walnut-containing food product. Nevertheless, more than 100 other food ingredients showed no reaction in the pecan ELISA including tree nuts such as almond, pistachio, Brazil nut, macadamia nut, and pine nut. The antibodies applied in the developed assay display pronounced specificity to pecan, but the accuracy and reliability of results are expected to be affected if the ELISA were used to detect pecan residues in a product that also contained walnut. It must be noted that the ability of the antibodies to also detect walnut be valuable as individuals who are allergic to pecan often try to avoid walnut as well.

Table 3.1. Cross-reactivity analysis of various food and food ingredients in the pecan ELISA

Ingredients	Protein Content (mg/ml)	Equivalent pecan level (ppm)	Ingredients	Protein Content (mg/ml)	Equivalent pecan level (ppm)
<u>Tree nuts</u>			<u>Legumes</u>		
Almond	7.77	< 0.3	Chickpea	8.92	< 0.3
English walnut	4.12	25.0	(garbanzo bean)		
Brazil nut	7.57	< 0.3	Green pea	6.89	< 0.3
Cashew	8.38	< 0.3	Lupine	21.55	< 0.3
Chestnut	1.85	< 0.3	Peanut	5.59	< 0.3
Chestnut (canned)	0.28	< 0.3	Soy lecithin	0.38	< 0.3
Coconut flakes	0.54	< 0.3	Soy milk	3.06	< 0.3
Hazelnut	9.80	0.38	Soybean flour	12.11	< 0.3
Macadamia nut	3.99	< 0.3	<u>Cereals</u>		
Pine nuts	5.45	< 0.3	Amaranth	2.15	< 0.3
Pistachio	11.60	< 0.3	Barley flour	1.65	< 0.3
<u>Seeds</u>			Buckwheat flour	8.78	< 0.3
Anise	4.84	< 0.3	Corn meal (yellow)	1.40	< 0.3
Caraway	ND	< 0.3	Oats	1.53	< 0.3
Celery	3.35	< 0.3	Potato flour	2.24	< 0.3
Celery	4.34	< 0.3	Quinoa flour	4.44	< 0.3
Fennel	3.60	< 0.3	Rice flour	1.08	< 0.3
Mustard Seed	11.53	0.7	Rye flour	1.34	< 0.3
Mustard (whole)	16.54	0.9	Sorghum flour	1.32	< 0.3
Poppy	4.82	< 0.3	Spelt	2.92	< 0.3
Sesame	6.12	< 0.3	Tapioca flour	0.64	< 0.3
Sunflower	5.48	< 0.3	Whole Wheat flour	1.88	< 0.3
Flax	5.67	< 0.3			

Protein content of extracts determined by the Lowry method, ppm = part per million ($\mu\text{g/ml}$), ND = not detected

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

Ingredients	Protein Content (mg/ml)	Equivalent pecan level (ppm)	Ingredients	Protein Content (mg/ml)	Equivalent pecan level (ppm)
<u>Baking</u>			<u>Spices/flavoring</u>		
Brown sugar	ND	< 0.3	Allspice	7.71	0.31
Butter (sweet cream)	0.39	< 0.3	Almond extract	ND	< 0.3
Cane syrup (pure)	0.13	< 0.3	Basil	8.63	< 0.3
Carob powder	2.59	< 0.3	Black pepper	3.37	< 0.3
Cocoa powder	6.90	< 0.3	Cinnamon	2.2	< 0.3
Coconut milk	0.53	< 0.3	Cloves	32.46	< 0.3
Corn syrup solids	ND	< 0.3	Cumin	7.71	< 0.3
Cream cheese	2.70	< 0.3	Garlic powder	4.68	< 0.3
Cream of tartar	ND	< 0.3	Ginger powder	6.55	< 0.3
Dark chocolate	3.23	< 0.3	Marjoram	10.19	< 0.3
Dried Egg white	34.87	< 0.3	Nutmeg	0.98	< 0.3
High fructose corn syrup	0.13	< 0.3	Onion powder	2.65	< 0.3
Honey	1.32	< 0.3	Oregano	11.04	< 0.3
Lemon juice	ND	< 0.3	Paprika	5.09	< 0.3
Light corn syrup	0.19	< 0.3	Vanilla extract	1.06	< 0.3
Liquid malt extract	6.84	< 0.3			
Maltodextrin	ND	< 0.3			
Molasses	4.39	< 0.3			
Orange (juice)	0.48	< 0.3			
Refined sugar cane	0.16	< 0.3			
Sorbitol	ND	< 0.3			
Yeast (active dry)	8.36	< 0.3			

Protein content of extracts determined by the Lowry method, ppm = part per million ($\mu\text{g/ml}$), ND = not detected

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

Ingredients	Protein Content (mg/ml)	Equivalent pecan level (ppm)	Ingredients	Protein Content (mg/ml)	Equivalent pecan level (ppm)
<u>Fruits/Veg</u>			<u>Oil</u>		
Apple (fresh)	0.28	0.32	Canola	ND	< 0.3
Apple (dried)	3.43	0.28	Corn oil	ND	< 0.3
Apricot (dried)	2.47	< 0.3	Olive	ND	< 0.3
Banana (dried chips)	0.35	< 0.3	Palm oil	ND	< 0.3
Banana (fresh)	0.89	< 0.3	Peanut	ND	< 0.3
Blueberry	0.66	< 0.3	Soybean	ND	< 0.3
Cherry	0.88	< 0.3	Hydrogenated vegetable	ND	< 0.3
Cranberry (dried)	1.61	< 0.3	Sunflower oil	ND	< 0.3
Dates (dried)	2.30	< 0.3	Walnut oil	ND	< 0.3
Kiwi	0.68	< 0.3	<u>Food coloring</u>		
Lemon	ND	< 0.3	Caramel color	32.28	< 0.3
Mango (dried)	0.91	< 0.3	FD & C Red 3	3.33	< 0.3
Mango (flesh and skin)	2.37	< 0.3	FD & C Red 40	11.00	< 0.3
Orange (flesh and skin)	0.09	< 0.3	FD & C Yellow 5	0.70	< 0.3
Peach	0.46	< 0.3	Yellow 6	11.64	< 0.3
Pineapple	1.01	< 0.3	<u>Functional ingredients</u>		
Plum (fresh)	ND	< 0.3	Guar gum	ND	< 0.3
Raisins	2.26	< 0.3	Xanthan gum	ND	< 0.3
Raspberry (fresh)	0.70	< 0.3	Carrageenan	ND	< 0.3
Strawberry (fresh)	0.73	< 0.3	Sodium Benzoate	ND	< 0.3

