EFFECT OF PULSED ULTRAVIOLET LIGHT, HIGH HYDROSTATIC PRESSURE AND NON-THERMAL PLASMA ON THE ANTIGENICITY OF ALMOND

By

YIQIAO LI

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To my lovely family

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Yiqiao Li

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Chair: Wade Yang
Cochair: Authur A. Teixeira
Major: Agricultural and Biological Engineering

Although there have been a number of studies showing the effect of various thermal and non-thermal treatments on the antigenicity of almond proteins, they have been proven to be ineffective due to the heat resistance and stability of almond allergen. In this study, the efficacy of pulsed ultraviolet light (PUV), high hydrostatic pressure (HHP) and non-thermal plasma (NTP) on reducing IgE-binding to almond allergen was studied. Almond extract was subjected to PUV, HHP and NTP at preset intensities and durations, and allergen levels and its IgE binding were analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblot, and enzyme-linked immunosorbant assay (ELISA).

Crude almond protein extracts (10 ml) were treated with PUV (3 pulses/s, 10 cm from lamp) for 0.5, 1, 2, 3, 4, 6, 7 and 10 min. The HHP was conducted at 600 MPa for 5, 15 and 30 min at three initial sample temperatures of 4, 21 and 70°C. The NTP treatment was performed at voltage 30 kV and frequency 60 Hz for 1, 3, and 5 min. IgE binding to almond allergen was analyzed via Western blot and indirect ELISA, probed

by human plasma containing IgE antibodies to almond and/or a rabbit anti-almond major protein (AMP) antibody.

The PUV treatment of almond resulted in reduction in protein solubility as illustrated by SDS-PAGE. Western blotting demonstrated reduced IgE binding in PUV treated samples, which may be attributed to the masking of allergens due to aggregation by cross-linking that increased the molecular weight of the protein aggregates. Indirect ELISA indicated that IgE binding was reduced by 76% following PUV treatment for 7 min compared to raw samples. Further testing with the whole almond kernels, instead of the protein extracts, subjected to PUV radiation also showed a decrease in IgE binding, as indicated by ELISA. This shows that PUV technology might be suitable for treating ground or whole almonds for allergen reduction. Unlike the PUV treatment, HHP and NTP treatments did not affect allergen levels or IgE binding at any of the conditions tested in this study.

In conclusion, PUV treatment of whole almond or almond extracts diminished *in vitro* immunoreactivity of almond allergens. If verified by further *in vivo* testing and experimentation, PUV treatment may provide a method for creating almond products with much reduced immunoreactivity.

CHAPTER 1
INTRODUCTION

According to the Food and Agriculture Organization (FAO 2011), global production of almonds is around 1.7 million metric tons at an average growth of 5% annually from 1993 to date, with a low throughput of 1 million tons in 1995 and a peak of around 1.9 million tons in 2002. In 2009, the top 6 almond production countries, i.e., United States, Spain, Syria, Turkey, Algeria and China, harvested 1.7 million tons. The U.S. production, which is concentrated in California, constitutes 30-40% of world's almonds. In 1999, the U.S. produced 360,000 metric tons of almonds, of which 210,000 tons were exported. However, in 2009, 10 years later, the U.S. production increased to 734,000 tons, of which 631,000 tons were exported, according to the 2009 statistics of California Almond Board (2009).

Almonds are globally popular and are often used as a snack food and ingredients in food products, such as bakery and confectionary products (Venkatachalam and others 2002). Although almond ranks third behind cashew nuts and walnut in the Food Allergy and Anaphylaxis Network (Sicherer and others 2001), its consumption is actually more than that of walnuts and cashew nuts (Albillos and others 2009). In tree nut production, almonds rank first in the world, and the U.S. has over 33% of the global almond production over the past 10 years.

Almond is a highly nutritious food and is a natural source of various nutrients. The high level nutrients in almonds, including fiber, minerals, and vitamins are essential for human health. Because of these health benefits, almond is popular worldwide (Sathe and Sze 1997). Every 100 gram of almonds contains 21.26 gram protein, 50.64 gram fat and 19.74 gram carbohydrate (Chen and others 2006). Sucrose and raffinose were the

main soluble sugars found in almond kernels (Nanos and others 2002),

In spite of their popularity and health benefits, tree nuts like almond can induce acute generalized allergy symptoms and even anaphylactic shock. Unlike some food allergies that are temporary and only affect young children, allergies to tree nuts are often lifelong and can be life-threatening (Roux and others 2001). Unlike other food allergies which can develop tolerance as children get older, only less than 10% of tree nut allergic people can outgrow tree nut allergy (Skripak and Wood 2008). Tree nuts affect 0.5% of the population (Roux and others 2001). There is currently no treatment for Immunoglobulin E (IgE)-mediated food allergy, and avoidance is not always possible, because allergens may be present in processed food due to cross-contamination by shared equipment or a consumer may unexpectedly consume a food which contains an allergen due to ingredient mislabeling. Hence, finding different approaches, including the post-harvest ways to eliminate or mitigate food allergen, is of great importance.

In almond, its major protein, AMP or amandin, is the major allergen as recognized by almond-allergic patients (Roux and others 2001). Almond allergens have molecular weights at 20, 22, 25, 48, and 65 kDa (Scheibe and others 2001). Human plasma containing IgE antibodies specific to almond bind the same peptides probed by rabbit anti-AMP polyclonal antibodies (Roux and others 2001).

There have been several technologies utilized so far for inactivation of almond allergens, including microwave heating, thermal processing (Venkatachalam and others 2002), chemical processing (Acosta and others 1999), and gamma irradiation (Su and others 2004). Those methods either partially remove or did not effectively alter the

allergens in almonds. However, no study is known about the inactivation of almond allergen by pulsed ultraviolet light (PUV), high hydrostatic pressure (HHP) and non-thermal plasma (NTP).

In this study, the effects of PUV, HHP and NTP treatments on almond antigenicity were investigated. The PUV treatments were applied to both the extracts from almond powder and the whole almond kernels. The HHP and NTP were used to treat the extracts from almond powder. SDS-PAGE, Western Blot and Indirect ELISA were conducted to evaluate the treated and untreated almond allergens. The goals of this study are to determine the effectiveness of PUV, HHP and NTP in inactivating almond allergen and the optimal treatment time for the sample to reduce IgE-binding reactivity. Statistical analysis was used to determine the IgE bindings after treatments and to determine which one is most effective in achieving the highest IgE binding reduction.

The benefit of finding an effective technique in modifying allergen and reducing the antigenicity of the allergen is countless. The individuals suffering from almond allergies will worry less about the presence of almond protein in various food products. The food industry will benefit from the new technique by producing almond with less allergen or without allergen while reducing the heating or sterilization procedures.

In PUV technology, the electrical energy is stored in a capacitor for a short period of time (few milliseconds) and is released as short-period (several nanoseconds), intermittent pulses. Inert gas in a lamp is ionized by the electrical energy, which produces broad spectrums of light contain wave lengths from near infrared to ultraviolet. Pulse rate is 1-20 pulses per second and the pulse width is 300 ns – 1ms. Although the total energy is comparable with conventional UV light, the short duration energy is

multiplied by many folds, and thus inactivation of microorganisms and alteration of allergen potency can occur. Studies have shown that pulses can be approximately 90,000 times more intense than the sunlight at the sea level (Chung and others 2008a). The UV fraction contributes to inactivate microorganisms by forming thymine dimmers, and the infrared region contributes to the heating as well as other possible photothermal effects. The PUV light penetrates deeper than conventional UV light (Krishnamurthy and others 2008). Groups of proteins may be disturbed by the high intensity pulses in the form of heat or photons. This energy increase can excite electrons of the groups to a higher energy level and subsequently return to ground state, which induces chemical changes and cross-linking reaction of proteins. Chung and others (2008) found IgE binding of the raw peanut extract was approximately 7-fold higher than the PUV treated peanut extract. This reduction of allergen reactivity by PUV is reported due to the change of structure and conformation of molecule. IgE-binding sites may be blocked after proteins undergo modification, causing a decrease in protein immunoreactivity.

High hydrostatic pressure treatment (HHP) has a pressure from 100 to over 800 MPa. Food placed in a pressure vessel which is filled with transmitting fluid before pressurization. The transmitting liquid will then be pressed by a pump or pressure intensifier. The pressure is uniformly distributed in all directions of the food (Spilimbergo and others 2002). The killing mechanism is that high pressure breaks non-covalent bonds (hydrogen, ionic, and hydrophobic bonds) that are present in polysaccharides, proteins, nucleic acid, and lipids. Those broken non-covalent bonds will inactivate enzymes that are responsible for DNA replication and transcription, thus stopping

microorganisms from reproduction. The disruption of non-covalent bonds will also damage cell membrane of the bacteria (Hoover and others 1989).

Non-thermal plasma (NTP) is electrically energized and highly energetic gaseous matter which can be generated by electrical discharge across an electrical field. The hypotheses of bacterial killing mechanism are oxidation of membrane lipids of bacteria cells by reactive oxygen species (Montie and others 2002), destruction of DNA by UV irradiation, erosion of spore surface by free radicals, and volatilization of compounds of spore by UV (Philip and others 2003). These mechanisms may also be applicable to allergen reduction of NTP in foods. The NTP species, including electrically neutral gas molecules, free radicals, photons, negative or positive ions and electrons, oxidize and decompose the inorganic and organic compounds. The inactivation effects of NTP may differ with the change of treatment time, frequency and voltage (Deng and others 2007). Non-thermal atmospheric pressure plasma has been used to inactivate pathogens on bacon and sterilization of *Listeria monocytogenes* on various materials (Kim and others 2011). Deng and others (2007) reported that NTP reduced E.coli on almond by approximately 5-log after 30 s at 30 kV and 2000 Hz.

CHAPTER 2
LITERATURE REVIEW

Food Allergy Overview

Food allergies are an abnormal immunological reaction to a food or food component, most of which are IgE-mediated hypersensitivity diseases (Poms and Anklam 2004). Food allergens can be defined as those substances in foods that induce the immunological reactions (Poms and Anklam 2004). There are more than 160 allergenic food materials, of which eight food materials lead to more than 90% of food allergies (Poms and Anklam 2004). Food allergic individuals can produce specific antibodies, when in contact with these antigens. Immunoglobin E (IgE) is a type of antibody that interacts with specific allergens in food (NIAID 2010).

The mechanism in IgE-mediated food allergies is well known. When the immune system is exposed to a specific food allergen, the antibodies are produced in response to stimulus of B cells. The IgE antibodies bind to the mast cells or basophils in the blood, which is a process called sensitization. Sensitization is symptomless. The next time the sensitized immune system is exposed to the same food allergen, it will cause an allergic reaction. The chemical mediators like histamine will release from the granules in the mast cells and basophils. The histamine can cause inflammation, pruritis, and contraction of muscles in the blood vessels, gastrointestinal tract, and respiratory tract (Taylor 2006).

Food allergy symptoms may include swelling of the tongue, lips, and throat; hives; difficulty breathing; abdominal cramping; a drop in blood pressure; vomiting or diarrhea. A small number of sensitive sufferers may experience severe anaphylactic shock. Although anaphylaxis is rare, it can be fatal if not treated immediately (Taylor and Hefle

2002). Till now, there has been no effective treatment to prevent food allergies. Most food allergies can be avoided with awareness, education, and proper management.

Tree nut allergy is one of the "big eight" food allergies. According to a recent epidemiologic survey, 0.5% of the population is affected by tree nuts. Unlike some food allergies that are temporary and only affect young children, allergies to tree nuts are often lifelong and can be life-threatening (Roux and others 2001).

Detection Methods

Shriver and Yang (2011) has recently published a comprehensive review on thermal and non-thermal methods to control food allergy, which includes a section reviewing on different detection methods. Briefly, the methods for detecting allergens in food are enzyme allergosorbent test (EAST), radio-allergosobent test (RAST), immunoblotting, rocket immune-electrophoresis (RIE), Polymerase Chain Reaction (PCR), and enzyme-linked immunosorbent assay (ELISA). ELISA is quantitative, accurate and relatively simple to conduct (Pom and others 2004). Indirect ELISA, Competitive ELISAs and Sandwich ELISAs are three commonly used types of ELISA, and the Indirect ELISA has been used in this study. The alteration of protein configuration and immunoreactivity can effect on the allergen detection.

Almond Allergen Overview

Almonds contain as many as 188 different proteins (Li and He 2004), including an albumin, a globulin (amandin) and emulsin (Wolf and Sathe 1998). The major allergen in almonds is defined as AMP or amandin (Roux and others 2001), which is an oligomeric protein containing prunin monomers. It is a legumin seed storage protein belonging to 11 S globulin families which belong to cupin superfamily. Cupins are generally very heat stable, because the double-stranded β-helix in cupins is a very

stable structural motif (Dunwell and others 2004). The molecular weights of peptide bands of amandin are 22, 28, 39, 41, 42 and 44 kDa (Sathe 1993). The major and water soluble storage protein in almonds (amandin or AMP) is composed of at least two types of polypeptides (38-41 and 20-22 KDa) (Acosta and others 1999). Almond allergen proteins are located in the 20, 22, 25, 48, 65 and 70 kDa regions (Scheibe and others 2001; Bargman and others 1992b). Secondary structure of hexameric amandin did not alter immensely at 90°C. The native amandin has higher thermal stability than the reduced amandin (Albillos and others 2009).The structure and conformation of the molecule play a critical role in shaping the allergenicity of a protein (Lee 1992). The allergenicity of various almond cultivars has been determined (Sathe and Sze 1997).

Treatments for Food Allergy

Many treatments for food allergy involve reducing the overall immune response of patients with food allergies. These include avoidance, oral and sublingual immunotheraphy, early allergen introduction, engineered recombinant and Chinese herbal medicine (Skripak and Wood 2008).

Pulsed Ultraviolet Light

PUV is an intense broad spectrum which is rich in UV-C light. UV-C light is effective in extending shelf life of fresh processed fruits, vegetables, and breads by reducing the microorganisms (Allende and Artés 2003; Butz and Tauscher 2002) and accumulation of antifungal compounds induced by UV (Erkan and others 2001).

Light source, dosage, exposure time, transmissivity and distance of the treated samples should be considered prior to PUV treatment. UV radiation loses 30% of its intensity 40 cm below the surface of distilled water (Bintsis and others 2000). The less distance from the sample to the light source, the more effective the PUV light treatment.

Food with low transmissivity has low penetration of PUV light (Guerrero-Beltran and Barbosa-Cánovas 2004). Color, solid content and thickness are factors that may affect the transmissivity of food. Thus, thin transparent liquid will make the PUV light more effective, which is the reason why almond extracts were used initially for PUV treatment in this study.

Prolonged UV light treatment causes formation of insoluble complex in food, depolymerization of starch, peroxidation of unsaturated fatty acid, carbohydrate crosslinking, protein crosslinking, and protein fragmentation (Fiedorowicz and others 2001). Carbohydrate crosslinking is due to the formation of superoxide radicals during PUV illumination. Prolonged PUV can change the bioactivity and quality of food components and nutrients.

