EFFECT OF SELECTED NONTHERMAL PROCESSING METHODS ON THE ALLERGEN REACTIVITY OF ATLANTIC WHITE SHRIMP (*LITOPENAEUS SETIFERUS*)

By

SANDRA KATE SHRIVER

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To all those who suffer from food allergies
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<td>BSA</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<td>EIA</td>
<td>Enzyme immunoassay</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>HHP</td>
<td>High hydrostatic pressure</td>
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<td>IgE</td>
<td>Immunoglobulin E</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<td>NTP</td>
<td>Nonthermal plasma</td>
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<td>OPD</td>
<td>O-phenylenediamine dihydrochloride</td>
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<td>PUV</td>
<td>Pulsed ultraviolet light</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide electrophoresis</td>
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EFFECT OF SELECTED NONTHERMAL PROCESSING METHODS ON THE ALLERGEN REACTIVITY OF ATLANTIC WHITE SHRIMP (LITOPENAEUS SETIFERUS)

By

Sandra Kate Shriver

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Food allergies are adverse reactions to food components and can cause moderate to severe symptoms including death. To date, there is no cure for food allergy and often the only relief comes from complete avoidance of the problem foods. Thus, a demand for methods aimed to reduce allergens from food products is becoming a popular topic of study. Food processing techniques have the ability to alter food components such as allergens. In this study, the efficacy of using emerging nonthermal methods to reduce the detectable levels of the major shrimp allergen, tropomyosin (36-kDa), and attenuate immunoglobulin E (IgE) binding to shrimp extract was examined. Pulsed ultraviolet light (PUV) technology, which has been shown to reduce allergen levels in peanut and soybean samples, was the focus of this research. High hydrostatic pressure (HHP) and nonthermal plasma (NTP) processing were also examined. Atlantic white shrimp (Litopenaeus setiferus) extract was treated with PUV (3 pulses/s, 10 cm from light source) for 4 min. For NTP treatment, plasma was generated using a voltage of 30 kV and a frequency of 60 Hz and treatment was carried out for 1, 3, and 5 min at ambient temperature. To determine the effects of HHP, shrimp extracts were treated for 15 min
at temperatures of 4, 21, and 70°C and a pressure of 600 MPa. Tropomyosin was compared in the untreated and treated shrimp samples by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblot, and enzyme-linked immunosorbent assay (ELISA). Results showed that levels of detectable tropomyosin and IgE binding to tropomyosin were reduced following PUV or NTP treatment. Dot blot and ELISA confirmed a decrease in tropomyosin reactivity following PUV or NTP treatment. Furthermore, PUV-treated samples were subjected to simulated human digestion to examine allergen stability. The PUV-treated samples remained less-reactive under simulated gastric and intestinal digestive conditions. Lastly, treating shrimp extract with HHP did not change allergen reactivity under the treatment conditions tested in this study. In conclusion, although HHP has little effect on shrimp allergen reactivity, PUV and NTP may be promising methods to reduce the allergenic potential of Atlantic white shrimp for the future manufacturing of less-allergenic food products.
CHAPTER 1
INTRODUCTION

The major heat-stable allergen of shrimp is a 34- to 38-kDa protein known as tropomyosin, also named Sa-II or Pen a 1 [1-3]. Tropomyosin plays an important role in muscle contraction, as well as regulation of cellular structure and motility [4]. Although present in both vertebrates and invertebrates, tropomyosin has been found to be allergenic only when it is derived from invertebrate sources, such as crustaceans, arachnids, insects, and mollusks [1]. Shrimp provides a major source of tropomyosin and causes many food-related complications to those with hypersensitivity. Studies [3, 5] have recognized eight IgE-binding epitopes along shrimp tropomyosin, and many of the sites are homologous to tropomyosin from other invertebrate sources, indicating cross-reactivity among different species [6].

Two other shrimp allergens have been recognized: myosin light chain (20-kDa) [7, 8] and arginine kinase (40-kDa) [9, 10]; yet the majority of shrimp allergenicity is attributed to tropomyosin alone. In previous studies [1, 3, 11], tropomyosin was recognized by 82% of patients with shrimp allergies and was shown to inhibit IgE-binding to whole body shrimp extract in 85-95% of patients.

In the United States, approximately 6% of children and 3.7% of adults are afflicted with one or more food allergies [12]. It is interesting to note that, overall, food allergies have a higher incidence in children than in adults. This is because those born with allergies have a tendency to outgrow them. Food allergy in children is often related to immature immunobarrriers, such as the mucosal barrier, or has been associated with maternal diet during pregnancy and lactation, delivery by cesarean section, infant diet, multivitamin intake, exposure of the child to tobacco smoke, and antacid use, as well as
genetic predisposition [13]. These food-related allergies in children often subside as the immune system matures and desensitization (or exposure to the allergen in small amounts) takes place. However, some food allergies can actually persist into adulthood or will not present themselves until later in life. For example, studies have shown that approximately 97% of children allergic to milk will outgrow this allergy by their teenage years, yet only about 20% of children allergic to peanuts will outgrow this allergy by adulthood [14]. Persistent adult food allergies include peanut, tree-nut, fish, and shellfish allergies [15].

Food allergies that do not manifest themselves until adulthood are termed “adult-onset” and are common with seafood, fruit, and vegetable hypersensitivities. A principal example of adult-onset food allergy is shellfish allergy. While allergy to shellfish comprises only 0.1% of food allergies in children, it is the most common food allergy for adults in the United States, with an incidence level of up to 2% [16].

Adult-onset food allergies are often of a different nature compared to food allergies in children. Some speculate that adult-onset food allergies have a genetic basis [17, 18] or may be the result of mucosal barrier dysfunction [14, 17]. Adults with impaired gastrointestinal mucosa may have increased permeability of the intestinal wall; thus, the amount of undigested proteins that persorb into the system can be higher and can ultimately cause an immune response [18]. Past studies have correlated higher intestinal permeability to patients with food allergy [19]. Furthermore, genetically predisposed individuals characteristically express Th2 cell phenotypes, which are known to regulate IgE synthesis and inflammation [18]. This contrasts with individuals without food allergy, where high Th1 cytokines, low Th2 cytokines, and undetectable
IgE levels are characteristic of the intestinal mucosa. Also, mutations in the genes which encode for IgE and increased reactivity of the molecules which present peptides to lymphocytes (HLA class II genotypes) have been described for food allergy patients [18, 20, 21].

There is also a considerable amount of evidence that an individual can be sensitized by the inhalation of allergens directly from the food source or inhalation of cross-reactive allergens from a different source, such as pollen or dust [22]. In the case of shellfish allergy, it is thought that shrimp-allergic individuals are sensitized by inhaling house mite or cockroach allergens [23-25]. Shrimp allergy is a type I hypersensitivity, which means that the initial encounter with an allergen sensitizes the individual to future exposures [26]. Because tropomyosin from these non-crustacean sources (dust mite and cockroach) is known to cross-react with shellfish tropomyosin [27-29], exposure by inhalation has been speculated to ultimately cause sensitization and IgE antibody production to the shellfish allergen. This has also been hypothesized for a number of adult-onset food allergies, including fruit and vegetable hypersensitivities which are thought to arise from pollen or latex inhalation [30].

The most frequent symptoms of shrimp-induced allergies include itching, hives, swelling of the lips and tongue, pulmonary symptoms, gastrointestinal symptoms, and anaphylactic shock [4]. To date, the only completely effective method to prevent shrimp-induced allergies is total avoidance. Avoidance is often difficult, however, due to unintentional cross-contamination or the addition of food allergens as ingredients in common foods. This underlies the importance of developing methods to inactivate or remove food allergens from food products.
Post-harvest approaches, such as gamma irradiation, high hydrostatic pressure (HHP), genetic modification, and high-intensity ultrasound have been studied for their effects on food allergens. More recently, a novel food technology, pulsed ultraviolet light (PUV), has been shown to reduce the allergen reactivity of peanut products [31] and soybean extract [32]. In a PUV system, electrical energy is captured and stored in a capacitor and then released in a short pulse containing ultraviolet (UV), infrared, and visible light spectra. Because the energy is initially built-up, the resultant burst can be several thousand times greater than conventional UV light [33]. Following release, the energy excites molecules in foods, which, as they return to ground state, liberate energy as heat or photons and/or induce chemical changes. The exact mechanism by which PUV treatment reduces the allergen reactivity of peanut and soy products is unknown, but it is speculated that it may be a combination of photophysical, photochemical, or photothermal changes [33]. Putative effects of PUV on food allergens have been attributed to Maillard reactions (photochemical) and/or protein aggregation or alteration (photothermal/photophysical) [31, 32]. Furthermore, although PUV treatment is considered nonthermal, instantaneous heating may contribute to the deactivation of molecules.

Nonthermal plasma (NTP) is a novel technology where electrically energized matter in a gaseous state is used to deactivate microorganisms in food systems [34]. Although the effects of NTP on proteins are not well studied, this technology may one day have potential in food allergen research. Electrical discharge into air produces positively or negatively charged ions, free radicals, electrons, and photons, which are highly reactive and could potentially react with food allergens. The electrons in
nonthermal plasmas reach such a high energy level that they are often termed “hot” [35], but actual treatment temperatures are less than 50°C.

Another nonthermal method, HHP, has been shown to alter allergen reactivity by affecting non-covalent bonds and/or by causing denaturation of food allergens [36, 37]. The benefits of HHP include the retention of organoleptic traits because thermal energy is not involved and because the pressure that acts on the food is isostatic, or constant at all points. However, mild to moderate heat can be applied in conjunction with the pressure to enhance the effectiveness of treatment. Like PUV and NTP, the effect of HHP on shrimp allergen reactivity has not yet been studied. The methods of PUV and HHP are described further in Chapter 2.

**Objectives**

Pulsed ultraviolet light treatment has recently been associated with the reduction of allergens in foods, namely peanuts [31], milk [38], and soybeans [32]. However, this nonthermal method had not yet been employed on other major foods that cause allergic reactions, such as shellfish. The focus of this research was to examine the efficacy of PUV exposure for reducing the reactivity of Atlantic white shrimp (*Litopenaeus setiferus*). In this study, we hypothesized that PUV treatment of shrimp would substantially reduce the allergen reactivity of the major shrimp allergen, tropomyosin. Specifically, the effectiveness of PUV treatment was dependent on many factors such as time and sample characteristics. Our goal was to find the minimum exposure time, using parameters established by Chung et al. [31] and Yang et al. [32], that could successfully reduce the reactivity of tropomyosin. Sample preparation (i.e. homogenization) was also optimized to facilitate light penetration. The allergen reactivity of tropomyosin was studied by analyzing protein profiles (SDS-PAGE) and allergen
reactivity (immunoblot), via immunoglobulin E (IgE) binding. In addition to changes in tropomyosin, IgE binding to the whole shrimp extract, that is all putative allergens as well as protein fragments, was analyzed by dot blot and enzyme-linked immunosorbent assay (ELISA).

Another objective of this study was to examine the putative mechanism by which IgE binding to shellfish allergens is inhibited by PUV treatment using observations, data obtained from the previously described analyses, and recently published literature on allergen modification. Furthermore, we wanted to determine whether digestive conditions caused allergen reactivity of the PUV-treated samples to change or, specifically, increase.

Lastly, this study also explored the effects of two novel processing methods, NTP and HHP, in regards to food allergen reactivity. Shrimp extract was subjected to NTP or HHP to determine whether allergen levels and/or reactivity were altered.

**Justification**

Postharvest treatments which effectively reduce the allergen potency of shrimp by putatively altering the reactive sites of the allergen can potentially be used to produce less-allergenic shrimp products. The impact of products that might one day be termed “hypoallergenic” could be tremendous both economically and socially when successfully conducted. Shrimp has been one of the most popular seafoods in America, with an annual consumption about 4.1 pounds per capita according to the National Oceanic and Atmospheric Administration (NOAA) [39]. This number represents more than 25% of all commercial fish and shellfish consumed in the United States. Shrimp consumption has almost tripled since 1980, greatly impacting the seafood industry [39]. Technologies such as PUV, NTP, or HHP would not only add value to shrimp products that could be
promoted as less allergenic, but would also enhance food safety, which will in turn impact many other sectors such as medical services, health care, insurance, government policy and retails.
CHAPTER 2
LITERATURE REVIEW

Current Food Allergen Research

Previous reviews [41, 42] regarding the effects of food processing on food allergen reactivity have investigated processing techniques, such as proteolysis, fermentation, or germination, in their ability to alter allergen reactivity. These processes, although desirable for some food products, are not relevant for all foods and, thus, do not have vast applications in reducing allergens from a variety of food items. Multiple thermal and nonthermal techniques have been researched, which may be utilized to reduce allergenicity of several food products without severely altering their inherent characteristics.

Changes in food allergen reactivity due to thermal treatment (i.e., moist and dry heat) have been extensively researched, and several mechanisms have been elucidated. Heating can alter proteins by inducing denaturation (loss in tertiary and/or secondary interactions), formation of new intra- or inter-molecular bonds, aggregation, and/or rearrangements of disulfide bonds, as well as other conformational modifications, which can ultimately lead to changes in allergen reactivity [43]. Antibodies, namely immunoglobulin E (IgE), produced by the immune system of an allergic individual bind to the allergen at a specific site (epitope), signaling a cascade of immunological events [44]. Altering protein configuration may change epitopes, which will no longer be recognized by IgE antibodies or trigger an immune response. Figure 2-1 includes a diagram that illustrates mechanisms by which food processing can alter food allergen

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reactivity. In some cases, thermal treatment has been shown to decrease allergen reactivity [43, 45, 46], yet in other cases, thermal treatment increases allergen potency [47-49], possibly by exposing new IgE-binding sites. Thermal treatment can be divided into two types: moist and dry heat. Moist heat includes boiling, frying, extrusion, autoclaving, and retorting, whereas dry heat encompasses baking, roasting, and microwaving.

Although thermal treatments can be used on many products to alter food allergen reactivity, thermal processing has also been known to modify organoleptic properties, including flavor, color, and nutrient content [50]. Nonthermal treatments are appealing in food processing due to their negligible effects on the properties of foods. Under nonthermal processing, foods can maintain their initial characteristics, appear fresher, and often maintain more nutritional benefits compared to thermally treated foods [51]. Nonthermal processing is important in food safety and quality for microbial and enzymatic inactivation [52-55], yet, more recently, research has shown that nonthermal treatments have been effective in altering allergen reactivity in several foods. These treatments include high pressure processing, pulsed ultraviolet light, pulsed electric fields, gamma irradiation, high intensity ultrasound, genetic modification, and physical, chemical, and enzymatic processing.

The objectives of this chapter are to review food allergies and food allergens, noting the importance of allergen structure in regard to reactivity; to provide a brief overview of methods used to detect changes in food allergens and the value of using different techniques; and to thoroughly explore thermal and nonthermal processing
techniques utilized to modify food allergen reactivity. A summary of major postharvest food treatments with emphasis on food allergen modification is presented in Table 2-1.

**Food Allergens**

Food allergies are caused by adverse immunological responses to proteins, or antigens, which are components of food matrices. In the case of foods, these antigens are referred to as food allergens [56]. The majority of food allergens are water-soluble glycoproteins between the molecular weights of 10-70 kDa [57] and are often characterized by having the following traits: the ability to sensitize a genetically predisposed individual by triggering the production of IgE antibodies, the ability to bind those particular IgE antibodies, and the ability to cause an allergic reaction following IgE binding [56]. However, some allergens, such as those involved in Celiac disease, can be non-IgE mediated, where the reaction is arbitrated by the lymphatic system [44]. For the purpose of this review, we will focus on IgE-mediated food allergies, since this pathway has been most elucidated and these allergies tend to be most severe.

