EFFECT OF PULSED LIGHT ON ALLERGENIC PROTEINS OF SHELLED WHOLE PEANUTS

By

XINGYU ZHAO

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To my husband Jiaqing Zhou, who supports me the most

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TABLE OF CONTENTS

page

ACKNOWLEDGMENTS 4

LIST OF TABLES 9

LIST OF FIGURES 10

LIST OF ABBREVIATIONS 12

ABSTRACT 15

CHAPTER

1 INTRODUCTION 17

Justification 18
Objective 19

2 LITERATURE REVIEW 21

Current Situation of Food Allergy 21
Food Allergy and Anaphylaxis 21
The Mechanism of Interaction between Antigens and Antibodies 23
History of Peanuts 26
The Nutrients and Health Benefits of Peanut 28
Peanut Allergy 30
Peanut Allergens 31
Processing Methods for Peanut Allergen Reduction 37
Effect of Thermal Treatment on Peanut Allergens 37
Thermal processing 37
Roasting 39
Boiling 41
Frying 42
Autoclaving 43
Non-Thermal Treatment on Peanut Allergens 44
Pulsed light 44
High pressure microfluidisation 46
γ-irradiation 47
Pulsed electric field 48
Chemical and biological methods 49

3 INVESTIGATION OF THE EFFECTIVENESS OF PULSED LIGHT ON PEANUT ALLERGENS 53

Introduction 53

Material and Methods 54
Materials 54
Pulsed Light Treatment of Peanut Kernels 55
Preparation of Peanut Extract after PL Treatment 56
SDS-PGAE of Peanut Extracts 57
Western Blot of Peanut Extracts 57
Dot Blot of Peanut Extracts 58
Indirect ELISA of Peanut Extracts 59
Results and Discussion 59
SDS-PAGE of PL Treated Peanut Kernels 59
Western Blot of PL Treated Peanut Kernels 62
Dot Blot of PL Treated Peanut Kernels 63
Indirect ELISA 64
Influence of PL Duration and Distance on IgE Binding Reactivity to Whole Peanut Allergens 65
Summary and Conclusions 66

4 EXAMINATION OF IGE IMMUNOREACTIVITY OF INSOLUBLE FRACTIONS OF PULSED LIGHT TREATED PEANUTS BY NOVEX NUPAGE ® ELECTROPHORESIS SYSTEM 71

Introduction 71
Materials and Methods 72
Sample Preparation 72
Protein Extraction 73
Denaturing Electrophoresis 73
Western Blot after Denaturing Electrophoresis 74
Non-Denaturing Electrophoresis 75
Western Blot after Non-Denaturing Electrophoresis 75
Indirect ELISA 76
Results and Discussion 76
Denaturing Electrophoresis 76
Western Blot 77
Non-Denaturing System 82
SDS-PAGE 82
Western Blot 83
Indirect ELISA 83

5 THE OPTIMIZATION OF PL EQUIPMENT AND THE INTERACTION EFFECT OF MAJOR PARAMETERS: TIME AND DISTANCE 90

Introduction 90
Material and Methods 91
Color Analysis 91
Indirect ELISA 92
Experimental Design 92
Results and Discussion 93

Motor Design 93
Factorial Analysis 94
Color analysis 94
Indirect ELISA 95
Multivariate ANOVA Analysis 97
Color analysis 97
Indirect ELISA analysis 97
Summary and Conclusions 98

6 *IN VITRO* SIMULATED GASTRIC AND INTESTINAL DIGESTIONS OF PL PROCESSED PEANUTS 104

Introduction 104
Material and Methods 105
Simulated Peptic Digestion 105
Simulated Intestinal Digestion 106
Results and Discussion 107
Simulated Pepsin Digestion 107
Raw peanut 107
Roasted peanut 108
Pulsed light sample preparation 109
Simulated Trypsin and α-Chymotrypsin Digestion 110
Raw and roasted peanut 110
Western blot 110
Pulsed light treated peanut 111
Discussion 112
Summary and conclusions 115

7 EXAMINATION OF QUALITY CHANGES (TEXTURE, COLOR, ANTIOXIDANT CAPACITY, LIPID OXIDATION) OF THE WHOLE PEANUTS BEFORE AND AFTER PL TREATMENT 122

Material and Method 123
Texture Analysis 123
Color Analysis 124
Total Antioxidants Capacity Evaluation 125
Extraction of total antioxidants 125
Hydrophilic-oxygen radical absorbance capacity (H-ORAC) assay 126
Lipophilic-oxygen radical absorbance capacity (L-ORAC) assay 127
Lipid Oxidation 127
Peroxide value 128
Thiobarbituric acid reactive substances (TBARS) assay 128
Results and Discussion 129
Texture Analysis 129
Temperature and Moisture Changes During PL Illumination 130
Color Analysis 132
Antioxidant Capacity 134

Lipid Oxidation 135
8 OVERALL CONCLUSIONS 143
LIST OF REFERENCES 146
BIOGRAPHICAL SKETCH 162

LIST OF TABLES

Table		page
5-1	F-test of distance and time interaction based on the L value	99
5-2	Tukey HSD results describing 5 min, 6 min, and 7min treatment groups of L value	99
5-3	HSD of interactions between combination groups of L value	99
5-4	F-test output of indirect ELISA readings	99
5-5	F-test of distance and time factors based on Indirect ELISA readings	99
5-6	Means separated by treatment time: 5min, 6min, and 7min of Indirect ELISA readings	100
5-7	Tukey HSD of interactions between combination groups ($p < 0.05$).	100
5-8	F-test of distance and time interaction based on the color readings ($p<0.05$).	100
5-9	Means separated by treatment time of color analysis L value ($p<0.05$)	100
5-10	F-test of time and distance interaction based on Indirect ELISA ($p<0.05$).	100
5-11	Means separated by Tukey HSD of indirect ELISA readings.	101
7-1	Texture comparison between the dry roasted and PL treated peanuts	138
7-2	Surface temperature and moisture loss of dry roasted and PL treated peanuts.	138
7-3	Comparison of color between dry roasted and PL treated peanuts.	139

LIST OF FIGURES

Figure page

2-1 Thermal effect on the protein structure changes. 52

3-1 SDS-PAGE profiles of raw and PL-treated peanut extracts at different durations of illumination. 67

3-2 Antigenic protein banding patterns in raw and PL treated peanuts via western blot *in vitro* analysis with pooled human plasma 68

3-3 Dot blot analysis of raw (control) and PL treated peanuts 69

3-4 Indirect ELISA of PL treated peanuts at 10 cm and 7 cm distance to lamp with pooled human plasma containing IgE at different illumination durations 70

4-1 Protein profiles of insoluble fractions of PL processed peanuts were analyzed by Novex denaturing electrophoresis. 85

4-2 Western blot of insoluble fractions of PL processed peanuts samples were compared to roasted peanut 86

4-3 Protein profiles of insoluble fractions of PL processed peanuts were analyzed by Novex non- denaturing electrophoresis 87

4-4 Insoluble antigenic protein fractions were analyzed by western blot on non-denaturing gel 88

4-5 Effects of PL treatment on total protein of peanut kernels with different duration at 10cm were determined by Indirect ELISA with pooled human plasma containing IgE antibodies 89

5-1 A model and a picture of a motor designed for uniformly roasting for PL treatment. 102

5-2 Factorial analysis: Tukey HSD of interactions between combination groups of color analysis values ($p < 0.05$). 103

5-3 Factorial analysis: Tukey HSD of interactions between combination groups of Indirect ELISA readings ($p < 0.05$). 103

6-1 Protein profiles and Western blot of pepsin digested raw peanut samples 117

6-2 Pepsin digested roasted peanuts were determined by SDS-PAGE and Western blot 118

6-3 Pepsin digestion of PL treated peanut at 12 min 119

6-4 SDS-PAGE and Western blot of mimic intestinal-digestion of raw and roasted peanuts 120

6-5 SDS-PAGE and Western blot of mimic intestinal- digestion of PL treated peanut at 12 min 121

7-1 H-ORAC of PL treated peanuts. Roasted and raw peanuts were considered as controls. Tukey HSD was applied to analyze the means separation. 140

7-2 L-ORAC of PL treated peanuts. Roasted and raw peanuts were considered as controls. Tukey HSD was applied to analyze the means separation. 141

7-3 Peroxide value of raw peanut, PL treated peanuts at 9 min, 12 min, 15 min on 0 day, 5 days, and 10 days shelf life at room temperature 142

7-4 TBARS of raw peanut, PL treated peanuts at 9 min, 12 min, 15 min on 0 day, 5 days, and 10 days shelf life at room temperature 142

LIST OF ABBREVIATIONS

Atmosphere	Atm
AAPH	2, 20-azobis (2-amidino-propane) dihydrochloride
AGEs	Advanced glycation end products
ANOVA	Analysis of variance
Ara h	Arachis hypogaea
BCA	Bicinochoninic protein assay
BSA	Bovine serum albumin
DPPH	1, 1, diphenyl, 2 picrylhydrazyl
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EM	Electromagnetic
FIR	Far infrared
HHP	High hydrostatic pressure
H-ORAC	Hydrophilic oxygen radical absorbance capacity
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
HSD	Honestly significant difference
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IR	Infrared
JMP	John’s Macintosh project

L-ORAC	Lipophilic-oxygen radical absorbance capacity
LSD	Least significant difference
MIR	Mid infrared
MRPs	Maillard reaction products
NIR	Near infrared
NMR	Nuclear magnetic resonance
OPD	O-phenylenediamine dihydrochloride
ORAC	Oxygen radical scavenging activity
PBS	Phosphate buffered saline
PEF	Pulsed electric field
POD	Peroxidase
PL	Pulsed light
PVDF	Polyvinylidene Difluoride
RMCD	Randomly methylated beta cyclodextrin
RNA	Ribonucleic acid
RT	Room temperature
SAS	Statistical analysis system
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide electrophoresis
TA	Texture analyzer
TBARS	Thiobarbituric acid reactive substances
TBS	Tris buffered saline
TBST	1X TBS with 0.05% Tween 20
TE	Trolox equivalent

UV	Ultraviolet radiation
WHO	World health organization
UNEP	United nations environment program

Abstract of Dissertation Presented to the Graduate School
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By

Xingyu Zhao

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Most peanuts are consumed in the dry roasted form in the United States. However, roasting has been affiliated with an increasing peanut allergenic reaction. In recent years, many processing technologies were applied to inactivate allergens in peanut seeds, which include the following: high hydrostatic pressure (HHP), pulsed electric field (PEF), γ-irradiation, and pulsed light (PL). This study focused on PL since it has been shown to reduce allergen levels in peanut, soybean, almond, and shrimp protein extracts. During this research project, this technology was used to identify a hypoallergenic peanut production method that retained similar qualities as conventionally roasted peanuts. The data was validated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot, and indirect enzyme-linked immunosorbent assay (ELISA). The results indicated that soluble allergens were notably reduced by PL technology in pooled human plasma. Insoluble fractions formed during the PL process as a result of the Maillard reaction were analyzed by the Novex gel system, which is specially designed for large protein molecules. The results have illustrated that PL was effective in the reduction of all insoluble allergenic proteins. To optimize the two major parameters, time and distance, the difference in the means were

separated by factorial analysis and multi-analysis of variance (ANOVA) based on effects of color values and indirect ELISA readings. The interaction between these two factors was significant ($p < 0.05$). The best treatment, determined by proper color/texture development, and low immunoreactivity was a12 min treatment at a10 cm distance. An *in vitro* digestion study was conducted after optimization using pepsin, trypsin, and α-chymotrypsin. To mimic digestion, fluids were prepared with the appropriate pH value for gastric digestion at pH 2.0 and intestinal digestion at pH 8.3. The results revealed that peanut allergens Ara h 1 thru 11 were completely removed in this study via pulsed light. The quality tests performed after PL illumination has proven that PL can produce similar texture and color as compared to conventionally roasted peanuts. The hydrophilic and lipophillic antioxidant capacities were significantly higher in 12 min PL treated peanuts than in the raw peanuts ($p < 0.05$).

CHAPTER 1
INTRODUCTION

Peanut allergies are among the most common food allergies affecting children and adults. It is estimated to affect between 0.5% and 1.1% of adults in Western and European countries and is the third most common allergy in children with 4% affection (Sicherer and others 2003). Most peanuts are consumed in a dry roasted form in the United States, which is known to trigger and increase peanut allergenic reactions (Maleki and others 2000a; Poms and others 2004). If peanut allergens can be masked during roasting, the peanut allergy problems can be alleviated for peanut allergy patients. In recent years, several technologies, such as HHP, PEF, and PL have been developed that show potentials for reducing food allergens (Johnson and others 2010; Li and others 2013).

The focus of this study is on the effect on allergen reduction of PL treated peanut kernels. Previous studies have demonstrated that PL can reduce allergen levels in shrimp extract, soybean extract, and peanut extract (Chung and others 2008; Yang and others 2010b; Shriver and others 2011). It was shown that PL at prolonged exposures ranging from 6 min to 9.5 min has an effect on protein solubility reduction. To further validate this technology, the optimization of PL applicator was conducted and *In vitro* tests, such as Western Blot, ELISA, simulated gastric and intestinal digestion with pooled human serum were conducted and validated for the IgE reactivity reduction to allergenic proteins in the PL treated peanuts.

A recent study by Yang and others (2012) elucidated the possible mechanism of this technology on protein changes after treatment. The putative mechanism of this novel technology was due to the photothermal, photochemical, and photophysical

effects (Shriver and others 2011; Yang and others 2012). It is suggested that proteins are modified during the illumination of PL, leading to protein cross-linkage, protein solubility reduction, and protein hydrolysis at the high instantaneous temperature environment (Shriver and others 2011; Li and others 2013). As a result, PL has gained more attention because of the effects of protein denaturation and epitope masking.

The overall goal of this study is to explore the viability of PL technology for reducing immunogenic potential of whole peanut products that have a similar quality to traditionally roasted peanuts. If successfully conducted, this study is of significant importance, because the data can provide a base for further clinical trails and the quality evaluation of the final products that will have a substantially low immunogenic reactivity. This study also provides a possible alternative for approaching the peanut allergy problem for peanut allergy patients without using immuno-intervention, or other chemical interruption, such as epinephrine (Turk and others 1983; Achaorbea and others 1988). So far, PL reduction of major peanut allergens has not been fully explored. This study will reveal more about this technology on the allergenic protein changes and even on the changes of those treated peanut proteins after gastric and intestinal digestions. The results could suggest for changes in food processing techniques, especially in the peanut industry, and pave the way leading towards producing hypoallergenic peanut and peanut-derived products.

Justification

Several studies have shown that the prevalence of peanut allergy both in the United States and Canada has increased from 1997 to 2007 (Finkelman 2010). One study showed that 1.4% of subjects in the United States reported they have peanut or tree nut allergy in a random telephone survey (Sicherer and others 2010b). This

percentage has not increased for the adults, but increased for children less than 18 years old from 0.6% in 1997 to 2.1% in 2007. Also, around 1% of individuals reported peanut allergy in a similar survey in Canada (Ben-Shoshan and others 2010; Sicherer and others 2010a). To reduce the IgE reactivity to peanut allergens, several methods have been effectively demonstrated on different peanut allergens, such as enzymatic hydrolysis, heat processing, chemical method, and high intensity ultrasound (Chung and others 2005; Chung and Champagne 2006; Chung and Champagne 2007; Chung and Champagne 2008; Sales and Resurreccion 2009). However, Maleki and others (2000) reported that roasted peanuts have been demonstrated to increase the IgE binding reactivity compared to the same variety of peanut when processed by other thermal processing methods. This means that roasted peanuts, which are the most common peanut product in the United States because of their flavor and taste, are more likely to trigger a severely anaphylactic reaction than fried or boiled peanuts (Beyer and others 2001). PL is regarded as a non-thermal, high-peak power technology that consists of intense flashes of broad-spectrum (Rowan and others 1999). Although no treatment is currently available for allergies except total avoidance, emerging technologies like PL have shown positive outcomes for allergenic foods such as peanut and shrimp (Shriver and others 2011). With successful development, this technology potentially could be used in commercial processing to create roasted hypoallergenic peanuts.

Objective

Objective 1: To investigate whether PL technology is effective in reducing major peanut allergens Ara h 1, Ara h 2 and Ara h 3 in whole kernel peanuts through *in vitro* tests including Western Blot and indirect ELISA.

Objective 2: Examination of IgE immunoreactivity of insoluble fractions of PL treated peanuts by Novex NUPAGE electrophoresis system.

Objective 3: Examination of the effects of PL treatment time and distance from the light source and their interactions on the allergenic properties and color development

Objective 4: To verify the reduced allergen reactivity of the PL roasted whole peanut by *in vitro* simulated gastric and intestinal digestions.

Objective 5: To examine the quality changes (texture, color, antioxidant capacity, and lipid oxidation) of the whole peanuts before and after PL treatments.

CHAPTER 2
LITERATURE REVIEW

Current Situation of Food Allergy

Food allergy is defined as an adverse immune response to food proteins. Symptoms range from acute, possibly life-threatening, allergic reactions such as atopic dermatitis, to chronic debilitating diseases such as eosinophilic gastroenteropathies. Epidemiology studies indicate the continuing rise of food allergies. In industrialized countries, 2% of the adult population and 5 – 8% of children are affected (Ortolani and others 2001; Kagan 2003). In the United States, approximately 3.7% of adults and 6% of children are known to have one or more food allergies (Sicherer and Sampson 2010). It is believed that one in 25 Americans is susceptible to food allergies (Westphal and others 2004; Sicherer and other 2004). Among adults, the major food allergen in the United States is shellfish (2%) while the major food allergen in European countries is fruits (0.5%) (Kanny and others 2001; Sicherer and Sampson 2006; Meyer 2008). The major allergen for children in the United States and the European countries are milk and egg with 2.5–3% (Kanny and others 2001; Sicherer and Sampson 2006; Meyer 2008). Despite being considered as the fourth most important public health problem by the World Health Organization, the only effective treatment for food allergens is total avoidance of the allergen-containing food. More than 160 foods can cause allergic reactions. Eight of them are considered major food allergens and include milk, egg, walnuts, fish, shellfish, soybean, wheat, and peanuts (Krska and others 2004).

Food Allergy and Anaphylaxis

The major food allergens are identified as class 1 type food allergies because they are represented by peanut, egg white, and cow's milk; their allergens are heat- and

acid-stable glycoproteins with those allergic sensitization *via* gastrointestinal tract and cause systemic reactions (Nowak-Wegrzyn 2007). They are known to be water-soluble glycoproteins with molecular sizes ranging from 10 to 70 kiloDaltons (kD) and are stable to heat, acid and proteases. Examples include proteins in peanut, milk, and egg (Bargman and others 1992; Breiteneder and Radauer 2004). Breiteneder and Radauer 2004 further suggested that most of plant food allergens are either storage or defense-related proteins.

Food allergy is a relatively rare and violent reaction of the immune system towards food proteins. It has been defined as an immunologically based adverse reaction in response to dietary antigens (Beyer and Teuber 2004). The allergen evokes an initial IgE antibody response followed by a secondary IgE antibody response, which is the signal to reflect an allergic reaction (Babu and others 2001).

Allergy symptoms range from a spectrum of severity. The most fatal reaction is anaphylaxis. Anaphylaxis is a life-threatening reaction caused by the rapid release of mediators (histamine) from mast cells and basophills due to the interaction of the allergen with the IgE that is attached to the cell (Morritt and Aszkenasy 2000). It impacts many areas of the body, including the skin, respiratory tract, gastrointestinal tract, and cardiovascular system. As a result of food allergies, it is estimated that 30 people per every 100,000 sensitive individuals are affected and about 30,000 cases of emergency room treatments in the United States are due to food allergies (The Food Allergy and Anaphylaxis Network 2006).

Sathe and others (2005) listed several ways in which allergens can be removed from the foods. These methods included physical removal of the specific allergen

(protein filtration or separation), chemical modification of the allergens, genetical suppression or elimination of genetical expression, and genetic modification or mutagenesis of the targeted allergens.

Chemical modifications involve the alteration of the amino acids with the goal of potentially changing the epitopes conformation on the targeted allergen (Sathe and others 2005). However, one disadvantage of this method is that it is not specific. On the other hand, mutagenesis allows for selective substitution of specific amino acid from the targeted epitopes and thus is more advantageous than the chemical modification.

Also, gene suppression has been reported to be successful in elimination of allergens in soybeans while gene silencing has been successfully employed in rice, soybean, and apple allergens (Sathe and others 2005). Another study on trying to inactive food allergens involved the use of proteolytic bacteria from fermented foods. Phromraksa and others (2008) used crude extracted bacterial enzymes from *B. subtilis* (SR and BD strains) and *B. subtilis* (BD strain) from fermented products to reduce wheat flour and milk immunoreactivity, respectively. They found that enzymes derived from these two bacteria could hydrolyze the major allergenic fragments of wheat flour (gliadin) and major allergenic epitopes of milk (β – Lactoglobulin) without influencing the nutritive value.