Proteins absorb light at 280 nm wavelength (Gómez - López and others 2005). Light with shorter wavelength is more effective in changing protein molecule. The PUV treatment could not easily break the peptide bonds in protein. Photons absorbed by cystine had a higher chance of inactivating a protein than photons absorbed in the aromatic amino acids. The absorbed photons ionize the protein (Setlow 2002). Aromatic amino acids (e.g., tyrosine and phenylalanine) can absorb UV radiation and recombine to form covalent cross-links in proteins (Gennadios and others 1998). Almonds have amino acid compositions such as tyrosine, phenylalanine and cystine (Ahrens and others 2003). Therefore, UV photons from the PUV radiation have great potential to be absorbed by almond amino acids and inactivate proteins in almond.

The UV portion of PUV forms oxygen radicals, superoxide radicals, hydrogen peroxide and super oxide radicals. Oxygen radicals lead to generation of ozone. Super

oxide radicals can induce protein cross-linking and protein fragmentation. Water molecules absorb UV photons and produces hydroxyl and hydrogen radicals (OH^- and H^+) which are powerful protein-modifying agents (Krishnamurthy and others 2008; Davies and others 1987). Hydroxyl radical were shown to induce alteration to the primary structure of bovine serum albumin (BSA). Oxygen radicals significantly alter the effects of hydroxyl radicals on protein primary structure. Protein aggregation by hydroxyl radical may involve intermolecular bityrosine formation (Davies and others 1987). However, there may be other covalent modifications involved in the process of PUV treatments.

Methods for Reducing Allergen Reactivities of Food

Food antigenicity can decrease, increase or remain unchanged in various food processing operations. The changes in antigenicity, if any, are due to the destruction of epitopes, generation of new epitopes or exposure of hidden epitopes (Besler and others 2001). Epitope is part of an allergen which is recognized by the immune system, including conformational epitope and linear epitope.

Post-harvest techniques can directly reduce the allergen potency of foods by masking or inactivating epitopes or altering the protein structures and conformations of the allergens. The techniques that have been researched on their effects on the reactivity of food allergens include thermal processing and non-thermal processing methods. Non-thermal processing includes, but is not limited to, enzymatic methods, fermentation, germination, genetic modification, chemical processing, physical method, gamma radiation, high intensity ultrasound, high pressure processing, and pulsed ultraviolet light (Sathe and others 2005).

Methods for Minimizing Antigenicity of Almond

For the thermal processing on almond like blanching and roasting, some reduction in reactivity was found for major allergenic protein (AMP) of almonds, however, there was still 60% of the reactivity retained. In other words, blanching, roasting and autoclaving of almonds did not markedly decrease the detectability of AMP (Roux and others 2001). Patients are still allergenic to thermal processed almond because of the heat stability of immunoreactive peptides (Roux and others 2001). Bargman and others (1992) described heat processing reduced major almond allergen with molecular weight of 70 kDa, while the almond allergen with molecular weight of 45-50 kDa was not affected (Bargman and others 1992). Non-amandin polypeptides are also heat stable (Venkatachalam and others 2002).

Thermal processing stability of almond allergen was also investigated by Acosta and others (1999) who described that antigenic stability of commercial processed almond samples (blanched, roasted and moist heat treatment) were respectively reduced by 50% ,56.6% and 87%. Although commercial almonds are often subjected to harsh heat processing, it was demonstrated that commercial processing (roasting, autoclaving, microwave heating and blanching) did not abrogate amandin immunoreactivity (Venkatachalam and others 2002).

Chemical processing includes the exposure to pH extremes. Acosta and others (1999) described that AMP dissolved in borate saline buffer (pH=12.5 and 1.5-2.5) caused a 53% and 57% antigenic decrease. This method has not been determined a desirable method for allergen reduction. Albillos and others (2009) studied AMP stability by the addition of urea at several concentrations. The concentration of urea needed to

achieve 50% denaturation of amandin was 2.59M (Albillos and others 2009). However, this study did not indicate if it has affected the allerginicity of amandin.

γ-irradiation has been reported to reduce the allergenicity of egg and shrimp. However, when the whole almond was treated with γ-irradiation, immunoreactivity of almonds was not affected by γ-irradiation or by γ-irradiation together with thermal processing (blanching, frying, autoclaving, microwaving and roasting) (Su and others 2004), which indicates that the almond protein is fairly stable and resistant to irradiation treatment.

Due to the disadvantage of these treatments, there is a need to look at novel food processing technologies to examine their capabilities for allergen reduction. Previous study has shown reduction of peanut protein allergens (Chung and others 2008b) and soybean allergens (Yang and others 2010) by PUV treatments. Until today there has been no published data on inactivation of almond allergens by PUV treatment.

Method for Mitigating Allergen Potencies of Other Foods

Thermal processing

Thermal processing is often applied in food processing to obtain better flavor and texture or to pasteurize or sterilize foods.

Thermal processing has been studied extensively and has been shown to both decrease and increase allergen reactivity of nuts. Boiling decreased the allergens in the peanut, because the allergens moved into the cooking water, thus reducing the overall antigenicity of peanut. Roasting increased IgE-binding capacity of Ara h1 and Ara h2 compared to raw and boiled peanut. However, there was no change in IgE immunoreactivity between whole protein extract from raw and roasted peanuts (Mondoulet and others 2005). Maleki and others (2000) described roasted peanuts

bound IgE from peanut allergic patients at higher levels than raw peanut. Ara h 1 and Ara h 2 were more resistant to digestion after undergoing Maillard reaction. In contrast, roasted hazelnuts triggered fewer allergic reactions than raw hazelnuts, indicating that hazelnut allergen is heat-liable (Hansen and others 2003). Roasting reduced the antigenicity of hazelnuts. Native hazelnuts needed less amounts of allergen extract concentration than the roasted hazelnuts to induce the same weal reaction and basophil activation (Worm and others 2009; Messens and others 1997).

Extrusion process, which is heating in combination with pressure, was used to treat soy proteins. Extrusion did not alter the antigenicity of two allergens located in 38 and 50 kDa regions, while the IgE reactivity of soybean protein at 31 to 34 kDa was not observed. Thus, the reactivity of total major soybean allergen was decreased (Wilson and others 2005).

Fish major allergen, parvalbumin, has been reported unchanged in antigenicity after undergoing high temperature (90°C) or proteolytic digestion in the gastro intestine (Arif and Hasnain 2010). However, canned tuna and salmon have significantly less protein content than the raw tuna and salmon, thus the antigenicity of canned tuna and salmon was drastically decreased, as demonstrated by ELISA and oral challenges (Bernhisel-Broadbent and others 1992). After boiling treatment, the IgE binding to overall shrimp extract was decreased despite that the antigenicity of tropomyosin is enhanced, as indicated by *in vitro* analyses (Liu and others 2010).

Taheri-Kafrani and others (2009) reported that β-lactoglobulin, a major milk allergen, has lost most of its antigenicity under the heating temperature at 85-95°C(Taheri-Kafrani and others 2009). Heat treatment considerably reduced the

antigenicity of whey proteins, but it did not reduce the antigenicity of casein (Lee 1992). Skin prick tests showed that boiled milk induced weaker immunoreactions of the children. Most of children allergic to milk showed tolerance to extensively heated milk (Nowak-Wegrzyn and others 2008).

Boiling at 100°C for 20 min could not eliminate the immunoactivity of ovalbumin and ovomucoid in eggs (Hoffman 1983). However, children allergic to egg have more tolerance to heated egg than unheated form (Nowak-Wegrzyn and others 2008).

Baking increased the wheat allergen reactivity because Maillard reaction increased the resistance of allergens to proteolytic digestion, which allowed them to reach gastrointestinal system without changing the epitopes and induce immune reaction (Simonato and others 2001).

Thermal processing does not eliminate the allergen potency of the food significantly to prevent a food allergy. Besides, it sometimes causes the undesirable flavors, qualities and properties of food.

Enzymatic methods

Proteolysis did not change IgE binding to peanut allergen, Ara h 2, despite the changes of secondary structure and reduction of disulfide bonds, because the linear epitopes of Ara h 2 were unaltered even when subjected to proteolysis (Sen and others 2002).

For the roasted peanuts and hazelnuts treated with pancreatic enzymes, the antigenicity of peanut proteins was persistent during gastric digestion, as shown in EAST and rat basophil leukaemia (RBL) cell mediator release assays, while the IgE reactivity of digested hazelnuts was reduced to less than 10% (Vieths and others 1999).

Enzymatic hydrolysis was found to significantly reduce the potency of soy allergen P34, but other proteins were also decomposed during this process. Protein hydrolysates had significantly lower antigenicity compared to the original proteins, but protein fragments or native proteins remaining in the hydrolyzed formulas could still trigger a severe allergenic reaction (Wilson and others 2005).

The mechanism of enymatic polyphenol oxidation was that polyphenoloxidase (PPO), as an enzyme, helped oxidize phenols to produce o-quinones derivative. O-quinones reacted with amino group, tryptophan residues or sulfhydryl groups in another protein, thus generating cross-linking of proteins. Researchers found PPO with phenolic compounds, such as caffeic acid and epicatechin reduced antigenicity of cherries major allergen Pru av 1 (Gruber and others 2004). PPO/caffeic acid effectively reduced IgE binding and allergen of peanut by cross-linking induced by PPO and/or caffeic acid (Chung and others 2005a).

Enzymatic digestion may fragment linear protein epitopes, making the antibodies unable to bind to the altered epitopes.

Fermentation

Fermentation hydrolyzed the soy protein into peptides by protease from the microorganisms, which led to the reduction in allergen reactivity. However, the flavor was altered and became unacceptable because of fermentation. The effect of fermentation on the reduction of allergen relies on the type of microorganism used and the level of hydrolysis (Wilson and others 2005).

Genetic engineering

Genetic engineering has been used to reduce the levels of allergens or allergenic epitopes. This technique prevents translation of chosen allergens by antisense gene

silencing or co-suppression (Shewry and others 2001). Herman and others (2003) reported the IgE binding to major soybean allergen, Gly m Bd 30 K, was eliminated by transgene-induced gene silencing. RNA interference method was used to silence the peanut allergens Ara h2 and Ara h6, which remarkably reduced IgE binding to the Ara h2 and Ara h6. However, expression of other peanut allergens Ara h1 and Ara h3 was not altered (Chu and others 2008).

Stability of the hypoallerginicity of genetically modified foods has not been determined. It is potentially risky to eat genetically modified foods if the suppression is incomplete or if the silencing does not exist any more. For many plant foods, lots of transgenes would be required to remove the allergens with no sequence homology. Also, removing the allergenic proteins from foods may alter functional and physical properties of the foods (Shewry and others 2001). It is also beneficial for this type of food allergen reduction technique to remove allergenic epitopes instead of allergen proteins. However, genetic modification has gone through serious scrutiny, as it may introduce new proteins which have the potential to be allergen (Wilson and others 2005).

Chemical methods

Chemical method in removing allergen involves cross-linking or formation of larger complex. Because of formation of cross-links, copper sulfate (CuSO4) significantly reduced allergen and IgE binding in peanut extract, while peanut extract incubated with ferric chloride did not reduce them, because peanut allergens induced the cross-linking of tyrosine residues with copper sulfate instead of ferric chloride (Chung and others 2005b).The Cu^{2+}/H_2O_2 treated peanut extracts showed reduced amount of allergens and allergenic potency due to the conversion of allergens into large polymers (Chung

and Champagne 2007). Phytic acid reduced peanut allergen antigenicity by 6-fold measured by competitive ELISA (ciELISA). The reason was that phytic acid generated insoluble complexes with peanut allergens through which their bioavailability was reduced (Chung and Champagne 2007). Chung and others (2009) found that phenolics irreversibly formed insoluble complexes with major peanut allergens due to the formation of hydrophobic coating on the protein by phenolics or cross-linking between free phenol groups and amino/tryptophan groups. The precipitations of the peanut allergen by phenolics caused a 10-to 16-fold reduction in IgE binding (Chung and Champagne 2009).

Physical methods

Four magnetic-bead systems (Ca2+, Fe3+, caffeic acid, hydrophobic) were found to effectively remove major peanut allergens and reduce IgE bindings. They separate the peanut allergens from other proteins, however, non-allergenic protein was significantly lost as well (Chung and Champagne; Chung and Champagne 2010).

Magnetic beads attached with chlorogenic acid (a phenolic compound) were found to significantly reduce major peanut allergen and slightly reduce other proteins. Peanut allergens complexed with ferric irons were captured by magnetic beads attached with chlorogenic acid, which caused reduced levels of allergens and IgE binding as well (Chung and Champagne 2009).

γ-irradiation

γ-irradiation led to the protein cross-linking, formation of disulfide bonds, protein fragmentation and aggregation, thus it has been reported to reduce the egg allergen ovalbumin at the dose of 100 kGy (Seo and others 2007). γ-irradiation led to

agglomeration and alteration of antigen reactivity of milk, as observed in ELISA and SDS-PAGE (Lee and others 2001).

The γ-irradiation treatment on the heat-stable shrimp protein decreased the IgE activity of the shrimp allergen (Byun and others 2000). In another study, γ-irradiation together reduced IgE binding of shrimp allergen when the dose of the irradiation was above 10 kGy, while γ-irradiation below 5 kGy showed an increase in the allergen potency of the shrimp allergen (Zhenxing and others 2007).

Hen's egg allergen, ovomucoid, has been treated with γ-irradiation alone or together with heat under different pH. Regardless of pH condition, the result showed that γ-irradiation with heat reduced more egg allergen than γ-irradiation or heat alone (Lee and others 2002).

High intensity ultrasound

High intensity ultrasound has been found to alter the protein structure. High intensity ultrasound reduced IgE binding of shrimp and purified shrimp allergen when treated with high intensity ultrasound for 30 to 180 min, as measured with ELISA, Gel-filtration HPLC and immunoblot assays (Li and others 2006).

High hydrostatic pressure

High hydrostatic pressure (HHP) was reported to reduce the antigenicity and nutritional value of soy protein. Sprout seeds treated with HHP grew sprouts with a significant reduction in antigenicity, while no reduction in nutrition value (Peñas and others 2011). HHP combined with enzymatic hydrolysis could be useful in reducing or mitigating the antigenicity of residual soy whey proteins (Penas and others 2006a). HHP applied during or prior to different enzymatic hydrolysis improved whey protein hydrolysis, thus helped reduce the antigenicity of hydrolysates from bovine whey protein

(Penas and others 2006b). HHP at 600 MPa effectively changed the antigenicity of the pork batter's protein of Hungarian fermented sausage (Hajós and others 2004). The allergens of rice grains were squeezed out of the matrix and extracted into the buffer when applied with HHP, therefore, the overall antigenicity of rice grains was reduced (Kato and others 2000). When HHP was applied to liquid whole hen's egg, 3.3-fold reduction in the antigenicity of egg was found, as illustrated by EAST inhibition. When HHP (600MPa) treated egg after heat treatment (70°C), the allergen reactivity of the egg reduced 8.9 fold because of the heat labile allergens in the egg (Hildebrandt and others 2010).

Conversely, HHP ranging from 200 to 600 MPa, or together with heat treatment was reported to increase the reactivity of major milk allergen (ß-lactoglobulin) (Kleber and others 2007), possibly because HHP induced the secondary and tertiary structure changes of meat and milk protein (Messens and others 1997), which made hidden epitopes assessable to antibodies.

Pulsed ultraviolet light

Pulsed ultraviolet light (PUV) has been reported to significantly reduce peanut, soybean, milk, shrimp, egg and wheat allergens.