Immunoglobulins that are produced by the body in response to an antigen must first bind the antigen in order to elicit an immune response. The regions to which IgE bind are called epitopes. Epitopes can be a portion of continuous amino acids along a protein, known as linear epitopes, or are formed by three-dimensional folding of the protein, known as conformational epitopes [58]. Altering the reactivity of the allergen often requires a disruption of the IgE-binding epitopes. In the case of linear epitopes, allergen reactivity can be altered by fragmentation or genetic modification of the amino acid sequence that comprises the epitope. Conformational epitopes can be destroyed if the allergen is modified structurally, such as in full or partial denaturation, aggregation, induced crosslinking, or chemical modification [41]. By altering the epitopes found on
food allergens, IgE antibodies may no longer be able to recognize the allergen, and, thus, initiation of the cascade of immunological events that lead to an allergic reaction are inhibited.

Many food allergens are resistant to denaturation, which allows them to retain the ability to elicit an immune reaction under conditions such as heating and enzymatic degradation [57]. Thus, proteins that are resistant to digestive conditions or high heat can enter the gastrointestinal tract with unaltered conformational and/or linear epitopes and cause an immune response. This is seen in the case of tropomyosin, a major shellfish allergen [3], and ovomucoid, a major egg allergen [59], which are both stable under conditions of high heat.

**Food Allergy**

Upon consumption, inhalation, or contact with food allergens, sensitive individuals can experience multiple symptoms, including itching, swelling, hives, respiratory distress, and even death [12]. It is estimated that 30,000 people require emergency treatment and 150 people die each year in the United States due to allergic reactions induced by food allergens [60]. Furthermore, the U.S. population has an increasing trend of food allergies over the years in both adults and children [61].

Over 100 different foods or food components have been documented to produce food allergy [62], yet, according to the Food Allergen Labeling and Consumer Protection Act of 2004, only eight major foods or food groups account for 90% of all food allergies; these foods include milk, eggs, fish, shellfish, tree nuts, peanuts, wheat, and soybeans [61]. Prevention of food allergies remains the ultimate objective for those who suffer from food sensitivities, which often requires complete avoidance of problem foods; however, because many of these major foods are ingredients or come in contact with
other food products during processing (e.g. contamination), absolute avoidance of food allergens is difficult. Thus, immunological methods, such as desensitization, have been the subject of past research [63]. More recently, however, postharvest removal of food allergens has gained attention, and thermal and nonthermal treatments of foods and food products are being extensively examined.

**Methods for Detecting Food Allergens**

Detection methods used for quantifying or qualifying food allergens are important in determining the efficacy of thermal and nonthermal processing techniques used to alter food allergen reactivity. The value of these processing techniques in the food allergen field must be verified by analyzing the treated food’s ability to trigger an immune response. Thus, for the purpose of this paper, a brief review of the methods used for allergen detection will help elucidate the language used in these analyses.

As mentioned, many food allergies are IgE mediated, and the reactivity of an allergen is often described by its ability to bind IgE antibodies. A food sample that has reduced IgE activity may indicate a modification or removal of food allergen(s). Allergen reactivity is most commonly determined by *in vitro* and *in vivo* testing. The benefit of *in vitro* testing is that the tests are often inexpensive, quick and do not pose a threat to human subjects. *In vivo* assays are the pinnacle of testing in that they provide a more accurate representation of the research, yet if human subjects are employed, there is a risk associated with exposure to potentially contaminated foods. Furthermore, it can be time-consuming and costly to utilize animal or human subjects. Table 2-2 summarizes the types of tests most often employed for food allergen analysis.
In Vivo Detection

Skin prick test

In vivo detection includes skin prick tests (SPT) and oral food challenges (OFC). The SPT method requires the formation of a wheal, or small red circle on the skin, created by injecting a small amount of allergen under the skin. Generally, the wheal size must be greater than 3 mm for the patient to be considered sensitive to the allergen, yet wheal sizes can vary by allergen and subject [64]. Using SPT to test for allergies is not considered quantitative and is used only to determine the presence or absence of an allergy. However, this test is not always an infallible method for determining allergy, for patients with atopic dermatitis may develop false-positive wheals [65]. Subjects must also abstain from using drugs, such as antihistamines, that will affect the results of the test. Furthermore, SPT is dependent on the quality of the allergen extract used for testing, and subjective results can differ between evaluators [66].

Oral food challenge

Oral food challenges (OFC) can provide more accurate information regarding food allergy. In this type of study, the subjects are required to consume foods that may potentially cause an allergic reaction. This can prove dangerous, since patients must be directly subjected to allergens, and those who are susceptible to anaphylaxis should not be included in this type of study [65]. Perry et al. [67] tested the safety of OFC studies and found that 28% of OFC participants had severe reactions. Oral food challenges can be performed openly or under single- or double-blind conditions. Once the participant consumes 10 g of the lyophilized protein without symptoms, allergy is ruled out but must be further confirmed [64]. These tests are often difficult to perform due to their
complexity, expense, and time-requirements. Animal models may be used to elude this problem, but these models are not always analogous to the human body.

**In Vitro Detection**

*In vitro* studies for determining allergen presence and reactivity include the measurement of serum IgE using radio-allergosorbent tests (RAST), enzyme-allergosorbent tests (EAST), and ImmunoCAP assays (Phadia, Uppsala, Sweden); sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); immunoblotting; and enzyme-linked immunosorbent assay (ELISA).

**RAST and EAST**

The RAST test, originally described by Wide et al. [68], utilizes antibodies bound to radioisotopes to quantify serum IgE. Briefly, the allergen is adsorbed to a solid phase and incubated with a serum sample. The IgE antibodies in the serum, which are specific to the chosen allergen, crosslink with the immobilized allergen. A secondary antibody that is conjugated to a radioisotope, such as I\textsuperscript{125}, reacts with the IgE antibodies, and radioactivity is measured. The results are then quantified using a standard curve [69].

The EAST test is similar in concept to RAST, yet EAST detection antibodies are conjugated to enzymes, such as alkaline phosphatase, and enzyme activity is measured [70].

The benefits of using RAST and EAST are that multiple samples can be tested at once and that a patient does not need to be present during the test [66]. However, quantification issues arise with standardization as qualitative differences with solid phase and sample preparation differ among analysts. Furthermore, IgG antibodies may cause interference, as they can compete with IgE antibodies for similar allergenic determinants [69].
**ImmunoCAP**

The ImmunoCAP test is similar in concept to RAST and EAST but offers improved sensitivity and is designed to overcome some of the obstacles seen in RAST and EAST [71]. The main highlight of an ImmunoCAP test is the three-dimensional solid phase, which minimizes nonspecific binding by non-IgE-binding antibodies. Furthermore, reagent preparation is designed to reduce loss of conformational epitopes [72]. ImmunoCAP tests can be performed in as little as 20 min [73].

**SDS-PAGE**

SDS-PAGE [74] is used mainly to determine the presence or absence of allergens or to determine a change in protein electrophoretic pattern following treatment. Changes in molecular weight, such as dimerization, can often be noted in SDS-PAGE. Allergens or proteins that become aggregated due to treatment conditions may become too large to travel through the pores of the polyacrylamide gel and thus get stuck in the wells or washed away. Conversely, if treatment causes protein fragmentation, fragments that are too small for the resolution of the gel will pass through the gel quickly and ultimately become lost in the buffer. Furthermore, studies have shown that proteins with induced intramolecular crosslinking may have smeared appearances in polyacrylamide gels [75]. This is due to intramolecular crosslinking that can hinder the protein from completely linearizing under denaturing conditions. Thus, even if the altered allergen is the same molecular weight, the protein may advance through the pores of the gel in an unexpected manner (due to the incomplete denaturation) and cause band smearing. The benefits of this analytic method are that it is inexpensive, and results can be obtained within hours, yet SDS-PAGE does not determine the IgE-binding reactivity of the allergens present.
**Immunoblot**

Immunoblotting for allergen analysis includes Western blot and dot blot analysis. Western blot analysis, as described by Towbin et al. [76], requires proteins to be separated by molecular weight using polyacrylamide gel electrophoresis. The proteins are then transferred to a membrane, often comprised of polyvinylidene flouride (PVDF) or nitrocellulose, and subjected to antibody detection methods. A disadvantage of Western blotting is that proteins are most often tested in their linear, or primary conformation; thus, conformational epitopes may not be represented [56]. Furthermore, new IgE-binding epitopes that were once hidden within the protein may be uncovered. It is also essential to note that since proteins are separated by molecular weight, those that are too large or too small for the resolution of the gel or blotting membrane may not be properly evaluated. The benefits of Western blotting are that protein bands can be individually analyzed to determine the changes in a specific allergen, and analysis is relatively fast, easy, and inexpensive.

For dot blot analysis, the sample is directly adsorbed onto a membrane (i.e., nitrocellulose) and analyzed via antibody detection [77]. A dot blot is a method of immunoblotting that does not involve denaturing conditions, and therefore, conformational epitopes may be preserved. However, adsorption to the membrane may result in protein unfolding. Because proteins are not separated by molecular weight, as in the case of Western blotting, the immunogenicity of the entire sample is analyzed. If the sample consists of an isolated protein, then single proteins may also be analyzed. However, like Western blotting, the resolution of the blotting membrane is the limiting factor for exceptionally high or low-molecular-weight proteins.
ELISA

ELISA can be used to test single proteins or whole sample reactivity, depending on the antibodies employed. Typical ELISAs performed for allergen analysis include competitive inhibition ELISA (ciELISA) and indirect ELISA. Briefly, proteins are adsorbed to a surface (i.e., 96-well plate) and detected using the appropriate antibodies [78, 79]. The ELISA method may be limited by the adsorbance of the protein to the plate. In some cases, hydrophobic interactions, which adhere the protein to the plate, can interfere with or mask conformational epitopes. Furthermore, if the hydrophobic interaction between the polystyrene material and the protein is too strong, denaturation and unfolding of the protein may occur [80]. Thus, care must be taken when choosing materials for ELISA.

Thermal Treatments

Moist Heat

Peanut

Affecting over 1% of the United States population, allergy to peanut is a persistent and severe allergy, which is often responsible for food-related anaphylaxis and death [81]. There have been eight named peanut allergens, Ara h 1 to Ara h 8, that are known to elicit IgE-mediated responses. Of these peanut allergens, Ara h 1, Ara h 2, Ara h 3, and Ara h 6 are the major allergens, which are known to elicit the greatest responses [82, 83].

Beyer et al. [45] illustrated a reduction in the allergen reactivity of Ara h 1 after subjecting whole, de-shelled peanuts to frying and boiling. The researchers speculated that the decrease in IgE binding was due to a decrease in the amount of allergen present. There was also a decrease in IgE binding to Ara h 2 and Ara h 3, even though
the allergens remained in the sample. Mondoulet et al. [43] also discovered a decrease in allergen content with boiling and thus a decrease in overall peanut antigenicity. The reduction was attributed to the allergens leaching from the peanuts and into the water during cooking.

Twin-screw extrusion cooking was performed on defatted peanut flour [46]. The researchers found that extrusion caused multiple peanut allergens to become insoluble, as determined by peptic digestion. They also noted a marked decrease in IgE binding to major peanut allergens, including Ara h 1. Peanut allergen Ara h 2, however, retained the ability to bind IgE and was partially hydrolyzed under peptic digestion.

Shellfish

An estimated 2% of the United States adult population are afflicted with shellfish allergies, making allergies to shellfish one of the leading causes of food-induced allergic reactions in that cohort [12]. The major shellfish allergen, tropomyosin (36 kDa), is a heat-stable protein involved in muscular contraction [3, 5]. Other recognized shellfish allergens include myosin light chain (20 kDa) and arginine kinase (40 kDa) [7, 9, 10].

Shellfish studies using shrimp have illustrated an increase in tropomyosin reactivity following boiling, yet overall shrimp antigenicity was decreased. Liu et al. [84] found that IgE binding to boiled shrimp samples was decreased compared to the raw samples, as illustrated with dot blot and ELISA. However, tropomyosin elicited a greater immune response following boiling treatment confirmed by further in vitro analyses. Carnes et al. [85] also discovered an increase in tropomyosin reactivity following boiling using in vivo SPT where boiled tropomyosin elicited larger skin wheals than untreated tropomyosin samples.
Fish

Fish consumption is popular for its nutritive proteins and unsaturated fatty acids, yet it may also be a source of food allergens for sensitive individuals. The major allergen in fish is parvalbumin (PV), which is a protein involved in the relaxation of skeletal muscle. Studies have shown that PV remains stable after treatments with high temperature (90°C) or proteolytic digestion [86]. Canning of fish requires high retort temperatures to inactivate potentially hazardous microorganisms and spoilage enzymes. Reduced allergen reactivity of canned tuna and salmon was found by methods of ELISA and oral challenges. However, this decrease may be accounted for by protein loss during food processing [87].

Milk

The most common allergy in children under 2 years old in the United States is cow’s milk allergy, where 1.6 to 1.8% are affected [88]. However, after the age of three, 85% of children have the tendency to outgrow cow’s milk allergy [89]. Thus, hypoallergenic milk products are vastly researched and have the potential to keep infants safe. Several attempts have been made to reduce the allergenicity of milk proteins using different technological processes including heat treatment. Researchers found that each allergen or protein fraction had different resistance to heat treatments. For example, α-casein was the most heat stable, β-lactoglobulin was relatively heat stable, and bovine serum albumin was the most heat labile [90]. Considerable reduction in the antigenicity of whey protein was observed during heat treatment [91], but casein antigenicity was not reduced when it was heated at 121°C for 15 min [90]. However, boiling whey for 30 min reduced the antigenicity and immunogenicity significantly; yet despite this reduction, infants still reacted to heat-treated whey protein [91].
In vivo studies [92] testing a group of 100 children with milk allergies concluded that extensively boiled milk was more tolerable than control milk. Skin prick tests illustrated that wheals of children exposed to extensively heated milk were significantly smaller than the control. Reduction in allergen potency was further confirmed using cow’s milk oral challenge methods. Tolerance to heated milk may partially be explained by the research of Taheri-Kafrani et al. [75], who studied the effects of heating on the major milk allergen, β-lactoglobulin (β-LG). These researchers noted changes in β-LG molecular weight following thermally induced denaturation and subsequent aggregation. The reorganization of β-LG is thought to mask the conformational or linear epitopes present on the major allergen, thus reducing overall IgE binding. They found that heating at 85-95°C had the most effect on IgE binding.

**Egg**

Allergy to egg is considered the second most common food allergy in children, following cow’s milk [93]. Egg contains 5 major allergens: ovomucoid (28 kDa), ovalbumin (43 kDa), ovotransferrin (78 kDa), lysozyme (14 kDa), and chicken serum albumin (69 kDa), which is the only major allergen present in the yolk [94]. Ovomucoid is considered the immune dominant protein and is noted by its stability to thermal conditions [59]. Ovomucoid and ovalbumin can still be detected immunologically despite of heat treatment of egg white at 100°C for 20 min (hard boiling) and 3 min (soft boiling) [95].

**Tree nuts**

Although tree nut allergies only affect approximately 0.5% of the U.S. population, unlike some food allergies, such as those to milk or egg, which can subside as a child grows older, tree nut allergies have the tendency to persist throughout one’s lifetime.
In fact, it has been estimated that less than 10% of tree nut sensitive persons will acquire tolerance [97]. Furthermore, tree nuts are often the cause of severe and even life-threatening allergic reactions [98]. Some common tree nuts include hazelnuts, pecans, cashews, chestnuts, and almonds.

Almonds are very popular sources of energy, which contain relatively high amounts of protein and have been associated with cardiovascular benefits [99]. They also contain potential food allergens, which include almond major protein (AMP), or amandin. Under most heating conditions—both moist and dry—almond allergens remained stable and retained the ability to elicit an immune response. Venkatachalam et al. [100] tested the effects of roasting, blanching, autoclaving, and microwave heating on almond allergens and found that, under most conditions, IgE binding is not significantly affected. However, there were mild reductions in IgE binding under extreme autoclaving (30 min), microwave heating (3 min), and roasting (320°F; 20 and 30 min).