The Mechanism of Interaction between Antigens and Antibodies

Allergic disorders are typically characterized as an abnormal or hyperactive immune response to environmental agents or food allergens. High levels of IgE in human plasma are responsible for the immune response (Galli and others 2008). IgE is a conserved member of the immunoglobulin family, and the titer of IgE is quite low (ng to mg / ml) in plasma of normal healthy individuals. IgE is widely distributed in epithelia

and mucosa, where it is bound to specific receptors on eosinophilic granulocytes and mast cells. When bound to these cells, IgE has a long half-life that can maintain from weeks to months, but unbound, free IgE in the plasma has a short half-life of about 6 h. This suggests that IgE plays a role in local immune defense mechanisms (Gould and others 2003).

Food proteins bind to the allergen-specific IgE molecules residing in the mast cells and basophils, causing them to release inflammatory mediators, including histamine (Beyerand and Teuber 2004). Small regions of allergenic proteins known as epitopes, composed of 5-7 amino acids or 3-4 sugar residues, cause the IgE-mediated allergy induced by antigens (Hendry and others 2001). Specifically, the crystallizable fragment of IgE binds strongly to high affinity receptors on mast cells and basophils, and together with the antigen, mediates the release of inflammatory agents from these cells. Afterwards, the allergen provokes an initial IgE antibody response followed by a secondary IgE antibody response, which signals an allergic reaction in the organism (Babu and Arshad 2001). The level of IgE is found in extraordinarily low concentrations in the serum of humans, varying from 20 to 500 ng / ml. Therefore, IgE is important since its biological activities are greatly amplified by binding to receptors on mast cells and basophils (Schiffer and others 1995).

An IgE molecule contains two identical light chains (23 kD) and two identical heavy chains (72 kD). Between the two antigen binding fragments, the molecule's surface has a depression that forms the antigen binding site. Individual protein allergens can have several recognition sites (epitopes) per allergenic protein. According to Ayuso and others (2002), the epitopes are not only fully characterized by their primary

structure, but also by their tertiary structure conformations. Food allergens and their epitopes are able to resist the effects of digestion and enzymatic reactions (Dollery and others 1987) and individual allergen systems are affected differently by the processing methods. This is because food allergens are complex mixtures of potentially immunoreactive proteins.

A short sequence of amino acids that lies at the base of each of the heavy chain regions of an immunoglobin is called a hinge region (Chintalacharuvu and others 2003). The hinge region is replaced by a constant domain, so that each heavy chain contains four constant domains. As a result, IgE has a molecular weight of 190 kD.

The structure of epitopes is generally categorized as either linear or conformational. Linear epitopes have a contiguous stretch of amino acids whereas conformational epitope involves noncontiguous amino acids forming a three-dimensional or structural motif. Individual patients may differ significantly in their sensitivity toward an allergen; however, the basis of such differential sensitivities remains to be elucidated. More than one epitope or IgE binding site is required per fragment of an allergen to cause IgE cross-linking. Therefore a molecule with a single IgE binding site must be bound or cross-linked to another molecule with an IgE binding site in order to cause histamine release. Understanding molecular properties of the epitopes is therefore important in learning the nature of IgE allergen interaction. In case of linear epitopes, amino acid residues that determine whether allergen would bind with IgE or not are known as critical amino acid residues. Any modification, deletion, or substitution of such critical amino acid residues may result in loss of IgE binding and may potentially result in reduction or elimination ofIgE immunoreactivity to peanut

allergens. If the epitope is conformational in nature, change in epitope conformation may permit modulation of allergic activity.

Food processing, under appropriate conditions, offers opportunities to alter the nature of epitopes. For example, epitope conformation may be modified as a result of protein denaturation treatments (e.g., various thermal processing treatments) leading to reduction or elimination, or in some cases, an increase, in IgE binding. Acid or enzyme hydrolysis of an allergenic protein may help delete critical amino acids of an epitope. Whether caused by protein denaturation or hydrolysis, loss of epitope and thus loss of IgE binding may help reduce or eliminate the bioactivity of an allergen. It should be emphasized here that processing, depending on the allergen and the processing method, may not affect the allergenic properties of all allergens. Physicochemical changes will alter the way in which allergens are broken down during digestion and may modify the form in which they are taken up across the gut mucosal barrier and presented to the immune system. Certainly, the structure of the food matrix can have a great impact on the elicitation of allergic reactions and fat-rich matrices may affect the kinetics of allergen release, potentiating the severity of allergic reactions (Grimshaw and others 2003). Understanding the impact of food processing and food structure on allergenic reaction is central to managing allergen risks in the food chain. However, our current knowledge of the impact of food processing on allergen structure indicates that there are no clear rules regarding how different allergens respond to food processing.

History of Peanuts

Peanuts are also named as groundnuts, earthnuts, and monkey nuts. The peanut plant (*Arachis Hypogea*) is belonged to Leguminosae family other than nuts family. Peanuts are self-pollinated plants and their pods are harvested by pulling the pods from

the ground. Normally, each peanut pod contains 2 - 3 oval shaped seeds with 2 lobes. Peanuts are originally from South America around 4000 to 5000 years ago and then exported to Africa and then moved to North America during the beginning of slave trade (Saavedradelgado 1989). By the nineteenth century, peanut industry had considerably expanded, which was then followed by a rapid demand for peanut oil, roasted and salted peanut, peanut butter and confections in the new twentieth century (The Peanut Institute 2007).

George Carver Washington, a researcher in Tuskegee University, Alabama, is regarded as the father of the peanut industry in United States. He started peanut research in 1903 and suggested farmers rotate their cotton farms with peanut and developed more than 300 different uses of peanut from recipes to industrial products (The Peanut Institute 2007).

Virginia type has the largest kernel and is mostly sold as roasted or processed in-shell. It accounts for 15% of total United States peanuts products and the roasted product is widely used as a snack (Conkerton and Ory 1976). Spanish-type peanuts have comparatively smaller kernels and are characterized by a reddish-brown skin and account for only 4% of the total production in United States. But they have the highest oil content compared to other varieties and are mostly used for candies and significant amount used for snacks, also for peanut butter. Valencia is characterized by having three or more kernels to a pod and is covered by a bright-red skin. It is the least produced in the United States accounting for only 1% of total production (The Peanut Institute 2007). Runner type peanuts have good flavor and better roasting

characteristics, which are widely grown in southeastern United States region (Pattee and others 1990).

Seven states are responsible for almost 99% of all peanuts grown in the United States. Georgia is the major producer (41%) followed by Texas (24%), Alabama (10%), North Carolina (9%), Florida (6%), Virginia (5%) and Oklahoma (5%) (Koppelman and others 2001). About 75% of the American peanut crop is used domestically, and the remainder is exported mainly to Canada, Japan and Western Europe. On average, the average consumption of peanut butter in USA is 2 kg per year (FDA/CFSAN 2000).

Except its main use as food, peanuts can also be used for several other purposes, and there are about 300 derivatives of peanuts (Morritt and Aszkenasy 2000). Hulls or pods can be used as fuel or in kitty litter; kernels can be crushed to obtain peanut oil, which can be used to soap manufacture. Peanut skins were used to make beverages at National Peanut Research Laboratory (FDA/CFSAN 2000). Peanuts can also be found in other products like cheese, milk, coffee, medicinal oil, flour, soap, cosmetics, dyes, plastics and linoleum (Hourihane 1997; Hill 2004; Husain and Schwartz 2012).

The Nutrients and Health Benefits of Peanut

Peanuts have more proteins than other legumes or nuts. It contains around 21-36% proteins in its seed (FDA/CFSAN 2000). One study indicated that the consumption of a peanut oil diet can reduce 17% of the risk of heart disease (The Peanut Institute 2007). It was further found that a peanut oil diet did not increase the tryglycerol concentrations or lower the good high - density lipoprotein (HDL) cholesterol levels compared to the other low - fat diets. Peanuts contain highly beneficial monounsaturated and polyunsaturated fats that have the potential to lower blood

cholesterol levels and in turn reduce the risk of coronary heart disease. High levels of phytochemicals in peanut could also lower the risk of coronary heart disease (Awad and others 2000). One of the phytochemicals found in peanuts was resveratrol. Phytosterols not only lower the risk of heart disease by interfering with the absorption of cholesterol, but also help lower the risk of cancer by inhibiting the tumor cell growth (Awad and others 2000). Other studies also found that phytosterols inhibited the cancer cell growth by 70% and reduced the prostate tumor growth by 40% (The Peanut Institute 2007). One of the most common forms of phytosterols is beta-sitosterol (SIT). Peanut oil contains more protective SIT than refined olive oil (Awad and others 2000).

Vitamin E content is rich in peanuts and has been associated with cancer reduction, immune system improvement and cardiovascular diseases prevention (Karunanandaa and others 2005). Important essential minerals like zinc, magnesium, phosphorus and potassium are also abundant in peanuts. Moreover, Harvard University's Public Health Center demonstrated the consumption of peanut butter and/or peanuts could reduce the risk of developing type two diabetes (The Peanut Institute 2007).

Phenolic phytochemicals in foods obtained increased interest from consumers and researchers for their antioxidant activity and health benefits. Their ideal chemical structure for free radical scavenging activities showed increased antioxidant activity compared to vitamin E and C on a molar level (RiceEvans and others 1997). Peanuts not only have healthy fatty acid profiles and serve good sources of fibers and protein, but also contain a number of components that are capable of directly scavenging free radicals. In peanuts, various polyphenolics (coumaric acid, derulic acid, resveratrol),

tocopherols, flavonoids (procyanicdins, catechin) and folate are found (Blomhoff and others 2006; Isanga and Zhang 2007). Peanut flours contain high protein but low fat ingredients prepared from roasted peanut seed. Peanut skins are established to be of rich source of phenolic compounds, including various procyanidins among others, which suggests that there is potential to produce neutraceutical ingredients from peanut skin (Nepote and others 2005; O'Keefe and Wang 2006).

Peanut seeds contain 51.9% oil in total mass. The composition of peanut fatty acid includes higher proportions of oleic acid (43.2%) and behenic acid (3.2%), which are higher than soybean seeds (28.8% and 0.3%). Peanuts also contain much lower proportions of linoleic acid (35%) and linolenic acid (0.1%) than soybean seeds. Overall, peanut oil is less susceptible to oxidation than soybean oil because of fewer double bonds present in peanut oil (Wang and others 2012).

Peanut Allergy

Among all the food allergies, peanut allergy is considered as one of the most serious allergies. Peanut allergy is the most severe fatal allergic reactions for patients who are sensitive, accounting for the majority of severe anaphylactic reactions for foods (Clarke and others 2006; de Jonge and others 2007). Bock and others (2001) also reported that peanut was the most dangerous food allergen source with responsibility of over 63% of the fatal reactions. In the United States alone, peanut-induced anaphylaxis affects around for 1.5 – 4.4 million people and causes about 50-125 deaths every year (Leung and Bock 2003; Sicherer and Leung 2006). Moreover, unlike other food allergies which tend to be outgrown in childhood, peanut allergy tends to be a lifelong problem for around 80% of the peanut allergic population (Viquez and others 2001; Clarke and others 2006; de Jonge and others 2007). However, avoidance of peanuts consumption

in foods is difficult, and those food products without proper label may have some contamination with peanuts. Allergic individuals always have several symptoms including oral allergy syndrome, mild hives, facial swelling, abdominal cramps and hypertension with anaphylactic shock.

Peanut Allergens

Peanut allergens are storage proteins belonging to two major globulin families, arachin (legumin) and conarachin (vicilin) (Viquez and others 2003). They are classified as either a member of the cereal prolamin superfamily or of the cupin superfamily (Dunwell and others 2001). Previous research has shown that there is no naturally occurring allergen-free peanut among a wide variety of commercially grown peanuts (Viquez and others 2001).

Among the eleven known peanut allergens Ara h 1- Ara h 11, Ara h 1, Ara h 2 and Ara h 3 are responsible for allergic reactions in more than 60% of peanut-allergic patients (Pele 2010). Moreover, out of these 3 major allergens, Ara h 1 and Ara h 2 are responsible for over 90% of the reactions in peanut-hypersensitive individuals (Viquez and others 2001).

Ara h 1 with a molecular weight of 63.5 kD is resistant to heat, stable to digestion, and highly structured at the secondary level (Koppelman and others 2003). This protein is affecting over 90% of the peanut sensitive population. Ara h 1 is known to have 30% to 45% similarity with other vicilin storage legumes such as soybeans, beans and garden pea (Pomes 2003). Ara h 1 has 23 allergenic epitopes that are immunodominant, being recognized by more than 80% of patients. This protein is very abundant in peanut representing around of 12 - 16% of the total protein. Ara h 1 has thermal stability and its allergenic properties are unaffected by thermal denaturation. It

is also resistant to proteolytic hydrolysis (Maleki and others 2000b; Wichers and others 2004).

Ara h 2 is a glycoprotein of about 17 kD with at least two major bands and an isoelectric point of 5.2 (Viquez and others 2001). Its amino acid sequence is composed of high percentage of glutamic acid, aspartic acid, glycine, and arginine which shows similarity to seed storage proteins of the conglutin family and the protein has at least 10 IgE epitopes (Viquez and others 2001). It also has 8 cystein residues that could form up to four disulphide bonds and is predominantly an α – helical protein (Lehmann and others 2003; Sen and others 2002), which is said to play an important role in its structure. Sen and others (2002) also found that the native non-reduced Ara h 2 protein migrated in a 12% SDS PAGE as a doublet protein with an average molecular weight of 12kD while the reduced Ara h 2 protein migrated as a slightly larger doublet with an average molecular weight of 17 kD. It is the most potent allergen and is recognized by the serum IgE of more than 95% of peanut-sensitive individuals (Kleber-Janke and others 2001). Ara h 2 has been reported to be active even at very low concentrations (< 100 pg/ml), while Ara h 1 and Ara h 3 are only active at 10 to 100-fold higher concentrations compared to Ara h 2 (Palmer and others 2002; Koppelman and others 2003; Knol and others 2003).

Ara h 3 belongs to the glycinin storage protein family. It consists of basic and acidic subunits with the molecular weight ranging from 14 to 45 kD. Koppelman and others (2003) reported that the basic subunits and to a lesser extent, the acidic subunits, bind to the IgE and may therefore act as allergenic peptides. They also found that the major IgE binding epitope is found on the N-terminal fragments of 42 and 45

kD. Ara h 3 has been reported to have homologous epitopes as soybean allergen Glycinin (Pomes 2003).

Peanut allergens have been reported to be difficult to avoid by sensitive individuals. This has been authenticated by reports showing that accidental ingestion of peanut by sensitive individuals has been increasing in recent years. Extensive use of peanuts in the food industry has made dietary avoidance very difficult. A study showed that about 55% of American children allergic to peanuts had accidental ingestion of peanut in 2005, indicating an annual increase rate of 33% (Yu and others 2006). This problem has been accelerated by the inability of many parents to carefully read the food labels. Kemp and Deshazo (2004) reported that around 54% of parents of allergic children fail to correctly read the labels, thus ending up with accidental ingestion of peanuts by children.

Dodo and others (2002) found that there was no significant difference in allergen content on four types of peanuts in their study on allergen content in various market type peanuts. Also Maleki and others (2000) found no difference in the level of IgE reactivity between high oleic peanuts (80% oleic acid) and normal peanut varieties (50% oleic acid). This implies that the risk of fatal reactions is the same for sensitive individuals, regardless of the peanut varieties consumed.

The severity and existence of peanut allergy in the Unites States has been explained in many different ways. It is thought that the development of peanut allergy has to do with the processing method they are handled. In the United States, roasting is the main processing technique while boiling is most common processing method in China and Indian. Cases of allergy in China and India are considerably lower as

compared to the United States (Sampson 2002). Therefore, roasting has been associated with the increasing cases of peanut allergy in the United States.

This theory is supported by a study conducted by Al-Mussawir and others (2002), who investigated the differences in the allergenic potency between boiled and dry-roasted peanuts. Their results showed that boiling reduced the allergenic potency of peanuts while dry-roasting increased it. Another study by Maleki and others (2000) found that extracts from roasted peanuts bound to serum IgE was 90 folds higher in peanut patients than that from raw peanuts. They concluded that the Maillard reaction was responsible for the observed effect. Further studies of Maleki and others (2003) pointed out that roasting peanuts caused a major peanut allergen, Ara h 2, to become a stronger inhibitor (at least 3.5 folds increase) to a digestive enzyme trypsin, thus making it more resistant to digestion. This study also found that Ara h 2 protected Ara h 1 from digestion, an action which is enhanced by roasting. In general, this shows that thermal processing such as roasting, curing and various types of cooking can cause non-enzymatic, biochemical reactions in foods, with the predominant reaction responsible for allergenic behavior being the Maillard reaction.

Maleki and others (2003) further established that heating causes alterations to the protein structure mainly due to the interactions of sugar components with amino acids, forming carboxymethyl lysine, malanoidin, and other compounds. These and other modifications to the protein are believed to have detrimental nutritional, physiological, and toxicological consequences. Chung and Champaigne (2002) reported that the protein-sugar complex formed during Maillard reaction may cross-link with proteins to form advanced glycation end-products (AGE,) which increase IgE binding.

They added that the formation of carboxymethyl lysine modification on the surface of the protein resulted in higher binding levels of allergens Ara h 1 and Ara h 2 with IgE.

To develop an effective processing method for inactivation of peanut allergen, several studies focused on investigating alternative treatments and prevention of peanut allergy. Nelson and others (1997) found that injection of peanut extracts can significantly improve the tolerance of anaphylactically sensitive subjects to the ingestion of peanut proteins. However, this tolerance cannot be maintained for individuals who are highly sensitive because of strong systemic reactions to injections. However, there is no evidence so far of its clinical application because of the risk of systematic reactions.

Activated charcoal has been investigated for treatment of peanut allergy. Routon and Kopper (2004) reported activated charcoal could absorb peanut allergens within seconds and prevent further serious reactions to IgE binding. Other methods which have been investigated to reduce the allergenic potency in peanuts include enzyme treatment. Chung and Champagne 2003 studied two enzymes, transglutaminase and peroxidase. They found peroxidase could reduce the allergenic potency of Ara h 1 and Ara h 2 in roasted peanuts while transglutaminase was not effective. However, both enzymes were not effective on raw peanuts. Chung and others (2005) treated peanut extracts with two metals, copper and iron, in order to assess their efficacy in allergen reduction. Treatment of roasted peanut extracts with copper in the presence of hydrogen peroxide resulted in the reduction of Ara h 1 and Ara h 2 levels. However, no effect was observed with iron treatment. Furthermore, in order to study the role of other peanut components onIgE immunoreactivity to peanut allergens, Chung and

Champagne (2006) studied the effect of phytic acid on the IgE binding to peanuts. In this study, they compared phytic acid-containing peanut allergens to native allergens. Their findings showed that the IgE binding for phytic-bound allergens was similar to that of native allergens, indicating that other peanut components such as phytic acid might not have any effect on the IgE binding of the peanut allergens. A follow-up study on the effect of phytic acid in masking peanut allergens was done by Chung and Champagne (2007). They attempted to explore the effect of phytic acid on reducing the allergenic potency of peanut butter. New findings were shown that phytic acid could form insoluble complexes with the major peanut allergens to interrupt digestion and subsequently prevent absorption.

Phenolic compounds which have effects on reducing the allergenic potency of peanuts has been explored by Chung and Champagne (2008). In this study, they used three different phenolic compounds: caffeic acid, chlorogenic acid, and feluric acid. They assessed their effectiveness on the reduction of peanut allergens in peanut extracts and peanut butter slurry. Results showed that out of the three compounds, only caffeic acid formed insoluble complexes with the major allergens Ara h 1 and Ara h 2 in treated samples, thus lowering their allergenic potency. However, chlorogenic acid and feluric acid had no effect. All these studies demonstrated the potential treatments in reducing peanut allergy in different ways. However, so far there has never been any practical application of these findings in the food industry, prompting the investigation of other technologies, which might be readily and easily applied in the food industry. Moreover, the reviewed studies did not list nutritive value changes after these treatments.

Processing Methods for Peanut Allergen Reduction

A number of thermal and non-thermal techniques have been investigated for allergens reduction in peanut and other foods. Thermal processing may alter the IgE binding sites of allergenic proteins in foods with increased or decreased allergenic effects (Wal 2003). The outcome depends on the structure and chemical properties of the allergen, the type of thermal processing applied (dry or wet), the temperature, and the duration of heating (Davis and Williams 1998). Thermal treatments by wet thermal methods include boiling, frying, extrusion, autoclaving, and retorting, while dry heat methods include baking, roasting, and microwaving. As mentioned earlier, roasting treatments enhance IgE-binding capacity while boiling peanut decreases IgE reactivity to peanut allergens by western blot (Maleki and others 2000a). In Chapter 2, only roasting, frying, boiling, and autoclaving methods will be reviewed on peanut allergen reduction with these thermal processing methods. Non-thermal processing methods exist and are labeled as emerging technologies because they have been used in recent years to reduce allergen reactivity of different foods. Non-thermal treatments provide several benefits, such as preserving the natural characteristics, nutritional values, and flavors as compared to the conventional thermal processing.