PUV has been found to markedly reduce some soybeen proteins, such as glycinin and β-conglycinin, while slightly reduce the soybeen proteins in 45kDa and 68-75kDa regions. Indirect ELISA showed that there were 50% reductions in IgE bindings of soybean protein after 6 min PUV treatement (Yang and others 2010).

Shrimp protein, tropomysin, has reduced antigenicity after PUV treatment for 4 min. ELISA shows that the overall shrimp antigenicity has reduced 30%. However,

when the shrimp protein was heated before PUV treatment, the reduction of the antigenicity of shrimp was less than PUV treatment alone (Shriver and others 2010).

PUV treatment together with boiling completely eliminated wheat allergens, whereas PUV treatment alone only slightly reduced the allergenicity of wheat allergen (Nooji and others 2010).

After 150 s of PUV treatment, the milk allergens do not appear in SDS-PAGE (Anugu and others 2009). ELISA showed that PUV treatment reduced antigenicity of isolated egg proteins (Anugu and others 2010).

For more information about different effects of various food processing on food allergens, the treatise by (Sathe and others 2005) can be referred to.

Statement of Problem

Food allergens elicit adverse allergic reactions of food allergic individuals. Food allergy represents a serious problem in the food industry. Allergic reactions to nuts have increased over the past few decades. Plant seeds are good sources of healthy and relatively inexpensive protein; however, 65% of main storage proteins in almond is allergen. Tree nuts like almond can induce acute generalized symptoms and even anaphylactic shock. Unlike milk and egg allergens, nut allergen is permanent and life-threatening. There is currently no treatment for Immunoglobulin E-mediated food allergy. Avoidance is not always possible because allergen may be present unintentionally in processed food due to cross-contamination in food processing equipments or consumers may unexpectedly take food with allergen due to mislabeling of the food products. Hence, there is a need to develop a technique to attenuate the allergen potency or eliminate the reactivity of food allergen to mitigate the health risk of

allergic individuals. If PUV is able to diminish almond allergens to a safe level, those allergic to almond can enjoy the benefits of almond, including taste and nutrition, without suffering from adverse reactions. It has been speculated that PUV reduces allergen reactivity by inducing protein aggregation or protein conformational changes that lead to the masking or changing of Immunoglobulin E (IgE) binding sites.

Objectives

The purpose of this study is to show IgE binding activity and expression of almond allergen protein after PUV, HHP, NTP treatments. For PUV treatment, almond extracts from blanched almond powder and whole almond kernel are used to compare the allergen removal differences.

- The first objective is to evaluate and compare the effect of boiling, PUV, HHP and NTP on reduction IgE binding activity of almond allergens.
- The second objective is to determine optimal exposure times for PUV, HHP and NTP treatment to achieve the best reduction in almond allergen potency.
- The third objective is to discuss the mechanism why IgE could be inhibited after those treatments.

The overall goal of this study is to determine how protein allergens in almond respond to PUV, HHP, NTP treatments at different conditions and determine which treatment is the most effective one in achieving the reduction of IgE binding activity.

CHAPTER 3
MATERIALS AND METHODS

Overview of Methods

In order to have an overview of the experiment procedures of this study, two flow charts (Figure 3-1 and Figure 3-2) were made. Preparation of almond extracts included two steps: (1) finely grounded almond flour (meal) was defatted; (2) proteins from the defatted samples were extracted.

Protein determination and normalization: (1) almond extracts were subjected to Branford assay to determine the protein concentrations, (2) according to Branford assay the protein concentrations were normalized before treatments.

Almond extracts were treated with boiling, PUV, HHP and NTP. The treated almond extracts were tested using SDS-PAGE, Western blot and Indirect ELISA. SDS-PAGE was to study the allergen level of the sample. Western blot was to study allergen reactivity of the sample. Indirect ELISA was to study the whole IgE binding activity of the sample.

Primary Antibody

The specific IgE antibodies to almond from a pool of single donor allergic human plasma and rabbit anti-AMP antisera were utilized for emzyme linked Immunosorbant assay and immunobloting. The almond protein specific human IgE concentration of 13.9kU/L was determined using an ImmunoCAP test (PlasmaLab International, Everett, USA). The control serum was non-allergic human plasma.

Almond Protein Extract and Nut Flour Preparation

Finely grounded almond flour (meal) from blanched whole almonds (Bob’s Red Mill, USA) was purchased from local market. It contains 6 grams of protein per 28

grams of flour. It was defatted by multiple extractions with cold acetone (meal to acetone ratio of 1:5 w/v) with constant magnetic stirring. After each extraction, the slurry was filtered through whatman filter paper #4 and the residue was used for the next extraction. Residue from final extraction and filtration step was dried in a fume hood. After the residue was dry in the fume hood, protein from the defatted samples was extracted with 0.1 M sodium phosphate buffer pH 7.0 (flour: buffer 1:20 w/v) for 30 min at 25°C with gentle shaking provided.

After whole almond kernels were treated with PUV, they were powdered manually using a mortar and pestle, and the flours were defatted and extracted the same way as mentioned above. The samples were then centrifuged at 15000g for 20 min and stored at -20 °C until further use. The supernatant was collected for protein analysis.

Protein Determination

The concentrations of samples were measured by Branford assay. Protein concentration was determined using 10 µl of sample and 300µl of Coomassie Plus Reagent in a 96-well plate at room temperature. The plate was read at 595 nm with a plate reader; diluted albumin (BSA) was used as standard. Take 10 ml out of the untreated sample respectively for boiling and PUV treatment. After treatment, some water was lost due to the increased temperature during PUV treatment. The volume in PUV treated sample was brought up by adding the water to the same volume of the untreated and boiled sample. So the total protein concentration including soluble and insoluble protein was unchanged. Then, samples were subjected to SDS-PAGE, Western blot and ELISA tests.

ELISA, SDS-PAGE and Western Blotting Reagents

Tris buffered saline (TBS), phosphate buffered saline (PBS), o-phenylendiamine dihydrochloride, StartingBlock TBS/T-20 blocking buffer, Gelcode Blue stain reagent, bicinochoninic (BCA) protein assay kit, beta-mercaptoethanol and Tween-20 were obtained from Pierce Chemical company (Rockford,IL). Precast trisglycine minigels (4-20%) and reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was obtained from BioRad Laboratories (Hercules,CA). Costar 96-well plate produced by Corning Incorporated (Corning, NY) was used for ELISA, Immobilon P was obtained from Millipore Corporation (Bedford, MA). Horseradish peroxidase-labeled goat anti-rabbit or goat anti-human antibody was purchased from Invitrogen (Carlsbad,CA). SuperSignal West Pico Chemiluminescent Substrate (Pierce) was purchased from Thermo Scientific.

Equipment

A non-contact infrared thermometer (OMEGA, USA) was used for monitoring the temperatures of the samples immediately after PUV treatment. A Steripulse XL-3000 manufactured by the Xenon Corporation (Woburn, MA) was used for PUV treatment. BioRad equipment was utilized for SDS-PAGE and Western blotting. A Spectramax 340 was used to analyze the 96-well plates. Laboratory-scale high-pressure unit (model Avure PT-1; Avure Technologies, Kent, WA) monitored with DASY*Lab* ® 7.0 software (DASYTEC USA, Bedford, NH) was used for HHP treatment. The experimental non-thermal plasma (NTP) system was used for NTP treatment.

Pulsed Ultraviolet Light Treatment of Almond Extracts

The Xenon Steripulse XL-3000 was used for carrying out PUV treatments. The distance between the sample and the PUV lamp in this unit can be changed by moving

the sample rack up and down. As in previous studies, the total energy at 14.6 cm was determined to be 0.92 J/cm^2/s (Krishnamurthy 2006). The distance 10 cm from the sample to quartz window was used. The distance from quartz window to lamp is 10.5 cm. So, the total distance from lamp to sample is 20.5 cm. Almond extract, 10mL each in an aluminum dish with a diameter of 7cm, was treated with PUV light at a preset time. For boiling treatment, vials containing the same amount of almond extracts as the PUV treated samples were placed in a boiling water bath (100°C) for 0.5, 1, 4, 6, and 7min.

High Hydrostatic Pressure Treatment of Almond Extracts

Each sample (1.5 ml) was transferred to a sterile polypropylene pouch (Fisher Scientific, Fair Lawn, NJ), heat-sealed, and sealed in a secondary pouch. Pressure treatment was carried out using a laboratory-scale high-pressure unit (model Avure PT-1; Avure Technologies, Kent, WA) monitored with DASY*Lab* ® 7.0 software (DASYTEC USA, Bedford, NH). The experiments were conducted at 600 MPa for 5, 15 and 30 min at three initial sample temperatures of 4, 21and 70°C using water as a hydrostatic medium. The pressure increasing rate was 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. Three independent trials were conducted.

Non-Thermal Plasma Treatment of Almond Extracts

The schematic diagram of the experimental non-thermal plasma (NTP) system used in this study is shown in Figure 3-8. It consists two major components, NTP reactor chamber and high voltage power supply, in addition to the electrical parameter measurement and control devices. The non-thermal plasma reactor was the planar configuration of the dielectric barrier discharge reactor with two dielectric layers. The electrodes in the NTP chamber were covered with dielectric plates (epoxy resin board).

The dielectric layer thickness was 0.16 cm (0.062 inch). The discharge chamber was a column with radius of 1 inches and height of 0.64 cm (0.25 inch). The prepared samples were placed in the discharge chamber and treated at following conditions: Voltage 30 kV, Frequency 60 Hz, and each sample has three treatment times: 1, 3, and 5 min. The sample depth is around 0.1 inch. The sample volume is 5 ml. The immunological tests were conducted after NTP treatment.

Electrophoresis of Treated Almond Extracts

The SDS-PAGE was run to show the profile of the protein of different molecular weights. The PUV treated samples were homogenized and the mixtures were then subjected to SDS-PAGE. The same amounts of proteins were loaded to each well on the same SDS-PAGE. The protein quantities loaded in the SDS-PAGE depended on the capacity of the lanes of SDS-PAGE. Each sample was combined with 2X laemlli sample buffer with 0.05% 2-mercaptoethanol to a concentration of 1X. Samples were boiled for 10 min and briefly centrifuged. Samples diluted in sample buffer was loaded onto a precast tris-glycine minigel (4-20%) positioned in a BioRad Mini-Protean tank apparatus and subjected to electrophoresis for 1.5 h at 190 volts. The gel was removed from the cassette and washed 3 times in Nanopure water. Twenty milliliters of Gel Code Blue reagent was added to the gel and incubated with gentle shaking at room temperature followed by distaining in distilled water.

Determination of IgE-Binding to Almond Extracts with Western Blotting

Western blotting is a technique for detecting a specific protein based on the ability of the protein to bind antibodies. The samples underwent electrophoresis as described above. The gel was removed from the cassette, equilibrated in transfer buffer for 1 h and transferred onto Immobilon P blotting membrane (Millipore Corporation, Bedford,

MA) using a semi-dry transfer apparatus at 15 volts for 30 min. The unbound sites on the membrane were blocked using Starting Block Tween for 15-30 min at room temperature with gentle rocking .The membrane were then incubated with patient antiserum (1:80) at 37 °C while rocking for 50 min. The membrane was washed twice with TBS-T, washed with rocking for 20 min each time. The blots were incubated at room temperature for 1 h with mouse anti-human IgE-HRP (Invitrogen, Carlsbad, CA) diluted in PBS at 1:1000. After secondary incubation, the blot was washed twice for 30 minutes each time with gentle shaking. All washes were with 1X TBS/0.05% Tween 20. Finally, SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used for detection.

Determination of IgE-Binding to Almond Extracts with Indirect ELISA

ELISA is an immunological technique used to detect the allergen in the samples. Costar Enzyme Immunoassay (EIA) Polystyrene 96-well plates (Corning, NY) were coated for 2 h at 37°C with raw, boiled, treated almond extract diluted in phosphate buffered saline (PBS) to a concentration of 20 µg/ml (100 µl per well). The plates were blocked with StartingBlock (200 µl per well) at 37°C for 1.5 h. Human plasma containing IgE antibody specific for almond allergens diluted in PBS was added in equal amounts to each well (1:4, 100 µl), and the plate was incubated at 37°C for 1 h. Pooled human plasma from one patient with no known allergies was used as control. After primary incubation, the plate was washed 3 times with TBST and patted dry. Each well was then incubated with secondary antibody, monoclonal mouse anti-human IgE conjugated to HRP (1:1000), at 37°C for 1 h (100 µl per well). After the plate was washed 3 times and patted dry, o-phenylenediamine dihydrochloride (OPD, Pierce) dissolved in Stable

Peroxide Substrate Buffer (Pierce) was added to each well (0.5 µg/µl, 100 µl per well). The reaction was stopped at 15-30 min with 2.5 N sulfuric acid (100 µl per well). Absorbance was read on Spectramax 340^{384} Spectrophotometer (Molecular Devices, Inc. Sunnyvale, CA) at 490 nm, or absorbance was read without stopping with sulfuric acid at 450 nm.

Statistical Analysis

Data was entered into Excel and sorted by treatment type, treatment time and absorbance reading. The data were then transferred onto SAS 9.0 (Cary, NC) software for analysis and comparison between treatments time within the same treatment type. Treatments are the independent variables. Results are the dependent variables. Differences were considered at $\alpha = 0.05$. In addition, a separation of means was conducted on treatment AOV results to see if there were any differences between the treatment time and treatment type. Least Significant Difference (LSD) Test was performed after AOV.