Protein content of extracts determined by the Lowry method, ppm = part per million ($\mu\text{g/ml}$), ND = not detected

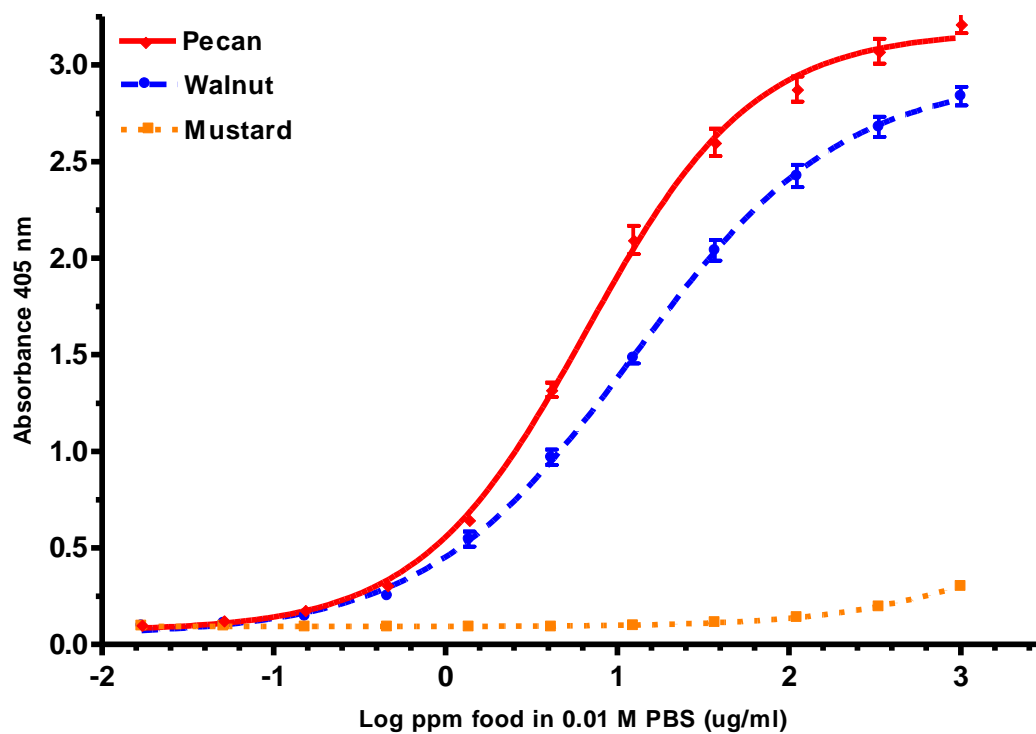


Figure 3.2. Evaluation of the cross-reactivity of walnut and mustard seed in the pecan ELISA. Standard curves were developed using 1000 ppm concentration extracts of each food serially diluted 3-fold in 0.01 M PBS (1 M NaCl). Each standard curve was derived from 4 measurements with an average standard error of < 0.10 AU.

Matrix Interference Studies

One of the common challenges of immunoassays for food analysis is matrix interference. The components of food matrices may impede critical procedural steps of immunoassays, such as allergen extraction or interference with antibody recognition of the targeted protein (Khuda et al., 2012). This interference can cause a shift in the overall standard curve thereby affecting the sensitivity of the assay. Three matrices were chosen to evaluate their possible effects on the developed pecan ELISA's sensitivity. Pecans are commonly used in baked products and confectionaries, increasing the risk of unintentional contamination to non-pecan varieties of the same food categories. Therefore, sugar cookies, vanilla ice cream, and dark chocolate were selected for the matrix interference study. To evaluate the effectiveness of the pecan ELISA in these different food matrices, the absorbance curves from the spiked food matrices were compared with the standard curve developed in PBS. The red, green, blue, and purple standard curves as shown in Figure 3.3 represent the standard curves generated by pecan spiked in PBS, vanilla ice cream, sugar cookies, and dark chocolate, respectively. Based on the statistical results using the ANOVA test, there were no significant differences ($p < 0.05$ significance level) in the means of the standard curves $F_{3,44} = 0.015$, $p = \text{ns}$, indicating that the pecan ELISA is efficient at detecting pecan in the presence of these complex food matrices. However, it is important to note that the dark chocolate matrix did cause the background value (the zero ppm pecan standard curve point) to increase which can contribute to a less sensitive limit of detection as well as a decline in the signal-to-noise ratio. Dark chocolate has the notoriety of being one of the more

complicated food matrices in terms of challenging the ELISA method. Not only does chocolate contain interfering components such as tannins and other polyphenols, the physical texture and fat content can cause difficulties in preparing homogeneous samples and antibody detection (Khuda et al., 2012). Despite the slight decrease in sensitivity due to the dark chocolate matrix, the nature of the sample did not significantly modify the recognition of pecan by the antibody. Overall, the comparable standard curves indicate that these food matrices did not significantly affect the assay's performance, and the sandwich ELISA described here is efficient in detecting pecan in their presence.