Soybean

Soybean consumption in the United States is on the rise due to its perceived health benefits and high source of inexpensive protein. Soy is also a popular meat alternative, and a large selection of soy-based vegetarian options is becoming available. At least 21 allergens have been identified in soybeans, and the major fractions containing allergens are the 11S and 7S globulins. However, the immunodominant allergen, P34, is found predominantly in the seed coteyldon [101]. Texturized soy proteins are created using extrusion techniques, under high temperature and pressure. Although these conditions did not change the allergen reactivity of two allergens (38- and 50-kDa), the major soybean allergen, namely P34, decreased in reactivity [101].
Dry Heat

Peanut

Although boiling may attenuate peanut allergens by removing proteins, studies have found that IgE binding to allergens obtained from roasted peanuts is markedly increased [43, 45], with results as evident as a 90-fold increase compared to the control [102]. Nordlee et al. [103] also found that dry-roasted, as well as oil-roasted, peanuts had increased allergen reactivity as determined by RAST inhibition assays.

Egg

Heated egg white was tolerated in about half of the patients tested in oral challenges administered by Urisu et al. [104]. Because ovomucoid is a heat-stable allergen, it appears to retain allergen reactivity in heated egg white. In fact, removing ovomucoid prior to heating the egg sample reduced the rate of allergic reaction in clinical trials. Other allergens within egg white (ovotransferrin and lysozyme) tended to denature and coagulate under thermal conditions and thus did not elicit as marked of an immune response.

Baked products (muffins and waffles), which contained egg, were tolerated by children with egg allergy in recent studies [105]. Serum IgE levels were not increased upon ingestion, and approximately 70% of the children faced with oral challenges of baked goods containing eggs did not show any immediate allergic symptoms [106].

Wheat

Food allergy to wheat is prevalent in both children and adults. Allergic reactions to wheat have been categorized as immediate (within 2 hours) or delayed (within 1 wk) [107]. Some allergic reactions shown in patients allergic to wheat are atopic dermatitis, nausea, abdominal pain, anaphylaxis, and gastrointestinal symptoms. Wheat proteins
are mainly divided into two groups based on their solubility: water/salt soluble albumin and globulin fraction and water/salt insoluble gluten fraction. The wheat gluten is further divided into alcohol soluble gliadin fraction comprised of α-, β-, γ- and ω-gliadin and alcohol insoluble glutenin fraction comprised of high-molecular-weight (HMW) and low-molecular-weight (LMW) glutenin subunits. Immunologic reactions to wheat have been identified in various wheat protein fractions. Simonato et al. [48] have determined α-amylase/trypsin inhibitor (12-18 kDa) as one of the major allergen present in water/salt soluble albumin and globulin fractions. Omega-5-gliadin has been reported as the major allergen in gliadin subunits responsible for allergic reactions in children [108].

Some wheat allergen reactivity appears to be stronger in baked wheat bread compared to unbaked dough [48, 49]. In one case, the researchers speculated that an increase in allergen reactivity was attributed to browning reactions that caused modification of the allergens and enhanced IgE binding [48]. Another study found that the digestibility of the allergens decreased after baking, possibly caused by aggregation and crosslinking, which made the proteins insoluble to digestion [49].

De Zorzi et al. [109] examined the effect of pasta drying temperature on wheat allergen potency. The results indicated that drying pasta at different temperatures (20-110°C, as used industrially) did not result in a hypoallergenic pasta product, as determined by Western blot and dot blot, even at the highest temperatures.

The potentially allergenic wheat gliadin proteins were subjected to microwave treatments by Leszczynska et al. [47]. Their results indicated a significant increase in IgE reactivity following exposure to microwaves at 40 kJ. Conversely, exposure to more
intense microwave energy doses of 80 kJ and 150 kJ negated increases in IgE reactivity, and levels of IgE binding were comparable with that of untreated sample.

**Tree nuts**

As previously mentioned, almond allergens remained stable under most heating conditions—both moist and dry. However, subjecting hazelnuts to roasting caused a decrease in allergen reactivity both *in vivo* and *ex vivo* [110, 111]. Skin prick tests, involving 132 hazelnut-allergic patients, created significantly smaller wheals compared to raw when roasted hazelnut extract was used. Furthermore, *ex vivo* basophil activation concluded that allergens from roasted hazelnut were less antigenic than those from raw hazelnut [110].

**Maillard Reaction**

Differences seen in dry versus moist thermal processing may be attributed to temperatures at which each heat treatment is executed. Dry-roasting is generally performed at higher temperatures (approximately 150°C), whereas boiling and frying are performed at relatively lower temperatures (100°C and 120°C, respectively) [112]. Maillard browning, or nonenzymatic browning, is a thermally driven reaction, where an increase in processing temperature often accelerates browning. During Maillard browning, reducing sugars react with the functional groups of amino acids under thermal conditions and form a variety of adducts that attribute to the odor, flavor, and color of cooked foods [113]. These adducts may change allergen reactivity by masking IgE-binding epitopes.

Gruber et al. [114] have illustrated that, by increasing the concentration of reducing sugars, cherry allergen reactivity is pronouncedly increased. Thus, it has been speculated that the Maillard reaction can alter the conformation of proteins, thus
masking IgE epitopes or alternatively creating new IgE-binding sites [102, 115]. Furthermore, studies have illustrated that IgE can have a higher affinity toward advanced glycation end products (products of the Maillard reaction) in dry-roasted peanuts [115].

Epitopal masking is also evident in studies performed by Taheri-Kafrani et al. [75]. By combining β-LG and several different reducing sugars under thermal conditions, it was found that glycated-β-LG had decreased IgE binding compared to the control. The glycation degree and amount of modified amino groups had a strong correlation with the change in IgE binding to β-LG, where ribose had the largest attenuation effect.

**Nonthermal Treatments**

**Ultra High Pressure Processing**

Ultra high pressure (UHP) or high hydrostatic pressure (HHP) has been employed in the food industry to inactivate microorganisms and enzymes and improve product texture by protein alteration and denaturation [116]. The effects of high pressure have been attributed to changes in noncovalent bonds, such as electrostatic and hydrophobic interactions, which causes conformational modification. Pressures greater than 200 MPa have been used to alter the secondary and tertiary structure of major proteins, such as those found in meat and milk; however, greater pressures are needed to induce protein changes in egg and soy (400 and 300 MPa, respectively) [117].

Because HHP has been shown to induce conformational changes in proteins, it has been theorized that HHP can alter allergen reactivity by altering the structure of food allergens. In a study by Kleber et al. [36], the researchers discovered that HHP had a negative effect regarding allergen reactivity of milk. Specifically, they found that the major milk allergen, β-lactoglobulin, was more reactive after treatment under HHP
parameters ranging from 200 to 600 MPa. Combining the treatment with heat further enhanced allergen potency. It was speculated that β-lactoglobulin unfolds under high pressure and heat, which allows for protein aggregation. In this case, conformational changes may actually expose new linear epitopes, or IgE-binding sites, and allow for greater allergen reactivity.

Conversely, Kato et al. [118] found that increasing HHP actually reduced the presence of rice allergens. By treating rice grains with pressures of 300 MPa for up to 120 min, it was found that rice endosperm cells were damaged. The researchers revealed that, following structural damage of rice grains due to pressurization, the surrounding buffer was able to permeate the rice grain, causing allergen solubilization and subsequent extraction into the buffer. However, the amount of allergen extraction was dependent on the solvent used and the solubility of the rice allergens; thus, solvent compatibility may restrict the efficacy of this method. The researchers also performed the study using proteases, which further enhanced the decrease in allergen reactivity.

When applied to pasteurized liquid whole hen’s egg, HHP caused the total egg allergen reactivity to decrease by 3.3 fold, as measured by EAST inhibition. When the samples were subjected to thermal treatment (70°C), a 1.5 fold reduction in allergen reactivity was noted. However, when samples were heat treated prior to HHP treatment, a reduction of 8.9 fold was observed compared to the control. In this study, heat treatment was performed at 70°C, and HHP was carried out at 600 MPa for 10 min at 20°C [37]. As described in the thermal treatment section of this review, heat-treated egg shows a decrease in allergen reactivity, particularly in those allergens, which are heat labile, such as lysozyme and ovotransferrin. However, the stability of ovomucoid to
thermal treatment can pose a problem. Thus, as demonstrated in this study, the combination of heat and HHP may allow for a greater reduction in overall food allergen reactivity.

In the case of soybeans, HHP (100–300 MPa for 15 min) reduced the immunoreactivity of an important soybean allergen, Gly m 1 [119]. According to the data, HHP-treated samples had less intense protein bands in SDS-PAGE; thus, a reduction in allergen content is noted. Furthermore, sprouts that grew from HHP-treated soybean seeds had significantly reduced antigenicity compared with ones grown from untreated seeds, and it was suggested that HHP can be used to produce hypoallergenic soybean sprouts without notable nutrient loss.

**Pulsed Ultraviolet Light**

Ultraviolet (UV) light has application in the food industry in continuous or pulsed modes. Pulsed ultraviolet (PUV) light can be several thousand times more intense than conventional, continuous UV light and thus can be effective in shorter periods of time [120]. In a PUV light system, high-voltage electrical energy is stored in a capacitor and released in a single burst, which passes through a lamp filled with inert gas, such as xenon. The gas becomes ionized and emits radiation as ultraviolet (54%), visible (26%), and infrared (20%) light, which occurs within several nanoseconds [121]. It is speculated that PUV light has photothermal, photophysical, and photochemical effects on food systems, which could alter allergen conformation [33] or cause protein aggregation [31], resulting in the loss of conformational epitopes. Specifically, PUV light can ionize molecules due to its high-energy contribution, and visible and infrared waves are responsible for vibration and rotation of molecules, respectively [122].
Currently, two types of PUV light systems, developed by Xenon Corp. (Wilmington, MA), are employed in research: a batch system, as used by the University of Florida and Pennsylvania State University, and a continuous system, as used by the University of Florida. The batch system allows the operator to adjust the treatment time and pulse frequency (typically 1-20 pulses/sec) for a single sample or group of samples. The continuous system is unique in that a conveyor belt passes the sample under two lamps in succession. Conveyor speed, pulse frequency, and treatment duration can all be adjusted, and the equipment can also be used for batch treatment. Furthermore, a hydraulic lift mechanism allows the operator to adjust the distance between the sample and the lamp. Figure 2-4 illustrates the two different PUV systems utilized in food safety research.

Traditionally, UV light is used for sterilization practices, yet more recently, PUV light has been shown to decrease allergen potency of peanut products [31], soybean extract [32], shrimp extract [123], egg extract [124], milk [38], and wheat extract [125]. When applied to peanut extracts and liquid peanut butter, PUV-treated samples had a decrease in IgE binding of 6- to 7-fold compared to the control [31]. It was speculated that PUV light treatment caused protein aggregation of the major peanut allergen Ara h 1, thus altering protein conformation and IgE-binding epitopes. These researchers discovered that proteins, which corresponded to the molecular weight of Ara h 1 (63 kDa), were insoluble in concentrated urea (2 M) or sodium chloride (1 M). The solubility of Ara h 2 (18-20 kDa) was unaffected, as noted by SDS-PAGE.

Yang et al. [32] also found a notable reduction when PUV light was applied to soybean extract. SDS-PAGE analysis illustrated a marked reduction in glycinin (14-34
kDa) and β-conglycinin (50 kDa). However, some soybean proteins (45 kDa and 68-75 kDa) reduced only slightly and were considerably more resistant to PUV light treatment. The researchers also found the formation of a larger molecular weight protein in the region of 150-250 kDa, indicating protein crosslinking and/or aggregation. According to analysis by indirect ELISA, a decrease in IgE binding of up to 50% following PUV light treatment of 6 min was noted in the soybean extract.

The treatment of shrimp protein extract with PUV light for 4 min notably reduced tropomyosin reactivity [123]. Overall shrimp reactivity was reduced up to 30%, as illustrated by ELISA. When the samples were heat treated (100°C) before PUV light exposure, allergen reduction was negligible. The authors believed that PUV light-induced effects of shrimp allergens were due to conformational changes of tropomyosin, particularly intramolecular crosslinking.

Studies by Anugu et al. [38] found that PUV light was also effective in reducing allergen reactivity of milk. The milk allergens casein and whey were undetectable in SDS-PAGE after 150 s of PUV light treatment. Furthermore, IgE binding was reduced by 7.7 fold in whey protein and 7.4 fold in α-casein, as determined by ELISA. These researchers further performed experiments on egg protein extract and illustrated that PUV light was effective in reducing egg allergens, as shown by SDS-PAGE [124]. Following 2 min of PUV light treatment, all IgE binding to major allergens was undetectable on Western blot, except for ovalbumin, which still exhibited some IgE binding. Total egg allergen reactivity was reduced by 9.5-fold at 2 min, as indicated by ELISA.
Wheat gluten immunogenicity was attenuated using PUV light for 45 s with and without heat (100°C) [125]. It was shown that PUV light was effective in reducing the amount of gluten present in the sample as shown by SDS-PAGE, and immunoblotting verified a reduction in allergen reactivity. Heat treatment alone had no effect on allergen reactivity; however, heat treatment in conjunction with PUV treatment dramatically enhanced the effects of PUV light on wheat allergens.

Although PUV light is a nonthermal technology at short treatment times, longer PUV light exposure may raise sample temperature to a pronounced level, and moisture loss has been noted due to evaporative loss [31, 32]. However, proper enclosures and/or the inclusion of heat sinks may help alleviate these phenomena [122]. Also, it must be noted that PUV light has limited penetration depth; thus, importance is placed on sample preparation to achieve uniform treatment. Oppenlander [126] describes several methods for PUV light treatment that can be used to overcome the issue of penetration depth.

**Gamma Irradiation**

Gamma irradiation is a nonthermal processing method that has been employed to reduce food allergen reactivity. It has been speculated that gamma irradiation structurally alters the IgE-binding epitopes found on egg [127], milk [128], and shrimp allergens [129, 130] by creating free radicals, which can cause protein fragmentation and aggregation. Seo et al. [127] described a reduction in the egg allergen ovalbumin when treated with gamma radiation (cobalt-60) doses of 100 kGy, yet samples treated with 10 kGy were not affected. The disappearance of this allergen was attributed to a change in molecular weight. Gamma radiation is thought to enhance protein crosslinking, including the formation of disulfide bonds, and cause hydrophobic
interactions that could lead to protein aggregation [131]. However, a dose of 100 kGy is rather high for food applications, and a maximum dose of approximately 10 kGy has been shown to be safe for human consumption [129]. Seo et al. [132] also found a decrease in ovalbumin reactivity in irradiated (10-20 kGy) white cake samples.

Studies on the effects of cobalt-60 irradiation on the major shrimp allergen, tropomyosin, found that the molecular weight band corresponding to tropomyosin (36 kDa) was undetectable in SDS-PAGE at treatment doses of 7 kGy and above [129]. Furthermore, IgE binding to tropomyosin was minimal at the maximum irradiation doses of 10 kGy. The authors noted protein denaturation as determined by turbidity, surface hydrophobicity, and colormetric reaction of the treated sample and speculated that the protein ultimately coagulated, explaining its disappearance on SDS-PAGE and decreased immunoreactivity.

In another study by Zhenxing et al. [130], gamma irradiation (1-15 kGy) in combination with heat treatment (100°C) was found effective in reducing IgE binding to irradiated shrimp allergens by 5 to 30 fold. Irradiation alone and heat treatment alone did not cause a notable decrease in allergen reactivity, showing a synergistic effect of synchronized thermal and radiation treatment for a decrease in shrimp antigenicity.