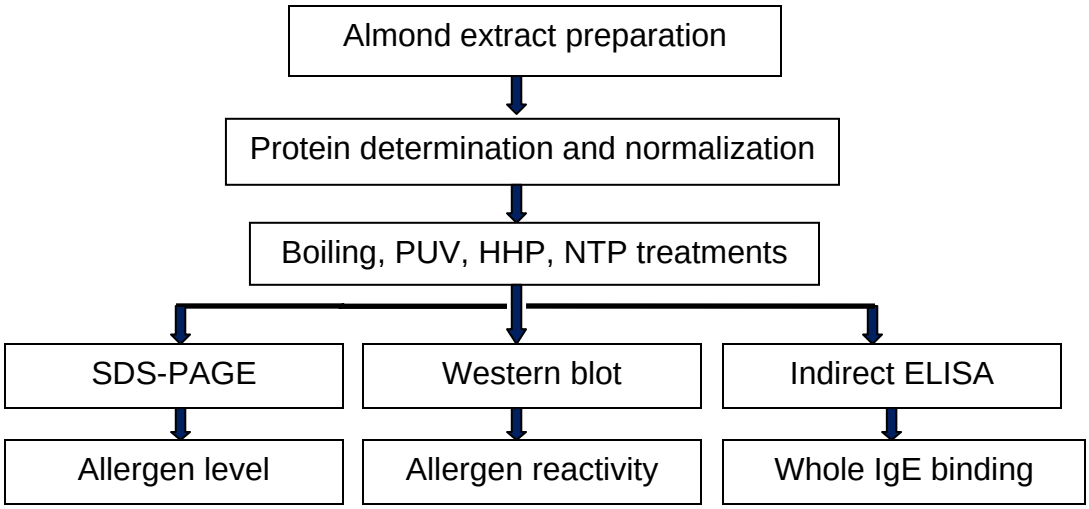

Figure 3-1. Flow chart (1)

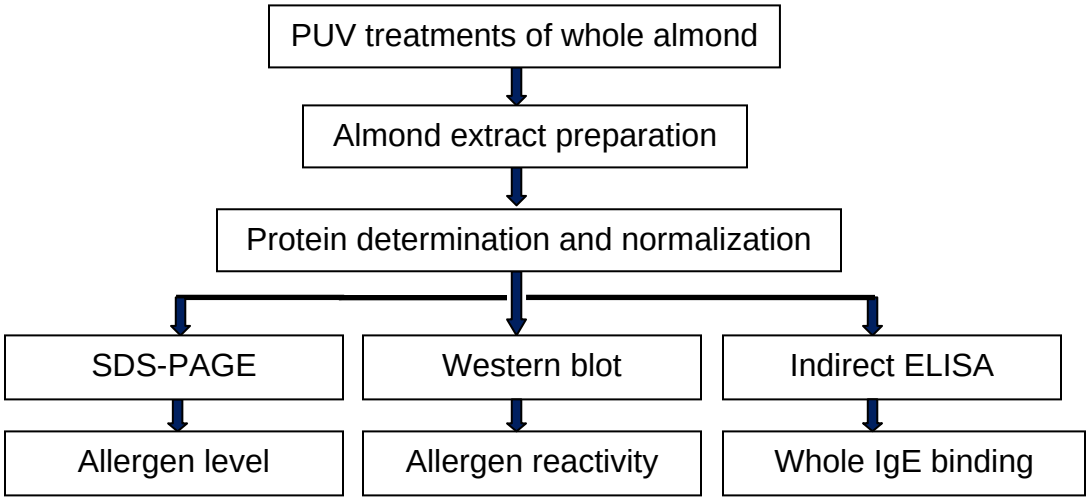

Figure 3-2. Flow chart (2)

Figure 3-3. Infrared thermometer (Photo courtesy of Dr.Wade Yang).

Figure 3-4. XENON-XL® 3000 PUV unit (Photo courtesy of Dr.Wade Yang).

Figure 3-5. PUV equipment developed by Xenon Corp (Photo courtesy of Dr.Wade Yang).

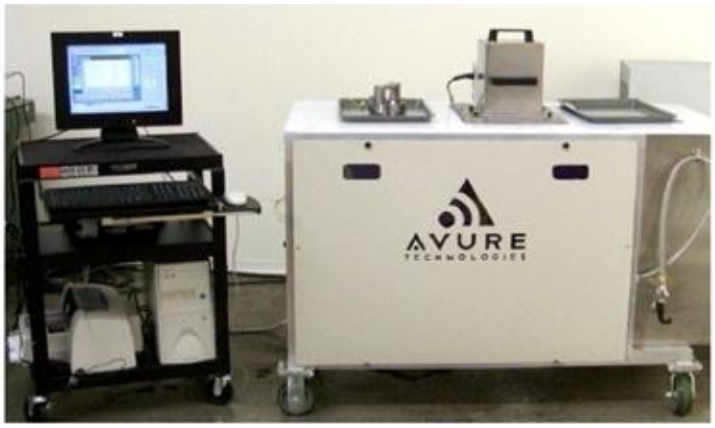

Figure 3-6. Laboratory-scale high hydrostatic pressure (HHP) unit (model Avure PT-1; Avure Technologies, Kent, WA) monitored with DASY*Lab* ® 7.0 software (DASYTEC USA, Bedford, NH) (Photo courtesy of Dr.Haiqiang Chen).

Figure 3-7. Laboratory-scale high hydrostatic pressure (HHP) vessel in which samples were placed (Photo courtesy of Dr.Haiqiang Chen).

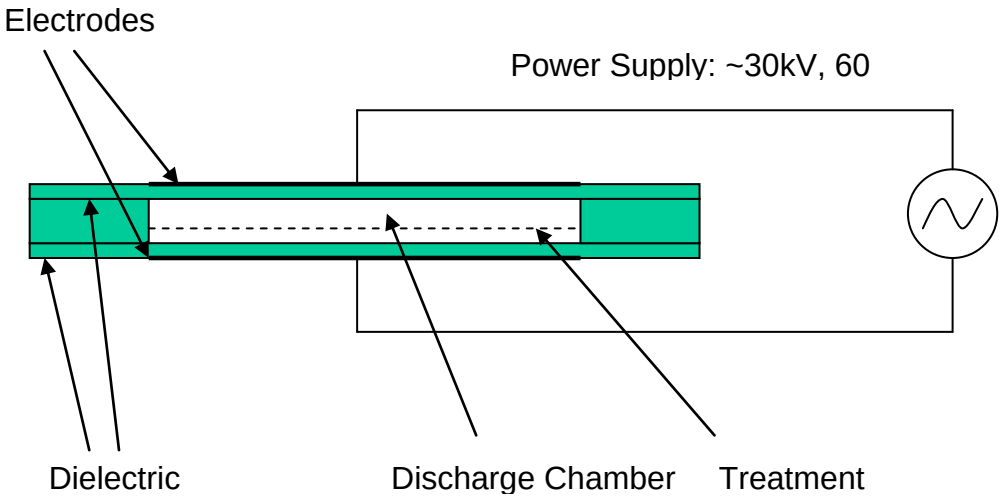

Figure 3-8. The schematic diagram of the experimental non-thermal plasma (NTP) system

CHAPTER 4
RESULTS AND DISCUSSION

Water Loss and Temperature Change after PUV Treatment

Before and after PUV treatments, the sample weight was checked by a digital balance. After PUV treatments, the temperatures of the samples were measured using an infrared thermometer. The results for temperature and water loss after PUV treatments are shown in Table 4-1. Temperatures read 56°C, 77°C, 80°C and 80°C, respectively, after 1, 2, 3 and 4 min PUV treatment. After PUV treatments, all almond samples exhibited changes in volume. As a result of water evaporation, the volumes were reduced by 6.6%, 11.6%, 20.6%, and 25.6%, respectively, after 1, 2, 3 and 4 min PUV treatment.

It is noted there was a slight time lag (around 5-10 s), between the completion of the PUV treatment and onset of temperature measurement, as it also takes a little time to take the samples out of the treatment chamber before temperature measurement with the infrared thermometer, otherwise the PUV treated samples should have a higher temperature if they were measured immediately after the treatments were completed.

The temperatures after PUV treatment for 3 min and 4 min appeared to be similar due to the longer time lag in sample treated with PUV for 4 min than 3 min. As mentioned earlier, the actual temperature might be higher than what is shown in the table.

A wood stick was accidentally left in the PUV chamber and treated for 8 min in the equipment, and the wood stick was ignited. The distance and pulse rate were the same as in the conditions for the almond extracts. The wood stick incident seemed to indicate that the instantaneous temperature of the sample during the PUV treatment for the 8

min duration could be very high. In this PUV unit, the energy intensity is about 0.12 J/cm^2 per pulse when the distance from lamp to the sample is 20.5 cm, according to the earlier study by (Krishnamurthy 2006). A Google search for a typical wood ignition temperature (burning point) returned with a temperature range of 160°C to over 200°C, which seemed to suggest that the localized, instantaneous temperature of food during an prolonged PUV treatment could go as high as 160°C to 200°C. If this was indeed so, PUV could no longer be categorized as a non-thermal technology when the treatment time is long, say, in minutes. For a short duration, say, in seconds, PUV will not cause much temperature rise of the treated sample, so it can still be called non-thermal.

In one measurement, the water loss of the sample treated with PUV for 7 min was about 32%, while in another measurement it was 50%. The phenomenon of water losses occurred in most of our samples treated for a longer time (i.e., in minutes). This can be explained by the foregoing significant localized, instantaneous temperature rise during prolonged PUV treatment.

In addition, it was also observed that the energy under the PUV lamp was quite uneven at different locations. As can be seen later, one sample slightly off from the center of the lamp vs. directly underneath the lamp, the percent allergen reduction was remarkably different. So, it is very important to treat the sample at the same location right below the center of the lamp every time as precisely as possible.

Braford Protein Determination Assay for PUV Treated Almond Extracts

Figure 4-1 shows that almond soluble protein concentration decreased with boiling and PUV treatments. PUV treatment appeared to have more effect on the change of soluble protein concentration than boiling treatment.

Although water contents decreased during PUV treatment, soluble protein concentrations actually decreased rather than becoming more concentrated, because large amount of proteins aggregated and precipitated. The extent of aggregation depended on the severity of treatment conditions. To compensate for reduced volume for PUV treated samples, all protein extracts were normalized before ELISA and SDS-PAGE assays.

SDS-PAGE and Western blot Results

SDS-PAGE and Western Blot for Almond Extracts Treated with PUV for 30 S And 1 Min

As can be seen in Figure 4-2, SDS-PAGE shows raw sample has strong bands between 37-50 kDa regions and has no bands in the region where the molecular weight is lower than 20 kDa, whereas PUV and boiling treated samples have bands lower than 20 kDa and have no band in 37-50 kDa regions. The fact that SDS-PAGE profile has been changed indicates a change of the molecular weight and configuration of proteins in these two PUV exposure times (i.e., 30 s and 1 min). However, PUV treatments under these mild conditions did not qualitatively alter the profile in Western blot, demonstrating that short time PUV treatment was not able to reduce the immunoreactivity of almond allergen. Comparing with egg or milk allergens, almond allergens appeared to be more antigenically stable under PUV treatment, which is consistent with results from other researchers on studies about antigenic stability of almond proteins under various food processing treatments**.**

SDS-PAGE and Western Blot for Almond Extracts Treated with PUV for 7 and 10 Min

The boiling sample increased the intensity of the 20 kDa band, meaning that boiling unfolded and linearized the protein, which enabled the protein to migrate further

to bottom of the SDS-PAGE gel .The pattern of the boiled sample profile in SDS-PAGE was almost the same as that of the raw sample. Almond protein was remarkably aggregated when treated with PUV for 7 min. The insoluble protein appeared as smear appearance in PUV treated samples (Figure 4-3). Band with molecular weight of 20 kDa was mostly noticeable in raw and boiled samples. This band signified one type of polypeptides of the almond major allergen. Its decrease in intensity was accompanied by appearance of high molecular weight smears. The smears were possibly because of cross-linking between almond proteins by oxidation of PUV. Intrachain cross-linking causes change of structure which leads to different mobility on SDS-PAGE.

From Figure 4-3A, it can be seen that IgE bound to the cross links in PUV treated samples in Western Blot (lane 1, 3, 4, 6). This seems to indicate that smeared bands (cross-links) have IgE-binding activity detected with human IgE. In this case, cross-linking did not seem to affect the antigenicity of almond allergen. If so, then the overall IgE binding of the PUV treated almond extracts may not be reduced because IgE binding to cross-links may compensate for the decrease in IgE binding to almond allergen. It is also possible that the overall IgE binding to PUV treated samples was reduced if the cross-links were not as immunoreactive as original allergens. However, we could not affirm that the IgE binding was reduced from Figure 4-3 A lane 3 and 4. On the other hand, lane 6 (also 7 min PUV treatment) showed a much lighter band than lane 3 and 4. The difference between the experimental procedure of lane 3 and lane 6 is that lane 3 was treated with larger volume (20 ml), while lane 6 was treated with less volume (10 ml). Sample volume is critical for PUV treatments because the thicker the sample, the less PUV light will penetrate into the sample. Lane 2 (also 7 min boiling

treatment) is lighter than lane 7. The difference of the sample for lane 2 and lane 7 is that the sample for lane 2 was prepared earlier than lane 7. After being stored for a period of time, protein lost its activity because of microbial or proteolytic degradation. More experiments need to be conducted in the future to probe further whether the cross-links are still active in IgE binding.

The “reactive” protein cross-links in PUV treated sample retained on the high molecular region of the SDS-PAGE, which might be the reason why the 20 KDa bands in PUV treated samples were lighter than the untreated. Part of the allergen might turn into cross links, resulting in lighter band in 20 kDa region. Western blot shows 20 kDa band intensity was reduced with PUV treatment and appeared to be most reduced at 7 min. Thus, 7 min was chosen for further study in ELISA.

SDS-PAGE for Almond Extracts Treated with PUV for 6 and 7 Min

Figure 4-4 shows the typical SDS-PAGE profile of the boiling treated almond extract compared to the PUV treated. Despite the formation of aggregates, the boiling treated extract displayed few missing bands compared to the PUV treated extract. Instead, it was similar to the control profile.

The SDS-PAGE shows a loss of protein bands, demonstrating PUV treatment progressively reduced the solubility of almond proteins. The PUV treated protein bands were less intense and had a stronger smear, compared to boiled and raw samples, presumably due to the reason that some proteins were broken down into smaller peptides or fragments and some proteins become larger aggregation due to the PUV treatment. This suggests that almond proteins were affected by PUV treatment. It was speculated that some PUV treated proteins could not run through the gel, because PUV rendered the proteins insoluble by cross-linking. Longer PUV treatment times resulted in

more reduced protein solubility and less intense protein bands. Intermolecular interactions are implicated in the formation of aggregates. Boiling did not reduce the protein solubility in a similar way as PUV as shown in SDS-PAGE. Furthermore, the PUV treatment changed the color of almond extracts from transparent white to light yellow and showed much more aggregation than the boiling treated sample. The reagents odium dodecyl sulfate (SDS) and β-mercaptoethanol solubilized the boiling treated protein aggregation but did not solubilize the PUV treated protein aggregation, indicating that the aggregation was not due to the disulfide bonds. It is clearly evident that almond allergen proteins were not able to preserve their structural properties after PUV treatment; otherwise they would not change their characteristic molecular weight.

We wanted to determine if thermal treatment affected almond allergens in the same way as PUV treatment in order to eliminate the possibility that the reduction of allergen resulted from an increase in temperature during PUV treatment. However, the instantaneous temperature of the sample during PUV treatment was unknown, and thus difficult to simulate the same temperature as the PUV treatments. As mentioned before, the temperature during the PUV treatments may be higher than the measured temperature, because the instantaneous temperature rises.

Western Blot for Almond Extracts Treated with PUV for 6 and 7 Min

Western blot (Figure 4-5) illustrates that IgE binding to almond extract probed by human IgE and anti-AMP rabbit antibody was reduced after PUV treatment and appeared to be more reduced at 7 min than 6 min. Figure 4-5 also demonstrates that epitopes relevant to human IgE recognition of the major amandin polypeptides (20 kDa to 25 kDa) were changed. Figure 4-5 shows the 20 kDa band in boiled samples was

almost the same as the band in raw sample in Figure 4-5 A and marginally weaker than the 20 kDa band in raw sample in Figure 4-5 B. This indicated that the effect of boiling treatment on IgE reactivity of almond allergen was not notable in Western Blot. Protein levels in PUV treated samples seen in SDS-PAGE were reduced yet not completely missing; however, there were no apparent bands in the PUV treated samples in 14 kDa region. The reduction of the IgE activity of almond allergen was indicated in the loss of the intensity of the bands in Western blot. Therefore, there was a larger decrease in IgE binding than the decrease in protein expression in PUV treated samples. This may be due to the fact that the allergen had undergone conformational changes following PUV treatment, which caused the IgE binding sites to become unavailable. Even though the molecular weight of some allergens did not change, the IgE binding sites of those allergens were no longer recognizable by antibody. Photo-thermal, photo-chemical and photo-physical energy produced by PUV might have caused modification of the allergen, including protein fragmentation or cross-linking. The reactivity of 70 kDa proteins with the human IgE in our investigations was consistent with the results of Bargman and others (1992), who reported that blanching essentially eliminated 70 kDa proteins. Figure 4-5 does not show the heat liable 70 kDa protein most likely because blanched almond powder was used in this experiment. This group also reported that the proteins from 45-50 kDa were stable and reactive after heat treatment. Conversely, in our study, the activity of the proteins from 45-50 kDa was reduced by PUV as shown in Figure 4-5, indicating that the reduction of the 45-50 kDa may not be due to the high temperature accumulated during PUV treatment.