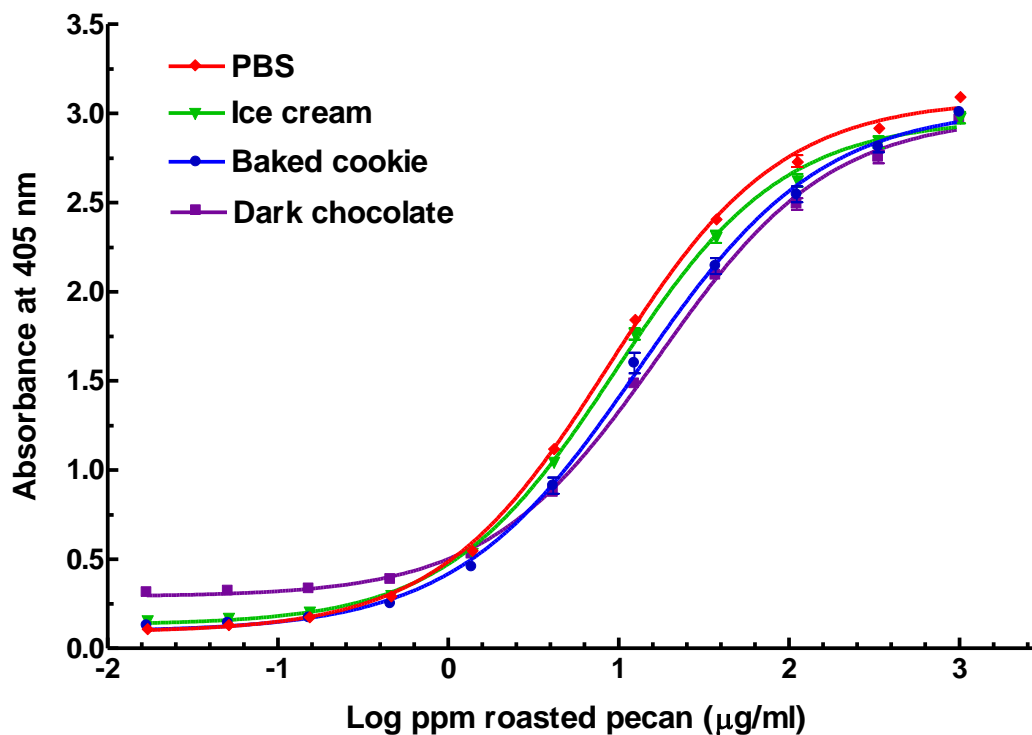


Figure 3.3. Roasted pecan spiked baked sugar cookie, vanilla ice cream, and dark chocolate standard curves as determined by the pecan ELISA. Ground roasted pecan extract was spiked into 0.01 M PBS (1 M NaCl) (red), baked sugar cookie (blue), vanilla ice cream (green), and dark chocolate (purple) to achieve a 1000 ppm pecan standard and appropriately diluted 1:3 (v/v). Each data point represents 18 readings (6 replicate trials with 3 analyses per replicate), with an average standard deviation of < 0.04 AU.

Effect of Extraction Buffer Additives on Dark Chocolate Matrix

The ability of an ELISA method to detect food allergens is affected by the efficiency with which these proteins are extracted from the sample. The buffer for sample preparation is a critical aspect for efficient extraction and accurate quantification of an analyte in food samples. Processed foods often require robust extraction buffers and protocols due to their complex nature. Although the previously evaluated dark chocolate matrix did not significantly affect the detection of pecan in the developed ELISA, the high background value was of concern. Therefore, further evaluation on the dark chocolate matrix was performed to determine if the nonspecific binding could be resolved and lower the high background value.

Chocolate is a problematic food matrix for the ELISA method. This issue has particularly affected the detection of peanut in various studies (Holzhauser et al., 2002; Hurst et al., 2002; Keck-Gassenmeier et al., 1999; Newsome & Abbot, 1999; Pomes et al., 2003). Dark chocolate or chocolate with high cocoa content commonly create problems to protein extraction due to the abundant amount of tannins and other phenolic compounds. These compounds can bind proteins during extraction and may also disturb or fully inhibit antibody-antigen reactions of ELISA (Holzhauser et al., 2002; Keck-Gassenmeier et al., 1999). The utilization of additives in the extraction buffer has demonstrated enhanced extraction efficiency and reduction of non-specific binding. Polyvinyl-polypyrrolidone (PVPP) is often used to circumvent the problems experienced with phenolic compounds in sample preparation (Charmont et al., 2005). A non-ionic detergent such as Tween-20 is an additive commonly used to help reduce non-specific

binding. Extraction buffer containing fish gelatin has been associated with improving allergen detection. For example, when Keck-Gassenmeier and colleagues (1999) used the extraction buffer supplied in a peanut commercial ELISA kit, only 2-3% of peanut added to dark chocolate could be recovered. By using a fish gelatin containing extraction buffer, recoveries of 60-90% and a detection limit of 2 mg peanut protein/kg product were achieved for this matrix. Holzhauser et al. (2002) also took this approach and incorporated 10% of fish gelatin into their sample extraction buffer which allowed them to successfully detect hazelnut in dark chocolate.

Another additive that has been employed to overcome the problem of high-cocoa containing matrices is non-fat dry milk (NFDM). Pomes and colleagues (2004) showed that the addition of NFDM to the extraction buffer could have the same effect as fish gelatin in previous studies by binding the tannins in chocolate that sequester target proteins. Their optimized extractions procedure for Ara h 1 in chocolate was 5% NFDM in PBS with 1 M NaCl for 2.5 hours at room temperature. These researchers also showed that no significant differences between the amounts of Ara h 1 extracted from chocolate products when extraction was carried out at 60°C for 15 minutes versus 2.5 hours at room temperature.

The described additives (PVPP, Tween-20, fish gelatin, NFDM) were chosen in this study to evaluate their effects on dark chocolate sample preparation when incorporated into the extraction buffer. Extraction of 1,000 ppm pecan standards and the development of curves in the pecan ELISA were carried out as previously described in the matrix interference study with the addition of the various additives into the buffer

prior to extraction. Figure 3.4 shows that the various additives did not decrease the high background absorbance characteristic of the dark chocolate matrix, and a slight shift of the curves to the right was displayed by the addition of fish gelatin. The comparison of the curves also indicated that the addition of NFDM in the extraction buffer contributes greatly to creating a suitable standard curve, given that the background absorbance doubled when no NFDM was present in the extraction buffer. Although the addition of PVPP slightly decreased the background value, an undesirable reduction in the dynamic range of the curve also resulted. The extraction buffer with only 1% NFDM provided the best results among the tested additives and was carried on to the subsequent study involving manufactured model foods. Although detection of pecan is not significantly hampered by the dark chocolate matrix, it is important that the high background effect be addressed when analyzing pecan in this matrix to ensure appropriate caution.