Effect of Thermal Treatment on Peanut Allergens

Thermal processing

Thermal processing was developed for enhancing food safety (pasteurization or sterilization), change of physical attributes or texture (drying, gelation), or to induce flavor profile modification (baking) (Davis and Williams 1998). However, thermal processing also affects protein structure. The rationale can be simply explained from the standpoint of altering protein structure. Significant alterations in protein structure occur

during heat treatments and these changes depend on two major factors: temperature and intrinsic characteristics of the protein and physic-chemical conditions of its environment (such as pH). Typically, loss of tertiary structure is starting from unfolding of proteins. In general, loss of secondary structure (55-70°C), cleavage of disulphide bonds (70-80°C), formation of new intra-/inter- molecular interactions, rearrangements of disulphide bonds (80-90°C) and the formation of aggregates (90-100°C) as shown in Figure 2-1 (Davis and Williams 1998). At the molecular level, linear epitopes of proteins are more difficult to eliminate than conformational epitopes by thermal processing or any other technique. Linear epitopes belong to the primary structure that is hard to be destroyed even under temperatures as high as 125°C. On the other hand, conformational epitopes are relatively easy to be cleaned out by altering the tertiary or secondary protein structure, which can occur within reasonable temperature ranges of 50-125°C (Lee 1992). Moreover, at higher temperatures, covalent bond formations between protein lysine residues and other constituents of the food matrix may take place and lead to the formation of other adducts (Chung and others 2003). Protein digestibility and gastrointestinal tract absorption are normally increased before heat treatment. In some cases, thermal processing may reduce the digestibility of a particular allergen, such as Ara h 1 and Ara h 2 in peanuts (Schmitt and others 2010). Thermal treatment may also lead to neo-antigen formations that are not originally existed. The generation of neo-antigens may enhance the allergenic issue in sensitized peanut allergy patients. One of the factors responsible for this effect is the Maillard reaction. Due to the interaction of sugar and protein components upon heating, which generates sugar conjugated protein derivatives, will increase the IgE immunoreactivity of the

protein effect (Davis and others 2001). Generally, roasting peanuts has shown to increase the IgE reactivity while boiling and frying have shown to decrease allergenic effects of peanuts.

Roasting

Peanuts are consumed either fried or boiled in China whereas peanuts are typically dry roasted in the United States (Beyer and others 2001). Runner type peanuts and the Florunner are the most common peanut varieties in the United States, which account for 73% of the total United States production (The Peanut Institute 2007). Runners are always consumed in a roasted form and used heavily in peanut butter production. Valencia, another common variety, is usually consumed in a roasted form in the United States (Beyer and others 2001). It was shown that roasting may strengthen the allergenic properties of peanuts as compared to unprocessed, raw peanuts (Maleki and others 2000a).

In the industry, peanuts are normally processed using dry roasting methods that include both batch and continuous processes. Batch roasters are usually natural gas-fired revolving ovens and the rotation of this oven produces evenly roasted peanuts products. The oven temperature is around 430°C (800°F) and internal temperatures of the peanut kernels reach to around 160°C (320°F) after 40min to 60min treatment. Different roasting temperatures and heating durations are used depending on the desired characteristics of the peanut batch.

Several types of continuous dry roasters are available, such as cross-flow roaster, vertical continuous roaster (Davidson and others 1999). Generally, peanuts move through an oven on a conveyor of by gravity feed. One design involves peanuts being fed on a conveyor into a stream of countercurrent hot air that roasts the peanuts.

The peanuts are agitated, allowing air to pass through the individual kernel and ensure an even roast. The major advantages of continuous systems are include higher capacity, greater control over the roasting conditions, and low labor costs due to an automated system (Fellows 2009).

For laboratory purposes, the roasting temperature ranges from 160°C to 176°C with different treatment times depending on the desired characteristics. (Schmitt and others 2010) used a temperature of 160°C (320 °F), for 5 – 50 min to investigate the thermal effects on peanut proteins. Protein aggregation and solubility reduction were observed during roasting process.

Since the IgE binding signal of roasted peanuts is higher than the boiled or fried peanuts, Maleki and others (2000) indicated that Maillard reaction is responsible for the higherIgE immunoreactivity. The Maillard reaction is important in the development of flavor and color of roasted peanuts. The amino groups of proteins are modified with reducing sugars, ketose, or aldehydes to form Amadori products. Subsequently, Amadori products are degraded into dicarbonyl intermediates. These compounds are more reactive than the parent sugars and react with other amino groups to form stable end products, called that named as Maillard reaction products (MRPs) or advanced glycation end products (AGEs). In addition, Maillard reaction may lead to the loss or modification of amino acids, such as lysine and cysteine, and can cause malanoidin formation and other non-cross-linking modifications to proteins (Baynes 1991; Kristal and Yu 1992). Maleki and others (2000) demonstrated that such collective modifications accounted for the increased IgE binding in roasted peanuts. Other byproducts that may contribute to the higher levels of IgE binding, such as lipid oxidation products, were not

investigated. Therefore, it is suggested that the increased allergenic properties of Ara h 2 in roasted peanuts result from the Maillard reaction. The modified protein products can further evoke an IgG response, which is known to be related with IgE production (Ikeda and others 1996; Duchateau and others 1998).

Boiling

Boiled peanuts are mainly consumed in China and other Asian and Middle Eastern countries, and have been popular also in the southern region of the United States. In recent years, boiled peanuts are found in grocery stores as a type of snack (Chukwumah and others 2007). Numerous studies point out that the sensitization and reactivity of boiled peanuts is much lower as compared to roasted peanuts as we discussed above. Typically, the peanuts are boiled at 100°C for approximately 20 min (Schmitt and others 2010).

The IgE binding of monomeric Ara h 1 and Ara h 1 trimer of boiled peanuts show a significant reduction compared to roasted peanut on Western blots. Heated Ara h 1 may form stable dimmers, trimers and larger complexes. The Ara h 1 trimer has a high stability and resists digestion processes that protect IgE binding sites. The IgE binding to Ara h 2 and Ara h 3 was, in parallel with Ara h 1, significantly lower in boiled peanuts (Beyer and others 2001). Furthermore, Maleki and others (2010) detected both soluble and insoluble fraction activities with IgE, which revealed that boiled peanuts have a significantly less IgE-reactivity in insoluble fraction than roasted peanuts. The overall decrease in IgE binding reactivity in boiled peanuts is not associated with structural modifications of peanut proteins, but mainly because of loss of the conversion of soluble low molecular weight proteins or peptide fragment into insoluble complexes. This would

explain the low prevalence of peanut allergy in some countries where peanuts are boiled (Mondoulet and others 2005).

Frying

The low prevalence of peanut allergy in China is also related to another popular form of peanut product, which is fried peanut (Beyer and others 2001). The cooking temperature is approximately 120°C. It is shown that the monomeric form of Ara h 1 is reduced in fried peanuts as compared with roasted peanut. The IgE binding intensities to Ara h 1 monomer and trimers were also significantly reduced in fried peanut relative to roasted peanuts (Beyer and others 2001). A further study detected both soluble and insoluble proteins in fried peanuts on SDS-PAGE and the IgE binding intensities on Western blot. Fried peanuts had higher or the same level of IgE binding as roasted peanut, but the soluble bands had a notable reduction of IgE reactivity (Schmitt and others 2010).

A recent study provided strong evidence for the disintegration of boiled, roasted, and fried peanuts in simulated gastric environment to investigate the influence of different thermal processing methods on peanut proteins, especially for allergens (Kong and others 2013). The absorption of moisture and the swelling of peanuts during gastric digestion were measured using magnetic resonance imaging (MRI). The disintegration rate of peanut proteins was rated as: fried > roasted > boiled > raw peanuts. Fried peanuts had the fastest disintegration rate with a half time of 3.6 h, but the raw sample extended to 10.7 h. Furthermore, the textures of boiled and fried are more softener than the roasted one in simulated gastric environment (Kong and others 2013).

Autoclaving

Previous studies have shown that autoclaving at 2.56 atmosphere (atm) for 30min could produce a relevant decrease in the IgE binding capacity of lentil and chickpea allergens. However, several immunoreactive proteins were still presented in these legumes upon harsh autoclaving (Cuadrado and others 2009). Instantaneous controlled pressure-drop treatment, which combines heat and steam pressure, has shown a remarkable decrease in the allergenic proteins of peanut at 5.92 atm for 3min (Beyer and others 2001).

In peanuts, one study sought to investigate the effects of autoclaving on the allergenic protein reactivity. Extreme conditions were applied at 2.56 atm for 30min at 138°C in this study. The results showed a significant reduction of IgE reactivity compared to conventional roasted peanut based on Western blotting and ELISA. These results pointed to a damaged α-helical structure. Many of the IgE binding epitopes in the major peanut allergens (Ara h 1, Ara h 2, Ara h 3) are located on the α-helical regions (Shin and others 1998; Barre and others 2007; Mueller and others 2011). About 22% of the sera from skin prick test results could recognize autoclaved peanut samples (Cabanillas and others 2012). The study demonstrated that the structure of peanut allergens has been changed and some of epitopes were destroyed after autoclave treatment.

In addition, the texture of autoclaved peanut is softer than that of roasted peanut. The digestibility of autoclaved peanut is more extensive by trypsin after 10 min treatment than that of roasted peanut. This study has demonstrated that heat treatments can increase the susceptibility to enzymatic digestion of peanut allergens (Morisawa and others 2009).

Non-Thermal Treatment on Peanut Allergens

Non-thermal processing is as an alternative method to conventional thermal processing. The goals of non-thermal processing are to strike a balance between food safety and minimal processing and to combine novel approaches with current processes installations to optimize resources. Non-thermal technologies can be used for decontamination, pasteurization and sterilization, but another emerging application of some non-thermal technologies is to inactivate the allergenic proteins in foods. Several non-thermal processed methods, such as PL, power ultrasound, HHP, ionized radiation, PEF.

Pulsed light

PL consists of a broad spectrum of white light with wavelengths from 200nm in the ultraviolet region to 1000nm in the near infrared region. The spectra are emitted within several nanoseconds from ionized inert gas, such as Xenon. The PL light is thousands of times more intense than conventional, continuous mercury UV light and sunshine at the sea level (Dunn and others 1995; Krishnamurthy and others 2007).

There are two types of PL systems used for food processing: a batch unit and a continuous unit. In the batch system, foods are manually loaded to the sample rack that can be adjusted by the operator to reach a proper distance from the light source. The pulse frequency is typically around 1 - 20 pulses / s. In a continuous system, a conveyor belt carries food samples through one or more lamps. The distance to the lamp, conveyor speed, pulse frequency, treatment duration, and lamp tilt angle of the belt can be adjusted to modify the process. A continuous unit is also capable of larger sample treatment and it can be used as a batch unit when the conveyor speed is set to zero.

PL is considered more effective in food processing due to its instantaneous high-energy pulses and greater capability to penetrate. PL is regarded as nonthermal technology with a short duration (e.g., seconds). However, some studies showed that extended PL treatment could incur a significant temperature increase and moisture loss of a food sample, which is due to the infrared spectrum (Yang and others 2012). In a PL system, electrical energy is captured and stored in a capacitor and is ultimately released in short pulses as UV light, infrared light, and visible light (around 54%, 20%, and 26%, respectively) (Shriver and Yang 2011). The resultant bursts can be several thousand times more intense than continuous UV light (Krishnamurthy and others 2007). As Shriver and Yang 2011 indicated, PL processes might increase the temperature for treated shrimp up to 68.5°C. Another previous study reported that pulsed UV light can reduce the human IgE reactivity on almond, soybean extract, peanut butter (Yang and others 2010a; Li and others 2011; Yang and others 2011).

It is believed that PL's photothermal, photophysical and photochemical effects could alter allergen conformation (Krishnamurthy and others 2007) or cause protein aggregation (Chung and others 2008; Yang and others 2010), destroying conformational and linear epitopes. Furthermore, radicals may be produced during PL treatment due to molecular ionization under its high-energy burst of lights. The visible and infrared waves in the PL spectra are responsible for vibration and rotation of molecules, respectively (Krishnamurthy and others 2009) UV light can also lead to protein aggregation and fragmentation. Infrared light can cause temperature increases in food sample and produce thermal effect on treated samples. Due to the protein aggregation, the digestibility of those aggregate has been reduced and the possibility to

cause allergy may reduce due to this reason. Since most epitopes of peanut allergens are linear form. PL can fragment the protein molecules to form smaller peptides. The linear epitopes of peanut allergens may be destroyed after PL illumination. To obtain more evidence to support PL on the changes of allergenic proteins, high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), skin prick test, oral administration studies are still needed.

Friedman and Brandon (2001) found that extended PL treatment caused formation of insoluble complex in food, depolymerization of starch, peroxidation of unsaturated fatty acid, carbohydrate crosslinking, protein crosslinking, and protein fragmentation. The PL 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 ionized 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). Chung and others (2008) conducted a study that was to investigate the effects of PL treatment on peanut extracts. Ara h 1 (63 kD) showed a significant disappearance in SDS-PAGE that indicated they form insoluble aggregates or precipitates. Ara h 2 (10-20 kD) allergens did not form aggregates because they still remained soluble form. Aggregates that formed in the treated peanut samples accounted for the peanut allergens disappearance.

High pressure microfluidisation

Dynamic high pressure microfluidisation is a new technology that combines mixing, ultrarefining, homogenization, emulsification and other units with the basic function of a microjet homogenizer and ultrahigh-pressure homogenizer. This

equipment has a strong shear force, high-speed bump, instantaneous pressure release, and cavitation effect. The pressure of this unit can achieve 200 MPa. A recent study explored the effects of this unit on Ara h 2 of peanut extracts. The results showed that the primary structure of Ara h 2 is not affected by high pressure. However, the teriary and quaternary structure of Ara h 2 have shown unfolded and more hydrophobic regions exposure. Under a pressure of 180 MPa, exposed hydrophobic groups form more stable aggregates. Some α – helices were destroyed and converted to β sheets, indicating the second structure of Ara h 2 has been destroyed. The disulfide bonds of Ara h 2 were converted into sulfhydryl groups, resulting in a reduction in the overall IgE binding signal (Hu and others 2011).

γ-irradiation

Irradiation is an ionic, no-heat process that is considered to be a preservation and functional modification method in polymer research (Abu and others 2005). In comparison with other processing methods, such as microwave, UV light, ultra high hydrostatic pressure and hydrothermal treatments, irradiation treatment is rapid, convenient, but more expensive (Bao and others 2005). Among non-thermal processing methods, γ-irradiation has been used to monitor food borne pathogens, reduce the population of microbial load and insect infestation, inhibit the germination of root crops and prolong the shelf life of perishable products. Furthermore, irradiation has been reported to have effects on reducing the IgE binding sites of ovalbumin, bovine serum, albumin and milk proteins, and shrimp tropomyosin (Kume and Matsuda 1995; Lee and others 2001; Byun and others 2002). Previous studies showed that a treatment on a shrimp allergen with a molecular weight 35 kD did not affect its activity (Hoffman and others 1981; Hefle 1996; Besler and others 2001). Later, γ-irradiation was combined

with heat treatment to have a reduced IgE immunoreactivity effect on shrimp (Li and others 2007). On the contrary, a combination of heat treatment and γ-irradiation (1 - 25 kGy) alone or followed by various thermal treatments would not change their allergenic potential (Su and others 2004). Another study reported that boiling followed by irradiation reduced IgE binding of treated peanuts, up to 26 folds (Kasera and others 2012). Later, to investigate the effects of gamma irradiation on the structure changes of Ara h 6, isolated Ara h 6 and irradiated at 1, 3, 5, or 10 kGy. A whole peanut protein extract was also treated by irradiation. The IgE immunoreactivity was measured by immunoblotting and indirect ELISA with anti Ara h 6 polyclonal antibodies. Results showed that irradiation induced significant changes in the secondary and tertiary structures of Ara h 6 and the IgE reactivity of Ara h 6 gradually decreased with the increased irradiation dose (Kasera and others 2012).

Pulsed electric field

Pulsed electric field (EF) is a novel processing technique used for food pasteurization. PEF involves the application of high voltage pulses (electric field strength of 0.1-40 kV/cm and total energy input of 0.5-1000 kJ/kg) to electroconductive foods that are located between the electrodes. The electric field could induce the movements of ions and permeablilization of cell membranes called electroporation. This biological effect is related to several applications, such as microbial inactivation (Garcia and others 2007), enhanced extraction (Schilling and others 2007), and improved mass transfer processes (Guderjan and others 2005). Results in this study showed that PEF treatment did not significantly affect the secondary structure of peanut allergens. It is more probable that highly localized thermal effects were responsible for the minor observed structural changes. The scarcity of studies on the effects of PEF on protein

structure is difficult to predict the principle of this method on allergenic proteins (Johnson and others 2010).

Chemical and biological methods

Peroxidase. Peroxidase (POD) is a heme-containing enzyme catalyzing the oxidation of a variety of phenolic compounds, such as converting ferulic acis into o-quinones. The o-quinones react with other phenolics, amino, or sulfhydryl compounds to form new cross-linked compounds. For instance, peroxidase helps to improve the functional properties by catalyzing the cross-linking of polysaccharides and/or proteins (Labat and others 2001; Dunnewind and others 2002). Proteins can become cross-linked with other proteins or polysaccharides in the presence of peroxidase. Proteins contain typrosine resides that carry a phenol group. It can react with the ferulic acid moieties of polysaccharides or the tyrosine residues of another protein to form protein-polysaccharide or protein-protein cross-links (Faergemand and others 1998; Labat and others 2001; Oudgenoeg and others 2001; Dunnewind and others 2002). One previous study reported that transglutaminase has an effect on the reduction of immunogenic and allergenic properties of soy proteins (Babiker and others 1998). This reduction in reducing IgE reactivity of soy proteins is due to the cross-linking effects that mask the binding sites on the allergenic proteins.

As mentioned earlier, roasted peanut have higher allergenic reactions than raw peanuts. Another study investigated the effects of POD on the IgE reactivity to the allergens of roasted peanuts. Protein extracts of roasted and raw peanuts were treated with POD for 1-16 h. SDS-PAGE results exhibited a significant decrease in density or levels of Ara h 1 and Ara h 2. Western blots and competitive inhibition ELISA results showed a significant reduction of IgE reactivity on Ara h 1 and Ara h 2 of roasted

peanuts. This reduction was due to the cross-linkage of these allergenic proteins. This may result in masking the IgE epitopes and, consequently reducing IgE binding. This study provided evidence that POD helped to reduce the IgE reactivity to the allergens in roasted peanuts, but not raw peanuts (Chung and others 2004).

Phytic Acid Phytic acid is the major storage form of phosphate and inositol in mature oilseeds (Reddy and others 2002). On a dry basis, around 1% phytic acid is stored in the whole oilseeds (Porres and others 2004). Phytic acid binds to and forms soluble and insoluble complexes with proteins (Okubo and others 1976; Lombardi-Boccia and others 1998). Insoluble complexes reduce the bioavailability of proteins. Chung and others (2007) investigated the effects of phytic acid on the allergencity of peanut allergens. Results in this study demonstrated that phytic acid assisted the complexes with the major peanut allergens (Ara h 1 and Ara h 2) and these complexes were insoluble at neutral or acidic pH. The levels of soluble allergens and IgE binding of a peanut extract were reduced 6-fold in allergenic potency. The similar effect was also observed in peanut butter, indicating that phytic acid may be a method for development of hypoallergenic peanut based products (Chung and Champagne 2007).

Polyphenol oxidase and caffeic acid. Polyphenol oxidase (PPO) or tyrosinase is a copper containing enzyme that is widely distribution in fruits and vegetables (Seo and others 2003; Matuschek and Svanberg 2005). It catalyzes the oxidation of phenols and converts them into o-quinones, which then polymerize with other phenolic compounds (caffecic acid) or amines to form browning products (Iyidogan and Bayindirli 2004). In proteins, PPO catalyzes the oxidation of tyrosine residue, which contains a phenol group, converting to an o-quinone derivative (Hurrell and others 1982; Matheis

and Whitaker 1984). The derived quinine on the protein can react with the amino group, sulfhydryl group, or the tryptophan residue of another protein to form cross-link complexes (Chung and others 2005). However, protein complexes would not form a cross-link if phenolic compounds such as caffeic acid or chlorogenic acid are present (Hurrell and others 1982; Matheis and Whitaker 1984).

Researchers explored the effects of PPO and/or caffeic acid and discovered that it can improve the functional properties of soy proteins (Rawel and others 2002), and also increase the heat stability of milk proteins (O'Connell and Fox 1999). Another study examined the role that PPO/caffeic acid played on peanut allergens. Results showed cross-link formation could mask the epitopes of peanut allergens after PPO/caffeic acid treatment. The IgE binding was markedly reduced based Western blots and ELISA results (Chung and others 2005).