The drawback of the Western blot assay is that it has to be transferred from the SDS-PAGE gel. The resolution of the gel is between approximately 10-250 kDa. Part of large insoluble protein aggregation cannot run into the gel. The pores of the gel are small and the current will not be able to push the whole sample into the gel. Because the aggregates are probably not entering the gel, the reactivity of the allergen that remains on the top of the SDS-PAGE gel cannot be tested by Western Blot. ELISA can address the issue by testing the entire almond sample.

SDS-PAGE and Western Blot for HHP Treated Almond Extracts

Figure 4-6 shows that unlike soybean whey and bovine whey protein hydrolysates, which were reduced in allergen reactivity following HHP, no reduction in allergen protein band intensity was noted following HHP treatment as observed in SDS-PAGE and Western blotting profiles. Figure 4-6 demonstrated that HHP was not able to reduce the immunoreactivity of almond protein under the conditions tested in this study.

SDS-PAGE and Western Blot for NTP Treated Almond Extracts (Test 1)

The final temperatures after 1, 3, 5 min treatment were 20, 22, and 24 °C, respectively. SDS-PAGE and Western blots of NTP treated samples are presented in Figure 4-7, which show that NTP treated samples did not show reduction in allergen protein or antigenicity. Allergens from 10 kDa to 15 kDa region shows in SDS-PAGE, while they did not show the band in western blot. This might be due to the possibility that NTP eliminated the IgE reactivity of 10-15 kDa proteins or it might be due to the fact that 10-15 kDa proteins somehow were not successfully transferred to the Western blot membrane.

Western Blot for NTP Treated Almond Extracts (Test 2)

As can be seen in Figure 4-8, the Western blot of NTP treated samples show that NTP treatment did not qualitatively alter the protein profile compared with the untreated raw sample. The raw sample did not show a protein band from 10-15 kDa, which indicated that the disappearance of the 10-15 kDa band in Figure 4-7 was not due to NTP treatment. As mentioned before, free radical is a strong protein-modifying agent. Although NTP creates free radicals such as hydroxyl radicals, it did not alter the antigenicity of almond protein.

Indirect ELISA Results

Indirect ELISA for Almond Extracts Treated with PUV for 1, 2, 3 and 4 Min

Indirect ELISA was performed to determine the overall reduction in IgE binding to the PUV treated samples. Figure 4-9 present the indirect ELISA for almond extract treated with PUV for 1, 2, 3 and 4 min, probed by human plasma containing IgE antibodies against almond. Results are expressed as absorbance readings from a spectrometer. Bars are labeled with letters. Values with the same letters are not significantly different. It is discernible that there were progressively increasing reductions in IgE binding activity as the PUV treatment time increased from 1 min to 4 min, but the reduction was not significant, indicating the immunological stability of almond allergen. Consistently, the protein bands become progressively weaker from 1 min to 4 min in SDS-PAGE (Figure 4-15).

Indirect ELISA for PUV Treated First Group of Almond Extracts

Figure 4-10 show Indirect ELISA for raw, boiled and PUV treated almond extracts using human plasma containing IgE antibodies against almond. Figure 4-10 suggest that PUV for 7 min was more effective than boiling in regard to reducing the IgE binding

reactivity to almond. The boiled and PUV treated samples had reduction in IgE binding of 40% and 76% of IgE binding to the almond allergen, respectively, compared with raw, as shown in the Figure 4-10. Acosta and others (1999) described that commercial blanched almond samples incurred 50% reduction in allergen level, compared with the untreated almond sample. Boiling and PUV treatment may change the almond protein to induce masking of allergenic epitopes, which reduced recognizable and assessable epitopes.

Indirect ELISA for PUV Treated Second Group of Almond Extracts

Figure 4-11 presents indirect ELISA for raw, boiled and PUV treated almond extracts using human plasma containing IgE antibodies against almond. Figure 4-11 illustrates that PUV reduced IgE binding, however, not as much as the previous result. It was because the sample was not placed directly under the lamp due to human error, so it may not have received the maximum exposure to PUV light. It can be inferred that the dose of the PUV light relative to the placement under the lamp. Furthermore, the water loss was inconsistent, because the energy under the PUV lamp was unevenly distributed at different locations. Thus, it is important to treat the sample at the exact same location every time, a little movement would make a great difference in the results. If samples were treated at exactly the same location, the result in Western blot and ELISA would have been more consistent.

Indirect ELISA for Almond Extracts from PUV Treated Whole Almond

Figure 4-12 presents Indirect ELISA of raw whole almond extract and the extract from whole almond treated with PUV for 4 min. Indirect ELISA was probed with human plasma containing IgE antibodies against almond. Figure 4-12 indicates that there was a significant qualitative reduction in IgE activity of protein extracts from PUV treated

whole almond. The PUV treated almond shows the same appearance and flavor as roasted almond. PUV can potentially be utilized for roasting and inactivation of allergen and pathogen of almond.

Indirect ELISA for HHP Treated Almond Extracts

Figure 4-14 illustrates Indirect ELISA of raw and HHP treated almond extract using human plasma containing IgE antibodies against almond. HHP has been demonstrated to alter allergen reactivity by modifying the structure of some food allergens. However, Figure 4-14 shows that HHP treatment alone did not significantly change the IgE binding compared with control, indicating the resistance of almond protein to HHP treatment. This ELISA result was consistent with the Western blot result.

Indirect ELISA for NTP Treated Almond Extracts

Figure 4-15 shows Indirect ELISA of raw and NTP treated almond extract using human plasma containing IgE antibodies against almond. The figure 4-15 shows the almond IgE reactivity was not significantly changed, which means the epitopes were still recognizable by human allergic sera after NTP treatment at the conditions tested in this study. The indirect ELISA result was consistent with SDS-PAGE and Western blotting results.

Whole Almond Treated with PUV for 4 Min and Almond Flours Treated with PUV for 7min

The whole almond treated with PUV for 4 min had a desirable and pleasurable roasted almond flavor. The measured temperature of the almond after PUV treatment was 87°C, while the temperature of the aluminum tray was 112°C. The instantaneous temperature during the PUV treatment was expected to be much higher than this. The results indicate that PUV may give us a potential way to roast the almond and reduce its

allergen. The almond flours treated with PUV for 7min had very strong burning smell and the sample was completely burnt because of the high temperature during the PUV treatment. This indicates that a shorter duration may be desirable for PUV treatment of the quantity of almond flour used.

The picture of PUV treated whole almond for 4 min and the PUV treated almond powder for 7 min is shown in Figure 4-13. Prolonged PUV exposure led to temperature build-up and changed the color due to non-enzymatic browning. The protein extracted from PUV treated almond kernels developed dark yellowish color. Peroxides that generated by PUV and Maillard reaction may have led to the discoloration.

Overall Results

The SDS-PAGE result indicates that almond exhibited a reduced solubility and almond allergen expression after PUV treatments. The Indirect ELISA results show that PUV reduced the almond antigenicity, even though almond allergen has been reported fairly stable and resistant to various food processing treatments. Indirect ELISA demonstrated that IgE binding to almond extract was reduced by 76% following PUV treatment for 7 min compared to raw samples. Western blot for almond extract treated with PUV for 7 min showed considerably reduced allergen bands. The loss of band intensity in Western blot was more notable than that in ELISA, which was associated with the insoluble allergen protein complex not being able to migrate through the SDS-PAGE gel, and thus not being able to be detected by Western blot. There was more reduction in IgE reactivity of almond extracts, when PUV treated on almond extract directly than on the whole almond and then prepared the extract, which was possibly due to the penetration of the PUV.

The results of this study suggest that the antigenicity of almond remained stable towards both HHP and NTP. There were no significant differences between raw almond extract and the HHP and NTP treated almond extracts in the ELISA and Western blot. The PUV technology was more effective in reducing the antigenicity of almond allergen when compared with HHP and NTP.

Table 4-1. Effect of PUV on water loss and temperature of almond extracts.

PUV treatment time	PUV 30sec	PUV 1min	PUV 2min	PUV 3min	PUV 4min	PUV 6min	PUV 7min	PUV 8min
water loss	3%	6.60%	11.60%	20.60%	25.60%	30%	50%	Some area dried out
Temperature after treatment	50°C	56°C	77°C	80°C	80°C	113°C	115°C	ignited a wood stick

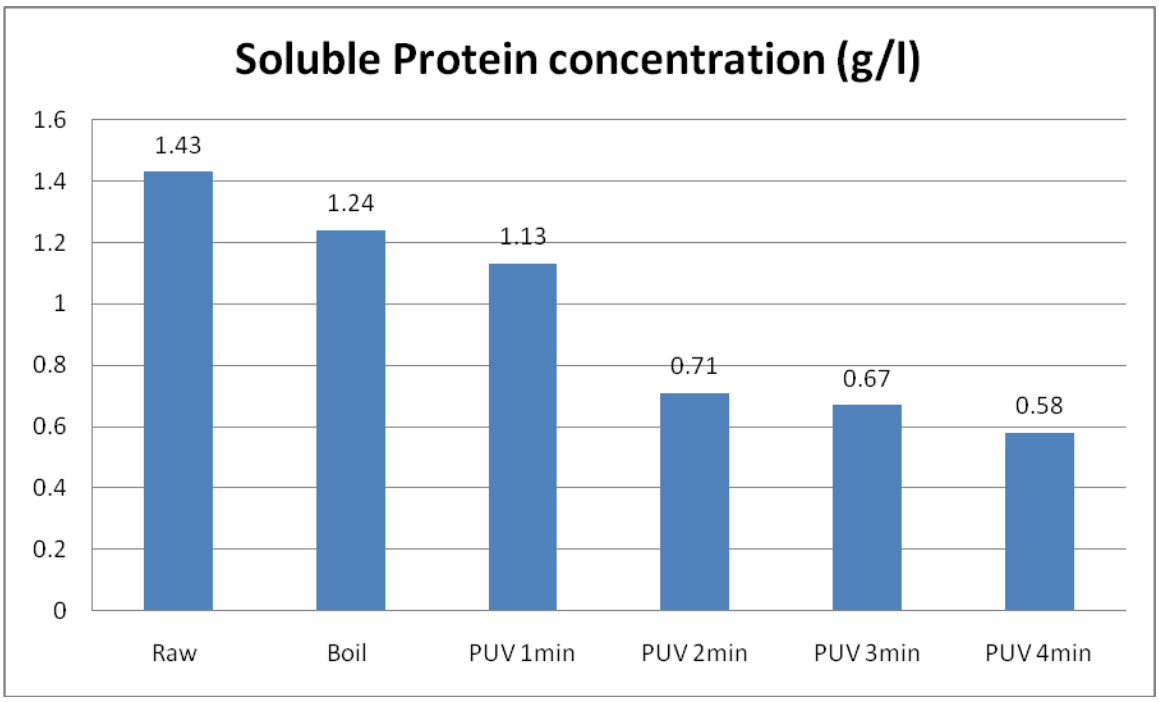

Figure 4-1. Braford assay of raw almond extracts treated with boiling for 4 min, PUV1 for 1 min, PUV2 for 2 min, PUV3 for 3 min, and PUV4 for 4 min.

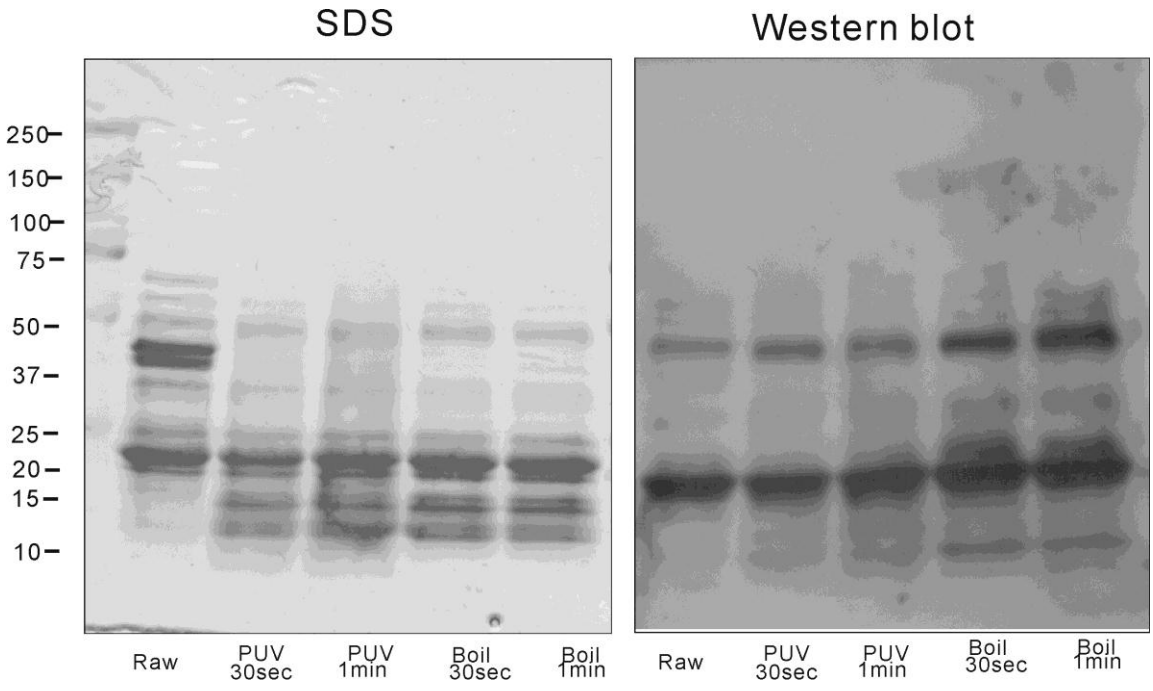

Figure 4-2. SDS-PAGE and Western blot of raw almond extracts treated with PUV for a short duration, PUV for 1min, boiling for 30 s, and boiling for 1 min.