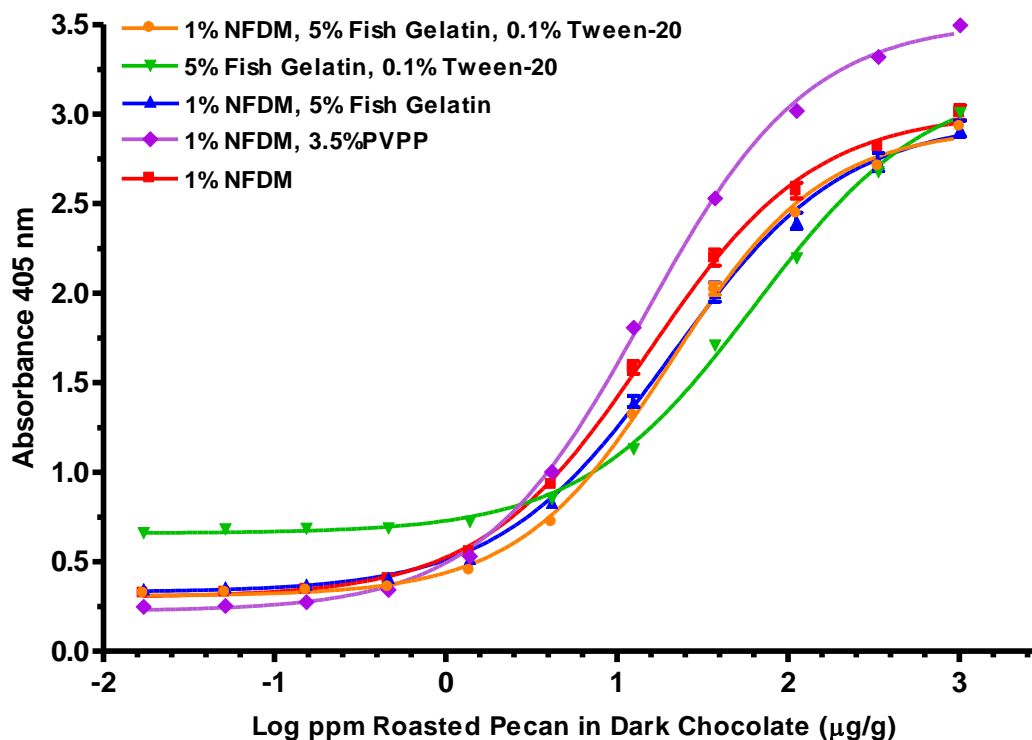


Figure 3.4. Comparison of extraction additives use for optimization of a roasted pecan spiked dark chocolate standard curves as determined by the pecan ELISA. A 1,000 ppm standard prepared by adding 5 mg of ground roasted pecan to 5 g of dark chocolate followed by extraction with 0.01 M PBS (1 M NaCl, pH 7.2) containing various additives. The 1000 ppm pecan extracts were serially diluted 1:3 (v/v) and analyzed in the pecan ELISA. Each standard curve was derived from 2 replicate trials with 3 analyses per replicate with an average standard error of <0.02 AU.

CONCLUSIONS

The animal IgG antibodies utilized in this ELISA are robust enough to detect raw and roasted pecan equally. Therefore, a standard curve produced by roasted pecan can be applied when detecting pecan in both the unprocessed and processed form. The antibodies displayed adequate specificity to pecan with no cross-reactions to more than 100 foods. However, the developed ELISA would not be reliable for the detection of pecan residues in a product that also contained walnut due to the high degree of cross-reactivity. Dark chocolate, ice cream, and baked cookie matrices spiked with pecan extract did not significantly affect the assay's performance although the dark chocolate matrix contributed to a higher background value. None of the tested additives were capable of significantly reducing the high background effect of the dark chocolate matrix, but the extraction buffer with only 1% NFDM provided the best results among the tested additives and should be utilized for sample preparation so long as the difficult matrix of dark chocolate is acknowledged.

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

Introduction

Technological progress in food manufacturing and recent discoveries in nutrition and food science have resulted in increasingly high food quality standards and enormous food variety. Globally, the food processing industry has a significant economic impact. With an increase in urban populations, demand for processed foods continues to increase (Sathe & Sharma, 2009). Adaptations to the modern fast-paced lifestyle have led to increased commercialization of processed, prepackaged food products to keep up with people's demand for convenience and variety. Some of the many changes in the way popular foods are produced include greater use of machines to reduce processing times, improve shelf life, and develop superior textural properties (Alvarez & Boye, 2012). As with any production operation, there are many drawbacks and concerns that accompany new innovations. One of the growing concerns is the unintended incorporation of hazards, such as food allergens, into processed foods. Therefore, food manufacturing plants need to establish effective allergen control plans to relieve consumers from unnecessary risk. Ice cream, bakery, and candy manufacturers are particularly prone to cross-contamination concerns due to common production practices such as sharing of production equipment for manufacturing of foods with different lists of ingredients and the inability to utilize wet-cleaning methods in bakery and confectionary processing plants. Reliable allergen detection tools are an important part of an allergen management plan because they monitor and validate sanitation procedures as well as establish accurate

product labeling. Although testing methods for many of the priority food allergens have been developed and commercialized with excellent sensitivity and selectivity, they are still subject to inaccuracies due to matrix and processing effects (Alvarez & Boye, 2012). Food allergens have a complex structure that can be altered or impaired during food processing. For example, proteins may denature which can disrupt the tertiary and secondary structure or modify conformational epitopes. Maillard reactions or partial hydrolysis as a result of food processing can modify linear epitopes. Food allergen proteins are susceptible to aggregation and loss of solubility. All of these modifications can affect the extractability of the protein as well as the interaction between the antibodies and the allergens, leading to erroneous results (Cucu et al., 2013). It is therefore essential to ensure that the maximum amount of the targeted analyte is extracted and detected in any analytical method.

The robustness of analytical methods for detecting a given food allergen is commonly evaluated on the basis of determined recovery after spiking allergen-free products shortly before the extraction step. Although this is considered an acceptable way to generate information about the method's performance in specific matrices, spiked samples may result in an artificially higher recovery, disregarding the possible effects of processing (Cucu et al., 2013). The most accurate representation of the recovery and response of a particular method can be achieved through incurred samples. In this method, known amounts of the food allergen are incorporated before or during processing, mimicking as closely as possible, the actual manufacturing conditions under which the sample matrix would be subjected. The processed model foods ensure that the

analytical method is able to detect the food allergen in the final product. It is expected that this approach would mitigate false negatives that could be detrimental not only to food allergic consumers but also food manufacturers if improper labeling transpires. Validation studies for allergen detection tests should be run using incurred samples when feasible even though the preparation of such samples may be more costly and difficult (Abbott et al., 2010).