Tannic acid. Tannic acid is another chemical that can cause protein cross links. Tannic acid is a water soluble polyphenol compound and an antioxidant that is normally stored in barks, legumes, coffee, tea, and other food products (Schofield and others 2001; Barbehenn and Constabel 2011).Tannic acid is used in the leather industry for manufacturing tanning leather, in burn treatments as a topical agent (Halkes and others 2001), as anti-bacterial agent in medical studies (Akiyama and others 2001), and as anti-ulcer agents (Souza and others 2007). Due to its ability to form protein cross link complex, tannic acid has shown effectiveness in eradicating mite allergens and cat allergens in house dust (Woodfolk and others 1995) and deproteinizing bacterial genomic and plasmid DNAs (van Huynh 2008). Tannic acid has a higher affinity for proline-rich proteins (Luck and others 1994). This study demonstrated that complexes

formed from tannic acid and peanut allergen interactions would be very stable under acidic and alkaline conditions. Results showed that tannic acid removed peanut allergens from peanut butter extracts at a pH of 7.2 by forming insoluble complexes with the allergens. The major peanut allergens (Ara h 1 and Ara h 2) also formed complexes with a tannic acid concentration over 0.5 mg /ml at pH 2 and 8. The data indicated that the insoluble proteins may be stable under gastric and intestinal conditions and ultimately pass through without any allergenic issue (Chung and Reed 2012). To verify the actual effects of all these enzymes and acids on peanut allergy, clinical trials and animal tests are still needed. No report exists as of now.

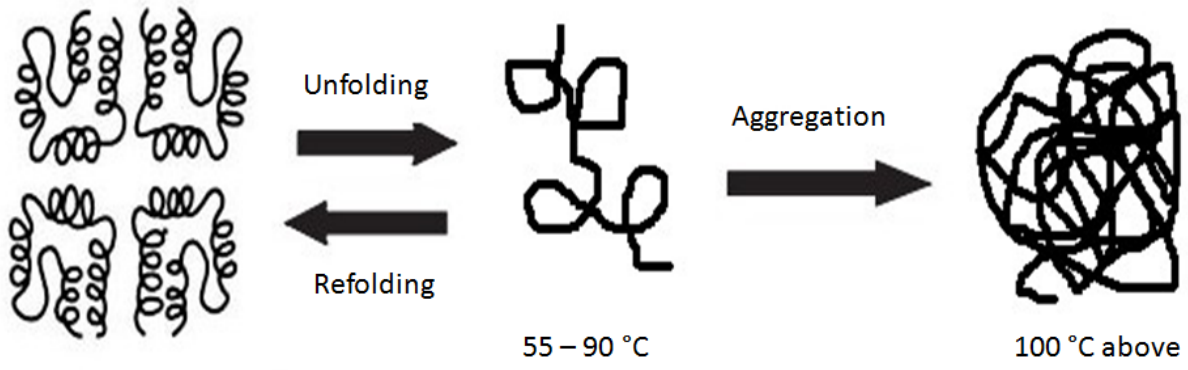


Figure 2-1. Thermal effects on the protein structure changes.

CHAPTER 3
INVESTIGATION OF THE EFFECTIVENESS OF PULSED LIGHT ON PEANUT ALLERGENS

Introduction

Recently, novel processing technologies such as PL have been shown to effectively reduce the allergen level of different foods. For example, Chung and others (2008) have shown that PL can effectively reduce the major allergens Ara h 1 and Ara h 2 in both peanut extracts and peanut butter slurry. Yang and others (2011) reported that PL significantly reduced the immunoreactivity of the major allergens Ara h 1, Ara h 2 and Ara h 3 in raw and roasted peanut extracts and peanut butter slurry. Yang and others (2010) showed a significant reduction of allergen levels by PL treatment of soybean extracts. A study by Shriver and others (2011) indicated that PL caused a notable reduction of allergen in shrimp extracts. Li and others (2011) have also demonstrated that PL can significantly reduce the IgE immunoreactivity of almond extracts.

The PL technology uses pulses of intense broad spectra containing approximately 54% UV, 20% infrared and 26% visible light (Shriver and Yang 2011). During PL emission, inert gas, such as Xenon, contained inside the PL lamp is excited by high voltage from a ground state to an excited state. When the Xenon molecules come back to a ground state, energy is released in photons and absorbed by the food molecules, which can lead to photo-thermal, photo-physical, and photo-chemical effects on foods (Shriver and others 2011).

The foregoing triple photo-effects are responsible for the putative mechanism for the interactions between PL and the food molecules (Krishnamurthy and others 2007). Previously, PL was considered as a non-thermal technology used for inactivation of

microorganisms in foods after a short exposure (e.g., seconds). However, recent research has pointed out that PL may also have photo-thermal effect besides its non-thermal nature, since extended exposures (e.g. minutes) can cause a pronounced temperature rise and moisture evaporation on shrimp and almond (Yang and others 2012). Furthermore, the photo-thermal effect can cause modification of protein allergen reactivity via protein cross-linkage, protein solubility reduction, and protein fragmentation under instantaneous high temperature (Li and others 2011; Yang and others 2012). In Chapter 3, the preliminary study on the variation of allergens in peanut kernels after PL treatment has been investigated.

Material and Methods

Materials

The materials used in preparing the peanut samples were aluminum weighing dish obtained from Fisher Scientific.Inc. (Allentown, PA, USA), Whatman filter paper #4 from Whatman (DeKalb County, IL, USA), acetone Fisher Scientific Inc. (Allentown, PA, USA), ethylene glycol tetraacetic acid (EGTA) from Sigma (St. Louis., MO, USA), and hexane from Fisher Scientific Inc. (Allerntown, PA, USA). The reagents used in the immunological tests included Coomassie Plus Protein assay from Bio-rad (Hercules, CA, USA), bovine serum albumin (BSA) from Invitrogen (Carlsbad, CA, USA), GelCode Blue gel staining reagent from Invitrogen (Carlsbad, CA, USA), Tris buffered saline (TBS) from Fisher Scientific Inc. (Allentown, PA, USA), phosphate buffered saline (PBS) from Fisher Scientific Inc. (Allentown, PA, USA), o-phenylendiamine dihydrochloride (OPD) and StartingBlock TBS/T-20 blocking buffer and Gelcode Blue stain reagent and bicinochoninic (BCA) protein assay kit from Invitrogen (Carlsbad, CA, USA), betamercaptoethanol and Tween-20 from Pierce Chemical (Rockford, IL, USA). Fresh

human plasma (IgE), which is specific to peanut allergy, was measured by ImmunoCAP testing and was shown as 86 kilounits per liter (kU/L). Precast trisglycine minigels (4-20%) and reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from BioRad Laboratories (Hercules, CA, USA). Costar 96-well plate produced by Corning Incorporated (Corning, NY, USA) was used for the enzyme-linked immunosorbant assay (ELISA) and the immobilon P blotting membrane was obtained from Millipore Corporation (Bedford, MA, USA). Horseradish peroxidase-conjugated mouse anti-human antibody was purchased from Invitrogen (Carlsbad, CA, USA). Super Signal West Pico Chemiluminescent Substrate was purchased from Thermo Scientific (Rockford, IL, USA).

Pulsed Light Treatment of Peanut Kernels

A PL applicator of Model# LHS40 LMP HSG from Xenon Corp (Wilmington, MA, USA) was used in this study for the PL treatments. The PL system consists of two lamps filled with Xenon gas, two cooling blowers, one treatment chamber with a conveyor belt, and a control module. Raw runner type peanuts were purchased from the local market and shelled by hand to yield the peanut kernels for use in this study. The PL system produces a broadband spectrum between 100 to 1100 nm and operates at 3 pulses per second, with a pulse width of 360 μs and approximately 54%, 26%, and 20% energy in the ultraviolet, visible, and infrared regions, respectively. As per the manufacturer's specification, this system can produce an energy level of 1.27 J per centimeter squared (J/cm^2) per lamp at 7.6 cm below the central axis of the PL lamp.

The PL parameters for treating the whole peanuts included the PL duration and the distance of the sample to the quartz window of the PL lamp. Shriver and others (2011) used the same system for allergen reduction of shrimp protein extracts, which

were placed in an aluminum dish of 7.2 cm diameter and illuminated at a distance of 10 cm from lamp. Two distances from lamp were chosen, 10 cm and 7 cm, along with PL durations ranging from 5 min to 9.5 min. The treatment times were selected based on the degree of roasting that occurred among the peanut samples, ranging from lightly roasted to burnt, during the preliminary tests.

In order for both sides of a peanut kernel to expose to PL illumination, there are two possible options for placing a kernel on the aluminum dish. One is to treat the whole kernel for half the preset duration and then flick the kernel upside down to continue with the 2^{nd} half treatment. The other is to naturally split the whole kernel into two halves and then treat both half kernels all at once for half the preset time as for a whole kernel. In this study, the second option was adopted. Three peanut kernels (cotyledon) were separated into two halves and placed linearly in the center of an aluminum dish. The dishes were loaded below the PL quartz window along its central line. The surface temperature of the samples was recorded using an Omega OS423-LS non-contact infrared thermometer from Omega Engineering Inc. (Stamford, CT, USA) before and after PL treatments. The samples were cooled by placing the aluminum dish on ice immediately after the surface temperature was recorded. Variations in moisture were also recorded by measuring the peanut mass on a balance before and after PL treatments. Triplicate measurements were conducted for PL treatment, temperature recording and moisture variations.

Preparation of Peanut Extract after PL Treatment

To perform the immunoassays, it was required that crude protein extracts be prepared in a manner that did not change the native properties of protein in the peanut kernels. The extraction followed the method by (Chung and Champagne 2001). For

each time treatment, the samples were ground with a Wiley mill in cold acetone until they become finely milled. After that, the meals were placed on the filter with Whatman filter paper #4 using hexane as an eluted solvent until they become white. These defatted peanut meals were air-dried at RT for 1 – 2 h.

To extract the soluble peanut proteins, the dry peanut powders were normalized by stirring 0.1 g of meals in 0.7 ml of 0.02 M sodium phosphate containing 10 mM EGTA obtained from Sigma (St. Louis., MO, USA) with the final pH adjusted to 7.4. These samples were placed at 4°C for 1 hr to fully collect soluble proteins and then centrifuged at 8500 x g for 10 min. The resultant supernatants were stored at -20°C for further immunoassays. Bradford protein assay was used to determine the concentration of soluble proteins in the PL treated samples.

SDS-PGAE of Peanut Extracts

To examine the soluble proteins in different samples, SDS-PAGE was performed based on the Laemmli method (Laemmli 1970). Basically, peanut extracts were loaded on 4% - 20% Tris-glycine gels (5 µg / well) combined with a sample buffer, which included 62.5mM Tris-HCL, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue and β-mercaptoethanol. This mixture was boiled for 5 min. The gel was subject to electrophoresis at 150 V for 1.5 h. Subsequently, the gel was stained with GelCode Blue reagent for 2 h and de-stained afterwards with distilled water for 2 h until there was a sharp contrast between the stained protein bands and the clear background.

Western Blot of Peanut Extracts

Western blot assay was performed according to the method described by (Towbin and others 1979). After separation of the proteins on SDS-PAGE gel, the protein bands were transferred to a PVDF blotting membrane for 30 min at 15V. On the

completion of transferring proteins, the membrane was washed by 20mM Tris saline buffer (TBS) with pH 7.4 and then blocked with StartingBlock blocking buffer. The membrane was incubated overnight at 4°C with pooled human plasma containing anti-peanut IgE antibodies (1:500) diluted in blocking buffer. After that, three separate washes with TBST (TBS 1X with 0.05% Tween 20) were done, using 5 min time periods. The blot was then incubated with a secondary antibody (1:1000) at room temperature (RT). Another series of washes was done with TBST, but this time, the time points were 5 min, 10 min and 10 min at room temperature. On the completion of the washing step, the membrane was incubated by SuperSiganl West Pico Chemiluminescent substrate for 5 min at RT. The protein bands signal was developed on X-ray film.

Dot Blot of Peanut Extracts

For dot blot analysis, a nitrocellulose membrane (0.45µm) was blotted with 2.5 µg of raw and PL treated peanut samples and then dried at 4°C. This was followed by blocking the whole membrane with StartingBlock blocking buffer. After that, the blot was incubated for 1 h at 37°C. After incubation with StartingBlock blocking buffer and subsequent washing, the blot was incubated with pooled human plasma IgE (1:500) and then with goat anti-human IgE-HRP (1:1000) for 1 h at RT for each step. Washes were done between each incubation step and after the final incubation. Protein dots were then developed on X-ray films following the exact procedure described above for Western blotting. The diluent used for the IgE and goat anti-human IgE-HRP was StartingBlock blocking buffer.

Indirect ELISA of Peanut Extracts

Diluted peanut extract prepared to a final concentration of 2 µg / 100µl with PBS buffer (pH 7.4) was coated on to a polystyrene 96-well plate for 2 h at 37°C. Each protein sample was added in triplicates with 100 µl per well. The plates were then washed with TBST three times and blocked with StartingBlock blocking buffer (200 µl per well) on a rocker at RT for 2 h. After another round of washing, 100 µl of pooled human plasma containing IgE antibodies (1:500 dilution in 1X PBS) was added to each well. The plate was incubated at 37°C for 1 hr. After the TBST wash, mouse anti-human IgE conjugated HRP antibody (1:3000) was added next in the same manner, with 100 µl per well. Incubation followed for 1 h. After another washing step, the protein-antibody bonded plate was developed with OPD substrate (0.5mg/ml) that was prepared by dissolving 0.1M citrate buffer (pH 5.5) and 0.03% hydrogen peroxide (100 µl per well). The developed time was between 15 – 30 min and was stopped with 2.5 N sulfuric acid (100µl per well). The absorbance was measured at 490nm using Spectramax 340384 spectrophotometer that was from Molecular Devices Inc. (Sunnyvale, CA, USA).

Results and Discussion

SDS-PAGE of PL Treated Peanut Kernels

Shown in Figure 3-2 is the soluble protein profile of raw and PL treated peanuts. Protein bands in the raw sample were differentiated as Ara h 1 (63 kD), Ara h 2 (17-20 kD), Ara h 3 (14-45 kD), Ara h 4 (37kD) and Ara h 5-Ara h 11 (9.8-17kD) (Finkelman 2010; Pele 2010), with the major four marked in the figure. Since Ara h 3 and Ara h 4 share a 91% identical sequence, the band at 37 kD could be Ara h 4 or a mixture of both (Kleber-Janke and others 1999). The two bands between 20 and 37 kD belonged to Ara h 3, which may consist of a series of polypeptides ranging from 14 – 45 kD.

For the 10 cm distance to lamp (left), compared to raw, 5 min and 6 min PL illumination did not cause a considerable reduction in the band density of Ara h 1, Ara h 2, Ara h 4 and the lower band of Ara h 3, but for the upper band of Ara h 3, pronounced reduction was observed at 5 min and total disappearance occurred from 6 min and up. From 7 min to 9.5 min, all the bands related to Ara h 1 to h 4 were either substantially reduced or totally disappeared.

For the 7 cm distance to lamp (the right group), 5 min PL illumination did not cause a considerable reduction in the band density of Ara h 1, Ara h 2, Ara h 4 and the lower band of Ara h 3, but for the upper band of Ara h 3, PL treatment for 5 min and up caused a total disappearance of this band. From 6 min to 7 min, all the bands related to Ara h 1 to Ara h 4 were either substantially reduced or totally disappeared. No experiments were carried out beyond 7 min, because the sample was already nearly burnt at 7.5 min.

The reduced or disappeared protein bands of Ara h 1 to Ara h 4 were considered to have formed insoluble aggregates or been cleaved into smaller polypeptides, because PL treatment can lead to insoluble complex formation between molecules, de-polymerization of starch, peroxidation of unsaturated fatty acid, carbohydrate cross-linking, protein cross-linking and protein fragmentation (Fiedorowicz and others 2001). The PL treatment could also cause aromatic amino acids to form cross-links in proteins due to the absorption of UV radiation, such as tyrosine and phenylalanine (Gennadios and others 1998). In a study reported by Chung and others (2008), peanut protein extracts were PL treated for 4 min and peanut butter slurry for 3 min, both at a distance of 14.6 cm to lamp. Their results indicated that the soluble peanut proteins could form

cross-links and consequently aggregates or precipitates, rendering them insoluble. However, those small soluble peanut proteins (17 - 20kD) still remained in a soluble state after PL treatment.

The insoluble aggregates usually show up on high molecular weight area of the gel if the entire homogenized peanut extract (without centrifuge) is loaded (Li and others 2011; Yang and others 2011), but they did not show up in Figure 3-2, because only the soluble proteins after centrifuge were used in this study and the insoluble aggregates were removed during centrifuging. However, in Figure 3-2, many new low molecular bands (below 17 kD) were generated after 7 min illumination (for 10 cm distance to lamp) and after 6 min illumination (for 7 cm distance to lamp), which is a clear indication of the fragmentation effect of PL on the peanut allergen proteins.

The PL illumination may also have an instantaneous photothermal effect during the treatment. Wekhof (2000) proposed that temporary overheating was produced during the PL illumination due to the absorption of all the UV light from the flash lamp when the overall energy exceeded 0.5 J cm^{-2}. Another study indicated that the overheating could be attributed to a difference in UV light absorption by bacteria and the surrounding medium (Takeshita and others 2003). Maleki and others (2010) demonstrated that thermal processing of peanuts alters the solubility of proteins and can lead to structural and chemical modifications, giving rise to a change in immunoreactivity of IgE. Thermal conditions can accelerate the Maillard reaction between reducing sugars and free amino groups of proteins, causing specific chemical modifications to protein molecules or inter- and intra-molecular covalent cross-linking or degradation of bonds (Maleki and others 2000a; Maleki and Champagne 2001; Maleki

and others 2003). However, the exact mechanism of PL treatment on protein structural changes is still not clear.

Western Blot of PL Treated Peanut Kernels

The foregoing SDS-PAGE results indicate that PL illumination was capable of reducing the immunoreactivity with IgE for major allergens in the PL treated peanut kernels to a low level. To characterize the immunoreactivity of IgE with the antigenic proteins of the PL-treated peanuts, Western blotting was performed *in vitro* using pooled human plasma that contained the IgE antibody specific to peanut allergens. The raw peanut sample was used as control.

Figure 3-3 shows the immunoreactivity signal of IgE on the Western blot membrane after X-ray film exposure. The raw peanut protein bands were detected at 63 kD (Ara h 1), 17-20 kD (Ara h 2), Ara h 3 (14 - 45 kD), Ara h 4 (37 kD) and Ara h 5 - h 11 (10 - 20 kD).

For the 10 cm distance to lamp (left), except for 5 min and 6 min PL illumination times where the allergens Ara h 1 - h 4 showed a marginal reduction, 7 min to 9.5 min PL treatments caused the major allergen bands to substantially diminish or even disappear. For 7 cm distance to lamp (right), Ara h 1 - Ara h 4 was significantly reduced or disappeared beginning from 5 min PL treatment. For Ara h 5 - Ara h 11, 5 min and 6 min PL illumination resulted in a considerable reduction, as can be seen from the much reduced band density in lane 5 and 6 in the 10 cm section (left). However, as the PL duration increased to 7 and beyond, the band intensity at 10-20 kD increased, because the protein fragments as discussed previously in Figure 3-2 were formed due to the degradation of Ara h 1 - h 4 caused by PL illumination. Some of the fragmental polypeptides might still carry effective epitopes that reacted with the IgE. However, from

Figure 3-3 (left) it can also be seen that when the PL duration further increased, the band intensity in the 10 - 20 kD range tended to diminish. This is especially evident for 7 cm distance from lamp (right).

Results in both Figure 3-2 and Figure 3-3 signify that with a shorter distance to lamp and longer durations of exposure, which could result in a higher energy release to the peanuts, PL illumination could significantly minimize the levels of allergen Ara h 1 - 11 in whole peanuts, even including minimizing the IgE reactivity of the fragments decomposed from Ara h 1 – Ara h 4 due to PL treatment. Maleki and others (2010) indicated that the decreased levels of allergens may be due to oligomerization or degradation resulting from the over-processing effects. Although in this study the PL treated peanuts might not have been over-processed, it is believed that oligomerization or degradation of peanut proteins might have contributed to the significant reduction of IgE immunoreactivity of PL treated peanuts.

Dot Blot of PL Treated Peanut Kernels

Dot blot is a technique for detecting, analyzing and also identifying proteins. It is similar to the Western blot in general, but differs in regard to the loading of protein samples as a dot on the membrane rather than separating them into different protein bands. Compared to the Western blot, Dot blot has an advantage in that it can detect more proteins in peanut extract samples, including the smaller proteins under 10 kD that would have otherwise missed in the Western blot or the bigger proteins that were unable to enter the SDS-PAGE gel and would not get transferred to the Western blot membrane. In this experiment, the peanut protein extracts of the PL treated and the raw peanuts (control) were loaded on a nitrocellulose membrane. The distance for the PL treatment was set at 10 cm with the durations being 5, 6, 7 and 7.5 min.

From Figure 3-4, it is evident that the IgE reactivity to total peanut allergens was significantly reduced at 7.0 min PL illumination and basically undetectable at 7.5 min.

As discussed previously, the photophysical, photochemical and also photothermal effects of PL could alter the protein structure to reduce the immunoreactivity with the human IgE antibody. To support dot-blot immunoassay, indirect ELISA was performed to provide further evidence.