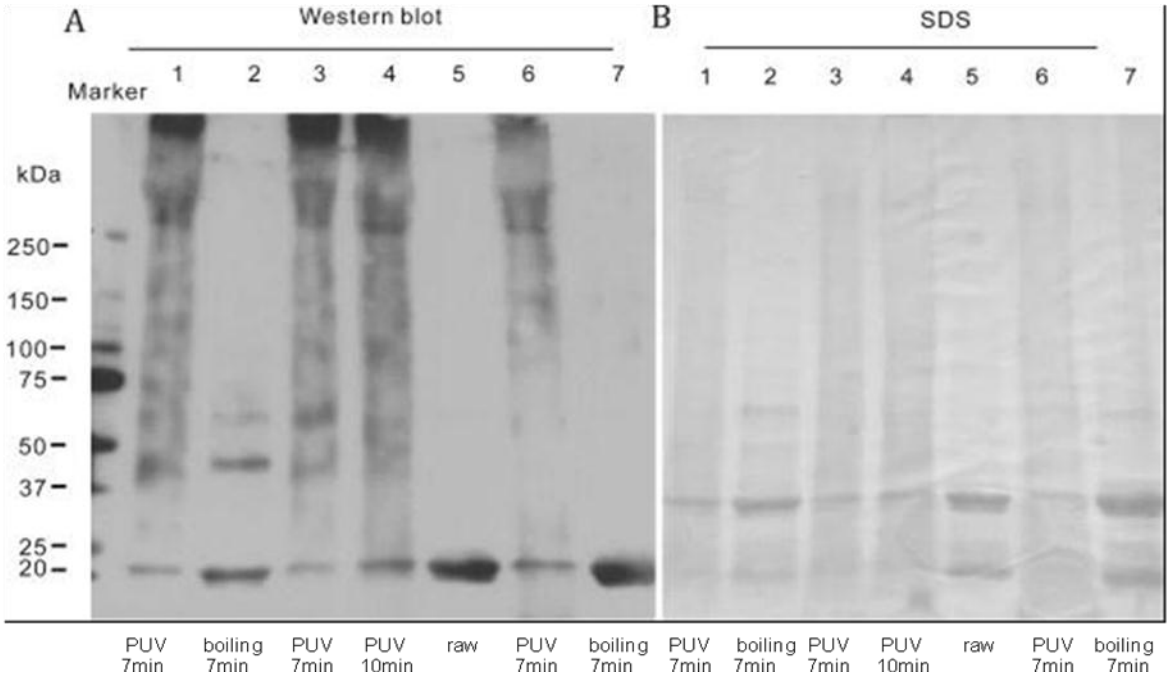

Figure 4-3. Western blot and SDS-PAGE profile of almond extracts treated with PUV for 7 min and 10 min. Lane 1 and 2 are from one sample, lane 3 and 4 are from another sample, and lane 5 and 6 are from the third sample.

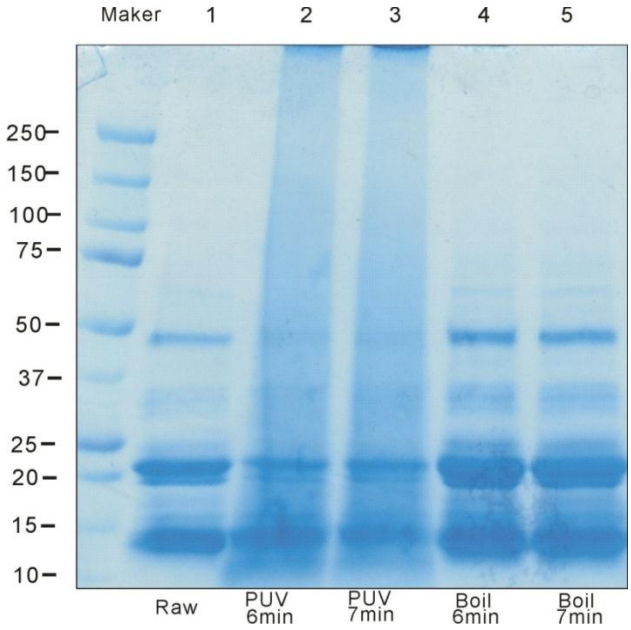

Figure 4-4. SDS-PAGE profile of almond extracts. 1, untreated supernatant; 2, PUV treated for 6 min; 3, PUV treated for 7 min; 4, Boiling treated for 6 min.

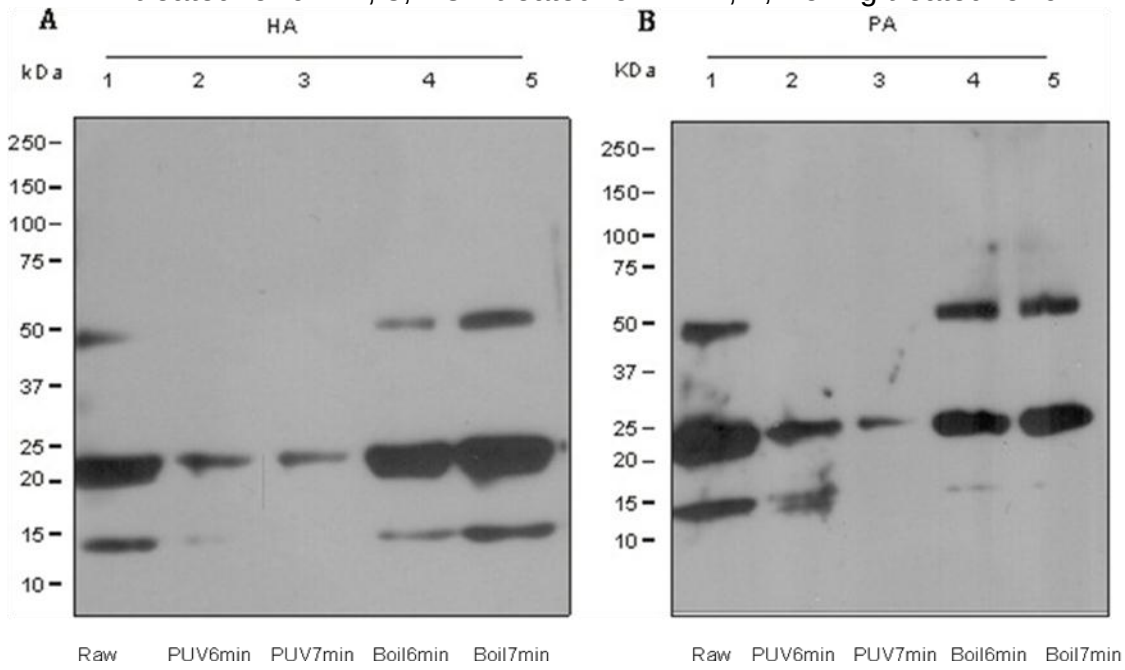

Figure 4-5. Western blots of almond extracts probed with human IgE (HA) and anti-AMP rabbit antibody (PA). 1, untreated supernatant; 2, PUV treated for 6 min; 3, PUV treated for 7 min; 4, Boil treated for 6 min; 5, PUV treated for 7 min. human IgE (HA) dilution (1:80), CAP 13.9KU/I. secondary dilution(1:1000). anti-AMP rabbit antibody (PA) dilution (1:40) anti-rabbit IGG dilution(1:2500).

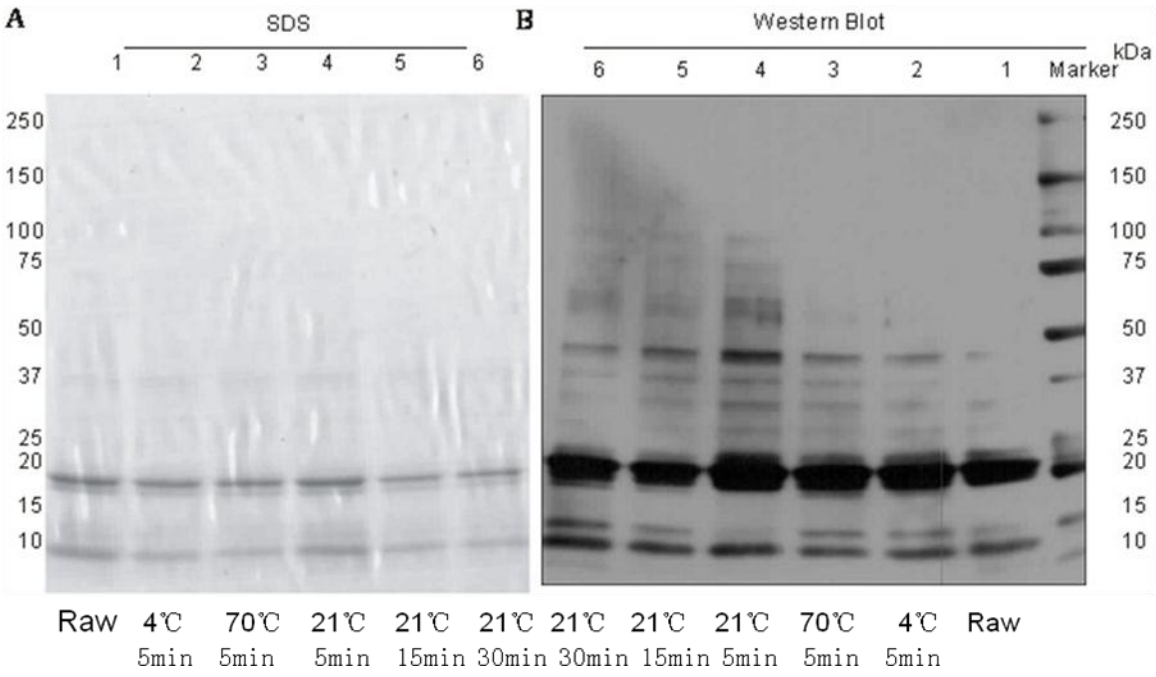

Figure 4-6. SDS and Western blots of HHP treated almond extracts probed with human IgE (HA). 1, untreated supernatant; 2, HHP treated at 4 °C for 5min; 3, HHP treated at 70 °C for 5 min; 4, HHP treated at 21 °C for 5 min; 5, HHP treated at 21 °C for 15 min; 6, HHP treated at 21 °C for 30 min. human IgE (HA) dilution (1:80), CAP 13.9KU/I. secondary dilution (1:1000).

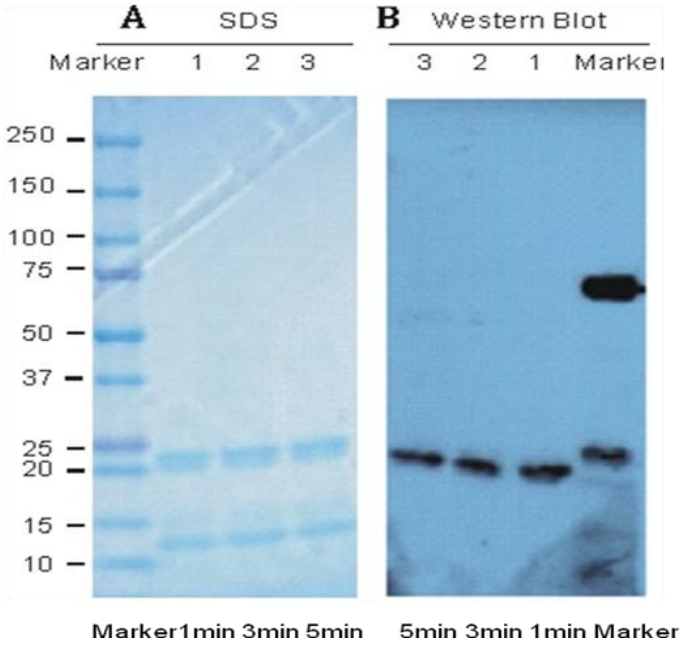

Figure 4-7. SDS-PAGE and Western blots of NTP treated almond extracts probed with human IgE (HA). 1, NTP treated for 1min; 2, NTP treated for 3 min; 3, NTP treated for 5 min. human IgE (HA) dilution (1:80), CAP 13.9KU/I. secondary dilution (1:1000).

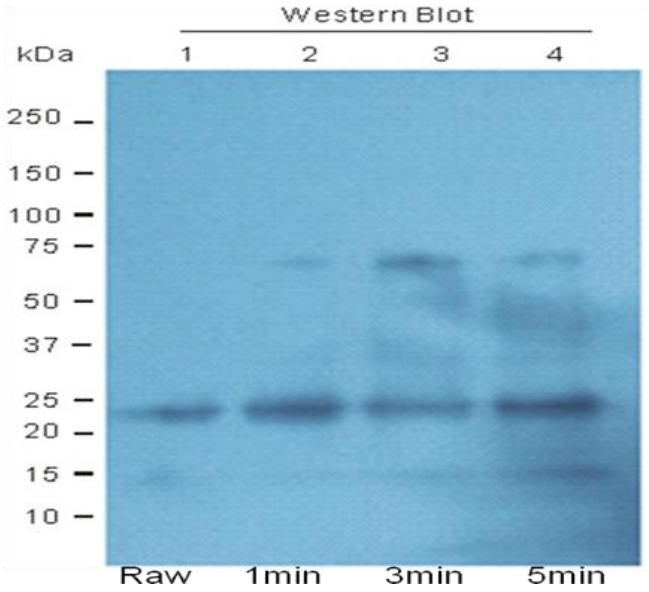

Figure 4-8. Western blots of NTP treated almond extracts probed with human IgE (HA). 1, untreated supernatant; 2, NTP treated for 1 min; 3, NTP treated for 3 min; 4, NTP treated for 5 min. human IgE (HA) dilution (1:80), CAP 13.9KU/I. secondary dilution (1:1000).

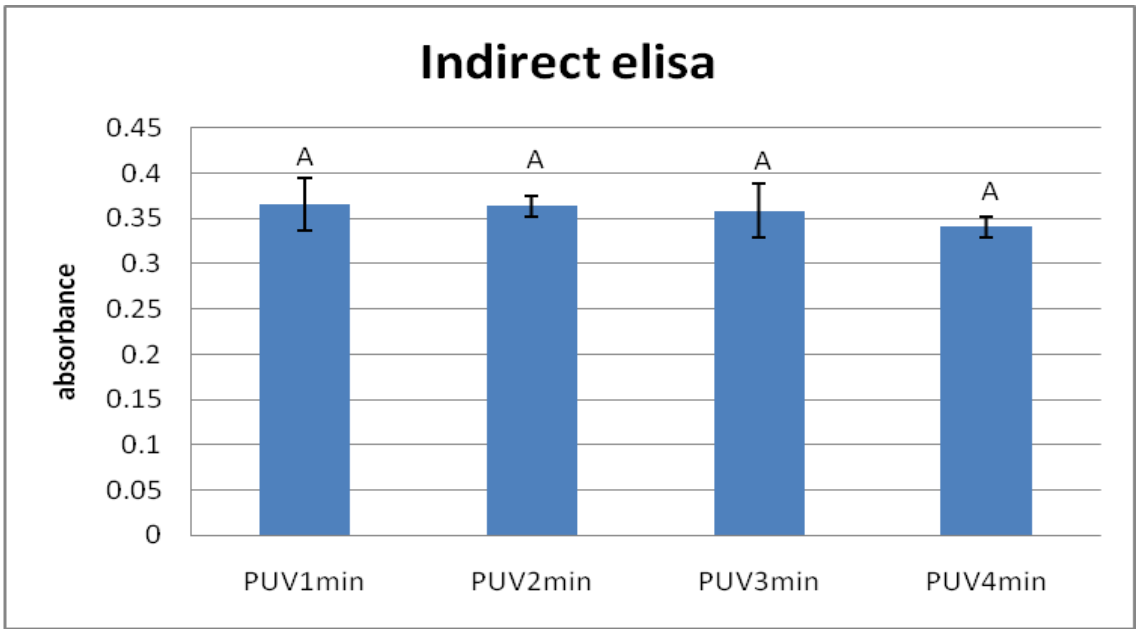

Figure 4-9. Indirect ELISA for almond extracts treated with PUV for 1, 2, 3 and 4min, probed by human plasma containing IgE antibodies against almond. Results are absorbance reading from spectrometer. The bar chart shows the mean of triplicates with Standard Error Mean error bar. Bars are labeled with letters. Values with different letters are significant different.