Isolated ovomucoid from hen’s egg was treated with a gamma irradiation dose of 10 kGy with or without heat [133]. Irradiation was more effective than heat alone in reducing the concentration of ovomucoid, which can be attributed to the heat-stability of ovomucoid. However, irradiation combined with heating decreased the concentration of ovomucoid to almost undetectable levels.
As for tree nuts, stability of the allergens to gamma irradiation was notable, even when combined with heat treatment. Almonds, cashews, and walnuts were subjected to gamma irradiation (1, 5 10, 25 kGy) with or without heat treatment (autoclaving, blanching, frying, microwave, and roasting) [134]. The researchers found that the allergens were stable under almost every condition.

**High Intensity Ultrasound**

High intensity ultrasound has been recently implemented for food processing such as peeling tomatoes [135], increasing shelf life and reducing the oil content of potato chips (Wambura and Yang 2009), reducing the amount of time necessary to parboil rice [136, 137], and many other processes. High intensity ultrasound processing utilizes mechanical waves within frequencies of 20 kHz to 100 kHz [138]. The high-energy waves promote formation of sonication bubbles in foods, which undergo compression and rarefaction intermittently, until they eventually collapse at critical bubble sizes. Following implosion, localized regions of high pressure and temperature (up to 1000 atm and 5000 K, respectively) surrounding these collapsed cavities can physically change the conformation of allergens and alter their reactivity. Furthermore, regions of high-shear stress and high-velocity gradients can create micro-streams that induce physical and chemical effects, and free radicals generated from water can contribute to protein modification [139].

To date, a modest amount of data is present on the effects of high intensity ultrasound on food allergen reactivity. However, Li et al. [140] found that treating shrimp with high intensity ultrasound reduced IgE binding to both isolated tropomyosin and crude shrimp protein extract from treated shrimp. The isolated protein and whole shrimp were treated with 30 Hz for 130-180 min. Following treatment, there was a larger
decrease in IgE binding to the isolated shrimp protein than to the extract prepared from the treated shrimp muscle. According to immunoblot analysis and ELISA, IgE binding to the treated isolated shrimp allergen decreased by approximately 81.3-88.5%, yet the protein extracted from the muscle of the treated shrimp had a reduction in IgE binding of approximately 68.9% (determined by ELISA only). It was further noted that during treatment of the allergen isolate, formation of a new protein fraction with a low-molecular-weight increased in quantity as treatment time elapsed. Thus, fragmentation of the shrimp allergen may occur during high intensity ultrasound under the conditions stated [140]. No indication of temperature increase or product quality was indicated following the relatively long ultrasound treatment of 130-180 min.

**Genetic Modification**

Genetic modification has gained increasing attention over the years, yet it has also been the subject of much scrutiny. This process prevents translation of selected allergens using post-transcriptional gene silencing or co-suppression [141]. Stability of hypoallergenic, genetically modified foods has not been determined; therefore, there may be a risk associated with eating genetically modified foods if the suppression is incomplete or if the silencing is no longer expressed. Also, removing allergens from foods may alter the foods’ functional and physical properties [141]. Many allergens are integral parts of development and metabolism and are required for growth and survival of organisms used for food. Furthermore, there is a public fear that altering proteins may actually create new allergens, or proteins that are no longer recognized by the body. Genetic modification of IgE epitopes rather than the entire protein may be the best method for this type of reduction technique if functionality can be maintained.
Rupa et al. [142] modified IgE-binding sites by post-translational glycosylation. They found that, by attaching carbohydrate moieties to the egg allergen, ovomucoid, at the known sites involved in IgE binding, they were able to inhibit allergic reaction. Using a murine model, they injected the mice subcutaneously with modified ovomucoid and determined IgE production and reactivity, both of which were reduced compared to the control. However, it should be noted that the proteins were produced in yeast cells and not within the egg itself. Thus, it is not known whether modification of the allergen will affect the entire egg product.

Chu et al. [143] used RNA interference methods to silence the major peanut allergens Ara h 2 and Ara h 6. They noted significant reduction in IgE binding to the silenced allergens; however, IgE binding to the whole sample persisted due to the presence of multiple other peanut allergens. It is interesting to note that silencing of Ara h 2 and Ara h 6 did not produce any noticeable defects in the peanut plant’s morphological traits. Dodo et al. [144] also noted a decrease in IgE binding using genetically silenced Ara h 2 allergens.

The silencing of the major soybean allergen, Gly m Bd 30, almost completely inhibited IgE binding in studies performed by Herman et al. [145]. Electron microscopic-immunocytochemical assays, two-dimensional protein analysis, and tandem mass spectrometric identification strongly supported that the modified soybean was not significantly different from the nonmodified protein, in respect to structural morphology and protein composition. Furthermore, comparative testing showed that no new allergens were formed during silencing.
Chemical, Physical, and Enzymatic Methods

Researchers have explored using chemical or enzymatic means to alter allergen reactivity. The addition of molecules to an allergen by chemical or enzymatic processes may alter the protein conformation or physically obstruct IgE antibodies from binding to conformational or linear epitopes. Enzymatic digestion can also alter linear epitopes via fragmentation. Fermented foods undergo changes by endogenous and microbial enzymes, which can ultimately modify allergen reactivity [146-148]. For a comprehensive review on allergen treatment with digestive enzymes or the effects of fermentation on food reactivity, refer to Besler et al. [42] or Sathe et al. [41].

Phytic acid

Chung et al. [149] investigated chemical methods for creating a hypoallergenic food product and observed a 6-fold reduction in peanut allergen reactivity following phytic acid treatment. Phytic acid forms insoluble complexes with proteins, which are not digestible or absorbed by the human body. Thus, these researchers postulated that the allergens treated with phytic acid would pass through the digestive system without eliciting an immune response.

Phenolic compounds

The addition of phenolic compounds to proteins can lead to the formation of soluble and insoluble complexes. Thus, the inherent binding affinity of phenols allows researchers to modify proteins and change allergen reactivity. Chung and Champagne [150] found that phenolic compounds, such as caffeic, chlorogenic, and ferulic acids, irreversibly form insoluble complexes with major peanut allergens due to putative crosslinking between phenol groups and free amino/tryptophan groups. The formation
of those compounds caused a 10- to 16-fold reduction in IgE binding in peanut products.

**Copper, hydrogen peroxide, peroxidase, and metals**

 Altering the structure of proteins can be attained by polymerization of allergens into larger compounds. Copper ions (Cu$^{2+}$), peroxidase, and hydrogen peroxide (H$_2$SO$_2$) have the ability to crosslink proteins via oxidation of tyrosine residues. It was shown that IgE binding was reduced in peanut extracts treated with these compounds, and crosslinking of the allergens was confirmed using monoclonal antibodies against dityrosine [151].

 The combination of metals with H$_2$SO$_2$ to reduce peanut allergen reactivity via metal-catalyzed oxidation was also explored [152]. Copper sulfate (CuSO$_4$) or ferric chloride (FeCl$_3$) in the presence of hydrogen peroxide was incubated with peanut extract from roasted samples. The researchers discovered that CuSO$_4$ significantly reduced allergen and IgE binding in peanut extract due to crosslink formation; however, peanut extract treated with FeCl$_3$ did not show any reduction in IgE binding or amount of allergen. Thus, not all metals can induce the crosslinking of tyrosine residues of peanut allergens.

**Polyphenol oxidase**

 Other methods of protein modification include the use of polyphenyl oxidase (PPO), an enzyme that catalyzes the production of o-quinones, which react with phenolic compounds and cause browning in fruits and vegetables. Gruber et al. [114] examined techniques for reducing allergen potency in cherries using PPO. They discovered that the addition of PPO with various phenolic compounds reduced allergen reactivity. Caffeic acid was the most potent inhibitor, preceding epicatechin, catechin,
and gallic acid. The addition of quercetin and rutin also displayed a decrease, yet it was less notable. However, PPO without the addition of a phenolic compound did not display a reduction in IgE binding. The researchers speculated that alteration of protein side chains caused by enzymatic reactions accounted for a loss in tertiary structure and IgE-binding epitopes. Chung et al. [153] also found that addition of PPO combined with caffeic acid reduced immunoreactivity of allergens.

**Stepwise polishing**

Stepwise polishing is a processing method used for the production of refined wheat flour. It is utilized to remove a defined percentage (up to 70%) of the tough, outer-layers of the grain. Stepwise polishing of whole wheat kernel resulted in hypoallergenic wheat flour, according to Handoyo et al. [154]. The wheat fraction containing 30% of whole grain showed lowest IgE reactivity. This is due to the removal of the wheat fraction (albumin/globulin) that is responsible for most allergenic reactions, which is located in the outer-layers. Thus, this study indicated that allergenic proteins may be eliminated by stepwise polishing, leaving a hypoallergenic product.

**Magnetic beads**

The use of magnetic beads to capture proteins has been a growing technology for the isolation of protein. In this method, allergens can be removed from a liquid product using antibodies specific to the protein of interest. The antibodies are first conjugated to magnetic beads and then are incubated with the sample. Removal of the proteins requires the use of a magnet to isolate the bead-protein complexes to facilitate sample extraction. Chung and Champagne [155] proposed using magnetic beads attached to IgE antibodies specific for peanut allergens to remove allergens from peanut extracts.
Although the beads captured peanut allergens, the system was not capable of producing a hypoallergenic product.

In another study employing magnetic beads for allergen extraction [156], four magnetic-bead systems (Ca$^{2+}$, Fe$^{3+}$, caffeic acid, hydrophobic) were found to effectively remove major peanut allergens and reduce IgE binding. However, a large amount of nonallergenic proteins were also removed from the samples, as seen by SDS-PAGE. Thus, these four methods may not have the potential to selectively remove food allergens without further altering the food composition.

In previous studies, the phenolic compounds chlorogenic and ferulic acid have been shown to complex with peanut allergens [150]. Thus, Chung and Champagne [155] conducted studies testing the efficacy of capturing food allergens using these compounds conjugated to magnetic beads. Magnetic beads covalently bound to chlorogenic acid significantly reduced the major peanut allergen Ara h 1 and slightly reduced Ara h 2. Using magnetic beads conjugated to ferric irons reduced Ara h 1 and Ara h 2 from peanut extracts and attenuated IgE binding by 28 to 47%.
### Table 2-1. A summary of thermal and nonthermal treatments for the reduction of food allergens.

<table>
<thead>
<tr>
<th>Examples</th>
<th>Key features</th>
<th>Changes in allergen potential</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thermal processing</strong></td>
<td></td>
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</tr>
<tr>
<td>Moist heat (i.e., boiling, extrusion, autoclaving, retorting, and frying)</td>
<td>Denaturation; Maillard reaction; protein conformational changes</td>
<td>Peanut: Ara h 1, Ara h 2, Ara h 3 decreased [43, 45, 46]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shrimp: overall decreased [84]; tropomyosin-only increased [84, 85]</td>
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<td></td>
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<td>Fish: parvalbumin unchanged (90°C) [86] or decreased (retorting) [87]</td>
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<td></td>
<td></td>
<td>Milk: casein unchanged [90]; whey decreased [91]; β-lactalbumin decreased [75]</td>
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<td></td>
<td></td>
<td>Egg: ovomucoid and ovalbumin unchanged [59, 95];</td>
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<tr>
<td></td>
<td></td>
<td>Almonds: unchanged [100]</td>
</tr>
<tr>
<td>Dry heat (i.e., roasting, microwave, baking, and air drying)</td>
<td>Denaturation; Maillard reaction; protein conformational changes</td>
<td>Peanut: increased [43, 45, 102, 103]</td>
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<tr>
<td></td>
<td></td>
<td>Egg: decreased [104-106]</td>
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<tr>
<td></td>
<td></td>
<td>Wheat: gliadin increased (40 kJ) [47]</td>
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<td></td>
<td></td>
<td>Wheat bread: increased [48, 49]</td>
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<tr>
<td></td>
<td></td>
<td>Wheat pasta: unchanged [109]</td>
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<tr>
<td></td>
<td></td>
<td>Almond: unchanged [100]</td>
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<tr>
<td></td>
<td></td>
<td>Hazelnuts: decreased [110, 111]</td>
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<tr>
<td><strong>Nonthermal processing</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High hydrostatic pressure</td>
<td>Denaturation; protein conformational changes; extraction of allergens</td>
<td>Milk: β-lactoglobulin increased [36]</td>
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<td></td>
<td></td>
<td>Rice: decreased [118]</td>
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<td></td>
<td></td>
<td>Soybean: Gly m 1 decreased [119]</td>
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<tr>
<td>Pulsed ultraviolet light</td>
<td>Photothermal, photochemical, and photophysical effects</td>
<td>Peanut: Ara h 1 decreased [31]</td>
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<tr>
<td></td>
<td></td>
<td>Soybean: glycinin and β-conglycinin decreased [32]</td>
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<tr>
<td></td>
<td></td>
<td>Shrimp: tropomyosin decreased [123]</td>
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<tr>
<td></td>
<td></td>
<td>Egg: decreased [124]</td>
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<tr>
<td></td>
<td></td>
<td>Milk: whey and casein decreased [38]</td>
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<tr>
<td></td>
<td></td>
<td>Wheat: gluten decreased [125]</td>
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<tr>
<td>Gamma irradiation</td>
<td>Protein aggregation; protein conformational changes</td>
<td>Egg: ovalbumin decreased [127, 132]</td>
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<td></td>
<td></td>
<td>Shrimp: decreased (with heat) [130]</td>
</tr>
<tr>
<td>Ultrasound</td>
<td>Localized regions of high pressure and temperature; high-shear stress</td>
<td>Tree nuts: almond, cashew, and walnut unchanged [134]</td>
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<td></td>
<td></td>
<td>Shrimp: decreased [140]</td>
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<tr>
<td>Genetic modification</td>
<td>Changes to primary protein structure</td>
<td>Egg: ovomucoid decreased [142]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Peanut: Ara h 2 and Ara h 6 decreased [143, 144]</td>
</tr>
<tr>
<td>Chemical, enzymatic and physical</td>
<td>Protein modification; protein crosslinking; removal of allergen</td>
<td>Peanuts: decreased [149-153, 155-157]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cherries: decreased [114]</td>
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<tr>
<td></td>
<td></td>
<td>Wheat: decreased [154]</td>
</tr>
<tr>
<td>Method of analysis</td>
<td>Measurement</td>
<td>Advantages</td>
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<td>-----------------------------------</td>
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<td>------------------------------------------------------</td>
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<tr>
<td><strong>In vivo</strong></td>
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<tr>
<td>Skin prick test (SPT)</td>
<td>Wheal diameter</td>
<td>Simple, inexpensive</td>
</tr>
<tr>
<td>Oral food challenge (OFC)</td>
<td>Presentation of symptoms</td>
<td>Considered the “gold standard” in diagnosing food allergy</td>
</tr>
<tr>
<td><strong>In vitro</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radio-allergosorbent test (RAST)</td>
<td>Serum-/Plasma-specific IgE values</td>
<td>Can run multiple samples at once</td>
</tr>
<tr>
<td>Enzyme-allergosorbent test (EAST)</td>
<td>Serum-/Plasma-specific IgE values</td>
<td>Can run multiple samples at once</td>
</tr>
<tr>
<td>ImmunoCAP</td>
<td>Serum-/Plasma-specific IgE values</td>
<td>Increased sensitivity compared to RAST and EAST; can test multiple allergens at once; rapid results (in as little as 20 min)</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)</td>
<td>Allergen presence; changes in protein profiles</td>
<td>Fast (within several hours) and inexpensive</td>
</tr>
<tr>
<td>Western blot</td>
<td>Allergen reactivity or presence</td>
<td>Relatively fast (within a few days) and inexpensive; can detect picogram quantities of antigen</td>
</tr>
<tr>
<td>Dot blot</td>
<td>Whole sample or allergen reactivity</td>
<td>Does not require SDS-PAGE and thus is faster than Western blotting; inexpensive; isolated allergens or crude samples may be analyzed</td>
</tr>
<tr>
<td>ELISA</td>
<td>Whole sample or allergen reactivity</td>
<td>Relatively fast (within a few days) and inexpensive; isolated samples or crude samples may be analyzed</td>
</tr>
</tbody>
</table>
Figure 2-1. Diagram of putative inactivation mechanisms caused by food processing on food allergens. Allergen in native conformation (a) can undergo denaturation (b), aggregation (c), crosslinking (d), or fragmentation (e) following food processing which can ultimately lead to changes in allergen reactivity. Genetic modification is not shown in this figure.