Indirect ELISA

Figure 3-5 shows the results of immunoreactivity for the PL-treated peanuts at different durations for both 10 cm (A) and 7 cm (B) distances from lamp. The raw peanut was used as positive control and normal human plasma was used as negative control.

For 10 cm distance to lamp (A), a significant reduction (α=0.05) was observed starting at 6 min PL exposure. When the PL duration reached 7.5 min, peanut allergens already diminished to a level similar to the negative control.

For 7 cm distance to lamp (B), a significant reduction (α=0.05) was observed starting at 5 min PL exposure. When the PL duration reached 6.5 min, peanut allergens already diminished to a level similar to the negative control and no significant increase in allergen reduction was observed with a further increased PL duration beyond 6.5 min.

This *in vitro* ELISA data shown in Figure 3-5 were consistent with the results of SDS-PAGE, Western and dot blot analyses stated previously, which all demonstrated that PL treatment could remarkably reduce the allergens of whole peanut kernels to a level near negative control.

Influence of PL Duration and Distance on IgE Binding Reactivity to Whole peanut allergens

For PL treatment of foods, the sample distance from the light source (which also relates to the PL intensity), number of pulses, thickness of the sample, and duration of illumination are considered as critical parameters for the optimization of a PL system (Soliva-Fortuny and others 2010). For the PL applicator used in this study, the number of pulses was set at 3 pulses/s, the thickness of treated sample was also constant, but the PL duration was varied between 5 min and a maximum of 9.5 min, depending on the distance to lamp, and the intensity of the light was varied by two distances to lamp: 7 cm and 10 cm. Shriver and others (2011) used a distance of 10 cm to achieve shrimp allergen reduction at different PL durations and a significant reduction was detected at 4 – 6 min. Chobert and others (2008) found that PL treatment at 4 cm distance to lamp led to protein aggregation and lipid oxidation. Based on the literature, distances of 10 cm and 7 cm were used in this study for PL roasting of whole peanuts for allergen reduction and quality retention as assessed by color and texture analyses.

From the SDS-PAGE (Figure 3-2) and Western blot analyses (Figure 3-3), the total protein profile as well as the specific protein bands related to Ara h 1 – Ara h 4 shows a significant reduction at 7 min PL treatment with 10 cm distance to lamp and at 6 min PL treatment with 7 cm distance to lamp. As the illumination energy increased, i.e., closer distance to lamp and longer PL duration, all the protein bands related to the 11 peanut allergens were minimized to an undetectable level. Li and others (2011) also showed that distance clearly influenced the reduction efficacy of PL on almond allergens due to the changes of energy incidence. It has been shown that the longer the distance between the sample and the lamp, the lower the lethality is for the process

(Gomez-Lopez and others 2005), and the longer the treatment time and the shorter the distance between the sample and lamp, the higher the inactivation (Gomez-Lopez and others 2007).

Summary and Conclusions

Pulsed light technique was investigated in this study for its efficacy on reducing the immunoreactivity of whole peanut kernels at two sample distances to light source (7 cm and 10 cm), a PL frequency of 3 pulses/s and PL durations of 5 min to 9.5 min. Results of SDS-PAGE, Western Blot, Dot Blot and ELISA with pooled plasma of allergenic patients show that PL was capable of reducing all major allergens of whole peanut kernels, including the polypeptide fragments degraded from Ara h 1 - Ara h 4 during PL treatments, to an undetectable level, when a proper combination of illumination durations and sample-to-lamp distances was chosen. A closer distance (7 cm) between the PL lamp and the sample resulted in stronger reduction of IgE immunoreactivity than 10 cm. To optimize this equipment, more trails of processed peanuts are required. Statistical analysis is needed to determine the interaction between treatment time and distance these two major factors of this PL applicator. The insoluble fractions in treated peanut seeds could not be detected by normal SDS-PAGE gels. The IgE reactivity of those insoluble fractions needed to be explored for the PL validation.

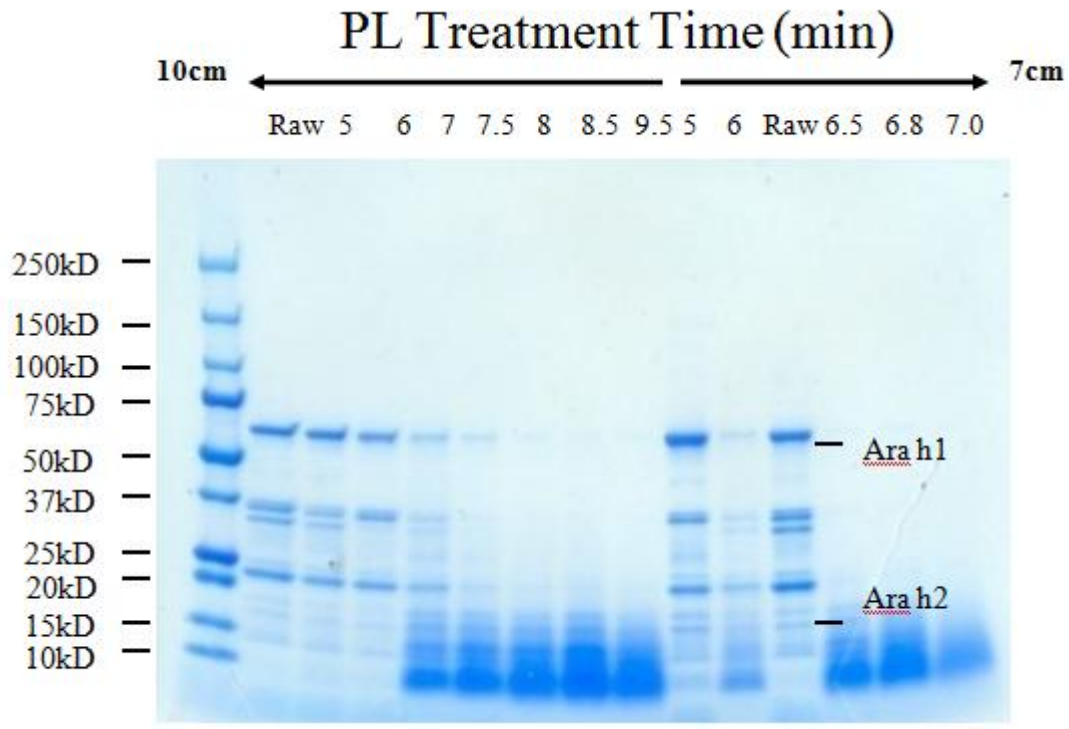


Figure 3-2. SDS-PAGE profiles of raw and PL-treated peanut extracts at different durations of illumination. The left shows the peanut samples PL treated at 10 cm distance to lamp, while the right 7 cm distance to lamp. The major peanut allergens Ara h 1 (63 kD), Ara h 2 (17 - 20 kD), Ara h 3 (14 - 45 kD) and Ara h 4 (37 kD) are labeled on the gel.

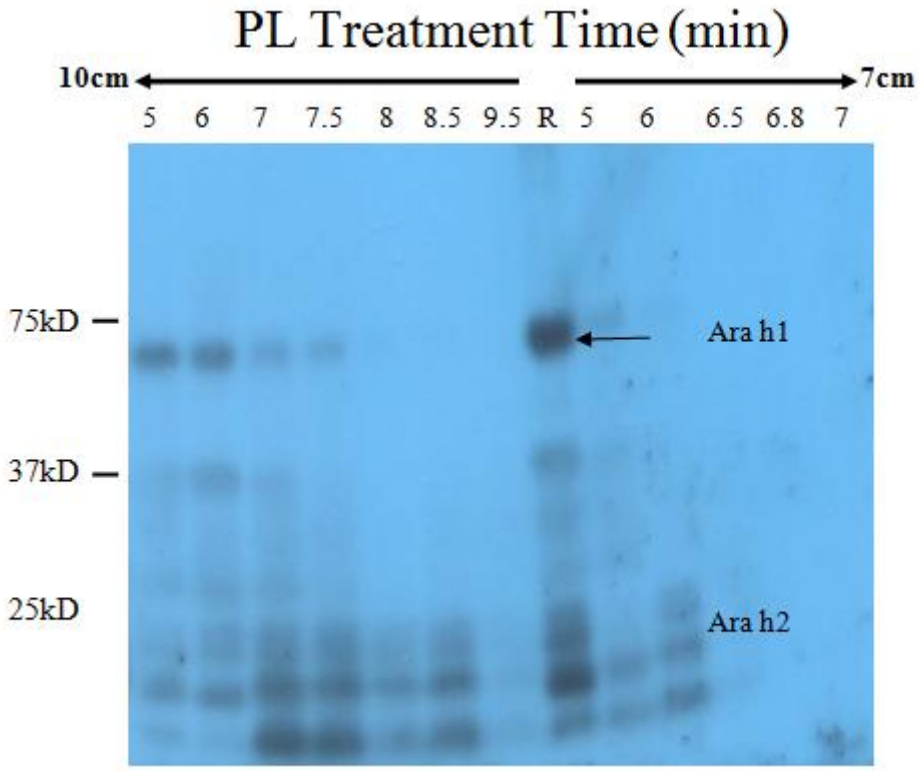


Figure 3-3. Antigenic protein banding patterns in raw and PL treated peanuts via western blot *in vitro* analysis with pooled human plasma. Four major allergens Ara h 1 to Ara h 4 are labeled in figure and R is raw peanut sample. The left arrow is for 10 cm distance to the quartz window and the right arrow is for 7 cm distance to the quartz window.

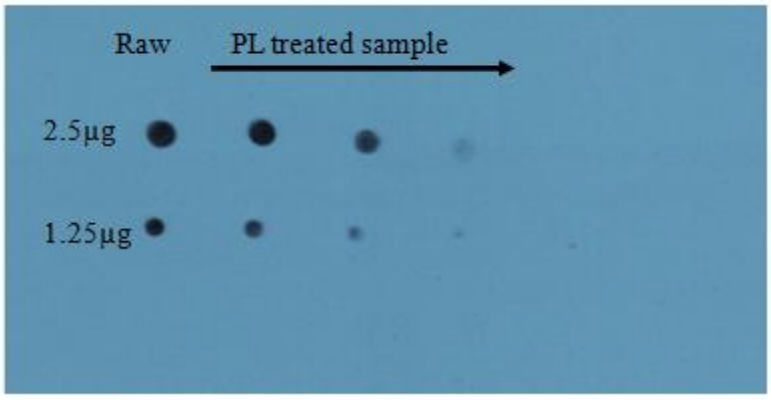


Figure 3-4. Dot blot analysis of raw (control) and PL treated peanuts. The total proteins loaded on the nitrocellulose membranes were 2.5 µg and 1.25 µg, respectively. The PL treatment was conducted at 5, 6min, 7 and 7.5 min at 10 cm distance from lamp.

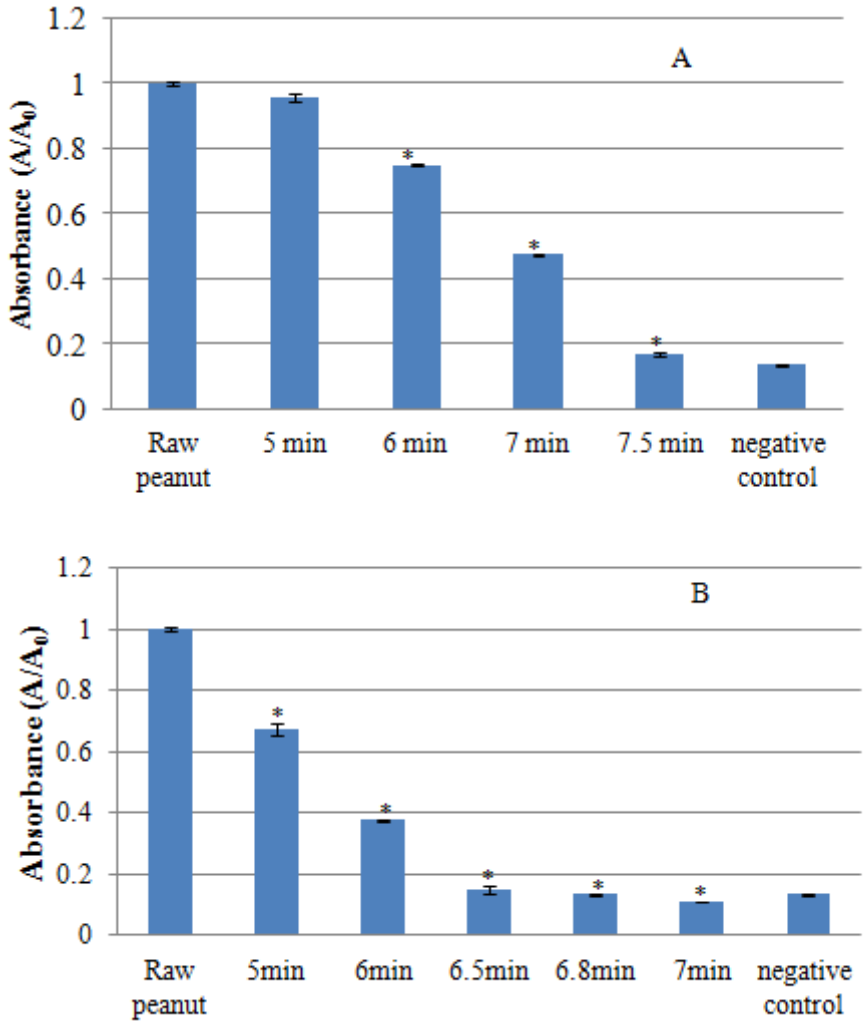


Figure 3-5. Indirect ELISA of PL treated peanuts at 10 cm (A) and 7 cm (B) distance to lamp with pooled human plasma containing IgE at different illumination durations. Absorbance readings were obtained at 450 nm. Raw sample was used as positive control. Normal human plasma was applied for negative control as shown in the last column. A = Absorbance of treated samples; A_0 = Absorbance of control. The "*" indicates significant signal reduction after the student t - test (α=0.05).

CHAPTER 4
EXAMINATION OF IGE IMMUNOREACTIVITY OF INSOLUBLE FRACTIONS OF PULSED LIGHT TREATED PEANUTS BY NOVEX NUPAGE ELECTROPHORESIS SYSTEM

Introduction

The objective of this experiment was to investigate the IgE immunoreactivity of insoluble fractions formed during PL treated peanuts. A previous study indicated that PL was a suitable non-thermal technology for inactivation of bacteria, virus, and molds consequently, this technology has been applied to a lot of food industries, such as the following: beverage pasteurization and surface sterilization of vegetables (Koutchma 2008). However, studies about allergen reduction in different food samples, such as shrimp and almond, has indicated that PL can no longer be considered as a non-thermal technology since temperatures significantly increase during processing (Yang and others 2012).

The energy simultaneously released within a short time and infrared light portion contributes the increased temperature of the food sample. Pulsed light has a very high energy as evident by the fact that the intensity of pulsed light is 20,000 times more than the sunlight (Mimouni 2001).The energy output of a PL applicator was ranging from 1.8 to 5.7 J/ cm^2 per pulse at 1.8 cm below the lamp surface when the voltage was varied from 2000 to 3800 V (Krishnamurthy and others 2009). The applicator has been used for this study was 2.7 J/ cm^2 per pulse at 10 cm (Shriver and others 2011).

In the case of heat denaturation of proteins, the process is generally considered irreversible. In brief, denaturation is defined as the process in which a protein is transformed from an ordered to a less-ordered state that is due to the rearrangement of hydrogen bonding without any change to covalent bonds. Denaturation is used to refer

to aggregation, coagulation, and gelation. Aggregation is a term to describe protein-protein interactions with formation of complexes of higher molecular weights that are normally occur in thermal processing (Tanford 1968; Maleki and others 2000a)

PL processed peanuts were analyzed using SDS-PAGE and western blot as described in Chapter 1. The proteins extracted from PL treated peanuts are soluble proteins that were quantified by the Bradford protein assay. The cross-linked proteins were hardly detected as described in Chapter 1 that is also because of the limitation of SDS-PAGE gel we purchased from Bio-Rad (the maximum protein molecules was around 250 kD) and the centrifuge step in protein extraction had filtered the insoluble fractions in protein samples. The denatured proteins and those amino acid residues trapped in the big complex *via* Maillard reaction during PL illumination cannot be detected with the gel for a general protein profile. To solve this problem, NuPAGE tris-acetate gels were selected. This type of polyacrylamide gel was designed to give optimal separation of large molecular weight proteins during gel electrophoresis. To detect the native state of PL treated proteins, the novex tris-glycine native running buffer was applied for native proteins detection and NuPAGE Tris-acetate SDS running buffer was used for denatured gel system in which the methods are described below.

Materials and Methods

Sample Preparation

Raw runner type peanuts were purchase from a local fresh market (Gainesville, Florida, USA). The same applicator as shown in Chapter 3 was used in this study for PL treatments. A motor-driven device was designed in this experiment as shown in Chapter 5. Eight kernels were placed in a transparent glass test tube with a hole (used for releasing the vapor pressure) at the bottom. This glass test tube with a stopper was

positioned at the top of the motor. The motor was placed on the conveyor belt of PL machine and adjusted to ensure that the rod of the motor was located in the center of the quartz window of the PL equipment. The treatment times were performed at 4 min, 5 min, 5.5 min, 6 min and 6.5 min. respectively.

Protein Extraction

The extraction followed the method performed by Chung and Champagne (2001). For each treatment time, the samples were ground to a fine powder using a Wiley mill (Model number and manufacturer address needed here) in cold acetone around 0 °C. Subsequently, the meals were placed on the filter with Whatman filter paper #4 using hexane as the eluting solvent until the meal was white in color. The defatted peanut meals were air-dried at room temperature for 1 – 2 h.

To extract the soluble peanut proteins, the dried peanut powders were normalized by stirring 0.1 g of meals in 0.7 ml of 0.02 M sodium phosphate containing 10 mM EGTA that obtained from Sigma Aldrich (St. Louis, MO, USA) with the final pH adjusted to 7.4. These samples were stored at 4°C for 1 h to fully generate. The homogenizer from ESGE Ltd. (Mettlen, Switzerland) was applied for the total protein homogenization. The Coomassie protein assay was used to determine the concentration of proteins in the PL treated samples for further SDS-PAGE and western blot assays.

Denaturing Electrophoresis

NuPAGE Novex Tris-Acetate gels produced by Invitrogen (Carlsbad, CA, USA) were used for electrophoresis. The types of the comb of the gels were 1.0 mm and 1.5 mm with each well of 25 µl and 37 µl. The gels were in pre-cast gel cassette with the size of 10 cm x 10 cm and then placed in XCell surlock TM Mini-cell unit purchased

from Invitrogen (Carlsbad, CA, USA) for gel electrophoresis. The prepared protein samples were mixed with LDS sample buffer (4 x) obtained from Invitrogen (Carlsbad, CA, USA) to get final concentration at 2.5 µg/ml and then the mixer of each sample was cooked for 10min before loading the gel. Each sample of different treatment samples was loaded as 25 µl and triplicate for each of them. The concentration of the gels we applied in this study is 3% - 8%. 1 x NuPAGE SDS Running buffer obtained from Invitrogen (Carlsbad, CA, USA) was prepared by using 20 x NuPAGE SDS Running buffer, which was using 50 ml 20 x NuPAGE SDS Running buffer and mixing with 950 ml deionized water. After assembled the XCell unit, the voltage was set at 150 V constant. The expected current for the whole gel was around 40 - 55 mA / gel. When the protein bands (blue color) achieved to the bottom of the gel, the voltage should be cut off by then. The gel will be stained by coomassie R-250 that was from Invitrogen (Carlsbad, CA, USA) for 2 h. Deionized water was used for background clearance after staining.

Western Blot after Denaturing Electrophoresis

After separation of the proteins on SDS-PAGE gel, the protein bands were transferred to a PVDF blotting membrane purchased from Millipore Corporation (Billerica, MA, USA) for 30 min at 15V. On the completion of transferring proteins, the membrane was washed by 20mM TBS at a pH 7.4 and then blocked with StartingBlock blocking buffer. The membrane was incubated overnight at 4°C with pooled human plasma containing anti-peanut IgE antibodies (1:500) diluted in blocking buffer. After that, three separate washes with TBST (TBS 1x with 0.05% Tween 20) were performed in 5 min time periods. The blot was then incubated with a secondary antibody (1:1000) at room temperature (RT). Another series of washes were performed with TBST.

However, in this experiment, the washing times were 5 min, 10 min, and 10 min at RT. On the completion of the washing step, the membrane was incubated by SuperSiganl West Pico Chemiluminescent substrate for 5 min at RT. The protein bands signal was developed on X-ray film.

Non-Denaturing Electrophoresis

The Novex Tris-Acetate gels were also applied in this experiment. The mini cell unit was the same as the denaturing electrophoresis system. Novex tris-glycine native running buffer (2 x) was used for the sample preparation. The ratio of native sample buffer for sample preparation was 1:1 v/v. Each well was loaded with 25 µL of sample and sample buffer mixer. After assembled the XCell unit, the voltage was set up at 150 V constant. The expected current for the whole gel was around 40 - 55 mA / gel. When the protein bands reached the bottom of the gel, the power supply was turned off. The gel was stained by coomassie R-250 for 2 h. Deionized water was used for background clearance after staining.