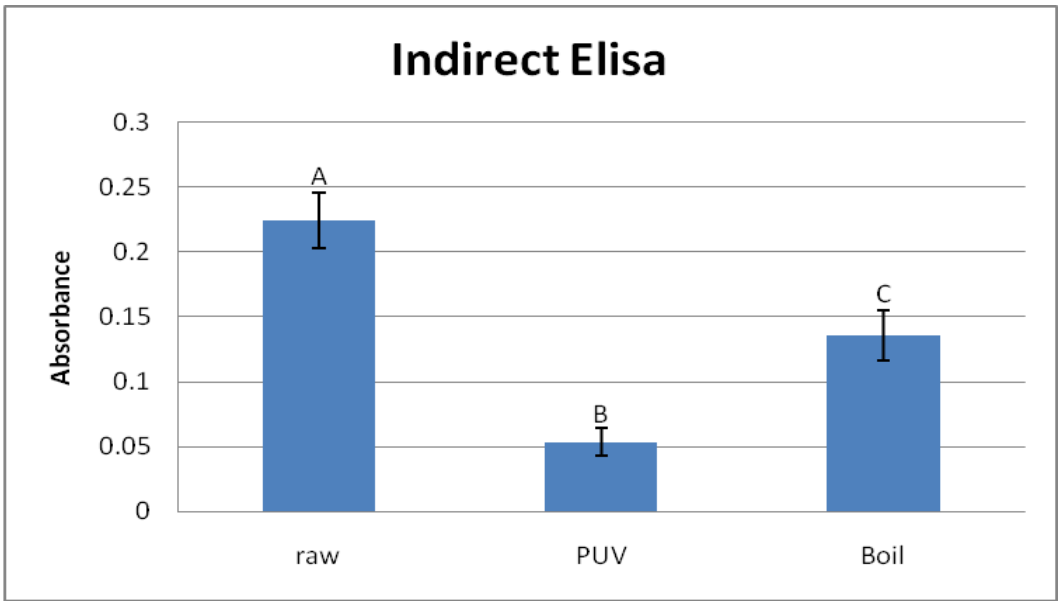

Figure 4-10. Indirect ELISA for raw, boiled and PUV treated first group of almond extracts using human plasma containing IgE antibodies against almond. Results are absorbance reading from spectrometer. The bar chart shows the mean of triplicates with Standard Error Mean error bar. Bars are labeled with letters. Values with different letters are significant different.

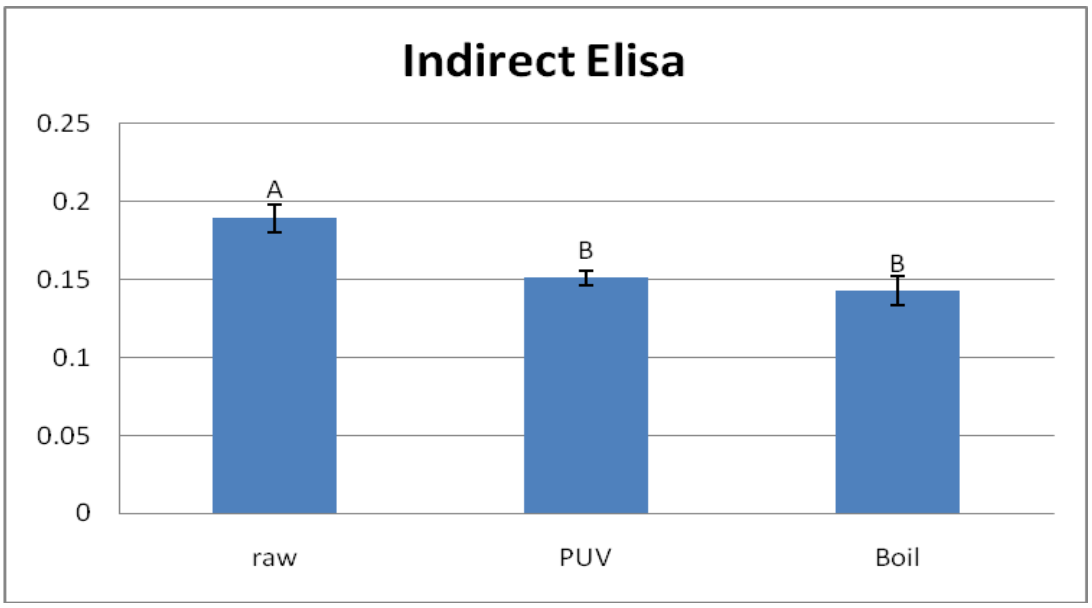

Figure 4-11. Indirect ELISA for raw, boiled and PUV treated second group of almond extracts using human plasma containing IgE antibodies against almond. Results are absorbance reading from spectrometer. The bar chart shows the mean of triplicates with Standard Error Mean error bar. Bars are labeled with letters. Values with different letters are significant different.

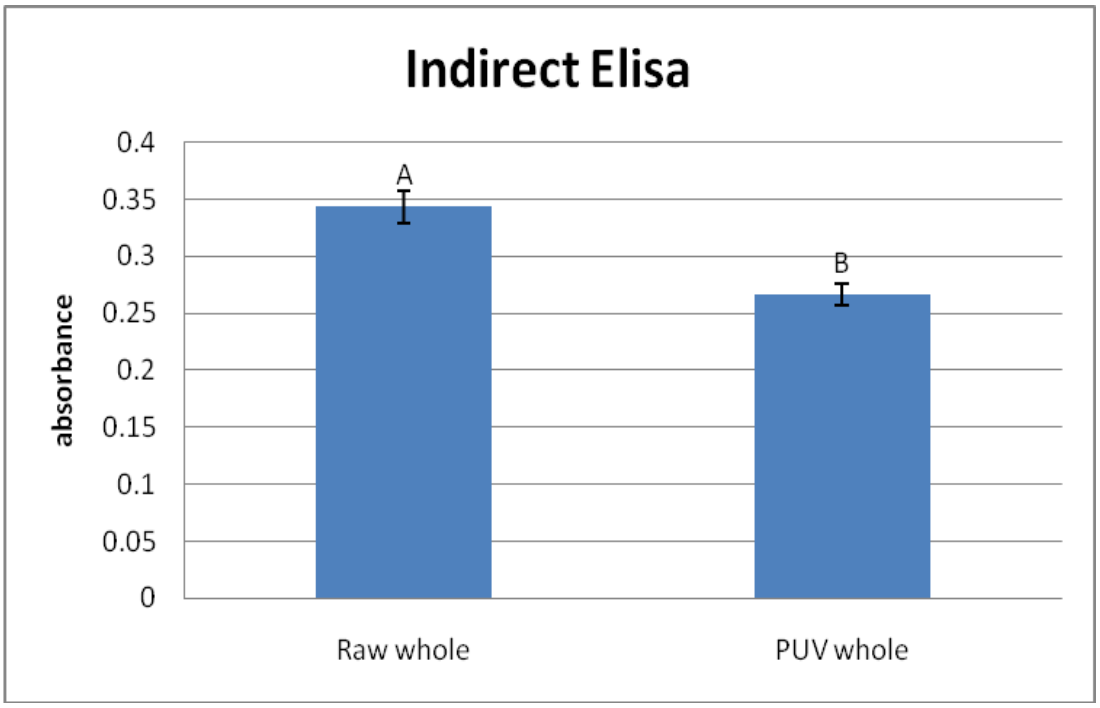

Figure 4-12. Indirect ELISA for raw whole almond extracts and extracts from whole almond treated with PUV for 4 min probed with human plasma containing IgE antibodies against almond. Results are absorbance reading from spectrometer. The bar chart shows the mean of triplicates with Standard Error Mean error bar. Bars are labeled with letters. Values with different letters are significant different.

Figure 4-13. Whole almond treated with PUV for 4 min and almond flours treated with PUV for 7min.

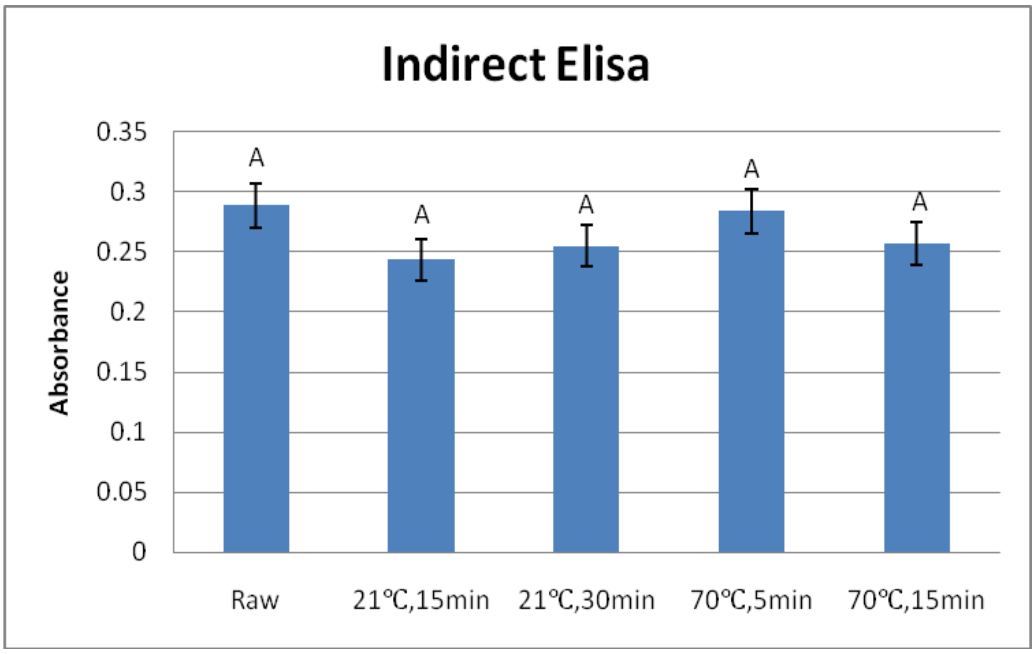

Figure 4-14. Indirect ELISA for raw and HHP treated almond extracts using human plasma containing IgE antibodies against almond. The bar chart shows the mean of triplicates with Standard Error Mean error bar. Bars are labeled with letters. Values with different letters are significant different.

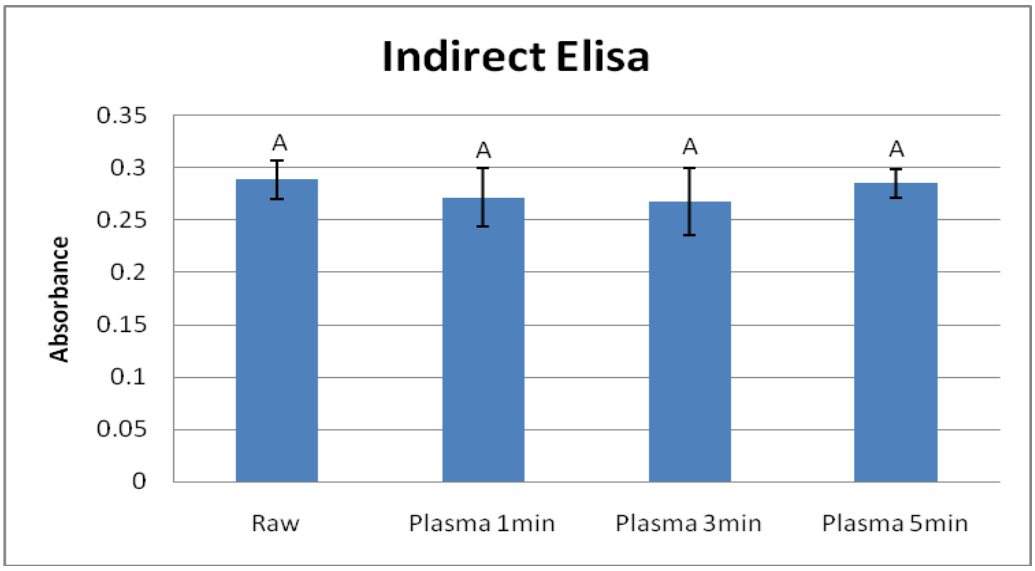

Figure 4-15. Indirect ELISA for raw and NTP treated almond extracts using human plasma containing IgE antibodies against almond. Results are relative values compare to the control. The bar chart shows the mean of triplicates with Standard Error Mean error bar. Bars are labeled with letters. Values with different letters are significant different.

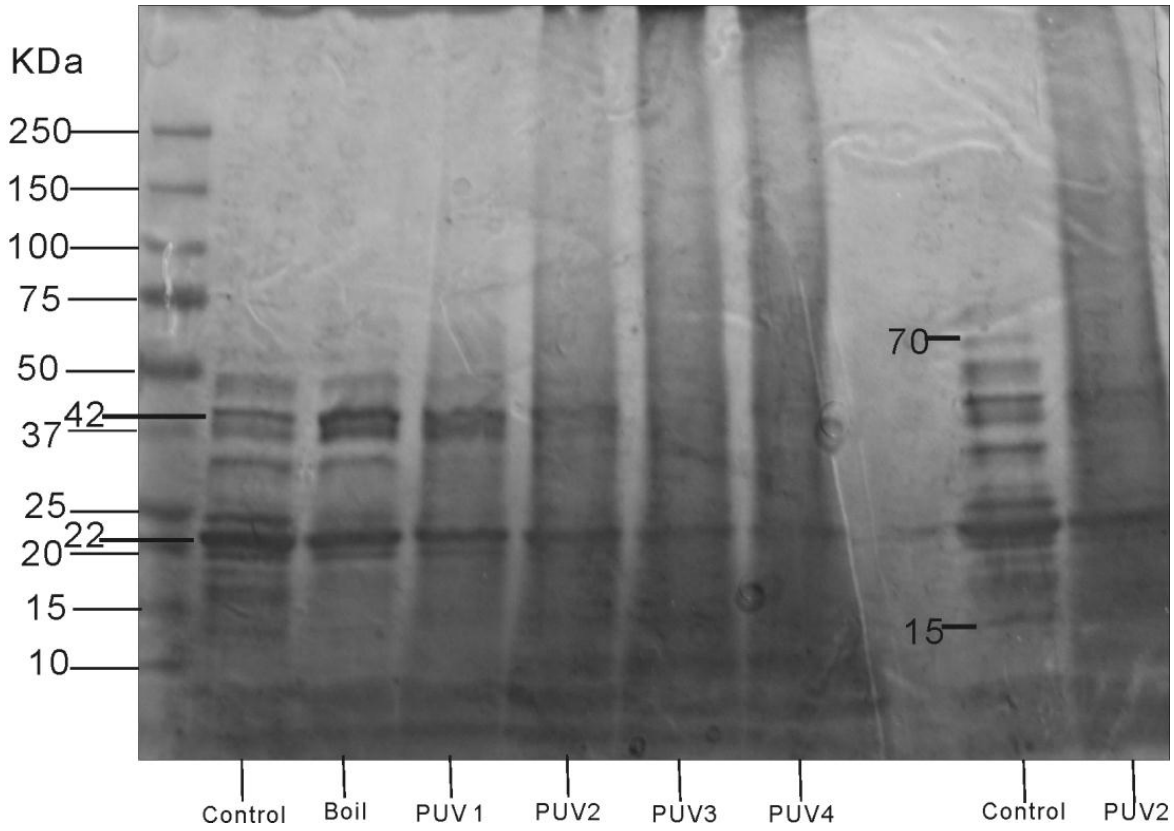

Figure 4-16. SDS-PAGE of raw almond extracts treated with boiling for 4 min and PUV for 1, 2, 3 and 4 min.