Figure 2-2. PUV equipment used for food safety research, developed by Xenon Corp.: (A) batch PUV system and (B) continuous PUV system.
CHAPTER 3
MATERIALS AND METHODS

Materials

Human Plasma

Human plasma containing IgE antibodies against shrimp was collected and pooled by PlasmaLabs International (Everett, WA). Because each shrimp-sensitive individual reacts to shrimp allergens differently, pooled plasma was chosen over plasma from an individual donor. Specifically, the plasma was obtained from 3 patients with a history of shrimp allergy. A shrimp-protein specific IgE concentration of 92 kU L\(^{-1}\) was determined via ImmunoCAP testing (Phadia AB, Uppsala, Sweden) performed by PlasmaLabs International. The plasma was utilized for immunoblotting and ELISA to determine the reactivity of shrimp allergens.

The IgE antibodies present in the plasma have the ability to bind to any putative shrimp allergen, including tropomyosin, myosin light chain, and arginine kinase. Binding of the IgE antibodies to the allergens is indicative of the reactivity of the allergen (i.e., more binding equals more reactive). To control for false positive or negative results, pooled plasma, obtained from three individuals with no known seafood allergies, was used as a control for all immunoassays performed.

Preparation of Shrimp Sample

Due to the poor penetration depth of PUV light and NTP, shrimp samples were homogenized before treatment. Furthermore, the samples were centrifuged to reduce errors in pipetting caused by particulates. The final shrimp sample used for treatment was a crude protein extract. Homogenization is most likely necessary when PUV or NTP treatment is performed on shrimp to enhance penetration depth and to account for
size variability in shrimp. Therefore, potential hypoallergenic shrimp products, such as shrimp flavored noodles or chips, would be a viable option for this allergen processing technique, rather than distributing whole hypoallergenic shrimp.

To ensure solubilization of the shrimp allergens, the buffer system and methods as described by Motoyama et al. [160, 161] were used with slight modification. Atlantic white shrimp (*Litopenaeus setiferus*) was purchased frozen, de-shelled, de-veined, and de-headed from Publix Super Markets, Inc. (Lakeland, FL). A mass of 25 g was thawed and ground in a food processor at high speed. Two hundred milliliters of 0.6 M KCl in 0.01M sodium phosphate buffer (pH 7) was added to the processed tissue, and the mixture was homogenized using a BioSpec (Bartlesville, OK) homogenizer at high speed for 2 minutes. The potassium chloride buffer was chosen to prevent protein denaturation and ensure solubilization of the muscular proteins. The samples were centrifuged at 20,000 x g for 30 min at 4°C and supernatant was used. Protein concentration was measured via Coomassie Plus (Bradford) Protein Assay (Pierce, Rockford, IL) using bovine serum albumin protein standards (Pierce). The homogenate was diluted to a concentration of 5 mg/ml with 0.6 M KCl in 0.01 M phosphate buffer (pH 7), and samples were stored on ice in a Styrofoam cooler at 4°C for no longer than 1 week.

**Preparation of Boiled Shrimp Sample**

Because PUV treatment can contribute heat to a system during extended exposure times, a boiled sample was used to control for thermal effects. A volume of 10 mL of prepared sample was placed in boiling water (100°C) for 4 minutes in a loosely capped 15 mL centrifuge tube to prevent moisture loss. Samples were cooled on ice.
directly following treatment, and were similarly stored on ice in a Styrofoam cooler at 4°C for no longer than 1 week.

**Treatments**

**Pulsed Ultraviolet Light**

Pulsed ultraviolet light treatments were performed following the methods of Chung et al. [31] and Yang et al. [32] with slight modification. A SteriPulse-XL RS-3000 Sterilization System (Xenon Corp., Wilmington, MA) was used to treat 10 ml of raw and boiled samples. Figure 2-2 illustrates the PUV equipment. Pulses were emitted at a rate of 3/s and a width of 360 µs at a distance of 10 cm from the samples. Preliminary analysis was performed using SDS-PAGE and Western blot to determine an effective exposure time. Treatment times of 0, 1, 2, 3, 4, 5, and 6 min were tested, and 4 min was chosen for all subsequent testing based on tropomyosin’s marked reactivity decrease at this exposure time. Protein concentration was measured in all samples after treatment using a Bradford protein assay. Three independent trials were conducted (n=3). Samples treated with PUV were analyzed with SDS-PAGE, Western blot, dot blot, indirect ELISA, and competitive inhibition ELISA. PUV-treated samples were also subjected to peptic and tryptic digestion to determine stability of the deactivated allergens.

**High Hydrostatic Pressure**

High hydrostatic pressure treatment was carried out by the laboratory of Dr. Haiqiang Chen at the University of Delaware, Dept. of Animal and Food Sciences (Newark, DE). Each sample (1.5 ml; 2 mg/mL) was transferred to a sterile polypropylene pouch (Fisher Scientific, Fair Lawn, NJ), which was heat-sealed and then sealed in a secondary pouch for added safety. Pressure treatment was carried out using
a laboratory-scale high-pressure unit (model Avure PT-1; Avure Technologies, Kent, WA) monitored with DASYLab ® 7.0 software (DASYTEC USA, Bedford, NH). The experiments were conducted for 5, 15 and 30 min at 600 MPa at three initial sample temperatures of 4, 21 and 70°C using water as the hydrostatic medium. Pressure increased at a rate of approximately 22 MPa/s, and pressure release was almost immediate. Pressurization time reported in this study did not include the pressure come up or release times. After pressure treatment, samples were stored at 4°C for less than 1 day before being shipped to University of Florida on ice for next day delivery. Three independent trials were conducted (n=3). Samples treated with HHP were analyzed with SDS-PAGE, Western blot, dot blot and indirect ELISA. Figure 3-1 illustrates the HHP equipment used and the treatment chamber.

**Nonthermal Plasma**

Nonthermal plasma treatment was carried out by the laboratory of Dr. Roger Ruan in the Department of Biosystems and Agricultural Engineering at the University of Minnesota (St. Paul, MN). Specifically, NTP was generated between two electrodes fitted with a dielectric barrier using air as the medium. The dielectric barrier was composed of epoxy resin board at a thickness of 0.062 in. The discharge chamber was a column with radius of 1 inch and height of 0.25 inch. A voltage of 30 kV and a frequency of 60 Hz was applied to the electrodes, and the shrimp extracts (2 mg/mL) were treated with NTP for 1, 3, and 5 min at ambient temperature. For this treatment only, each condition was tested once (n=1) and the samples were analyzed in triplicates. The samples treated with NTP were analyzed with SDS-PAGE, Western blot, dot blot, and sandwich ELISA. Figure 3-2 illustrates set-up of the NTP equipment.
Simulated Peptic Digestion

For simulated gastric digestion, pepsin was dissolved in simulated gastric fluid (SGF) as described by the United States Pharmacopoeia (USP) [162]. Briefly, 3.2 g of porcine pepsin (Sigma) with an activity of 272 U/mg was dissolved in 1 L of 0.03 M NaCl titrated to a pH of 1.2 with HCl. Following the methods of Liu et al. [163], digestion was performed using a ratio of 0.33 U of pepsin activity per μg of protein (1:50 w/w). A volume of 30 μL of SGF was added to 970 μL of shrimp protein extract (5 mg/mL; untreated and PUV-treated) that had been adjusted to a pH of 1.2 with 13.5 μL of 8 N HCl. Digestion was carried out at in a 1.5 mL microcentrifuge tube at 37°C with continuous shaking. At 0, 5, 15, 30, 60, and 120 min, aliquots of 200 μL were terminated using 22 μL of 1 N NaOH, as described by Fu et al. [164]. Two 1 mL (control and PUV-treated) samples were digested for 120 min for subsequent trypsic digestion; these samples were terminated with 110 μL of 1 N NaOH. Three independent trials were conducted (n=3). The samples (4.39 mg/mL) were stored on ice and under refrigeration (4°C) for no longer than one week and analyzed by SDS-PAGE, immunoblot, and ELISA.

Simulated Intestinal Digestion

Simulated intestinal fluid (SIF) was prepared according to the USP [162] and consists of 0.05 M KH₂PO₄ at a pH of 6.8. Trypsin and α-chymotrypsin (Sigma) with enzyme activities of 325 U/mg of protein and 62 U/mg of protein, respectively, were added to the SIF at concentrations of 3.2 mg/mL. Digestion conditions followed the procedure of Liu et al. [163], in which 0.20 U of trypsin activity per μg of shrimp protein (1:50 w/w) and 0.04 U of α-chymotrypsin activity per μg of shrimp protein (1:50 w/w) was used to digest the shrimp protein. Digestion was performed in volumes of 1 mL.
Aliquots of 200 μL were removed and terminated at 0, 5, 15, 60, and 120 minutes. Trypsin inhibitor (Sigma) was used to terminate the reaction at concentrations of 1:1 w/w of trypsin inhibitor to trypsin and chymotrypsin as described by Bublin et al. [165]. Three independent trials were conducted (n=3). The samples were stored on ice and under refrigeration (4°C) for no longer than week and analyzed by SDS-PAGE, immunoblot, and ELISA.

**Analyses**

**Temperature Measurement**

Temperature was measured following PUV treatment using an Omega OS423-LS non-contact infrared thermometer (Omega Engineering, Inc., Stamford, CT). Temperature was unable to be measured during treatment, because previous research has determined that type K thermocouples are not ideal due to heating of the metal probe by PUV exposure, which ultimately confounds the sample readings.

**SDS-PAGE**

Protein profiles were analyzed by SDS-PAGE as described by Laemmli [158]. Reduction in band intensity in SDS-PAGE following treatment is generally indicative of protein fragmentation or protein aggregation. Also, proteins may become a different molecular weight if they become dimers or oligomers via crosslinking. Thus, SDS-PAGE was performed to analyze changes in protein bands.

As determined by protein concentration, 12 μg of protein was combined with 2X Laemmli sample buffer (BioRad Laboratories, Hercules, CA) which contained 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% Bromophenol Blue, and 0.05% β-mercaptoethanol. Samples were boiled for five minutes. The samples were loaded onto a pre-cast tris-glycine minigel (BioRad, 4-15%) positioned in a BioRad Mini-Protean
tank apparatus and subjected to electrophoresis per the manufacturer’s recommendations for 1.5 h at 150 V. A volume of 25 mL of GelCode Blue reagent (Pierce) was added to the gel. The gel was incubated with gentle shaking for 2 h at room temperature (RT). The gel was destained with water for 2 h with frequent changing of the water to decrease background staining. The protein bands were scanned with a Canon Pixma MP160 scanner.

**Western Blot**

To determine reactivity of protein bands, Western blotting was performed using human IgE antibodies. Furthermore, tropomyosin was monitored using an anti-tropomyosin antibody obtained from an invertebrate Mayfly. Human IgE antibodies react with, but are not limited to, tropomyosin, and are used to determine the immunoreactivity of allergens. IgE antibodies can show reactivity of other putative allergens such as myosin light chain and arginine kinase. Anti-tropomyosin is produced to only react with tropomyosin and is used to determine the presence or absence of tropomyosin, but does not determine reactivity.

Treated and untreated samples underwent electrophoresis as described by SDS-PAGE. Following electrophoresis, the gel was removed from the cassette, equilibrated in transfer buffer for 15-30 min and transferred onto Immobilon P blotting membrane (Millipore Corporation, Bedford, MA) using a Trans-Blot SD Semi-Dry Transfer Cell (BioRad) at 15 volts for 30 min. The blot was blocked in StartingBlock T-20 (TBS) Blocking buffer (Pierce) for a minimum of 1 h with gentle rocking. The membrane was then incubated for 2 h at RT to overnight at 4°C with pooled human plasma containing anti-shrimp IgE (1:5). After two, 10 min washes, the blot was incubated in mouse anti-
human IgE-HRP (Invitrogen, Carlsbad, CA) for 1 h. Again, the membrane was washed twice for 10 min with gentle shaking. All samples, primary antibodies, and secondary antibodies were diluted in StartingBlock (TBS) T20. All washes contained 1X TBS/0.05% Tween 20 (TBST). A 1-Step Chloronaphthol substrate (Pierce) was added to the blot for 30 min. The blot was rinsed with water and quickly scanned.

To visualize changes in tropomyosin band intensity, the above procedure was replicated, replacing the primary antibody with rat monoclonal anti-tropomyosin (1:1000) and the secondary antibody with rabbit polyclonal anti-rat IgG-HRP (1:40,000). An enhanced chemiluminescence (ECL) substrate was used for detection.

**Dot Blot**

While SDS-PAGE and Western blot separate proteins based on their molecular weight in order to analyze individual protein bands, dot blot and indirect ELISA test the “whole” sample. In the case of allergen reactivity, that means that dot blot and indirect ELISA can describe total allergen reactivity, or the sum of all allergens, in a crude protein sample. This is an important concept in that SDS-PAGE and Western blot have resolution minimums and maximums, depending on the pore sizes of the gel and/or membrane. Dot blot partly overcomes this issue by removing the electrophoretic procedure, however the pore size of the membrane may still be an issue. The ELISA analysis does not require polyacrylamide gels or membranes; thus molecular weight is less of an issue and proteins that are too large or small for SDS-PAGE and Western blot analysis can be studied.

A nitrocellulose membrane was blotted with 1.5 µg of raw, boiled, PUV-treated, and boiled+PUV-treated shrimp extract protein and allowed to dry at 4°C. Following
blocking, the blot was incubated in human plasma containing anti-shrimp IgE diluted in blocking buffer (1:80) overnight at 4°C. The blot was incubated in mouse anti-human IgE-HRP diluted in blocking buffer (1:1000; 1 h; RT). A 4-chloro-1-naphthol substrate was added to the blot and incubated for 30 min to visualize protein spots. Spots appearing more pronounced after incubation indicated an increase in the amount of IgE bound to the shrimp proteins.

**Indirect ELISA**

As mentioned, indirect ELISA was used to test the whole-sample reactivity. Costar EIA polystyrene 96-well plates (Corning, NY) were coated overnight with raw, boiled, PUV-treated, and boiled+PUV-treated shrimp extract diluted in phosphate-buffered saline (PBS) to a concentration of 20 μg/mL (100 μL per well in triplicates). The plates were blocked with StartingBlock (200 μl per well) at room temperature for 2-3 h. Pooled human plasma containing IgE antibody specific for shrimp allergens diluted in PBS (1:10) was added in equal amounts to each well (100 μL), and the plate was incubated at room temperature with gentle shaking for 1 h. Pooled human plasma from 3 patients with no known allergy was used as a control. Each well was then incubated with secondary antibody, monoclonal mouse anti-human IgE conjugated to HRP (1:1000), for 1 h (100 μL per well) with gentle shaking. Before each subsequent step, the wells were washed using TBST. An OPD substrate (0.5 mg/mL) dissolved in 0.1 M citrate buffer (pH 5.5) and 0.03% hydrogen peroxide was added to each well (100 μL per well). The reaction was stopped at 15-30 min with 2.5 N sulfuric acid (100 μL per well), and absorbance was measured at 490 nm using a Spectramax 340 spectrophotometer (Molecular Devices, Inc. Sunnyvale, CA).
Preliminary testing using the indirect ELISA procedure and plasma concentrations of 1:1, 1:5, 1:10, 1:20, and 1:30 determined the concentration needed for this assay (1:10). Secondary antibody dilution was also optimized by testing a series of dilutions (1:500, 1:1000, 1:2000, 1:3000, and 1:4000) and determining minimal background at a dilution of 1:3,000.