Western Blot after Non-Denaturing Electrophoresis

After separation of the proteins on SDS-PAGE gel, the protein bands were transferred to a PVDF blotting membrane for 30 min at 15V. On the completion of transferring proteins, the membrane was washed by 20mM TBS with pH 7.4 and then blocked with StartingBlock blocking buffer. The membrane was incubated overnight at 4°C with pooled human plasma containing anti-peanut IgE antibodies (1:500) diluted in blocking buffer. After that, three 5min separated washes with TBST were done. The blot was then incubated with a secondary antibody (1:1000) at RT. Another series of washes was done with TBST, but this time, the time points were 5 min, 10 min, and 10 min at RT. On the completion of the washing step, the membrane was incubated by

SuperSiganl West Pico Chemiluminescent substrate for 5 min at RT. The protein bands signal was developed on X-ray film.

Indirect ELISA

Diluted total peanut extracts prepared to a final concentration of 2 µg / 100µl with PBS buffer (pH 7.4) was coated on to a polystyrene 96-well plate for 2 h at 37°C. Each protein sample was added in triplicates with 100 µl per well. The plates were then washed by TBST for three times and blocked with StartingBlock blocking buffer (200 µl per well) on a rocker at RT for 2 h. After another round of washing, 100 µl of pooled human plasma containing IgE antibodies (1:500 dilution in 1X PBS) was added to each well. The plate was incubated at 37°C for 1 h. After the TBST wash, mouse anti-human IgE conjugated HRP antibody (1:3000) was added next in the same manner, with 100 µl per well. Incubation followed for 1 h. After another washing step, the protein-antibody bonded plate was developed with OPD substrate (0.5mg/ml) that was prepared by dissolving 0.1M citrate buffer (pH 5.5) and 0.03% hydrogen peroxide (100 µl per well). The developed time was between 15 – 30 min and was stopped with 2.5N sulfuric acid (100µl per well). The absorbance was measured at 490nm using Spectramax 340384 spectrophotometer.

Results and Discussion

Denaturing Electrophoresis

Denaturing SDS-PAGE results were shown in Figure 4-1. Roasted and raw peanut samples were considered as controls that were used to compare with the PL treated samples. The protein profile ranged from 37 kD to 460 kD. Raw peanut sample had different molecular proteins in the seeds, which was shown on SDS-PAGE. The smaller proteins (< 37 kD) were eluting from the gel. Roasted peanuts were prepared at

165°C for 15 min. The protein- protein cross-linkages were formed during the roasting. The trimer of Ara h 1 was formed and could be detected on the SDS-PAGE, which had the molecular weight around 190 kD. The PL treatment times were selected at 4 min, 5 min, 5.5 min, 6 min, 6.5 min, respectively. The smear was formed during the PL processing, which was observed on the SDS-PAGE. The blue protein smear was dyed by coomassie blue and shown an increased amount with a longer treatment on SDS-PAGE. The treatment at 6.5 min did not have many sharp protein bands as compared to the raw samples. The smears were distributed from the top to the bottom of the gel. The smears were considered to result from the photo-thermal effect of PL that caused the aggregates or protein cross-linkage formation. The aggregations of proteins were macromolecules and some of them could not enter into gel system even the Novex system was utilized that was designed for large molecules electrophoresis. From the Figure 4-1, it was illustrated that some proteins stayed on the top of the gel without entering into the electrophoresis system.

Western Blot

The Figure 4-2 showed the results of western blot that generated from Novex gel electrophoresis. The IgE immunoreactivity of the samples treated for 4 min was shown to exhibit a strong signal from 460 kD to 37 kD. The extended treatments after 4 min showed a distinct reduction of amount on overall protein bands. However, for the 6.5 min treatment, it was difficult to detect the IgE reactivity. The roasted peanut was considered a control. The dark area of the roasted sample of the western blot result illustrated that a strong IgE immunoreactivity of those protein-protein cross-linkages that were detected during the thermal processing. A study conducted by Maleki and others (2000) explained that the reason for this phenomenon is that thermal processing such

as roasting, curing, and various types of cooking can cause multiple, non-enzymatic, biochemical reactions in foods. A major reaction that is related to these reactions is Maillard reaction browning, which is considered as an important method in the development of flavor and color in peanuts. The modifications of the amino groups of proteins by Schiff bases formation with reducing sugars undergo rearrangement to form Amadori products. Amadori products would finally degrade into dicarbonyl intermediates. These compounds are more reactive than the parent sugars and they could assist the amino groups of proteins to form cross-links, stable end products called advanced Maillard reaction products (MRPs) or advanced glycation end products (AGEs). Proteins including allergenic proteins in peanut seeds could also be trapped in these macromolecules. This study also demonstrated that the smears that appeared on the SDS-PAGE were formed during the thermal processing were due to the cross-linkage and non-cross linkage adducts produced by Maillard reaction. Western blot results showed that Ara h 3 or Ara h 4 could be detected in the 4 min, 5 min, 5.5 min, 6 min treated samples but not in 6.5 min. The reasons for this phenomenon attributed to the amount proteins of loaded sample were comparatively large or some 37 kD protein cross-linkage may formed during the PL process or PL could not inactive Ara h 3 or Ara h 4 before 6 min treatment. Chapter 3 illustrated that soluble peanut allergenic proteins were inactivated after PL treatment, which demonstrated that PL treatment could notably mask all the allergen proteins in treated peanuts due to the fragmentation and cross linkage of treated proteins. The temperature profile we also measured during the processing will be discussed in Chapter 7. The significant increase of temperature after PL treatment could be observed on each peanut kernel. Infrared thermometer was used

to record the temperature changes before and after treatment. Normally, the room temperature was around 23-25°C. The burnt peanut samples could achieve 130°C. These data will be presented and discussed in Chapter 7.

The putative mechanism of PL can be explained as photothermal, photochemical and photophysical effects on the food samples, such as shrimp, peanut, soybean (Krishnamurthy and others 2007; Chung and Champagne 2008; Yang and others 2010b). Protein fragmentation and cross-linkage were two major reasons for destroying and masking epitopes of peanut allergen proteins. Infrared light was one composition of PL, with which the temperature of the food samples could be increased after processing.

Previous studies have been shown that roasted peanut proteins such as Ara h 1 and Ara h 2 bind higher levels of IgE than raw peanuts (Koppelman and others 1999; Maleki and others 2000a). Another study revealed that thermal processing of peanuts could alter the solubility of the proteins and leads structural and chemical modifications that contribute to the IgE binding increase. Overall, the thermal effect caused the peanut proteins to be less soluble in addition to large molecular weight smears being formed with increased time of heating. Aggregation of proteins was due to the protein covalent modifications other than disulfide linkages. They have been covalently linked since proteins held together with non-covalent interactions such as hydrophobic, electrostatic, or disulfide bonds would separate into monomeric components after boiling with SDS sample dye containing dithiothreitol (Schmitt and others 2010). Structural and chemical alternations that occur during heat treatment caused the peanut proteins to become less soluble contribute to enhanced IgE binding. Enhanced IgE binding was detected in the

insoluble fractions, which were believed to result from the Maillard reaction (Gruber and others 2005).

In addition, heat transfer occurs through one of three methods, conduction, convection, and radiation. Foods and biological materials are heated primarily to extend their shelf life or to enhance taste. In conventional heating, heat is produced outside of the object to be heated and is conveyed to the material by convection of hot air or by thermal conduction. Under the infrared (IR) radiation, the heat energy generated can be absorbed by food matrixes. This radiation method transfers thermal energy in the form of electromagnetic (EM) waves and encompasses that portion of the EM spectrum (Krishnamurthy and others 2008). Recently, more focus on thermal processing because of its higher thermal efficiency and fast heating rate/response time in comparison to conventional heating. IR radiation can be classified into 3 regions, near-infrared (NIR), mid-infrared (MIR), and far-infrared (FIR), corresponding to the spectral ranges of 0.75 to 1.4, 1.4 to 3, and 3 to 1000 μm (Sakai and Hanzawa 1994).

Amino acids, polypeptides, and proteins revealed two strong absorption bands localized at 3 to 4 and 6 to 9μm. Water and organic compounds such as proteins and starches, which were the main components of food, can absorb FIR energy at wavelengths greater than 2.5μm (Sakai and Hanzawa 1994). Thermal inactivation could damage DNA, RNA, ribosome, cell envelope, and proteins in microbial cell. Sawai and others (1995) demonstrated that the inactivation of *E.coli* was due to sub-lethally injured cells production after irradiation. IR heating showed that the cell wall was more vulnerable than conventional heating. The order of magnitude of infrared damages was shown as follow; protein > RNA > Cell wall > DNA. However, FIR radiation quantum

energy was reported it was insufficient for hydrolysis of hydrogen links in proteins and other biological macromolecules. FIR irradiation caused destruction of either polypeptide chain of protein or appearance of oligopeptide. The secondary structure of three proteins including alcohol dehydrogenase, peroxidase and trypsin has demonstrated that there was no significant change after FIR radiation. However, it was detected that changes occurred in albumin α-helices (Govorun and others 1991).

Ultraviolet radiation (UV) is a part of the non-ionizing region of the electromagnetic spectrum that encompasses about 8 – 9 % of the total solar radiation (Coohill 1989). UV is traditionally divided into three wavelength ranges: UV-A (320-400nm) representing approximately 6.3 % of the incoming solar radiation and is the less hazardous part of UV light; UV-B (280-320nm) this UV's wavelength represents only around 1.5 % of the total spectrum, but can induce a variety of damaging effects in plants; UV-C (200-280nm) is extremely harmful to organisms, but not relevant under natural conditions of solar irradiation. UV irradiation can cause not only the modification or destruction of amino acid residues, but also leads to inactivation of whole proteins and enzymes. Inactivation of proteins and enzymes by UV light is due to photolysis of aromatic amino acids or disulfide groups if affected residues are included in active site (Grossweiner 1984).

UV-C irradiation is known for its lethal activity against most microorganisms including bacteria and viruses. As one important study indicated, UV-C could change the structure of whey proteins and increased concentration of free thiol groups with UV exposure. This study also demonstrated that UV-C irradiation can significantly change the tertiary and quaternary structure of whey proteins, accessibility of thiol groups, and

oxidation of tryptophan and tyrosine (Kristo and others 2012). Moreover, another report indicated that UV-C irradiation cause off-flavors production on the whole goat milk (Matak and others 2007). The UV light portion of pulsed light is consisted of most UV-C. This UV-C has a harmful effect on the enzymes and proteins in the food samples.

Non-Denaturing System

To obtain the actual effect on the native allergenic proteins rather than the denatured forms after dithiothreitol treatment, a non-denaturing system was utilized (Figure 4-3 and Figure 4-4). In this study, the native SDS-PAGE sample buffer was applied and there was no step for boiling sample/ sample buffer mixture to uncurl the protein molecules. The samples loaded on the gels were maintained in their native states as the proteins were stored in treated peanut seeds. This study provided a further evidence for IgE reactivity examination of PL treated peanut allergens.

SDS-PAGE

Figure 4-3 showed a protein profile of PL treated peanut protein samples at 4 min, 5 min, 5.5 min, 6 min, 6.5 min on Novex gel. Roasted and raw peanuts were designated the controls. The raw peanut sample illustrated sharp protein bands than roasted peanut that was primarily due to the Maillard reaction in roasted peanut sample. The smears were clearly detected in PL treated samples and roasted sample. The 37 kD was detected in each peanut sample, which may be Ara h 3 or h 4. The allergen Ara h 1 (63 kD) was also detected in each sample except the 6.5 min treatment. However, Ara h 1 cannot be detected after 5.5 min treatment. This may be due to the fact that some protein cross linkages and complex were formed during the processing and those macromolecules have similar molecular weight around 63 kD. Moreover, the fragmentation of Ara h 1 occurred but those native proteins did not separate when

mixed with the sample buffer. The whole Ara h 1 was still appeared on the SDS-PAGE gel. Ara h 2 cannot be detected on this gel because the smaller molecular weight that was held in this gel was 37 kD.

Western Blot

The results of western blot were illustrated in Figure 4-4. The IgE binding of each sample was detected on the transferred membrane. The signals were detected before 6.5 min through the top area to the bottom area of the PL treated samples. However, roasted peanut samples were darkened in color on their surfaces. The specific band of different allergenic protein could not be differentiated on the designated Western blot membrane. These observations could have been related to the different molecular size of protein cross-linkage formation. Most of the proteins were trapped in the Maillard reaction and finally had different molecular weight and distributed through the entire gel. All these proteins were curled and maintained as native state. The linear epitopes were masked inside without exposure. The conformational epitopes were the most important factors that produce the IgE reactivity.

Indirect ELISA

As previously discussed, the SDS-PAGE and western blot were applied to detect the insoluble fractions above 37 kD. The smaller insoluble fractions were easily eluted from the gel. To include all the proteins and investigate the IgE reactivity to allergenic proteins, the total peanut proteins were extracted by using a homogenizer. The roasted and raw peanut proteins were designated as the controls. The roasted peanut sample was normalized as “1”. This experiment was operated with pooled human IgE. The results were presented in Figure 4-5. It was observed that the IgE reactivity of the roasted sample was significantly higher than the other samples with student t-test

examination. The samples treated for 6.5 min showed the lowest signal value than the others. Overall, the results of this study clearly demonstrated that PL is quite effective on both soluble and insoluble protein fractions inactivation.

Summary and conclusion

The foregoing evidences pointed to the inactivation effect of PL on insoluble allergenic proteins. In conjunction with the previous results on soluble proteins, it could be concluded that the inactivation effects of PL on both soluble and insoluble fractions were eminent. In order to further verify the PL effect, *in vitro* digestion study should be conducted to investigate the changes of IgE binding reactivity and immunogenic potential of the allergenic proteins when they are in digestive environment, such as different pH values and different enzymatic hydrolyses.

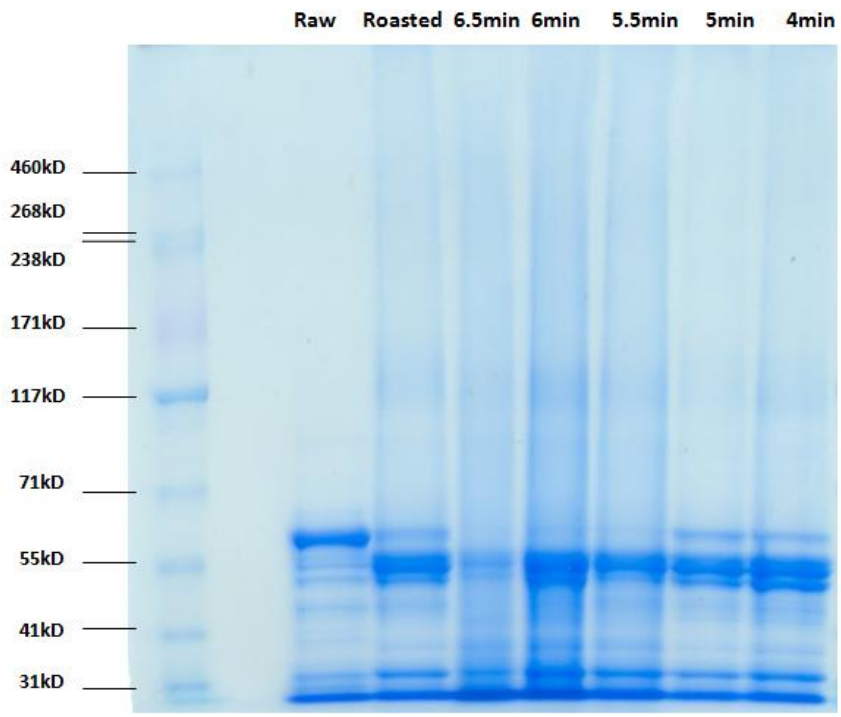


Figure 4-1. Protein profiles of insoluble fractions of PL processed peanuts were analyzed by Novex denaturing electrophoresis. R was referred to a raw peanut sample. Ro was referred to roasted peanut sample. Different durations of PL treatment were expressed as 4 min, 5 min, 5.5 min, 6 min, and 6.5 min. The range of protein standard was from 30 kD to 460 kD. The extended treatments were at 5 min, 5.5 min, 6 min, 6.5min.

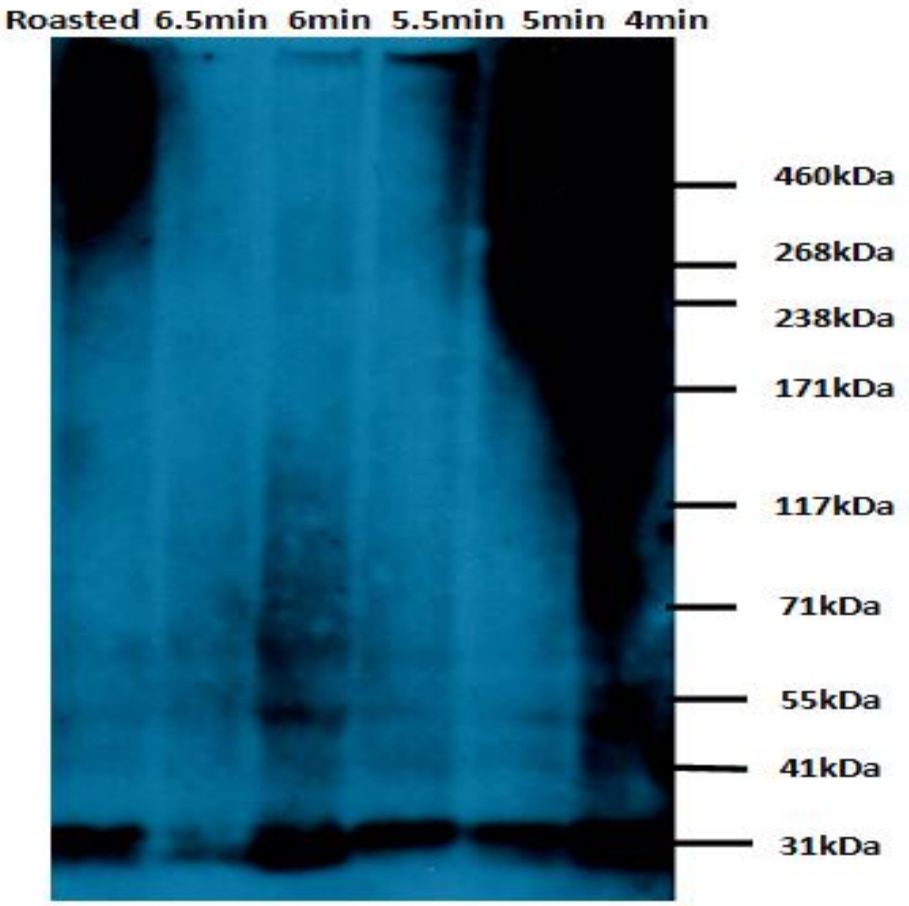


Figure 4-2. Western blot of insoluble fractions of PL processed peanuts samples were compared to roasted peanut. Antigenic protein bands were labeled from 30 kD to 460 kD. Notable reduction of IgE binding can be detected in extended time pointed, such as 5.5min, 6min, and 6.5min.

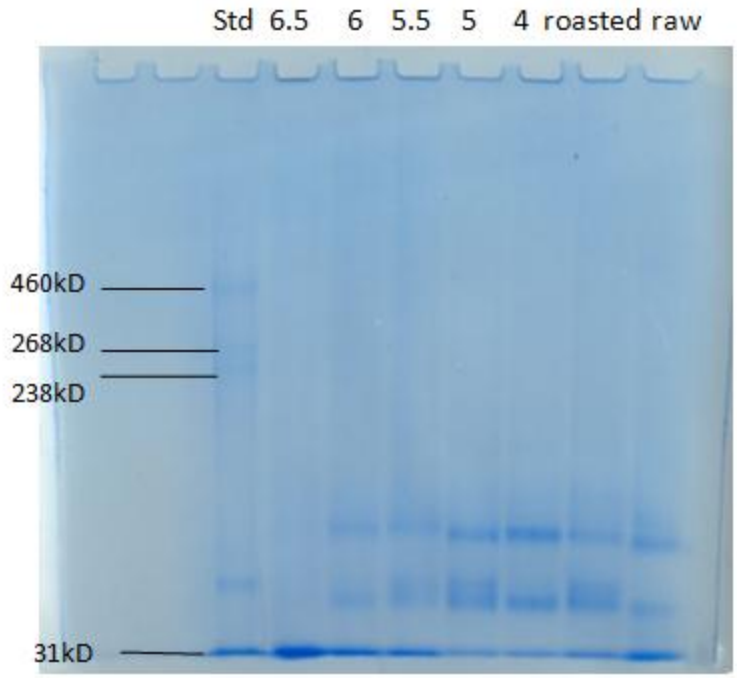


Figure 4-3. Protein profiles of insoluble fractions of PL processed peanuts were analyzed by Novex non- denaturing electrophoresis. R was referred to a raw peanut sample. Ro was referred to roasted peanut sample. Different durations of PL treatment were expressed as 4min, 5min, 5.5min, 6min, and 6.5min. The range of protein standard was from 30 kD to 460 kD.