Because pecan, like other tree nuts, is an important food allergen and is frequently included as an ingredient in desserts and baked products, ice cream and cookies were chosen as model foods to validate the developed ELISA's performance. Priority was not only given to these two food matrices because they are most likely to be contaminated by pecan, but also because it provided the opportunity to investigate the applicability of the method for foods that have gone through a freezing or baking process. Therefore, the present study focused on assessing the impact of processing on the performance of the pecan ELISA by producing vanilla ice cream and sugar cookie incurred food matrices on a laboratory-scale that closely mimicked treatments likely applied during industrial processing.

MATERIALS AND METHODS

Preparation of Model Foods

Vanilla Ice Cream

The recipe for vanilla ice cream was adapted from the website of allrecipes.com – vanilla ice cream (<http://allrecipes.com/recipe/vanilla-ice-cream-2/detail.aspx>), and the

model food was prepared as previously described by Lim (2011) with slight modifications. The ingredients used for formulating vanilla ice cream were heavy whipping cream, half and half cream, white granulated sugar and vanilla extract. The basic formulation for the ice cream is shown in Table 4.1. All ingredients were purchased from local grocery stores in Lincoln, Nebraska and pre-screened for the possible presence of pecan or cross-reactive proteins using the developed ELISA. Each ingredient was added according to the unit of mass (gram, g) to facilitate consistency. Before preparing nine individual batches of vanilla ice cream (670 g each), each containing different added levels of pecan (0, 1, 2, 2.5, 5, 10, 25, 50, 100 ppm), a 112,100 ppm powdered pecan-sugar mix was prepared (ground pecan in white sugar) by grinding 1.121 g of ground pecan with 112.1 g of sugar in a KrupsTM coffee grinder. This highly concentrated spike material was generated to help mitigate problems regarding non-homogeneity when low levels of analyte are incorporated into large sample batches. Four sub-samples were collected from the 112,100 ppm powdered pecan-sugar mix and each was extracted 1:10 (w/v) with phosphate buffered saline (0.01 M PBS, 1 M NaCl, pH 7.2) containing 1% non-fat dry milk (NFDM). After extracting at 60 °C for 1 hour in a shaking water bath (Julabo SW22, Julabo USA, Inc., Allentown, PA), the extracts were centrifuged at 3,612 x g (4100 rpm) for 30 minutes using a table top centrifuge (Sorvall® LegendTM RT, Kendro Laboratory Products, Newton, CT). The supernatant was collected and filtered through a 0.45 µm membrane. The clarified supernatant was then analyzed using the pecan ELISA to verify the uniformity of mixing. The homogenous 112,100 ppm powdered pecan-sugar mixture was then appropriately added with the other ice cream

ingredients in each batch to achieve eight different incurred pecan levels. The batches of ice creams were prepared independently in ascending order by starting from the lowest level (0 ppm) and continuing to the highest level (100 ppm). The ice cream maker was thoroughly cleaned between each batch of ice cream to avoid any pecan protein carryover by washing with warm soapy water followed by rinses of distilled water and left to thoroughly air dry. Before making the ice cream, the Speedee Freeze™ Double Insulated Canister belonging to the ice cream maker (Deni Scoop Factory Compact Automatic Ice Cream & Frozen Dessert maker, Model 5000, Keystone Manufacturing Company, Buffalo, NY) was kept in the freezer overnight every time before use. In addition, designated amounts of heavy whipping cream, half and half, and sugar were prepared and stored in the refrigerator to chill until use. On the following day, a specific amount of vanilla extract was added to the chilled ice cream mixture, and the ice cream maker was set up by placing the stir paddle inside the frozen canister, locking on the Clear-View™ lid, and attaching the motor on top. The unit was turned on, and the chilled ice cream mixture was carefully poured into the rotating canister. After 10 minutes of rotation, when the ice cream achieved a soft, pudding-like texture, specific amounts of powdered sugar and the powdered pecan-sugar mix were added to attain the desired level of pecan in each batch of ice cream. Rotation continued for an additional 10 minutes to homogenously distribute the pecan while the ice cream formed a firmer texture. The finished ice cream was kept in labeled Ziploc bags and stored in the freezer at -20°C. The frozen vanilla ice cream from each level were tested in 3 independent trials for recovery of pecan using the developed ELISA.

Table 4.1. Formulation for naturally incurred pecan in vanilla ice cream

Naturally incurred standards in vanilla ice cream (g, g)									
Ingredients	100 ppm ^a	50 ppm	25 ppm	10 ppm	5 ppm	2.5 ppm	2 ppm	1 ppm	0 ppm
Heavy whipping cream	282.7	282.7	282.7	282.7	282.7	282.7	282.7	282.7	282.7
Half & half cream	282.7	282.7	282.7	282.7	282.7	282.7	282.7	282.7	282.7
White sugar	89.65	89.65	89.65	89.65	89.65	89.65	89.65	89.65	89.65
Vanilla extract	9	9	9	9	9	9	9	9	9
Powdered sugar	5.38	5.68	5.83	5.92	5.95	5.965	5.968	5.97	5.98
Powdered pecan-sugar mix	0.6	0.3	0.15	0.06	0.03	0.015	0.012	0.006	0
Total	670	670	670	670	670	670	670	670	670

^appm = parts per million; µg/g

Sugar Cookies

Similar to the ice cream preparation, all ingredients for the sugar cookies were purchased from local grocery stores in Lincoln, Nebraska and pre-screened for the possible presence of pecan or cross-reactive proteins using the developed ELISA. A concentrated spike level of pecan in sugar was also prepared prior to making the sugar cookies for easier incorporation into other ingredients and even distribution of pecan in the sugar cookies. Using a Krups™ coffee grinder, 0.68 g of pecan and 50 g of granulated white sugar were mixed and finely ground together to create a 13,606 ppm powdered pecan-sugar mix. Ten grams of this mix was subsequently combined with 992 grams of Betty Crocker™ sugar cookie mix in a Kitchen Aid™ 5 Quart Artisan Mixer (Kitchen Aid, St. Joseph, MI) and mixed for 30 minutes to obtain a final 135.8 ppm pecan concentration. Afterwards, four sub-samples were collected from the powdered pecan-sugar mix and each was extracted and analyzed in the pecan ELISA as described in the ice cream preparation. After homogeneity of the 135.8 ppm working spike was verified, appropriate amounts were added with the other sugar cookie ingredients in each batch to achieve eight different incurred pecan levels (1, 2, 2.5, 5, 10, 25, 50, 100 ppm). The ingredients for the sugar cookies included commercial Betty Crocker™ sugar cookie mix, butter, and egg. The specific formulation for each incurred standard is shown in Table 4.2.