**Sandwich ELISA**

Sandwich ELISA was used to further test the effects of processing on tropomyosin protein. Costar EIA polystyrene 96-well plates were coated with anti-tropomyosin antibody diluted in phosphate-buffered saline (PBS) to a concentration of 5 μg/mL (100 μL per well in triplicates) for 2 h at 37°C. The plates were blocked with StartingBlock (200 μl per well) at room temperature on a shaking platform for 2-3 h. The wells were washed 2 times with TBST. Control and NTP-treated samples were added to each well in triplicates at a concentration of 100 ng/mL (100 μL per well) and incubated at RT with shaking. The wells were washed 3 times with TBST. Pooled human plasma containing IgE antibody specific for shrimp allergens diluted in PBS (1:10) was added in equal amounts to each well (100 μL), and the plate was incubated at room temperature with gentle shaking for 1 h. Pooled human plasma from 3 patients with no known allergy was used as a control. Following 3 washes, each well was then incubated with secondary antibody, monoclonal mouse anti-human IgE conjugated to HRP (1:3000), for 1 h (100 μL per well) with gentle shaking. An OPD substrate (0.5 mg/mL) dissolved in 0.1 M citrate buffer (pH 5.5) and 0.03% hydrogen peroxide was added to each well (100 μL per well). The reaction was stopped at 15-30 min with 2.5 N sulfuric acid (100 μL per well), and absorbance was measured at 490 nm using a Spectramax 340 spectrophotometer.
Preliminary testing using the sandwich ELISA procedure and plasma sample concentrations of 1, 10, 100, and 1000 ng/mL determined the concentration needed for this assay (100 ng/mL). Two concentrations of anti-tropomyosin antibody were tested as well (1 μg/mL and 5 μg/mL), and 5 μg/mL was chosen for subsequent experiments. Secondary antibody dilution was also optimized by testing a series of dilutions (1:500, 1:1000, 1:2000, 1:3000, and 1:4000) and determining minimal background at a dilution of 1:3000.

**Statistical Analysis**

Statistical analysis was conducted using one-way analysis of variance (ANOVA) with the SAS 9.2 software package (Cary, N.C.). Significant differences (α = 0.05) between means of the untreated (control) and experimental (boiled, PUV-treated, and [boiled+PUV]-treated) groups for total IgE binding were determined using least significant difference (LSD) and Duncan’s Multiple Range tests. The ImageJ 1.44 densitometry software by the National Institute of Health (NIH) was used to verify changes in band intensity for SDS-PAGE and ELISA.
Figure 3-1. High hydrostatic pressure (HHP) equipment used for shrimp allergen treatment. Illustration includes the control station and HHP unit (a), the sample chamber (b), and the sample being loaded into the chamber (c). Equipment was produced by Avure Technologies (Kent, WA).

Figure 3-2. Diagram of the experimental set-up of the nonthermal plasma equipment. A power supply of approximately 30 kV and 60 Hz supplied the electrodes. Dielectric material was attached to the innerside of the electrodes, which enveloped the discharge chamber. Samples were placed in the treatment chamber as thin films.
CHAPTER 4
RESULTS AND DISCUSSION

Pulsed Ultraviolet Light

Initial Determination of PUV Treatment Time

To establish an appropriate treatment time for shrimp extract, several PUV exposure times were tested. SDS-PAGE and Western blot analyses were used to determine the minimum exposure time at which PUV treatment was effective when applied at 3 pulses/s, 10 cm from the light source. SDS-PAGE analysis (Figure 4-1) illustrates that tropomyosin bands (36-kDa) remained present following PUV treatments of 0 (control), 1, 2, 3, 4, 5 and 6 min, but were reduced in samples treated for 4-6 min.

Changes in reactivity of tropomyosin at each time were determined via Western blot analysis. Figure 4-2 illustrates that IgE binding to tropomyosin was reduced in as little as 3 minutes, with marked reduction at 4-6 min. Therefore, a minimum PUV treatment time of 4 min was chosen for subsequent experimentation.

Temperature and Volume Changes

Pulsed ultraviolet light treatment is considered nonthermal when used for brief periods of time; however, following PUV treatment of 4 min, shrimp extract surface temperatures of 68.3 ± 2.5°C were measured using an infrared thermometer. It must be noted that instantaneous temperatures may be higher in the sample during treatment, which was not measured in this study. Previous trials using thermocouples to monitor temperature during treatment were unsuccessful, because extended PUV exposure of the metal probe confounded the readings. Autoclave tape (produced by Fisher Scientific), which changes color when exposed to temperatures of at least 120°C, was subjected to PUV treatment for 4 min in an aluminum dish with or without liquid (water).
Under both conditions, the autoclave tape changed color (e.g., turned black). This indicates that instantaneous temperatures were at least 120°C or higher. For shorter PUV treatment times, a type-K thermocouple produced by Omega Engineering, Inc. (Stanford, CT), has been utilized [166]. Keklik et al. [166] reported an increased temperature of 10.5 ±1.2 °C after 30 s at 9.5 cm when using PUV treatment for decontamination of shell-eggs. Table 4-1 illustrates representative data from the time course experiments regarding changes in surface temperature and moisture loss.

To control for possible thermal effects, a boiled-only control was used to ensure that changes in allergen reactivity following PUV treatment were not simply a result of thermal increases. Thus shrimp extracts were subjected to boiling water for 4 min for comparison with the control and PUV-treated samples. Boiled shrimp extracts were also treated with PUV to determine if synergistic or antagonistic effects exist between thermal and PUV treatments.

Following boiling, PUV, and [boiling+PUV] treatments, moisture loss in each sample was measured: boiled-only (5.83 ± 2.3%), PUV-only (29 ± 3.6%), and boiled with PUV (39.7 ± 2.5%). Moisture loss was higher in the PUV-treated samples because the samples were not enclosed during PUV treatment (the purpose was to ensure the maximum penetration depth). By contrast, moisture loss was minimal in the boiled sample because the samples were loosely capped during boiling. Moisture loss is indicative of possible higher instantaneous temperatures during PUV treatment than the measured surface temperatures shortly after treatment. Chung et al. [31] also noted volume reductions of approximately 40% following PUV treatment. Using a closed system for PUV treatment may be beneficial for preventing moisture loss; however, the
PUV penetration into the sample may be compromised. Another solution would be to fit PUV equipment with cooling devices to inhibit temperature increases and evaporative losses. Alternatively, if temperature increases are adequate, they may potentially be used in conjunction with food preparation, reducing the need to thermally process products before or after treatment. To correct for moisture loss in the samples, total protein measurements were taken after treatments, and these values were used for subsequent experiments.

**Sensory Remarks**

Overall, changes in sample color were not remarkable; however, samples with longer exposure times appeared slightly yellow. Figure 4-3 illustrates changes in shrimp extract color following 0, 1, 2, 3, 4, 5, and 6 min PUV-treatment. Also, a mild odor was sensed following immediately after treatment, which could be associated with ozone production by UV interaction with oxygen or thermal increases (cooking) of the sample. Off-odors, that disappeared shortly after PUV treatment, have been observed in previous studies with leaf lettuce [167]. Furthermore, following an incubation of 5 days at room temperature, PUV-treated shrimp samples maintained a fresher appearance and smell compared to untreated samples. After this incubation, the untreated sample turned a bright, neon green; whereas, the PUV-treated sample did not have a notable change in color. Because PUV is extensively researched for its microbial deactivation properties, it is not surprising that the PUV-treated shrimp extract stayed fresher longer at ambient temperature. Figure 4-4 illustrates the color change in the untreated sample that was assumed to be associated with microbial spoilage.
Changes in Tropomyosin Levels

**SDS-PAGE.** As illustrated in the SDS-PAGE profile (Figure 4-5), a 36-kDa band representing tropomyosin was present following treatments with boiling, PUV, and boiling+PUV. A decrease in tropomyosin was observed in both PUV- and [boiled+PUV]-treated samples (lanes 3 and 4), whereas the boiled extract (lane 2) did not show a change in tropomyosin density compared to the control. These results were verified using densitometry analysis. Resistance to thermal denaturation or degradation is characteristic of the tropomyosin protein, as it is recognized for its heat stability [3]. However, instantaneous pulses of energy generated during PUV treatment may cause more intense localized heating [168, 169], which may result in the reduction of tropomyosin.

**IgE-Binding Analyses**

**Western blot**

Figure 4-6 (a) demonstrates a notable decrease in IgE binding to tropomyosin following PUV treatment, as evidenced by a decrease in band intensity. This is consistent with the reduction in SDS-PAGE, yet is more pronounced in Western blot analysis. Heat treatment, however, did not appear to affect tropomyosin reactivity, and in boiled samples, IgE binding was similar to the control. IgE binding to tropomyosin was also reduced in samples that were boiled in conjunction with PUV treatment.

Considering tropomyosin’s heat stability, the reductions in band density and IgE binding seen in the PUV-treated sample were not likely to be caused by hydrothermal effects alone. However, mechanisms such as localized photochemical and photophysical energy produced by PUV may have the potential to reduce IgE-binding to shrimp allergens. For instance, PUV energy can potentially cause protein modifications,
which include protein fragmentation, denaturation, or intra- or inter-molecular protein crosslinking. Fragmented protein sections that are smaller than the resolution limit of the gel may travel through the acrylamide pores more quickly, causing them to ultimately be lost in the buffer. Conversely, proteins that have been modified by intermolecular crosslinking (i.e., protein aggregation) are often too large to migrate through the gel, and are not clearly detectable via SDS-PAGE or Western blot, but may appear congested in the wells of the gel. However, proteins modified via covalent modification such as intramolecular crosslinking can maintain approximately the same molecular weight, yet may often migrate through polyacrylamide gels with slight difficulty, as they are not completely linearized under reducing conditions. Thus the protein bands corresponding to modified proteins can have a smeared appearance in Western blot analyses [75]. This was apparent in Figure 4-6 (b), where an anti-tropomyosin monoclonal antibody was used to detect tropomyosin (not to determine the IgE reactivity). Tropomyosin bands were notably smeared in PUV-treated samples; therefore, it appears that PUV treatment might have caused shrimp allergen modification via putative covalent modification. Importantly, it was noted that the smeared tropomyosin band was not detected with human IgE antibodies, as illustrated in Figure 4-6 (a). This indicates that conformational modification to tropomyosin might have changed the reactivity of the allergen by altering IgE-binding epitopes.

Previous experiments [75] have shown that covalent modifications, such as glycation, have been attributed to the Maillard reaction, where reducing sugars interact with proteins in a non-enzymatic browning reaction [115]. Nakamura et al. [170] found that IgE binding ability of squid tropomyosin was suppressed with the progression of the
Maillard reaction. Thus, allergens may be irreversibly modified and IgE-binding sites may become masked after amino acids undergo modification during non-enzymatic browning, causing a decrease in allergen reactivity. Conversely, previous experiments testing scallop tropomyosin [171] and peanut allergens [115, 171] displayed an increase in allergen potency with the progression of the Maillard reaction. The effects of the Maillard reaction appear to be dependent on the sample and the amount and type of reducing sugars present, as well as many other variables, such as treatment temperature. The concentration of reducing sugars and free amino acids in shrimp varies among species and changes in response to climate; it has been reported that concentrations can be particularly high during colder seasons [172].

Non-enzymatic browning can be accelerated by increases in temperature, such as those noted during extended PUV treatments. Thus modification of proteins via non-enzymatic browning could be a putative explanation for PUV effects on shrimp allergen reactivity. Taheri-Kafrani et al. [75] illustrate protein-band smearing, similar to that of Figure 4b, of milk allergens that have been modified via the Maillard reaction. Band smearing may also explain the reduction of the 36-kDa band corresponding to tropomyosin in SDS-PAGE (Figure 4-5).

**Dot blot**

Dot blot analysis was performed to determine the allergen reactivity of the shrimp protein extract as a whole, that is, tropomyosin and other possible allergic components that are not detectable on Western blots (i.e., a molecule which is too large or too small). IgE binding to whole shrimp extract was greatly reduced following PUV treatment (Figure 4-7). However, there was an increase in IgE binding following boiling treatment. Interestingly, PUV appeared to attenuate the boiling effect, as shown in the
boiled+PUV-treated sample; IgE binding was notably reduced compared to the boiled-only sample.

The effect of thermal processing on food allergens has been studied extensively, and, in different studies, heating has been shown to either decrease or increase allergen potency. For example, Maleki et al. [102] described a 90-fold increase in IgE binding of roasted peanuts over raw peanuts, whereas roasting actually decreased the overall allergen reactivity of hazelnuts [111]. It has also been noted that children with milk allergies show a tolerance to extensively heated milk [92]. However, because tropomyosin is considered a heat-stable protein, increases in IgE binding may not be explained by changes in tropomyosin alone. It must be considered that other proteins may have the potential to become reactive when exposed to heat. Arginine kinase (40-kDa) [9] and myosin light chain (20-kDa) [8] have both been found to play a minor role in shrimp allergy. It is possible that one, or both, of these proteins could be denatured or modified during thermal treatment, exposing linear IgE epitopes and causing an increase in shrimp reactivity. As seen in Figure 4-6 (a), a band just above the tropomyosin band, at approximately 40-kDa, appears to become reactive following heat treatment (Lane 2) but is not present in the control (Lane 1). This band could possibly correspond to arginine kinase or a dimerized form of myosin light chain; however, this would need to be verified. The effects of boiling on shrimp reactivity are consistent with the results of Carnes et al. [85] who also found that boiled shrimp extracts were more immunoreactive in both in vivo skin prick trials and in vitro direct ELISA results.

**Indirect ELISA**

Indirect ELISA was also used to determine the reactivity of the whole shrimp protein extract. ELISA results (Figure 4-8) supported the dot blot analysis, illustrating a
decrease in IgE binding to PUV-treated samples, compared to the untreated, of approximately 23%. Furthermore, boiling was shown to increase IgE binding by approximately 34%, whereas boiling followed by PUV treatment showed a negligible change in IgE binding.

Reductions in IgE binding were likely explained by changes in tropomyosin seen in Western blot analysis. In the boiled+PUV-treated sample, the increase in IgE binding following boiling, presumably by heat-labile shrimp allergens, and the decrease in IgE binding following PUV treatment, by the effect of PUV on tropomyosin, appear to negate each other; thus IgE binding to the boiled+PUV-treated sample was similar to the control. As a result, the effects of boiling before PUV treatment on the IgE-binding levels were not as pronounced as in PUV-treated samples alone, which may be considered an antagonistic effect.

**Pulsed Ultraviolet Light and Simulated Digestion**

**Simulated Gastric Digestion**

**SDS-PAGE**

Control and PUV-treated shrimp extracts were tested for their stability under gastric conditions (e.g. pepsin and low pH) for 2 h. This experiment was intended to determine whether the reactivity of PUV-treated shrimp allergens remained decreased even after simulated digestive conditions. As illustrated in Figure 4-9, tropomyosin remains present in equal amounts compared to the undigested control following peptic digestion in SGF. The SDS-PAGE profiles of control and PUV-treated shrimp extracts showed a succession of overall protein reduction with time; however, tropomyosin appeared to remain constant. These data are concurrent with SDS-PAGE profiles of SGF digested tropomyosin from Grass prawn and Pacific white shrimp described by Liu
et al. [163]. In the mentioned study, purified tropomyosin from the two species of shrimp was subjected to in vitro gastric digestion in SGF at a ratio of 1:50 (pepsin to protein, w/w) for up to 60 min. The tropomyosin band intensity remained unchanged in both species of shrimp under these conditions.