CHAPTER 5
CONCLUSION AND RECOMMENDATION

Concluding Remarks

Tree nut is one of the 8 major allergenic foods that are responsible for approximately 90% of all allergic reactions. Almond, as a kind of tree nut and a source of allergen, has been a concern for a proportion of consumers. Almond allergies have been proven to be severe in nature, lifelong, and can even cause fatal reaction. Since avoidance is not always possible, it would be ideal if allergens could be eliminated or minimized in the processing steps before the products reach consumers. This study compared three novel food processing technologies (PUV, HHP and NTP) in reducing almond allergens and IgE binding activity.

- PUV was more effective than HHP and NTP in almond allergen reduction
- Short-time PUV treatment was not effective in allergen reduction
- Longer PUV treatment performed better in allergen reduction (e.g., 76% reduction after 7 min PUV treatment)
- The whole almonds treated with PUV showed significant reduction in allergen
- The mechanism of PUV treatment was likely to be that PUV changed the structure based epitopes or hid epitopes due to its photo-thermal, photo-chemical or photo-physical effect, with photo-thermal effect being more prominent

Short-time PUV treatment (e.g. 30 and 60 s) is not effective and shows no significant change in almond allergen. Longer PUV exposure (e.g. 7 min) performed better in reducing the IgE activity in extracts from almond powder, resulting in around 76% reduction in IgE activity. Long time PUV treatments incurred much instantaneous heat generation due to the PUV's photothermal effect, resulting in significant volume reduction of the extracts and the burning of the almond powder. However, Non-thermal plasma treatment (NTP) and high hydrostatic presure (HHP) treatment (600 Mpa) were

not effective in reducing the IgE activity of almond allergens at all conditions tested in this study. Neither Western blot nor ELISA has showed significant changes in the case of NTP and HHP.

The results on the extracts from whole almond kernels treated with PUV have shown a significant reduction in allergen potency, compared with extracts from untreated whole almonds. This finding may lead to a new horizon of PUV applications to almond products, i.e., to achieve roasting and allergen reduction in unison. In addition, the extracts from whole almonds treated with PUV for 4 min has significantly lower protein concentration than the extracts from whole raw almonds, even though they are extracted at the same condition.

Based on this study, the main mechanism of reduction of IgE activity to almond extracts by PUV treatment is that PUV induced the cross-linking, fragmentation, denaturalization and aggregation, which changed the structure based epitopes or masking of epitopes. Aggregation masked epitopes which rendered epitopes unaccessible. Denaturalization changed the conformational based epitopes, and fragmentation changed linear epitopes which caused epitopes to be unrecognizable. There are three possible mechanisms for cross linking: (1) Maillard reaction and denaturation due to photo-thermal effect of PUV led to the formation of cross-links, (2) PUV photons absorbed by water led to generation of free radicals which triggered crosslinking, (3) PUV photons absorbed by protein amino acids caused crosslinking.

The benefit of an effective allergen modification and reduction technique is countless, which can lead to safer products and the wellbeing of food allergic consumers. Although PUV did not eliminate the allergens in almonds, it has showed a

great potential for significantly reducing the almond allergens, which may lead to the production of hypoallergenic almond products.

Recommendations

Molecular information of the cross links caused by PUV treatments needs to be studied in the future. The beta- mercaptoethanol was used before running the SDS-PAGE, so the cross-linking was not a disulfide bond.

The high instantaneous heat generated during PUV treatments might be one effect of changing the allergen, and the thermal energy might be utilized to process the food. PUV treatment for 7 min was the most effective in reducing the allergenic potencies of almond allergens. However, the temperature after PUV treatment for 7 min was high, which rendered PUV thermal in this case. If new PUV equipment could prevent samples from increasing the temperature, then the temperature of the sample would not have to be considered as a factor of changing the allergen. The PUV equipment may be put in a cool chamber to maintain the temperature of the sample and reduce the water evaporation. However, the heat generated by PUV may be used to preheat food during preparation, thus reducing the energy required in the thermal treatment of foods. It is a beneficial idea to reduce the antigenicity and inactivate the pathogens of foods while saving the energy.

Further research on PUV and PUV treated almonds needs to be conducted in the future. It is suggested that PUV treated almond extracts need to go through the sensory test and ingredient analysis to insure the retention of nutrients, quality and flavor. As a kind of electromagnetic radiation, PUV may face the issue of consumer acceptance. The Food and Drug Administration has issued approval in the use of PUV in food processing, however, food should be treated with PUV of minimum dose reasonably

required to reduce the acceptable antigenicity level. Further research might be conducted on the effects of the HHP together with enzyme on the IgE reactivity of almond. However, it is possible that protein hydrolysis may cause changes in food flavor and sensory attributes. Which wavelength region of PUV is playing an important role in altering almond protein structures can be investigated in future studies. Answers to these questions need to be confirmed. The almond IgE-binding epitope structure and its biological activity can be determined in future studies. Furthermore, more studies could be done in PUV treatment on whole nut kernels.

It has been reported that insoluble protein can be soluble at acidic pH or by glycolytic enzymes (Kopper and others 2005). So, allergen may be released in acidic stomaches or intestines. The insoluble protein containing allergen may retain antigenicity after being resolubilized and digested in to smaller peptides, and some almond allergen epitopes may stay intact after gastric digestion, resulting in allergic reactions. Thus, *in vivo* studies are needed after *in vitro* studies in assessing the antigenicity of allergen in gastrointestinal environment and in assessing the antigenicity of almond allergen.

APPENDIX
STATISTICAL ANALYSIS

Table A-1. Statistical analysis of ELISA result for first group of almond extracts treated with PUV.

A) ANOVA. B) Means by treatment

A)

The ANOVA Procedure

Dependent Variable: result result

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.08741303	0.04370652	23.11	<.0001
Error	15	0.02836556	0.00189104		
Corrected Total	17	0.11577859			

R-Square	Coeff Var	Root MSE	result Mean
0.755002	31.58913	0.043486	0.137661

Source	DF	Anova SS	Mean Square	F Value	Pr > F
treatment	2	0.08741303	0.04370652	23.11	<.0001

14:53 Wednesday, January 27, 2011 3

The ANOVA Procedure

B)

t Tests (LSD) for result

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	15
Error Mean Square	0.001891
Critical Value of t	2.13145
Least Significant Difference	0.0535

Means with the same letter are not significantly different.

t Grouping	Mean	N	treatment
A	0.22415	6	raw
B	0.13533	6	Boil
C	0.05350	6	PUV

Means and Descriptive Statistics 1

14:53 Wednesday, January 27, 2011

treatment	Mean of RESULT	Std. Dev. of RESULT	Std. Error of RESULT	Variance of RESULT

Boil	0.13533	0.047266	0.019296	.002234063
PUV	0.05350	0.026720	0.010908	.000713963
raw	0.22415	0.052202	0.021312	.002725086

15:01 Wednesday, January 27, 2011 1

The ANOVA Procedure

Class Level Information

Class	Levels	Values
treatment	3	Boil PUV raw

Table A-2. Statistical analysis of ELISA result for second group of almond extracts treated with PUV.

A) ANOVA. B) Means by treatment

A)

The ANOVA Procedure

Dependent Variable: result result

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.00751161	0.00375581	9.91	0.0018
Error	15	0.00568351	0.00037890		
Corrected Total	17	0.01319513			

R-Square	Coeff Var	Root MSE	result Mean
0.569272	12.09408	0.019465	0.160950

Source	DF	Anova SS	Mean Square	F Value	Pr > F
treatment	2	0.00751161	0.00375581	9.91	0.0018

15:01 Wednesday, January 27, 2011 3

B)

The ANOVA Procedure

t Tests (LSD) for result

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	15
Error Mean Square	0.000379
Critical Value of t	2.13145
Least Significant Difference	0.024

Means with the same letter are not significantly different.

t Grouping	Mean	N	treatment
A	0.18942	6	raw
B	0.15098	6	PUV

```
                    B       0.14245      6    Boil

Means and Descriptive Statistics                                    1
                                                  15:01 Wednesday, January 27, 2011

                    Mean of    Std. Dev.    Std. Error      Variance
    treatment        RESULT    of RESULT     of RESULT     of RESULT


      Boil         0.14245     0.022714    .009272807    .000515910
      PUV          0.15098     0.011997    .004897670    .000143923
      raw          0.18942     0.021837    .008915059    .000476870
```

Table A-3. Statistical analysis of ELISA result for almond extracts treated with HHP. A) ANOVA. B) Means by treatment

A)

```
                              The ANOVA Procedure

                           Class Level Information

Class           Levels    Values

Treatment_           5    HHP(21?,15min) HHP(21?,30min) HHP(70?,15min) HHP(70?,5min) raw

                         Number of observations    45

                              The ANOVA Procedure

Dependent Variable: Result   Result

                                        Sum of
   Source                    DF         Squares     Mean Square    F Value    Pr > F

   Model                      4      0.01385029      0.00346257       1.21    0.3213

   Error                     40      0.11437462      0.00285937

   Corrected Total           44      0.12822491

                 R-Square     Coeff Var      Root MSE    Result Mean

                 0.108016      20.14337      0.053473       0.265462

   Source                    DF        Anova SS     Mean Square    F Value    Pr > F

   Treatment_                 4      0.01385029      0.00346257       1.21    0.3213
```

B)

```
                              The ANOVA Procedure

                          t Tests (LSD) for Result

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

                          Alpha                          0.05
                          Error Degrees of Freedom         40
                          Error Mean Square          0.002859
                          Critical Value of t         2.02108
                          Least Significant Difference  0.0509
```

Means with the same letter are not significantly different.

t Grouping	Mean	N	Treatment_
A	0.28847	9	raw
A	0.28374	9	HHP(70?,5min)
A	0.25689	9	HHP(70?,15min)
A	0.25489	9	HHP(21?,30min)
A	0.24332	9	HHP(21?,15min)

Means and Descriptive Statistics

Treatment	Mean of RESULT	Std. Dev. of RESULT	Std. Error of RESULT	Variance of RESULT
HHP(21?,15min)	0.24332	0.050854	0.016951	.002586097
HHP(21?,30min)	0.25489	0.052208	0.017403	.002725651
HHP(70?,15min)	0.25689	0.053480	0.017827	.002860091
HHP(70?,5min)				
raw	0.28847	0.053943	0.017981	.002909807

Table A-4. Statistical analysis of ELISA result for almond extracts treated with NTP. A) ANOVA. B) Means by treatment

A)

The ANOVA Procedure

Class Level Information

Class	Levels	Values
treatment	3	Plasma 1min Plasma 3min Plasma 5min

Number of observations 27

The ANOVA Procedure

Dependent Variable: result result

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.00143279	0.00071640	0.12	0.8863
Error	24	0.14174753	0.00590615		
Corrected Total	26	0.14318033			

R-Square	Coeff Var	Root MSE	result Mean

0.010007 27.95013 0.076851 0.274959

Source	DF	Anova SS	Mean Square	F Value	Pr > F
treatment	2	0.00143279	0.00071640	0.12	0.8863

B)

The ANOVA Procedure

t Tests (LSD) for result

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	24
Error Mean Square	0.005906
Critical Value of t	2.06390
Least Significant Difference	0.0748

Means with the same letter are not significantly different.

t Grouping	Mean	N	treatment
A	0.28503	9	Plasma 5min
A	0.27179	9	Plasma 1min
A	0.26806	9	Plasma 3min

Means and Descriptive Statistics

1

02:33 Wednesday, February 24, 2011

treatment	Mean of RESULT	Std. Dev. of RESULT	Std. Error of RESULT	Variance of RESULT
Plasma 1min	0.27179	0.082206	0.027402	.006757756
Plasma 3min	0.26806	0.096090	0.032030	.009233375
Plasma 5min	0.28503	0.041561	0.013854	.001727310

Table A-5. Statistical analysis of ELISA result for almond extracts from raw and PUV treated whole almond.

A) ANOVA. B) Means by treatment

A)

The ANOVA Procedure

Class Level Information

Class	Levels	Values
treatment	2	PUV Whole Raw Whole

Number of observations 24

NOTE: Due to missing values, only 18 observations can be used in this analysis.

10:55 Monday, February 8, 2011 2

The ANOVA Procedure

Dependent Variable: result result

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.02686562	0.02686562	18.47	0.0006
Error	16	0.02327012	0.00145438		
Corrected Total	17	0.05013574			

R-Square	Coeff Var	Root MSE	result Mean
0.535858	12.51741	0.038136	0.304667

Source	DF	Anova SS	Mean Square	F Value	Pr > F
treatment	1	0.02686562	0.02686562	18.47	0.0006

B)

The ANOVA Procedure

t Tests (LSD) for result

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.001454
Critical Value of t	2.11991
Least Significant Difference	0.0381

Means with the same letter are not significantly different.

t Grouping	Mean	N	treatment
A	0.34330	9	Raw Whole
B	0.26603	9	PUV Whole

Table A-6. Statistical analysis of ELISA result for almond extracts treated with PUV for 1 min, 2 min, 3 min and 4 min.

A) ANOVA. B) Means by treatment

A)

The ANOVA Procedure

Class Level Information

Class	Levels	Values
treatment	4	PUV1min PUV2min PUV3min PUV4min

Number of observations 24

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The ANOVA Procedure

Dependent Variable: result result

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.00234829	0.00078276	0.25	0.8574
Error	20	0.06159665	0.00307983		
Corrected Total	23	0.06394493			

R-Square	Coeff Var	Root MSE	result Mean
0.036724	15.54226	0.055496	0.357067

Source	DF	Anova SS	Mean Square	F Value	Pr > F
treatment	3	0.00234829	0.00078276	0.25	0.8574

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B)

The ANOVA Procedure

t Tests (LSD) for result

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	20
Error Mean Square	0.00308
Critical Value of t	2.08596
Least Significant Difference	0.0668

Means with the same letter are not significantly different.

t Grouping	Mean	N	treatment
A	0.36588	6	PUV1min
A			
A	0.36328	6	PUV2min
A			
A	0.35855	6	PUV3min
A			
A	0.34055	6	PUV4min

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BIOGRAPHICAL SKETCH

Yiqiao Li was born in 1988 in China. She graduated from Zhejiang Sci-Tech University with B.S. in packaging engineering. She attended the University of Florida from 2009-2011 and graduated with her M.S. in agricultural and biological engineering. She also received minors in packaging science and food science.

Yiqiao Li was offered a research assistantship at the University of Florida to pursue her master's degree in agricultural and biological engineering, under the supervision of Dr.Wade Yang. She did her research in Department of Food and Human Nutrition where she gained invaluable experience about the research. She has also been an active member in organizations in her field, such as the Institute of Food Technologists (IFT).