Each of the nine different levels was prepared individually starting from 0 ppm to the next lowest pecan level. To achieve each desired level, appropriate amounts of the 135.8 ppm working standard were combined with the appropriate amount of Betty

Crocker™ sugar cookie mix, and the dry ingredients were mixed for 30 minutes in the Kitchen Aid™ mixer before adding the rest of the ingredients. After all the ingredients were combined, the batter was mixed for a total of 15 minutes. To help attain well distributed incurred levels of pecan for each batch, the mixer was stopped at 5 minute intervals and the dough was manually folded by bringing the dough at the bottom of the mixing bowl to the top using a spatula. The mixing bowl and utensils were thoroughly cleaned with warm soapy water followed by rinsing with distilled water, and finally air dried between each batch to prevent any carryover of pecan proteins. All levels of cookie dough were kept in labeled Ziploc bags and stored at 4°C until use. For each individual batch, the sugar cookies were weighed out to approximately 30 g and rolled into balls prior to placing the dough on aluminum covered baking sheets. Each level of pecan incurred cookies was separately baked at 375°F for 15 minutes. The baked cookies were allowed to cool and then were weighed to determine the moisture loss of the cookies after baking. The remaining sugar cookie dough and three fresh baked cookies from each level were taken and tested in 3 independent trials for recovery of pecan using the developed ELISA.

Table 4.2. Formulation for naturally incurred pecan in sugar cookies

Naturally incurred standards in sugar cookies (g, g)									
Ingredients	100 ppm^a	50 ppm	25 ppm	10 ppm	5 ppm	2.5 ppm	2 ppm	1 ppm	0 ppm
Pecan-free sugar cookie mix (negative control)	0	248	372	446.4	471.2	483.6	486.1	491	496
Pecan sugar cookie mix (135.8 ppm)	501	250.5	125.3	50.1	25.05	12.52	10.02	5.01	0
Powdered sugar	0	2.5	3.75	4.5	4.75	4.875	4.9	4.95	5
Butter	118.3	118.3	118.3	118.3	118.3	118.3	118.3	118.3	118.3
Egg	61	61	61	61	61	61	61	61	61
Total weight	680.3	680.3	680.3	680.3	680.3	680.3	680.3	680.3	680.3

^appm = parts per million; µg/g

Extraction and Evaluation of Manufactured Model Foods

Vanilla Ice Cream

Three subsamples from each level were collected, and the 0 ppm ice cream was used as the negative control. A 1,000 ppm pecan standard or positive control was prepared by adding 5 mg of ground roasted pecan to 5 g of the negative control (0 ppm) vanilla ice cream. All three subsamples from each incurred level as well the positive and negative control samples were allowed to melt before extracting 1:10 (w/v) with 0.01 M PBS (1 M NaCl, pH 7.4) containing 1% NFDm. Extractions were carried out in a 60°C shaking water bath (Julabo SW22, Julabo USA, Inc., Allentown, PA) for 1 hour. Sample extracts were then centrifuged at 3,612 x g (4,100 rpm) for 30 minutes at 10°C in a tabletop centrifuge (Sorvall® Legend™ RT, Kendro Laboratory Products, Newton, CT). The supernatant was collected and filtered through a 0.45 µm membrane. The clarified supernatants were analyzed in the pecan ELISA for recovery of pecan.

A standard curve was developed by serially diluting the positive control 1:3 (v/v) in the negative control ice cream extract to achieve 12 pecan standards (1000, 333, 111, 37, 12.3, 4.11, 1.37, 0.45, 0.15, 0.05, 0.02, and 0 ppm pecan). The standards were used alongside the vanilla ice cream samples during the ELISA analysis to determine the relative amount of pecan recovered in each sample. Standard curves were constructed using a four parameter logistic equation in GraphPad Prism v.4.03 software (GraphPad Software Inc., San Diego, CA). The percent recovery of pecan from the vanilla ice cream at each level was calculated as a ratio of the recovered ppm level to the ppm level of added pecan, and was expressed as the mean ± standard error of the mean based on the

results obtained from triplicate extractions of each model food that was analyzed in 3 trials on separate days.

Sugar Cookies and Cookie Dough

Prior to extraction and analysis, three subsamples of baked sugar cookies from each level (0 to 100 ppm) were individually ground to a fine particle size using an Osterizer® blender (Sunbeam Corporation, Delray Beach, FL). The 0 ppm cookie dough and baked sugar cookies were used as negative controls. Positive control standards were prepared by adding 5 mg of ground roasted pecan to 5 g of the negative control (0 ppm) cookie dough and baked sugar cookie to obtain a 1,000 ppm pecan spiked cookie dough standard and a 1,000 ppm pecan spiked baked sugar cookie standard, respectively. All subsamples of cookie dough and baked sugar cookies from each incurred level along with the positive and negative control samples were extracted as described above for ice cream.

The standard curves for the cookie dough and the baked sugar cookies were individually developed by serially diluting the positive control 1:3 (v/v) in the negative control extract to achieve 12 pecan standards (1000, 333, 111, 37, 12.3, 4.11, 1.37, 0.45, 0.15, 0.05, 0.02, and 0 ppm pecan). The appropriate standards were tested alongside the cookie samples during the ELISA analysis to determine the relative amount of pecan recovered in each sample. The standard curves were constructed using a four parameter logistic equation in GraphPad Prism v.4.03 software (GraphPad Software Inc., San Diego, CA). The percent recovery of pecan from the cookie dough and baked sugar cookies were calculated as a ratio of the recovered ppm level to the ppm level of added

pecan. The moisture loss during baking was also taken into account to calculate the actual ppm level of added pecan available in the sugar cookies after baking. The final calculated percent recoveries of pecan from each manufactured model foods were expressed as the mean \pm standard error of the mean based on the results obtained from triplicate extractions of each model food that was analyzed in 3 trials on separate days.