**Western blot**

Western blot analysis was used to determine the reactivity of the digested PUV-treated shrimp extract following simulated gastric digestion. Figure 4-10 shows that the undigested sample and the digested samples are not different in regards to tropomyosin reactivity. Thus, pepsin digestion did not appear to change (i.e. increase or decrease) tropomyosin reactivity. Furthermore, tropomyosin levels, as detected by an anti-tropomyosin antibody, were unchanged as illustrated in Figure 4-11. The anti-tropomyosin antibody also detected the band smearing, as seen previously in Figure 4-6; thus, peptic digestion did not appear to alter the allergen modification caused by PUV treatment. Stability to digestive conditions is characteristic of some food allergens and may actually contribute to their reactivity [30, 173]. An undigested allergen may have a greater potential to cross the digestive membranes and elicit an immune response. Huang et al. [174] noted a similar stability of crab tropomyosin to SGF. They also found that increasing the pepsin to protein ratio from 1:50 to 1:1 (w/w) caused IgE binding to crab tropomyosin to noticeably decrease. However, it is estimated [30, 175] that humans have an average pepsin release of approximately 10 mg per day with a daily average intake of 75 g of protein, making the pepsin to protein ratio (w/w) approximately 1:7500 for 24 hours. This suggests that a ratio of 1:1 is highly unlikely. Furthermore, because optimal pepsin activity is related to pH, namely a low pH of 1.2 to 3.5 [30], the pepsin activity in individuals may vary due to physiological differences [175].
**Indirect ELISA and dot blot**

Indirect ELISA and dot blot were used to determine the reactivity of the shrimp extract following digestion in SGF. Figure 4-12 illustrates a mild reduction in allergen reactivity during peptic digestion; however, this reduction is not markedly different from the control. Dot blot analysis (Figure 4-13) shows that the IgE binding in the untreated shrimp extract remains unchanged; yet, the PUV-treated sample slightly decreases after 60 and 120 min digestion in SGF. These results agree with the SDS-PAGE and Western blot data and elude to the postulation that peptic digestion alone may not increase the reactivity of PUV-treated samples.

The explanation of why tropomyosin is stable to pepsin has not yet been elucidated. However, research on one of the major peanut allergens (Ara h 2) has shown that disulfide bonds may enhance stability to digestive conditions [176]. In this study, the researchers reduced disulfide bonds before digestion and found that the allergen was no longer resistant. Circular dichroism further confirmed the importance of disulfide bonds in regards to resistance to digestion.

**Simulated Tryptic Digestion**

**SDS-PAGE**

Control and PUV-treated shrimp extracts were subjected to trypsin and α-chymotrypsin in SIF for up to 2 h to determine the stability of tropomyosin. Unlike in simulated gastric digestion, tropomyosin did not appear to be stable in SIF, as illustrated in SDS-PAGE profiles (Figure 4-14). Compared to the undigested samples, tropomyosin was not present in detectable levels in the control or the PUV-treated shrimp extracts. Compared to a study by Liu et al. [163], tropomyosin from Atlantic white shrimp was degraded much faster than tropomyosin from Grass prawn and Pacific white shrimp.
when trypsin and chymotrypsin were combined. In the mentioned study, the effects of trypsin and chymostrypsin were separated; however, physiologically this is not the case.

**Western blot**

According to Western blot analysis (Figure 4-15), IgE binding to tropomyosin was not detectable in the control or the PUV-treated shrimp extracts compared to the undigested extracts. Tropomyosin bands were also not detected with the tropomyosin-specific antibody (Figure 4-16). A smaller reactive protein band also began to appear after 60 min at approximately 10-kDa in both the control and the PUV treated Western blots (data not shown). To determine whether these bands were tropomyosin fragments, the Western blot was probed with the tropomyosin-specific antibody; however, these bands were not detected with this antibody. These results may either indicate that the bands were not tropomyosin fragments, or that the fragments did not contain the epitope to which the tropomyosin-specific antibody binds. Previous studies [174] using trypsin and chymotrypsin also noted smaller molecular weight bands which disappeared after 3 h of digestion.

**Indirect ELISA and dot blot**

Indirect ELISA (Figure 4-17) illustrates a decrease in shrimp reactivity as time progresses, with almost no detectable reactivity at 120 min for both the control and PUV-treated samples. Unlike the Western blot and dot blot (Figure 4-13), the reactivity did not decrease completely after just 5 min. The disparity between the Western blot data and the indirect ELISA was likely due to the fragmentation, or enzymatic breakdown, of tropomyosin. If the protein was cleaved by the proteases, it would no longer appear at the same molecular weight and would be too small to be resolved on a polyacrylamide gel (i.e., undetectable on Western blot); however, the tropomyosin
fragments might still contain IgE-binding epitopes. As time progresses, these epitopes would begin to disappear, as well, as the digestion reached completion.

**Peptic and Tryptic Digestion**

**SDS-PAGE**

Samples subjected to SGF for 2 h were treated with SIF to simulate the natural digestive succession. According to SDS-PAGE analysis, it was found that no detectable tropomyosin remained following 5 min or longer under every condition (Figure 4-18). Furthermore, most proteins were digested under these conditions, which is predictable considering that intestinal digestion is the last step in human digestion.

**Western blot**

In concurrence with SDS-PAGE, IgE binding to tropomyosin was undetectable following 5 min or longer under every condition tested (Figure 4-19). However, a smaller molecular weight band (approximately 10-kDa) appeared after 5 min and became more intense as time lapsed (data not shown). This band appeared to be the same protein as seen in the SIF-only trials and was not detected with the tropomyosin-specific antibody (Figure 4-20). It is possible that this band represented a fragment of tropomyosin and contained an IgE-binding epitope or that this was a fragment of another shrimp allergen, possibly myosin light chain or arginine kinase.

**Indirect ELISA and dot blot**

Indirect ELISA (Figure 4-21) also determined a reduction in shrimp extract reactivity following SGF+SIF digestion. The decrease in reactivity was a step-wise progression with time and was nearly undetectable at 120 min. However, as shown by dot blot analysis (Figure 4-13), IgE binding to PUV-treated samples was undetectable following 5 min treatment. The step-wise progression in digestion was not apparent in
SDS-PAGE or immunoblot analysis but was apparent in ELISA. Thus, although the band corresponding to tropomyosin disappeared after just 5 min under conditions of simulated intestinal digestion, the tropomyosin fragments might not have been fully degraded and might have maintained IgE-binding epitopes. As described above, it appeared that these fragments were gradually broken down as time elapsed, because the IgE-binding epitopes were progressively destroyed. This would explain the disparity between SDS-PAGE, Western blot, and ELISA.

Overall, under the conditions of digestion described: SGF, SIG, and SGF+SIF, PUV-treated samples did not return to their initial reactive state. Thus PUV treatment may be suitable for allergen treatment in the future; however, this postulation must first be tested and further verified with in vivo trials.

Nonthermal Plasma

SDS-PAGE

To assess the effect of NTP on shrimp allergen levels, the extracts were separated using SDS-PAGE. The effect of NTP on tropomyosin reduction was marked, as illustrated by Figure 4-22. Tropomyosin appeared in the control shrimp extract, yet it was greatly reduced following NTP treatments of 1, 3, and 5 minutes. Interestingly, other proteins within the SDS-PAGE profile did not seem to be affected with treatment. Unlike PUV treatment, it appeared that NTP had a propensity towards the tropomyosin protein, but not other proteins. Because NTP technology is novel and mainly used for microbial deactivation, there is no literature to compare these results.

Western Blot

IgE binding to tropomyosin in NTP treated samples was substantially reduced after 1, 3, and 5 min treatment (Figure 4-23). This is likely due to the reduction in
tropomyosin levels seen in Figure 4-22. Furthermore, there appeared to be no reactivity from other allergen bands (myosin light chain or arginine kinase). Furthermore, unlike the putative effects of PUV treatment on shrimp extract, NTP treatment did not appear to cause intramolecular crosslinking. There was no band smearing when the Western blot was probed with the tropomyosin-specific antibody (Figure 4-24).

Dot Blot

To determine the reactivity of whole shrimp extract, including allergic components that were not resolved during SDS-PAGE and Western blot analysis, dot blot analysis was performed (Figure 4-25). Dot blot analysis, confirmed by densitometry, showed a decrease in IgE binding to shrimp extract following 1, 3, and 5 min of NTP treatment. Reductions in IgE binding were similar in 1 and 3 min treatments, with a remarkable decrease in shrimp extract reactivity at 5 min. These data were strikingly similar to the sandwich ELISA results described below.

ELISA

Because it appeared that NTP was specific for the tropomyosin protein, a sandwich ELISA utilizing an anti-tropomyosin capture antibody and human IgE detection antibodies was performed to verify its specificity (Figure 4-26). The data concurred with the dot blot analysis (Figure 4-25). IgE binding to tropomyosin was markedly reduced after 5 min, and IgE binding in 1 min and 3 min samples were significantly different from the control ($\alpha = 0.05$). A reduction of approximately $76.3 \pm 3.2\%$ was noted in the 5 min sample. Thus in this trial, tropomyosin was largely deactivated with NTP treatment, and future trials with longer treatment times could be promising. Furthermore, indirect ELISA was used to verify the decrease in reactivity for the whole shrimp extract. Figure 4-26 shows a marked decrease in reactivity following 5
min NTP treatment. This emerging technology is attractive considering the mild effect on other food proteins as seen in SDS-PAGE (Figure 4-22), yet future trials must validate these data.

**High Hydrostatic Pressure**

**SDS-PAGE, Western Blot, and Dot Blot.** The effects of HHP on tropomyosin levels were unremarkable, as illustrated by Figures 4-28, 4-29, and 4-30, and there were no noticeable differences between samples treated with HHP and the control. To determine the effects of HHP at different temperatures on IgE binding, Western blot and dot blot analysis were performed (Figures 4-29 and 4-31). IgE binding was unchanged compared to the control in all treated samples. Although high hydrostatic pressure has been shown effective in altering the allergen structure and/or reactivity of some food allergens [36, 119, 177], it appeared to have little effect on shrimp extract. Also, the addition of heat did not enhance the effects of pressure, as noted by the sample treated with HHP for 15 min at 70°C. As mentioned, tropomyosin is considered a heat-stable protein, and, in this experiment, we found that it also appeared to be stable to conditions of high pressure. These characteristics may be explained by covalent linkages that stabilize the conformation of tropomyosin under conditions of heat or high pressure, since heat and HHP can affect non-covalent linkages, such as electrostatic interactions and hydrogen bonds, but do not affect covalent bonds. Research on covalent linkages which stabilize the major peanut allergen, Ara h 2, has been described [176]; however, it is yet to be verified for tropomyosin.
Table 4-1. Representative sample data from the time-course experiment displaying changes in temperature and percent moisture loss following PUV treatment.

<table>
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<th>Treatment time (min)</th>
<th>Initial mass (g)</th>
<th>Final mass (g)</th>
<th>Initial temp (°C)</th>
<th>Final temp (°C)</th>
<th>Loss (%)</th>
<th>Change in temp (°C)</th>
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<td>69.0</td>
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Figure 4-1. SDS-PAGE profile of shrimp extract treated with PUV at 0 (control), 1, 2, 3, 4, 5, and 6 min. Molecular weight marker (M) is shown. The band corresponding to tropomyosin (36-kDa) is highlighted with an arrow.
Figure 4-2. Western blot analysis of shrimp extracts treated with PUV at 0 (control), 1, 2, 3, 4, 5, and 6 min using pooled human plasma from 3 individuals containing IgE antibodies against shrimp. Tropomyosin bands (36-kDa) are highlighted with an arrow.

Figure 4-3. Changes in sample color following PUV treatment. A volume of 10 mL of shrimp extract were treated for 0, 1, 2, 3, 4, 5, and 6 min. Samples appear to become more yellow as exposure time increases.
Figure 4-4. Analysis of untreated (raw) and PUV-treated shrimp samples following one week at room temperature. The raw sample appears a bright neon green; whereas the PUV-treated does not change color and appears fresher. These results are assumed to be microbiologically related considering the disinfection effects of PUV. Refer to Fig. 4-3 for comparison of color.

Figure 4-5. SDS-PAGE profile comparing untreated (1), boiled (2), PUV-treated (3), and [boiled+PUV]-treated (4) shrimp extract samples. Molecular weight marker (M) is shown. An arrow highlights the bands corresponding to tropomyosin (36-kDa).
Figure 4-6. Western blot under reduced conditions using (a) pooled human plasma containing IgE antibodies against shrimp and (b) monoclonal anti-tropomyosin (IgG) antibody, for untreated (1), boiled (2), PUV-treated (3) and [boiled+PUV]-treated (4) shrimp extracts. Tropomyosin (36-kDa) bands are highlighted with an arrow.

Figure 4-7. Dot blot analysis of untreated, boiled, PUV-treated, and [boiled+PUV]-treated shrimp extract using pooled human plasma containing IgE antibodies against shrimp.
Figure 4-8. Indirect ELISA illustrating changes in IgE binding compared to untreated, boiled, PUV-treated, and [boiled+PUV]-treated shrimp extracts using pooled human plasma containing IgE antibodies against shrimp. \( A \) = absorbance of the sample; \( A_0 \) = absorbance of untreated sample. Data are expressed as mean ± SEM (n = 5). Results are relative values, normalized to the untreated sample; untreated is standardized and set to 1. Values that are significantly different (\( \alpha = 0.05 \)) from the untreated sample are annotated as **.

Figure 4-9. SDS-PAGE analysis of control (a) and PUV-treated (b) shrimp extracts that have been subjected to simulated gastric digestion. The protease used was pepsin in SGF. Samples were digested for 0, 5, 15, 30, 60, and 120 min. The tropomyosin band (36-kDa) is highlighted with an arrow.
Figure 4-10. Western blot analysis of control (a) and PUV-treated (b) shrimp extracts that have been subjected to simulated gastric digestion. The protease used was pepsin in SGF. Samples were digested for 0, 5, 15, 30, 60, and 120 min. Human IgE antibodies were used to determine the reactivity of tropomyosin. The tropomyosin band (36-kDa) is highlighted with an arrow.

Figure 4-11. Western blot analysis of control (a) and PUV-treated (b) shrimp extracts that have been subjected to simulated gastric digestion. The protease used was pepsin in SGF. Samples were digested for 0, 5, 15, 30, 60, and 120 min. The detection antibody used was a monoclonal anti-tropomyosin (IgG). Band smearing is noted in the PUV-treated samples. The tropomyosin band (36-kDa) is highlighted with an arrow.
Figure 4-12. ELISA of control (a) and PUV-treated (b) shrimp extracts that have been subjected to simulated gastric digestion. The protease used was pepsin in SGF. Samples were digested for 0, 5, 15, 30, 60, and 120 min. Human IgE antibodies were used to determine shrimp allergen reactivity. Relative IgE binding is expressed as $A/A_0$, where $A$ is the absorbance of the sample divided by the absorbance of the control/untreated. Data are expressed as mean ± SD.

Figure 4-13. Dot blot analysis of digested untreated (U) and PUV-treated (P) shrimp extracts. Samples were digested for 0, 5, 15, 30, 60, and 120 min in either SGF, SIF, or SGF followed by SIF (SGF+SIF). Proteases used were pepsin (SGF) and trypsin and chymotrypsin (SIF).
Figure 4-14. SDS-PAGE analysis of control (a) and PUV-treated (b) shrimp extracts that have been subjected to simulated intestinal digestion. The proteases used were trypsin and α-chymotrypsin in SIF. Samples were digested for 0, 5, 15, 30, 60, and 120 min. The tropomyosin band (36-kDa) is highlighted with an arrow.

Figure 4-15. Western blot analysis of control (a) and PUV-treated (b) shrimp extracts that have been subjected to simulated intestinal digestion. The proteases used were trypsin and α-chymotrypsin in SIF. Samples were digested for 0, 5, 15, 30, 60, and 120 min. Human IgE antibodies were used to determine the reactivity of tropomyosin. The tropomyosin band (36-kDa) is highlighted with an arrow.
Figure 4-16. Western blot analysis of control (a) and PUV-treated (b) shrimp extracts that have been subjected to simulated intestinal digestion. The proteases used were trypsin and α-chymotrypsin in SIF. Samples were digested for 0, 5, 15, 30, 60, and 120 min in SIF. The detection antibody used was a monoclonal anti-tropomyosin (IgG). The tropomyosin band (36-kDa) is highlighted with an arrow.