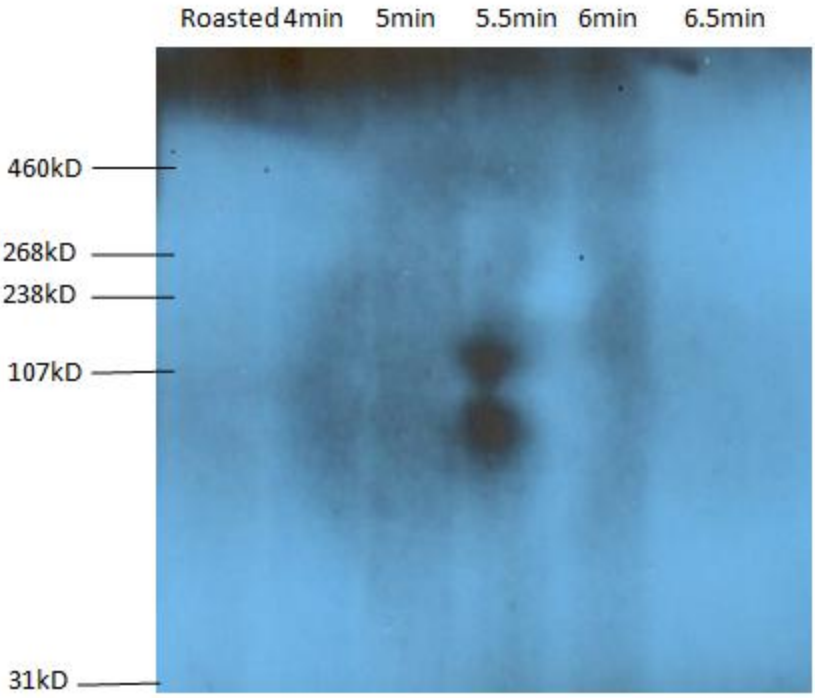


Figure 4-4. Insoluble antigenic protein fractions were analyzed by western blot on non-denaturing gel. The IgE binding of large molecules that formed during PL treatment and roasting were examined up to 460 kD. Notable reduction was detected at 6.5min PL treatment. Ro was referred to roasted peanut sample. PL treatment was at 4min, 5min, 5.5min, 6min and 6.5min.

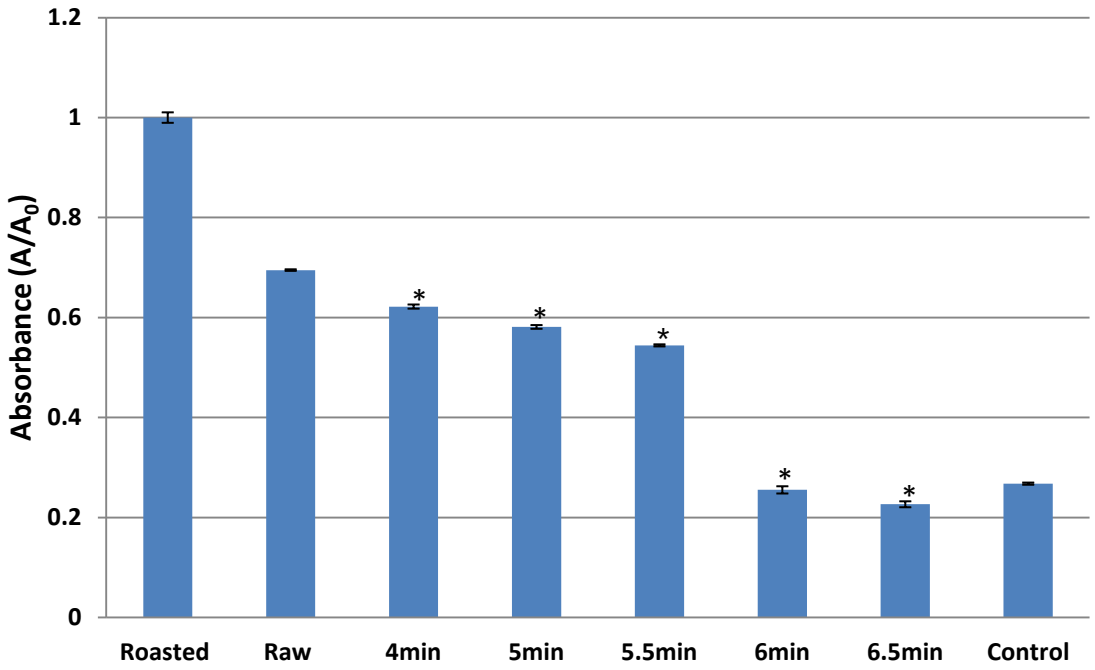


Figure 4-5. Effects of PL treatment on total protein of peanut kernels with different duration at 10cm were determined by Indirect ELISA with pooled human plasma containing IgE antibodies. Absorbance readings were at 450nm. Roasted sample was considered as a positive control. Normal human plasma was applied for negative control which is the last column in the graph. A= Absorbance of treated samples; A_0 = Absorbance of control. Results are normalized, roasted sample is standardized and set to 1. "*" stands for significant signal reduction (α = 0.05).

CHAPTER 5
THE OPTIMIZATION OF PL EQUIPMENT AND THE INTERACTION EFFECT OF MAJOR PARAMETERS: TIME AND DISTANCE

Introduction

The limitation of PL applicator in this study was observed when we conducted initial experiments that were in Chapter 3. The non-uniform irradiation of this equipment on the treated peanut kernel was observed, which meant some part of peanut had deeper roasting than other parts of one peanut kernel. The reason for this phenomenon was because the single lamp in quartz window, which indicated the light resources were scattered from the lamp. The unit area of peanut surface had received different amount PL and then induced different roasting effects of peanut surface. In Chapter 5, a new designed motor was applied for uniformly roasting. This motor would be fixed in a certain location on the conveyor belt to ensure the constant of PL treatment.

The time treatment and distance were two major factors that could affect the effects of PL roasting. To optimize these two factors and obtain proper quality of processed peanuts were important in this study because our goal for this technology is to produce hypoallergenic peanuts that could replace the conventional roasted ones. Statistical analysis for color development and IgE reactivity readings could be applied for this optimization. Proper roasted color with low IgE reactivity was an ideal state for PL treated peanut samples. From this analysis, the best treatment time and proper distance could be confirmed in this study. The peanut products with these two fixed parameters were used for next digestion study. Another benefit for this optimization study is to choose a good peanut product for further hypoallergenic peanut butter producing.

In this part of the experiment, the optimal treatment time and distance would be determined. The treated peanuts would be further used for the digestion study.

Material and Methods

Raw runner type peanuts were purchased from a Publix store in Gainesville, FL, USA. The same PL applicator was used in this study for PL treatment as indication in Chapter 3. A motor was assembled in the Egg Processing Lab of University of Florida with a steel rod, control pad, wires, motor, and a steel plate. It would allow for sample rotation during the treatment. Normal glass tubes from Fisher Scientific (Rockford, IL, USA) used to hold the sample during the treatment.

Color Analysis

To quantify the color changes, a machine vision system consisting of a Nikon D 200 digital camera housed inside a light box [42.5cm (W) x 61.0cm (L) x 78.1 cm (H)] (Wallat and others 2002) was used to measure the color of peanut kernels with a D65 (daylight) lamp and 10° observer angle. Each image includes traditional roasted peanuts (control) and PL peanuts as measured samples. Each image was calibrated against a yellow color reference tile (L=87.09, a=7.71, b=70.75) that was obtained from the Lab Sphere X-Rite Company (North Sutton, NH, USA). The software LensEyeSK® v10.0.0 from Engineering and Cyber Solutions Inc. (Gainesville, FL, USA) was applied to collect and analyze the images. To obtain precise calibration, the initial image was obtained using the yellow-round reference tile and then converted to a rectangular (~150 x 95 pixels) processed image that was without interference from the black border of the yellow tile. Next, the processed images were subjected to background corrections to produce the corrected images. The final corrected images were calibrated with a standard yellow color (L_{ref}, a_{ref}, b_{ref}). The L, a, and b values from the Hunter color

system were recorded individually for each peanut sample. The Hunter L, a, and b color space is organized in a cube form. The maximum L is 100 (white) and the minimum L is 0 (black). A positive a value denotes red, a negative a value for green; a positive b value for yellow, and a negative b value for blue. This color analysis was triplicated for each sample.

Indirect ELISA

All the reagents and the methods are the same as those mentioned in Chapter 3.

Experimental Design

The two major treatment conditions investigated were treatment time and the distance between the sample and the central axis of source of PL lamp. A factorial analysis was applied in this research. Factorial analysis is used for analysis of the effect of multiple factors at different levels and helps find out whether the two main factors are significant. In addition, the interactions between these two factors were also analyzed. The factors used in setting this design are discussed below. Two distance 10 cm and 7 cm from the light source were applied. Treatment time points were selected at 5 min, 6 min, and 7 min. This time point selection was based on the maximum treatment time at 7 cm, which is 7 min. At this point, the peanut samples were considered burnt. Medium roasted peanut kernels were normally judged by the Hunter color Lightness (L) of 50 ± 1.0 (Greene and others 2006). The color analysis of the treated peanuts was used for subsequent factorial analysis and the Hunter color system's L value for each peanut kernel was measured for peanut color evaluation. Indirect ELISA was another test to evaluate the allergenicty of treated peanuts. The reading value 0.4 was considered as a threshold, below this value was considered as low allergenic reaction. Both color analysis and indirect ELISA were two types of indicators for PL treatment evaluation.

Treatment time was evaluated first since previous studies showed that the effect of PL on the food samples is highly dependent on the treatment time. Distance from the central axis of the PL lamp was the second factor considered. The intensity of PL is proportional to the distance from the PL source. The intensity of light energy is highest at the closest position of the sample to the center axis of the lamp and decreases as the distance between the two increases. Since the durations and distance have been selected, they were expected to provide significant differences on peanut allergenic potency and provide an insight on the most effective energy levels on the reduction of peanut allergenic potency with proper color generation.

After factorial analysis, the distance would be fixed at 10 cm for the further study. To explore the proper treatment time that could produce a low IgE reactivity with a good color after roasting, a one factor completely randomized statistical design was applied for this purpose. Multivariate ANOVA analysis was also conducted using JMP Pro 10.0 (Cary, NC, USA).

Results and Discussion

Motor Mechanism

A significant challenge was to achieve uniform treatment over the roasted peanut samples. A motor-driven device was designed to handle the peanut samples. As mentioned previously, some parts of the peanut samples had darker spots than other parts of the peanut if the kernel were not rotated during PL treatment.

The components of this motor device are shown in Figure 5-1. The motor shaft was connected to a steel rod. On top of the steel rod, a glass tube with a stopper made of aluminium foil was placed. Peanut seeds were placed inside the glass tube prior to

treatment. Once the motor is powered on, the steel rod rotates. Seven or eight full peanut seeds are rotated in glass tube with full exposure to the PL.

After the preliminary optimization of the distance and durations of this rotator, another problem that caught our attention was the inner pressure generated from the temperature increase of the peanut seeds. The glass tube was always detached from the steel rod during the treatment. Then another improvement of the glass tube was to release the inner pressure during the PL treatment. Each glass tube was burning on the alcohol lamp to make a hole at the bottom. This remodeled glass tube was prepared before the PL treatment.

Factorial Analysis

Statistical analysis was performed using JMP Pro 10.0 software. Statistical analysis is based on two-factor factorial completely randomized statistical design at $p < 0.05$. In this analysis, distance was considered as first factor and time was considered as the second factor. The color analysis and indirect ELISA were considered as two responses. The combinations of this factorial analysis were 2x3=6 treatments. The interaction of distance and time was also determined at $p<0.05$.

Color analysis

The results of the F-test in the color analysis show that distance and time effects are significantly different ($p < 0.05$). In addition, there is a significant interaction between the distance and time. All these results are shown in Table 5-1.

Table 5-2 shows the least significant difference (LSD) that is applied for means separation. From the separated means, they present different levels A, B, and C. Time as another factor can cause significant effect on the color generation. The means of L value in each different time group show a significant difference at $p < 0.05$.

Table 5-3 gives the Tukey honestly significant difference (HSD), which examined the interactions between time and distance factors. There are six combinations: 7 cm with 5min, 6min, and 7min treatments and 10 cm with 5min, 6min, and 7min treatments. The least significant means describe the effects caused by these two factors, which are significantly interacted (Figure 5-2). There is some interaction observed between the two factors. The Tukey HSD results indicate that 5min treatment at 10 cm and 5min treatment at 7 cm do not have significant differences; however, the rest of the combinations show a notable significant difference between the groups.

Indirect ELISA

The output of factorial analysis of indirect ELISA is shown in Table 5-4. The alternative hypothesis is true and the null hypothesis is rejected at $p < 0.05$. The significant difference between two factors is shown in Table 5-5. The significant interaction between these two factors is also significant, which indicates that these two factors do not have parallel effects on the reduction of IgE reactivity to the allergenic proteins in the treated peanuts.

The means of indirect ELISA readings are categorized as two groups by distance, 7 cm and 10 cm. The means of these two groups are displayed in Table 5-5. The first one is the mean of each group. The IgE reactivity is significantly low in the 7 cm group. The plot gives a clear comparison with these two means. This result shows that lower distance has a positive relationship with lower IgE reactivity. In other words, the potency of PL has an increased effect on allergen reduction.

Time was also examined as a factor by the tukey HSD test. These three groups are separated as A, B, C, which means they are significantly different. The mean of each time group is presented in Table 5-6, The IgE reactivity readings of the 7 min

group at two different distance parameters is significantly low than the other 5min and 6min groups.

The interaction of time and distance is shown in Figure 5-3. The two lines in the graph do not run parallel. Thus, the interaction between time and distance is significant in ELISA test. The results of the tukey HSD analysis are shown in Table 5-7. The 7 min group at 10 cm and the 6 min group at 7 cm treatment group do not have a significant difference, but the rest of combinations are significantly different. It is worth mentioning that the 5 min treatment groups at two different distances are significant different. The same results are observed at 6 min and 7 min treatment groups. Overall, these results show that the effect of time is not the same as the effect of distance.

In summary, the color analysis of the 7 min treatment peanuts is similar to the medium roasted peanut with a L mean value around 58, as compared to standard value of 50. The ELISA analysis shows treatment at 6 min and 7 min have more than 60 % IgE reactivity reduction as compared to the raw samples. This factorial analysis demonstrates that the 7 min treatment at two distance parameters (7 cm and 10 cm) is more similar to medium roasted peanuts used in the industry. Since the 7.5 min treatment at 7 cm distance from light source produced burnt peanut samples, it is impossible to extend the time treatment beyond 7min for a given distance of 7 cm. However, it is possible to extend the time treatment at a 10 cm distance because these produce peanuts with better texture and color and low IgE reactivity.

Previous studies about PL inactivating allergens in different food samples applied 10 cm as a fixed distance since it has a reasonable duration from food treatment. The maximum energy level of the emitted radiation was 0.27 J/cm^2 per pulse, as per the

factory calibration. In Chapter 5, another one-factor completely randomized design was applied to explore the optimization of distance and time at 10 cm to find out the time treatment with low IgE reactivity readings and proper texture.

Multivariate ANOVA Analysis

After optimizing the motor-driven rotator, 10 cm was selected as a fixed distance for this analysis and further study. The time periods for this treatment were at 10 min, 11 min, 12 min, and 15 min. The output of statistical analysis is shown below.

Color analysis

Table 5-8 shows that the sum of squares of model is 2438.290 and the error is 311.994. The degree of freedom is 15. The mean squares of model and error are 812.763 and 26.00, respectively. The p value is less than 0.0001, which is significant at $p < 0.05$. This means that it rejects the null hypothesis and accepts the alternative hypothesis.

A Tukey HSD test (Table 5-9) demonstrated that the separation of the means of each group between 12 min treatment and 15 min treatment groups. However, the 10 min and 11 min treatment groups are similar in the color analysis. For the means of L value in the different groups, the 12 min treatment group has an average L value of 52.33. This is the closest value that is achieved with commercial medium roasted peanuts. The 15 min treatment group was over roasted (burnt) and is indicated in Table 5-9.

Indirect ELISA analysis

The output is shown as Table 5-10. The sum of squares of model and error are 0.0023 and 0.002. The total degree of freedom is 23. The mean squares are 0.008 and 0.0001. The F ratio is shown as 77.19. This number is over 1, which means that it

rejects the null hypothesis but accepts the alternative hypothesis. Furthermore, different groups are significantly different in the F- test calculations.

The least squares means is listed in Table 5-11. The overall means of each group are quite low. The 12 min and 15 min groups could not be detected at observable values on the ELISA plate. The plot of the means of each group demonstrated that a longer treatment is positively related to the lower IgE reactivity of peanut samples.

The tukey HSD test results are shown in Table 5-11and show that the separated means in each time treatment are significantly different. The 10 min, 11 min, 12 min, 15 min treated groups are significant difference of IgE binding. Based on this, a longer treatment is more potent on allergen inactivation.

Summary and Conclusions

In summary, the 12 min treatment group showed the best color with an average L value of 52.33 as compared to other groups. In addition, the 12 min treatment group presented a significantly low IgE reactivity compared to the 10 min treatment group and the raw peanut samples. The reduction at 12 min was 90% with respect to the raw peanuts. Thus, the 12 min group was selected for the *in vitro* digestion study because of its low IgE reactivity readings and the possibility of use in commercial settings.

Table 5-1. F-test of distance and time interaction based on the L value

Source	Nparm	DF	Sum of squares	F ratio	Prob > F
distance (cm)	1	1	831.1689	86.7176	<.0001*
time (min)	2	2	62.2860	62.2860	<.0001*
distance* time	2	2	12.6550	12.6550	0.0001*

Table 5-2. Tukey HSD results describing 5 min, 6 min, and 7min treatment groups of L value

Level		Least sq Mean
5 min	A	72.066667
6 min	B	63.7858
7 min	C	58.0358

Table 5-3. HSD of interactions between combination groups of L value

Level		Least sq mean
10, 5	A	74.001667
7, 5	A B	70.131667
10, 6	B	68.043333
10, 7	B	66.258333
7, 6	C	59.5283333
7, 7	D	49.813333

Table 5-4. F-test output of indirect ELISA readings

Source	DF	Sum of squares	Mean square	F Ratio
Model	5	0.39431196	0.078862	927.0173
Error	12	0.00102085	0.000085	Prob > F
C. Total	17	0.39533282		< .0001*

Table 5-5. F-test of distance and time factors based on Indirect ELISA readings

Source	Nparm	DF	Sum of squares	F ratio	Prob > F
distance (cm)	1	1	0.13304201	1563.892	<.0001*
time (min)	2	2	0.25922388	1523.572	<.0001*
distance* time	2	2	0.00204607	12.0256	0.0014

Table 5-6. Means separated by treatment time: 5min, 6min, and 7min of Indirect ELISA readings

Level		Least sq mean
5 min	A	0.59106667
6 min	B	0.47553333
7 min	C	0.29921667

Table 5-7. Tukey HSD of interactions between combination groups (p <0.05).

Level		Least sq mean
10, 5	A	0.66946667
10, 6	B	0.55400000
7, 5	C	0.51266667
10, 7	D	0.40026667
7, 6	D	0.39706667
7, 7	E	0.19816667

Table 5-8. F-test of distance and time interaction based on the color readings (p<0.05).

Source	DF	Sum of squares	Mean square	F Ratio
Model	3	2438.2904	812.763	31.2607
Error	12	311.9941	26.000	Prob > F
C.Total	15	2750.2845		<.0001*

Table 5-9. Means separated by treatment time of color analysis L value (p<0.05)

Level		Least sq Mean
10 min	A	73.76500
11 min	A	65.582500
12 min	B	52.337500
15 min	C	41.512500

Table 5-10. F-test of time and distance interaction based on Indirect ELISA (p<0.05).

Source	DF	Sum of squares	Mean square	F Ratio
Model	3	0.02387682	0.007959	77.1922
Error	20	0.00206211	0.000103	Prob > F
C.Total	23	0.02593893		<.0001*

Table 5-11. Means separated by Tukey HSD of indirect ELISA readings.