RESULTS AND DISCUSSION

Recovery of Pecan from Vanilla Ice Cream

The standard curve used to determine the relative amount of recovered pecan from the ice cream model was prepared by spiking pecan into the negative control and serial diluting the 1,000 ppm pecan-spiked standard 1:3 (v/v). Because food matrices may alter the detection of pecan, applying a standard curve produced in the negative control food matrix to determine the relative amount of pecan recovered in that same matrix generates results that are more accurate. Figure 4.1 shows the standard curve developed in negative control vanilla ice cream as compared to the standard curve produced in PBS containing 1% NFDM. Using an unpaired t-test at the $p < 0.05$ significance level (GraphPad Prism® v.4.03 software), no significant difference was shown between the ice cream and PBS curves, $t(22) = 0.295$, $p = \text{ns}$.

The recovery of pecan from vanilla ice cream at various levels obtained from 3 trials is shown in Table 4.3. Overall, excellent recovery of pecan was obtained with a mean percent recovery of $103\% \pm 4.28\%$, indicating that the mixing and freezing process

required to produce the ice cream does not affect the antibodies in the developed pecan ELISA from reliably detecting pecan, even at low ppm pecan concentrations.

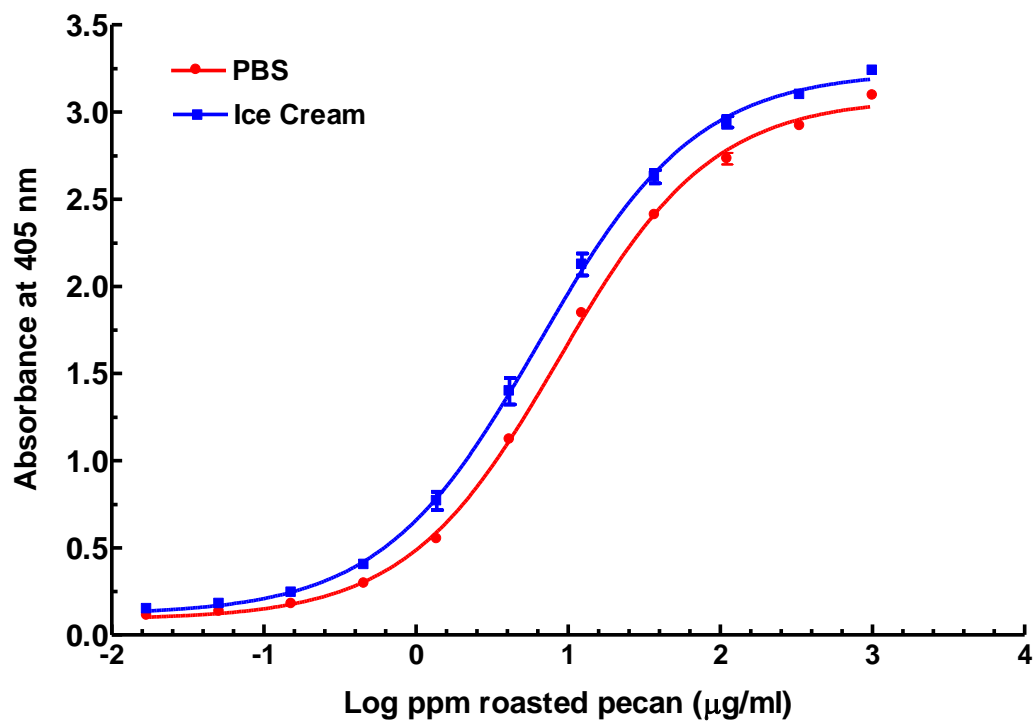


Figure 4.1. Comparison of vanilla ice cream and PBS (1 M NaCl; containing 1% NFDM) standard curves. A 1,000 ppm standard prepared by adding 5 mg of ground roasted pecan to 5 g of vanilla ice cream negative control or 5 ml of 0.01 M PBS (1 M NaCl) was serially diluted 1:3 (v/v) and analyzed in the pecan ELISA. Each data point represents the mean of 18 readings, with a standard deviation of < 0.04 AU.

Table 4.3. Recovery of pecan from the manufactured vanilla ice cream model, as determined by the pecan ELISA.

VANILLA ICE CREAM		
Incurring pecan level (ppm ^a)	Mean ppm recovery ^b	% recovery ^c
0	BLQ ^d	NA ^e
1	1.04 ± 0.02	104 ± 1.90
2	1.82 ± 0.05	91.0 ± 2.62
2.5	2.95 ± 0.14	118 ± 5.82
5	5.18 ± 0.23	104 ± 4.65
10	9.77 ± 0.21	97.7 ± 2.06
25	23.8 ± 0.09	95.4 ± 0.38
50	54.2 ± 5.55	108 ± 11.1
100	107 ± 5.70	108 ± 5.70

^appm-parts per million (mg of ground pecan per kg)

^bData are mean ± standard error (n=3 trials)

^cPercent recovery calculated as ratio of average ppm pecan recovered to the available pecan in the finished product

^dBLQ – below limit of quantification

^eNA – not applicable

Recovery of Pecan from Cookie Dough and Baked Sugar Cookies

The standard curve used to determine the relative amount of recovered pecan from the prepared cookie dough and baked sugar cookies were prepared by spiking pecan into the corresponding negative controls and serial diluting the 1,000 ppm pecan-spiked standard 1:3 (v/v). Producing standard curves in negative control food matrices instead of simple buffers to determine analyte recovery in model foods takes into account the effect that food components in that particular matrix may have on detection, allowing a more appropriate estimate of the recovery. The standard curves developed in negative control cookie dough and baked sugar cookies compared to the standard curve produced in PBS containing 1% NFDM are shown in Figure 4.2. The results of a one-way ANOVA test (GraphPad Prism® v.4.03 software) confirms that there is no significant difference among the curves, $F_{2,33} = 0.021$, $p = \text{ns}$ ($p < 0.05$ significance level).