Figure 4-17. Indirect ELISA of control (a) and PUV-treated (b) shrimp extracts that have been subjected to simulated intestinal digestion. The proteases used were trypsin and α-chymotrypsin in SIF. Samples were digested for 0, 5, 15, 30, 60, and 120 min in SIF. Human IgE antibodies were used to determine allergen reactivity. Relative IgE binding is expressed as A/A₀, where A is the absorbance of the sample divided by the absorbance of the control/untreated. Data are expressed as mean ± SD.
Figure 4-18. SDS-PAGE analysis of control (a) and PUV-treated (b) shrimp extracts that have been subjected to simulated gastric digestion followed by simulated intestinal digestion. The protease first used was pepsin in SGF, followed by a mixture of trypsin and α-chymotrypsin in SIF. Samples were digested for 120 min in SGF followed by 0, 5, 15, 30, 60, and 120 min in SIF. The tropomyosin band (36-kDa) is highlighted with an arrow.

Figure 4-19. Western blot analysis of control (a) and PUV-treated (b) shrimp extracts that have been subjected to simulated gastric digestion followed by simulated intestinal digestion. The protease first used was pepsin in SGF, followed by a mixture of trypsin and α-chymotrypsin in SIF. Samples were digested for 120 min in SGF followed by 0, 5, 15, 30, 60, and 120 min in SIF. Human IgE antibodies were used to determine the reactivity of tropomyosin. The tropomyosin band (36-kDa) is highlighted with an arrow.
Figure 4-20. Western blot analysis of control (a) and PUV-treated (b) shrimp extracts that have been subjected to simulated gastric digestion followed by simulated intestinal digestion. The protease first used was pepsin in SGF, followed by a mixture of trypsin and α-chymotrypsin in SIF. Samples were digested for 120 min in SGF followed by 0, 5, 15, 30, 60, and 120 min in SIF. The detection antibody used was a monoclonal anti-tropomyosin (IgG). The tropomyosin band (36-kDa) is highlighted with an arrow.

![Western blot analysis](image)

Figure 4-21. Indirect ELISA of control (a) and PUV-treated (b) shrimp extracts that have been subjected to simulated gastric digestion followed by simulated intestinal digestion. The protease first used was pepsin in SGF, followed by a mixture of trypsin and α-chymotrypsin in SIF. Samples were digested for 120 min in SGF followed by 0, 5, 15, 30, 60, and 120 min in SIF. Human IgE antibodies were used to determine allergen reactivity. Relative IgE binding is expressed as A/A₀, where A is the absorbance of the sample divided by the absorbance of the control/untreated. Data are expressed as mean ± SD.

![Indirect ELISA](image)
Figure 4-22. SDS-PAGE illustrating protein profiles of shrimp extracts treated with nonthermal plasma (NTP) for 1, 3, and 5 min compared to untreated shrimp extract (C). Tropomyosin band (36-kDa) is highlighted with an arrow.

Figure 4-23. Western blot illustrating IgE binding to shrimp allergens treated with nonthermal plasma (NTP) for 1, 3, and 5 min compared to untreated shrimp allergen (C). Tropomyosin band (36-kDa) is highlighted with an arrow.
Figure 4-24. Western blot illustrating tropomyosin levels of shrimp extract treated with nonthermal plasma (NTP) for 1, 3, and 5 min compared to untreated shrimp extract (C). The antibody used was a monoclonal anti-tropomyosin (IgG) which determines the presence or absence of tropomyosin but does not determine the reactivity of this allergen. Tropomyosin band (36-kDa) is highlighted with an arrow.

Figure 4-25. Dot blot illustrating changes in IgE binding to shrimp extracts treated with nonthermal plasma (NTP) for 1, 3, and 5 min compared to untreated shrimp extract. Allergen reactivity was determined using pooled human plasma containing IgE antibodies against shrimp. Shrimp extract was blotted at 1.25 and 2.5 μg of protein.
Figure 4-26. Sandwich ELISA illustrating changes in IgE binding to shrimp extracts treated with nonthermal plasma (NTP) for 1, 3, and 5 min compared to untreated shrimp extract. The capture antibody was anti-tropomyosin and allergen reactivity was determined using pooled human plasma containing IgE antibodies against shrimp. Data are expressed as mean of triplicates ± SD. A = absorbance of sample; A₀ = absorbance of untreated sample. Results are relative values (n=1), normalized to the untreated sample; untreated is standardized and set to 1.

Figure 4-27. Indirect ELISA illustrating changes in IgE binding to shrimp extracts treated with nonthermal plasma (NTP) for 1, 3, and 5 min compared to untreated shrimp extract. Allergen reactivity was determined using pooled human plasma containing IgE antibodies against shrimp. Data are expressed as mean of triplicates ± SD. A = absorbance of sample; A₀ = absorbance of untreated sample. Results are relative values (n=1), normalized to the untreated sample; untreated is standardized and set to 1.
Figure 4-28. SDS-PAGE illustrating protein profiles of shrimp extracts treated with high hydrostatic pressure for 30 min, at 4 and 21°C, and 15 min, at 70°C compared to untreated shrimp extract. Tropomyosin band (36-kDa) is highlighted with an arrow.

Figure 4-29. Western blot illustrating IgE binding to shrimp allergens treated with high hydrostatic pressure for 30 min, at 4 and 21°C, and 15 min, at 70°C compared to untreated shrimp allergen. Tropomyosin band (36-kDa) is highlighted within a box.
Figure 4-30. Western blot illustrating tropomyosin levels of shrimp extract treated with high hydrostatic pressure for 30 min, at 4 and 21°C, and 15 min, at 70°C compared to untreated shrimp extract (C). The antibody used was a monoclonal anti-tropomyosin (IgG) which determines the presence or absence of tropomyosin but does not determine the reactivity of this allergen. Tropomyosin band (36-kDa) is highlighted with an arrow.

Figure 4-31. Dot blot illustrating changes in IgE binding to shrimp extracts treated with high hydrostatic pressure for 30 min at 4 and 21°C and 15 min at 70°C compared to untreated shrimp extract. Allergen reactivity was determined using pooled human plasma containing IgE antibodies against shrimp. Shrimp extract was blotted at 1.25 and 2.5 μg of protein.
CHAPTER 5
CONCLUSIONS

Research devoted to reducing allergens from food products is gaining popularity due to the increasing prevalence of food-related allergies worldwide. By using thermal and nonthermal processing methods, researchers have modified allergen reactivity by changing protein structure and altering IgE-binding sites. In the case of thermal processing, dry heat often appears to affect allergen reactivity via Maillard browning, whereas moist heat largely affects allergens by protein denaturation. For nonthermal processing, there are multiple mechanisms that have been explained which can affect food allergen reactivity, but the underlying concept is modification to the IgE-binding epitopes. For example, conformational epitopes can be altered by the aggregation or crosslinking of proteins; linear epitopes can be fragmented, masked by modification, or altered by genetic modification. Many nonthermal processing techniques, such as HHP, high intensity ultrasound, PUV, gamma irradiation, genetic modification, and, more recently, NTP, are attractive because often they have the ability to alter allergen reactivity without significantly changing the inherent properties of the foods. The relationship between protein conformation and allergen reactivity is a principal concept that can be utilized by researchers dedicated to developing methods for food allergen reduction.

In this study, the effects of three food-processing methods (PUV, NTP, and HHP) on Atlantic white shrimp allergen reactivity were examined. A marked decrease in IgE binding to shrimp extract following PUV and NTP treatment has been demonstrated. The decrease in shrimp allergen reactivity caused by PUV and NTP treatment was likely due to a reduction in the detectable level of tropomyosin, as well as, allergen
modification. Furthermore, the appearance of protein band smearing in PUV-treated samples was likely due to the covalent modification of tropomyosin and may contribute to the decrease in IgE binding. Boiling increased IgE binding to the whole shrimp extract in PUV-treated samples; however, the effect of boiling was greatly reduced when it was combined with PUV treatment. Furthermore, simulated digestion did not cause an increase in the reactivity of PUV-treated shrimp allergens. In fact, decreases in allergen reactivity following gastric and intestinal digestion were noted.

Interestingly, NTP treatment markedly reduced tropomyosin levels yet did not noticeably affect other proteins. Samples treated with NTP for 5 min had a significant decrease in IgE binding to tropomyosin. Thus, the NTP technology may be promising for the future of less-allergenic shrimp products due to its propensity towards tropomyosin.

With HHP processing, there was no notable change in shrimp allergen reactivity. Specifically, protein profiles of the HHP-treated samples were similar to the control. Also, IgE binding to tropomyosin and whole shrimp extract was unchanged. Because tropomyosin is considered heat-stable and was found to be stable to peptic digestion and HHP, it may be speculated that the allergen is stabilized by covalent bonds.

Although nonthermal processes are often advantageous over thermal processes, they also have limitations. For example, PUV and NTP have limited penetration depth; thus samples must be carefully prepared in order for treatment. Furthermore, in PUV and NTP treatment, vitamins may be diminished by photodegradation or free radical generation. Also, when these technologies are used for short periods of times, negligible rises in temperature are noted; however, at longer exposure times, increases
in temperature may become significant—especially in the case of PUV. Lastly, off-odors or ozone may be formed by NTP or PUV, yet in the case of PUV, this has not led to degradation in product quality. These drawbacks may be assessed and overcome in future studies.
Overall, PUV and NTP were found to be capable of reducing the allergenic potency of shrimp extracts. Due to a limited penetration depth, the future of PUV and NTP in regards to whole-body shrimp treatment is unclear; however, treated-shrimp homogenate could be used for multiple food products such as shrimp snacks, shrimp-flavored noodles, and shrimp seasoning. The efficacy of using PUV or NTP treatment for whole-body shrimp in terms of allergen reduction needs to be determined using future research and testing. The possibility of using the temperature rise during PUV exposure as a cooking tool for whole shrimp may also be elucidated in this testing.

The mechanism of PUV or NTP can be further explained using circular dichroism and fluorescence studies, where the tropomyosin protein would be isolated and changes in protein structure could be elucidated. The stability of the tropomyosin protein to heat and HHP may be due to covalent bonding. To test this, covalent bonds can first be reduced, using chemicals such as β–mercaptoethanol or dithiolthreitol, and HHP or thermal treatment can be applied to determine if reducing covalent bonds causes a reduction in the stability of the protein.

Nutritional testing, including lysine bioavailability would be useful to determine both nutritional value and the effects of the Maillard reaction, following food processing. Also, vitamin content including the reduction of certain vitamins (A and C) and the increase in other vitamins (D) could be measured. Because shrimp has high cholesterol content and studies have shown that UV-light can convert cholesterol to vitamin D, further studies on this conversion should be carried out.
To reduce the effects of thermal increases, PUV equipment could be fitted with heat sinks. Also, different filters can be placed on the quartz window of the PUV equipment to separate out wavelengths such as infrared and to remove the thermal effects of infrared radiation. This may also be useful in determining which wavelengths have the largest effects during PUV treatment of allergens.

Lastly, the use of the term “hypoallergenic” for food products still needs to be defined to ensure food safety, and further research and optimization are needed before the PUV and NTP technologies can be adopted for “hypoallergenic” food production. Sensory testing would be necessary to determine changes in the organoleptic traits of shrimp following these food-processing techniques and to determine consumer acceptability. Finally, animal studies would be needed to verify the reduction in allergenic potency of the PUV-treated shrimp extract.
APPENDIX A
PUV ENERGY AND PENETRATION DEPTH

Table A-1. Broadband energy measurement during pulsed UV-light treatment for batch processing. The information in this table was extracted from the work of Krishnamurthy [178]. The approximate energy in J/cm² per pulse for shrimp extract batch treatment was estimated using this information.

<table>
<thead>
<tr>
<th>Distance from quartz window² (cm)</th>
<th>Distance from centre axis of lamp (cm)</th>
<th>Energy³ (J/pulse)</th>
<th>Energy⁴ (J/cm² per pulse)</th>
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<tr>
<td>2.60</td>
<td>8.40</td>
<td>7.19</td>
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<tr>
<td>3.80</td>
<td>9.60</td>
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</tr>
<tr>
<td>12.40</td>
<td>18.20</td>
<td>3.47</td>
<td>0.19</td>
</tr>
</tbody>
</table>

¹Radiometer was calibrated at 254 nm and measured the broadband energy in the wavelength range of 100 to 1100 nm.
²The distance between the quartz window and the centre axis of the UV-strobe is 5.8 cm.
³Energy was averaged over 30 pulses; three independent measurements were taken and average is reported
⁴Surface area of the radiometer detector head was 18.096 cm²
APPENDIX B
STATISTICAL ANALYSIS

Table B-1. LSD test for PUV-treated shrimp extracts.

\textit{t} Tests (LSD) for AbsRatio

\textbf{NOTE:} This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

\begin{itemize}
\item Alpha \hspace{1cm} 0.05
\item Error Degrees of Freedom \hspace{1cm} 16
\item Error Mean Square \hspace{1cm} 0.00842
\item Critical Value of \textit{t} \hspace{1cm} 2.11991
\item Least Significant Difference \hspace{1cm} 0.123
\end{itemize}

Means with the same letter are not significantly different.

\begin{center}
\begin{tabular}{lcc}
\text{t Grouping} & \text{Mean} & \text{N} & \text{treatment} \\
\hline
A & 1.34006 & 5 & Boiled \\
B & 1.00000 & 5 & Raw \\
B & 0.90094 & 5 & PUV + Boiled \\
C & 0.77174 & 5 & PUV \\
\end{tabular}
\end{center}

Table B-2. Duncan's multiple range test for PUV-treated shrimp extracts.

\textit{Duncan's Multiple Range Test} for AbsRatio

\textbf{NOTE:} This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

\begin{itemize}
\item Alpha \hspace{1cm} 0.05
\item Error Degrees of Freedom \hspace{1cm} 16
\item Error Mean Square \hspace{1cm} 0.00842
\item Number of Means \hspace{1cm} 2 \hspace{1cm} 3 \hspace{1cm} 4
\item Critical Range \hspace{1cm} 0.1230 \hspace{1cm} 0.1290 \hspace{1cm} 0.1327
\end{itemize}

Means with the same letter are not significantly different.

\begin{center}
\begin{tabular}{lcc}
\text{Duncan Grouping} & \text{Mean} & \text{N} & \text{treatment} \\
\hline
A & 1.34006 & 5 & Boiled \\
B & 1.00000 & 5 & Raw \\
B & 0.90094 & 5 & PUV + Boiled \\
C & 0.77174 & 5 & PUV \\
\end{tabular}
\end{center}
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Sandi Shriver was born in 1984 in Melbourne Beach, FL. Growing up, she loved
the beach, surfing, and golfing. After graduating at the top of her high school class in
2003, she attended the University of South Florida from 2003-2007 and graduated
summa cum laude with her Bachelor of Science in biomedical sciences and a minor in
biomedical physics. Following graduation, Sandi took a year to travel to Europe and
Australia to gain experience with different cultures and lands. She then worked in a
laboratory devoted to cardiovascular health, learned invaluable research skills, and,
thanks to her mentor (who was also a registered dietician) discovered a love for food
science. Sandi was offered an assistantship at the University of Florida to pursue a
master’s degree in food science in 2009. She was funded for two years by the Yeomans
Fellowship. In May 2011, she graduated with her M.S. in food science and human
nutrition.

During the course of her master’s degree, Sandi was a representative for the food
science division of the Food Science and Human Nutrition Graduate Student
Association (FSHN GSA). She also formed and captained her department’s intramural
sports teams. She was a member of IFT, and she earned second place in her division
for the poster competition at the IFT Annual Meeting of 2010. She was also first author
on two manuscripts.