The recovery of pecan obtained from 3 trials in the unbaked cookie dough and baked sugar cookies at various levels is shown in Table 4.4. Compared to the ice cream model, somewhat lower recovery of pecan residues was obtained from the baked sugar cookies with a mean percent recovery of $87.0\% \pm 5.45\%$ after compensation for the moisture loss after baking ($8.90\% \pm 1.80\%$). The exposure to high heat during the baking process can affect the immunological and physiochemical properties of proteins within a food matrix. This can lead to a loss in solubility or interference of antigen-antibody reactions and epitope binding due to aggregation, changes in conformation, or protein-carbohydrate interactions such as the Maillard browning reaction (Davis et al., 2001; Koppelman et al., 2002; Mills et al., 2009). These events can possibly reduce the

quantitative extraction of protein from the matrix as well as decrease antigen detection. Various studies have demonstrated low recovery of allergens after the baking process. For example, the mean percent recovery from two different ELISAs developed to detect cashew and pistachio residues had a mean percent recovery of 84% and 54%, respectively, after incurred sugar cookies were baked (Gaskin & Taylor, 2011; Lim, 2010). Cucu et al. (2012) also examined the performance of a developed competitive ELISA using incurred cookie models. The results showed a significantly lower (10-18%) recovery of hazelnut after baking. In addition, the mean percent recovery from a developed buckwheat ELISA was approximately 60% from baked muffins incurred with buckwheat (Panda et al., 2010). Compared to these studies, the overall recovery of pecan by the developed ELISA was quite sufficient for the cookies before and after the baking process. Although there was a decrease in recovery of pecan encountered at the lower incurred levels (1-2.5 ppm) for the baked sugar cookies, the impact was relatively low, and the developed pecan ELISA would still be suitable for monitoring purposes.

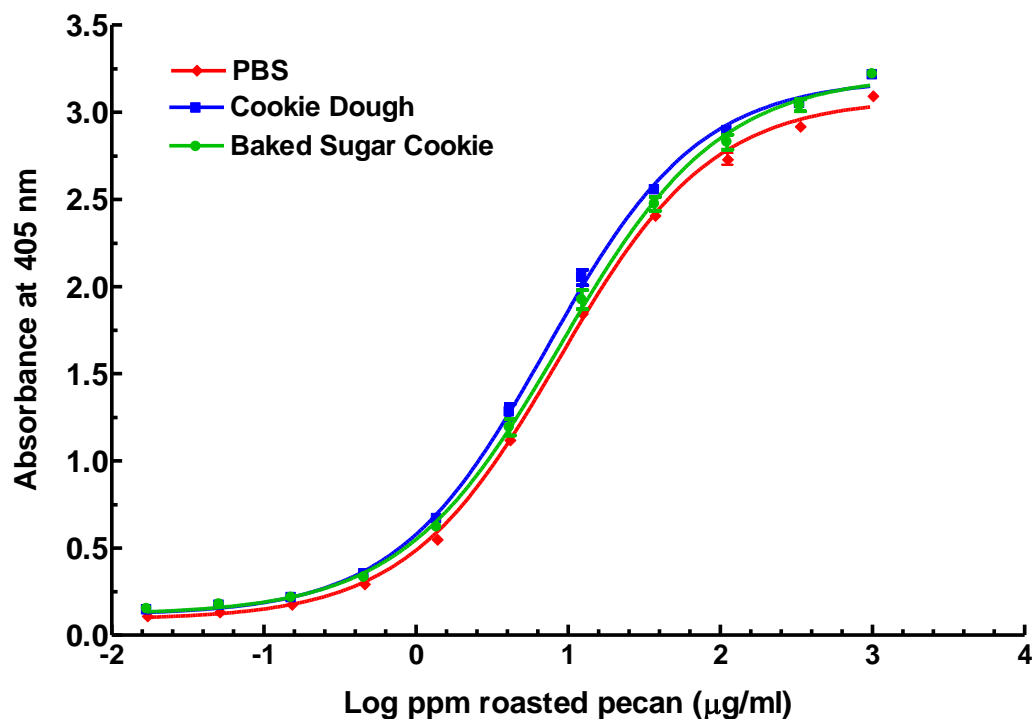


Figure 4.2. Comparison of cookie dough, baked sugar cookie and PBS (1 M NaCl; containing 1% NFDM) standard curves. A 1,000 ppm standard prepared by adding 5 mg of ground roasted pecan to 5 g of cookie dough or baked sugar cookie negative control was serially diluted 1:3 (v/v) and analyzed in the pecan ELISA. Each data point represents the mean of 18 readings, with a standard deviation of < 0.04 absorbance unit (AU).

Table 4.4. Recovery of pecan from the manufactured sugar cookie model, as determined by the pecan ELISA.

Incurred Pecan level (ppm) ^b	Dough (Before baking)		Sugar Cookie (After baking) ^a	
	Mean ppm Recovery ^c	% Recovery ^d	Mean ppm Recovery ^c	% Recovery ^d
0	BLQ ^e	NA ^f	BLQ ^e	NA ^f
1	0.93 ± 0.04	92.4 ± 4.29	0.65 ± 0.05	59.2 ± 4.80
2	1.87 ± 0.05	93.4 ± 2.34	1.82 ± 0.09	82.9 ± 4.24
2.5	2.66 ± 0.10	104 ± 3.30	1.95 ± 0.20	70.8 ± 7.30
5	4.76 ± 0.18	95.2 ± 3.40	5.22 ± 0.36	95.1 ± 6.62
10	10.3 ± 0.23	104 ± 2.37	10.4 ± 0.75	94.9 ± 6.87
25	24.8 ± 0.18	98.4 ± 1.20	29.4 ± 1.98	107 ± 7.22
50	48.8 ± 2.38	97.6 ± 4.76	52.7 ± 2.10	96.0 ± 3.82
100	105 ± 8.24	105 ± 8.24	110 ± 6.64	96.8 ± 3.01

^aAmount of pecan available after baking was calculated from moisture loss of cookie samples after baking (mean moisture loss from cookie samples= 8.90 % ± 1.80%)

^bppm-parts per million (mg of ground pecan per kg)

^cData are mean ± standard error (n=3 trials)

^dPercent recovery calculated as ratio of average ppm pecan recovered to the available pecan in the finished product

^eBLQ – below limit of quantification

^fNA – not applicable

CONCLUSIONS

Because contamination of food products with allergens is most likely to occur before any food processing conditions are applied, the spike-and-recovery method commonly used to assess a method's performance does not necessarily represent results regarding real-life situation (Cucu et al., 2012). The naturally incurred standards into model foods demonstrated in this study is a more relevant approach for validating an assay's performance. The high recovery means of $103\% \pm 4.28\%$ and $87.0\% \pm 5.45\%$ from both the manufactured vanilla ice cream and sugar cookie models, respectively, confirm that the extraction protocol used in this study is sufficient in extracting soluble pecan proteins and that the processes applied to the foods did not significantly affect the antibodies' ability to detect the pecan proteins. The developed ELISA is highly sensitive and can be utilized by the food industry to detect and quantify pecan residues in processed foods.

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