Level		Least sq Mean
10 min	A	0.13766667
11 min	B	0.10455000
12 min	C	0.08373333
15 min	D	0.05091667

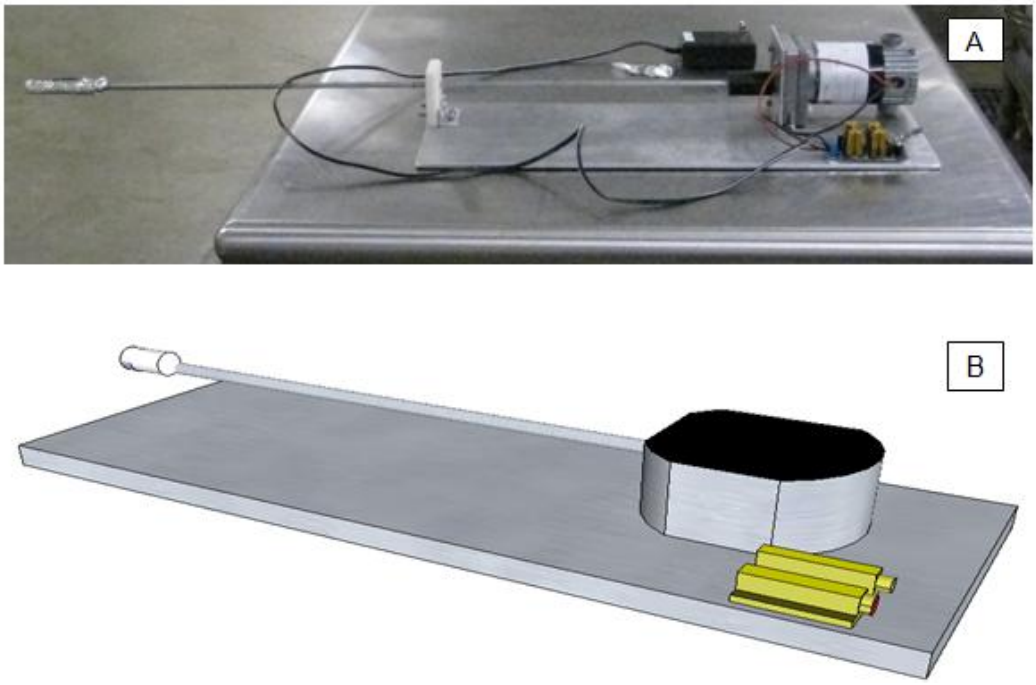


Figure 5-1. A model and a picture of a motor designed for uniformly roasting for PL treatment. A) A motor designed for uniformly roasting is placed in pilot plan of food science department, University of Florida; B) A model describes the structure of this motor. Black box is motor. Yellow part is control pad. The rod is the drive for peanut rotating. The peanut samples are placed at the top of the rod in a glass tube.

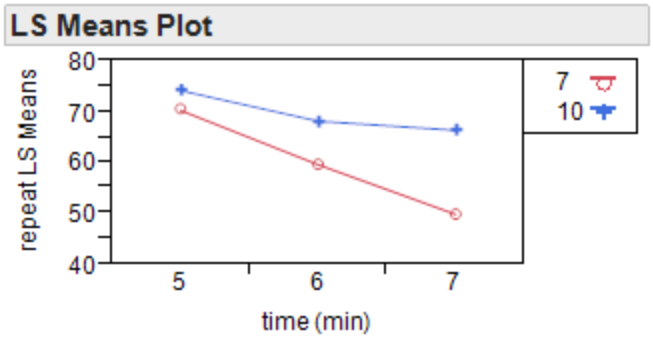


Figure 5-2. Factorial analysis: Tukey HSD of interactions between combination groups of color analysis values ($p < 0.05$).

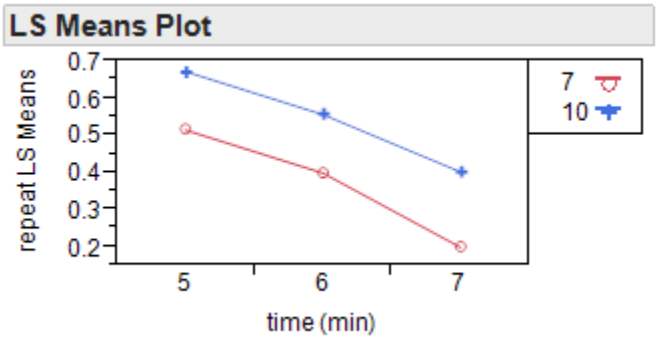


Figure 5-3. Factorial analysis: Tukey HSD of interactions between combination groups of Indirect ELISA readings ($p < 0.05$).

CHAPTER 6
IN VITRO SIMULATED GASTRIC AND INTESTINAL DIGESTIONS OF PL PROCESSED PEANUTS

Introduction

Although PL had a notable effect on the allergen reduction of peanut kernels as discussed in the foregoing chapters, it is still unknown whether PL treated peanuts could sustain the gastrointestinal digestion without changing its immunogenic potential. Literature has shown that digestion in the gastrointestinal tract by digestive enzymes can lead to changes in the allergenic potency of food allergens. Therefore, it is important to determine the allergenic changes of PL treated whole peanuts following digestion through simulated gastric and intestinal enzymes, which would determine more thoroughly the effect of PL on peanuts allergens when subjected to gastrointestinal digestion.

Many of the major allergens in foods are resistant to digestion. It is what distinguishes them from minor allergens, which are present in foods, but digested in the GI track. One study demonstrated that the digestion of peanut extract with pepsin did not affect the IgE binding properties, even though substantial proteolytic breakdown and fragments formed (Vieths and others 1999). The digestion of purified peanut allergens was investigated by (Koppelman and others 2010). However, the digestion of the total protein extracts from the processed peanut kernels, such as roasted peanut and PL treated peanuts, has not been examined.

Some studies have indicated that the food allergens digested by digestive enzymes in the stomach or intestines could modify the protein conformation (Untersmayr and Jensen-Jarolim 2006; Shriver and Yang 2011). It is important to

determine the allergenic potency of PL treated peanut extracts to understand the IgE reactivity changes of these peanut samples.

As discussed in the Chapter 3, Ara h 1 was considered to have molecular weight of 63.5 kD that normally occurs in a trimeric form of around 180 kD (Burks and others 1991; Becker 1997). It is formed via non-covalent interactions after thermal treatments (Shin and others 1998; Koppelman and others 1999). The trimeric Ara h 1 often forms aggregates, forming multimers of up to 600-700 kD (Van Boxtel and others 2006; van Boxtel and others 2007). We have demonstrated this in Chapter 3 and Chapter 4. Ara h 2 migrates as a doublet at around 20 kD (Burks and others 1992). Ara h 3 is a more complex allergen. It is initially identified as a 14 kD protein (Eigenmann and others 1996), but subsequent purification of Ara h 3 shows that this protein consists of a triplet around 42-45 kD, and another distinct band at 25 kD, and some bands in the range of 12-18 kD (Koppelman and others 2003; Piersma and others 2005). Lastly, Ara h 6 was found to have a molecular weight around 15 kD (Koppelman and others 2005). In this study, the incidence of these allergens in simulated gastric and intestinal fluids was analyzed by SDS-PAGE, Western blotting, and indirect ELISA.

Material and Methods

The runner type peanuts treated by PL at 12 min were chosen in this study due to the presentable color, texture, and comparatively low allergenic potency. Roasted and raw peanuts were considered as controls. The processing methods for peanuts are the same as shown in Chapter 3.

Simulated Peptic Digestion

This procedure followed Laemmli (1970b). The simulated gastric fluid (SGF) was prepared with a pH adjusted to 1.2, and also included 0.063 N HCl, 35 mM NaCl and

4000 U pepsin. The porcine pepsin was purchased from sigma (St Louis, MO, USA). The SGF was pre-warmed in the incubator at 37°C and 80 µL of 5mg/ml extracted peanut proteins were added in 1.52 ml SGF at time point t=0. The pepsin: substrate protein ratio was 10 U of pepsin:1µg of substrate protein. The pepsin activity was prepared at 3300 U/ml. A substrate protein concentration of 250 µl 1mg/ml peanut protein extracts and 760 µg/ml pepsin were applied. 200 µl of the reaction samples was collected at different time points: 0.5, 2, 5,10, 20, 30, and 60 min. Digestion reaction was stopped at appropriate times by mixing with 100 µl of 200 mM $NaHCO_3$ (pH=11.0) and also 100 µl 2X concentrated electrophoresis buffer that contained 40% glycerol, 20% SDS, with 5% β-mercaptoethanol, 0.33 M Tris (pH 6.8) and 0.05% bromophenol blue. All the samples were heated for 5 min at 100°C.

The sample at time point t = 0 was prepared by adding bicarbonate and Laemmli buffer and heating the mixture of SGF prior to the addition of test proteins. After the tested proteins were added, the samples were heated again to ensure full protein denaturation. Potential pepsin digestion was tested by adding 80 µl of water to SGF and incubated for 60 min.

Simulated Intestinal Digestion

This procedure followed Koppelman and others (2005b). Simulated intestinal fluid (SIF) was prepared by adding 65 mM Tris buffer at pH 8.3 containing 1 mM EDTA. The trypsin and α-chymotrypsin, having enzyme activities of 325 U/mg of protein and 62 U/mg of protein, respectively, were added into the SIF at a concentration of 3.2 mg/ml. These two enzymes were purchased from sigma (St Louis, MO, USA). They were diluted in the SIF with a ratio of 0.20 U and 0.04 U per µg of treated peanut proteins. Digestion was conducted in volumes of 1 ml for each peanut sample. Aliquots of 200 µl

SIF mixtures were terminated and then removed at 5min, 10 min, 20 min, 30 min, 40 min, 60 min, and 90 min. The termination was acheived by adding 200 µL of 2x SDS-PAGE sample buffer (containing 40% glycerol, 20% SDS, 0.33 M Tris (pH 6.8) and 0.05% bromophenol blue). Three independent trials were performed.

Results and Discussion

Simulated Pepsin Digestion

Raw peanut

The SDS-PAGE and Western blot results of pepsin digested raw peanuts are presented in Figure 6-1. The left lane is the protein standard, ranging from 10 kD to 250 kD. From left to right, the first two samples were the protein profile of pepsin. The fragments were located at 37 kD, 15-20 kD, and 10 kD. The next two lanes are the samples from t = 0. The densities of these two lanes are darker than the first two lanes, indicating the incorporation of peanut proteins with pepsin enzymes.

No proteins were observed at molecular weights above 37 kD due to enzymatic reactions. All the digested samples showed darker and thicker bands at around 37 kD. Compared to the zero time treatment, the smaller bands of proteins disappeared while the 37 kD protein band showed an increased density. The treatment at 2.5 and 5min showed the protein bands of 25 kD, 17-18 kD were not as strong as the 0 min treatment and pure pepsin control, which indicated that pepsin may combine with the peptide substrate and form a bigger complex. The treatments at 10 min, 20 min, 30 min did not show a palpable difference of protein profiles as compared to the pure pepsin group. However, the smaller areas (under 25 kD) were clearer and sharper. It is believed to arise from the complex formation or fragments produced by enzymatic digestion.

The Western blot results are shown in Figure 6-1. Western blot provides image of the IgE binding for the specific proteins. Pooled plasma from five patients was used in this study. The strong signals on the Western blot membrane displayed the allergenic potency of the digested proteins. The 0 min treated sample had two major bands - one was around 37 kD and the band was appearing at 17 kD which was Ara h 2. After two min treatment, the Ara h 2 protein completely disappeared. By 60 min digestion, most of IgE binding signals were not present.

Roasted peanut

For roasted peanuts, SDS-PAGE results are presented in the same order as the raw peanut sample (Figure 6-2). The first lane is protein standard ranging from 10 kD to 250 kD. The following lanes are the pepsin control group, t=0 treatment group, 2.5 min, 5 min, 10 min, 20 min, 30 min, and 60 min treatment groups.

Smears were present on top of each sample and throughout the gel. The treatment group t = 0 had an unclear area in the small protein range under 25 kD. The 37 kD area was markedly increased due to the combination between enzyme and proteins or protein aggregation, as discussed above. The bigger protein bands, such as Ara h 1 (63 kD), could not be detected on the SDS-PAGE gel. The possibility of this phenomenon can be explained to dis-assembled Ara h 1 or protein aggregation.

Western blotting also provided a further view of IgE binding with allergenic proteins (Figure 6-2). The roasted peanut control had a strong signal of IgE reactivity at 17 kD, which was Ara h 2. The Ara h 1 band was shown at 63 kD. Other various bands were also detected on the membrane. For t=0 and t=20 min treatments, the digested samples showed protein reactivity at 37 kD and 10-25 kD. The treatment at 30 min and 60 min only showed the Ara h 2 signals and no other allergen bands.

Pulsed light sample preparation

The 12 min PL treated samples were evaluated. The protein profile and allergen reactivity were examined by SDS-PAGE and Western blot (Figure 6-3). The standard protein is located on the leftmost column, ranging from from 10 – 250 kD. Next is the pepsin group that shows the protein bands of pepsin. The rest of the samples are the experimental groups from t=0 treatment to t=60min.

Compared to the raw and roasted peanuts, two major differences were observed on the SDS-PAGE. One is that the smears were distributed throughout the whole gel from the top to the bottom; another is that the smaller protein bands area were blurred, which means different size proteins were distributed randomly in this area. Once digestion started, the density at 37 kD was notably increased. Extended treatments at 30 min and 60 min decreased the amount of smaller proteins (< 25 kD). Ara h 1 disappeared, which may be due to denaturation because of low pH values or enzymatic digestion.

Western blot results (Figure 6-3) demonstrated the IgE immunoreactivity of allergen proteins. PL treated samples was different as compared to raw and roasted peanut samples. The pooled plasma could detect major peanut allergens Ara h 1 –Ara 3 and also other allergens Ara h 4 - Ara h 11. However, Ara h 2 was the only allergenic protein visible on the Western blot. The other bands were not present. The digestion of 60 min had a distinct reduction of Ara h 2 IgE binding. These results indicate that PL could inactive most of the peanut allergens. After 60 min pepsin digestion, the IgE binding of Ara h 2 virtually disappeared.

Simulated Trypsin and α-Chymotrypsin Digestion

Raw and roasted peanut

Chymotrypsin is a digestive enzyme found in pancreatic juice. It acts in the duodenum as an enzyme for protein proteolysis, the breakdown of proteins and polypeptides (Chervenka and Wilcox 1956). Trypsin is a serine protease that is found in the digestive system of many vertebrates where it hydrolyses protein. Trypsin is produced in the pancreas as the inactive form: proenzyme trypsinogen. Trypsin cleaves peptide chains mainly at carboxyl side of the amino acids lysine or arginine (Rawlings and Barrett 1994). In this study, these two enzymes were used for simulated intestinal digestion.

SDS-PAGE and western blot results are presented in

Figure 6-4. The left lane is a protein standard ranging from 10 kD to 250 kD. The first seven samples were digested remains of raw peanut samples and the second set of seven samples was digested remains of roasted peanut samples. This digestion study did not show a difference between raw peanuts and roasted peanuts. The large protein molecules (above 50 kD) disappeared on the SDS-PAGE gel. Ara h 2 and other peanut proteins were observed under 25 kD. Ara h 1, Ara h 3 and Ara h 4 were not detected on the SDS-PAGE gel, which indicated the digestive effect of intestinal enzymes on these allergens.

Western blot

IgE binding activity was only detected (Figure 6-4) at 16-18 kD (Ara h 2 band) of both raw and roasted samples. The treatment at 60 min and 90 min of roasted peanut had a prominent reduction signal compared to the lower treatment times. The IgE immunoreactivity of raw peanut samples showed a distinct reduction after the 20 min

treatment. Thus, the digestion efficiency of raw peanut samples was higher than roasted peanut.

Pulsed light treated peanut

The PL treated peanut was digested at different time points, starting at 0 min and going to 90 min. The right lane in the SDS-PAGE (Figure 6-5) is the protein standard ranging from 10 kD to 250 kD. Next are trypsin and α-chymotrypsin controls. Different fragments of these two enzymes were detected on the SDS-PAGE gel around 70 kD, 37 kD, and 20 kD. The smears of pulsed treated peanut accumulated throughout the whole gel, especially for the 0 min treatment. With the extended digestion of intestinal enzymes, the smears were gradually reduced. The sample of 90 min treatment did not show smears except for the top area. No specific bands were observed from the PL treated samples.

Western blot results (Figure 6-5) matched the results of the SDS-PAGE. The starting treatment at 0 min had a strong IgE immunoreactivity of smears throughout the whole sample lane on the western blot. Ara h 1 and Ara h 3 could not be detected on the western blot, but Ara h 2 showed strong signals and bands on the western blot membrane. The 5 min and 10 min treatment groups had distinct IgE immunoreactivity reduction as compared to the 0 min treatment group. After 10 min, the total IgE immunoreactivity of the longer treatment groups almost disappeared. Ara h 1- Ara h 11 could not be detected on the Western blot and even Ara h 2 was inactivated completely. No smears were present on the gel after the 0 min treatment.

Discussion

In the SDS-PAGE, Ara h 1 was not shown. The trimeric (Shin and others 1998) and the oligomeric (Van Boxtel and others 2006) organization of Ara h 1 on the

quaternary folding level contains disulfide bridges that link the proteins, but there is a dissociation of these complexes. It is not known what is the reason, but multimers are not present on the SDS-PAGE of raw and roasted samples. One study pointed out that some peptides of approximately 5 and 10 kD are stable for up to 2- 8 min (Koppelman and others 2010). Our study also has shown smaller peptides were generated during the digestion process. Another study described that the digestion of Ara h 1 with low pepsin concentration and found that there were no associated peptides of Ara h 1 by chromatography. They found that peptides of relatively high molecular weight, half the mass of the Ara h 1, were detected on the chromatograms (van Boxtel and others 2007). The absence of any band on the Western blot could be explained as Ara h 1 was degraded rapidly or the loss of immunoreactivity of Ara h 1 after limited digestion. It was shown that the digestion of pepsin leads to Ara h 1 degradation in a very short time.

This study also detected the changes of Ara h 3 after pepsin digestion. The SDS-PAGE results of raw and roasted peanut samples indicated that the 37 kD area was much thicker than normal. A study investigated the changes of purified Ara h 3 after pepsin digestion. Their results showed that the acidic and basic subunits were still associated by a disulfide bridge and the molecular weight was 70 kD (Piersma and others 2005). However, our results did not show the big molecular bands on the gel. Another study conducted by van boxtel and others (2008) analyzed the size excluded proteins chromatography under denaturing conditions. The results demonstrated that the majority of the Ara h 3 was found in the range of 7-14 kD. After 60 min digestion, the majority of molecular weights were centralized at less than 7 kD (Schmitt and others 2004). This explained why the longer digestion samples have more protein fragments in

in the range less 25 kD. The IgE immunoreactivity of these protein fragments were not shown on the Western blot. However, the 37 kD (Ara h 3) bands were clearly appearing before a 20 min digestion. This may be due to the destroyed epitopes after the digestion process.

In contrast to Ara h 1 and Ara h 3, Ara h 2 was more stable (Figure 6-2, Figure 6-3 and Figure 6-4) except for the PL treated samples. The pepsin and trypsin/chymotrypsin digestion results demonstrated that Ara h 2 were not reduced in roasted peanut samples after both pepsin and trypsin/chymotrpsin digestion. Ara h 2 was markedly reduced after 2 min pepsin digestion and had a complete reduction at 5 min digestion of raw peanut sample. However, the trypsin/chymotrypsin digestion of raw peanut samples only had the Ara h 2 protein bands on the SDS-PAGE gel. The Western blot results showed that IgE immunoreactivity of Ara h 2 existed throughout the whole digestion of the raw samples except at the 90 min mark. Sen and others (2002) investigated the digestion of Ara h 2 and their results described a digestion-resistant peptide. This peptide is stable with minor differences at the N-terminal and/or C-terminal part. This shows that proteolysis is restricted by the Ara h 2 structure rather than the specificity of the applied proteases. Evidence from Thomas and others (2004) showed that intact Ara h 2 migrated on the SDS-PAGE as a single band of approximately 14 kD. However, it is known that Ara h 6 has the same molecular weight, but is characterized as having 2S albumin units (Suhr and others 2004; Koppelman and others 2005). The Ara h 2 bands of our results did not have migration on the SDS-PAGE gel and western blot membrane.

The pattern of Ara h 6 was very similar to Ara h 2. The molecular weight of Ara h 6 was approximately 15 kD based on SDS-PAGE and 14.98 kD from mass spectroscopy (Koppelman and others 2005). Ara h 2 and Ara h 6 are both 2S albumins with a high degree of amino acid identity (Kleber-Janke and others 1999; Suhr and others 2004; Koppelman and others 2005). Our results cannot differentiate Ara h 2 and Ara h 6 because we used crude peanut proteins. One study detected the changes of protein patterns and IgE binding with purified peanut allergens. They reported that Ara h 6 has a very similar pattern to the Ara h 2. The results also revealed that Ara h 6 disappeared with a rate faster than the larger isoform of Ara h 2. Their results described that Ara h 6 was substantially digested after only 1 min at the highest pepsin concentration. Fragmentation was observed at the lowest pepsin concentration and some Ara h 6 was intact after 30 min. Similarly to Ara h 2, the intramolecular disulfide brides of Ara h 6 maintained the digestion fragments as a single molecule. Although the digestion of Ara h 6 was more rapid than that of Ara h 2, a similar large peptide remained for the experiment even after 1 hour. Combined with our results, the bands of Ara h 6 and Ara h 2 overlapped. No bands migrated from our pepsin digestion results. In order to determine the protein bands around 15-17 kD, chromatography is needed for future studies (Koppelman and others 2010).

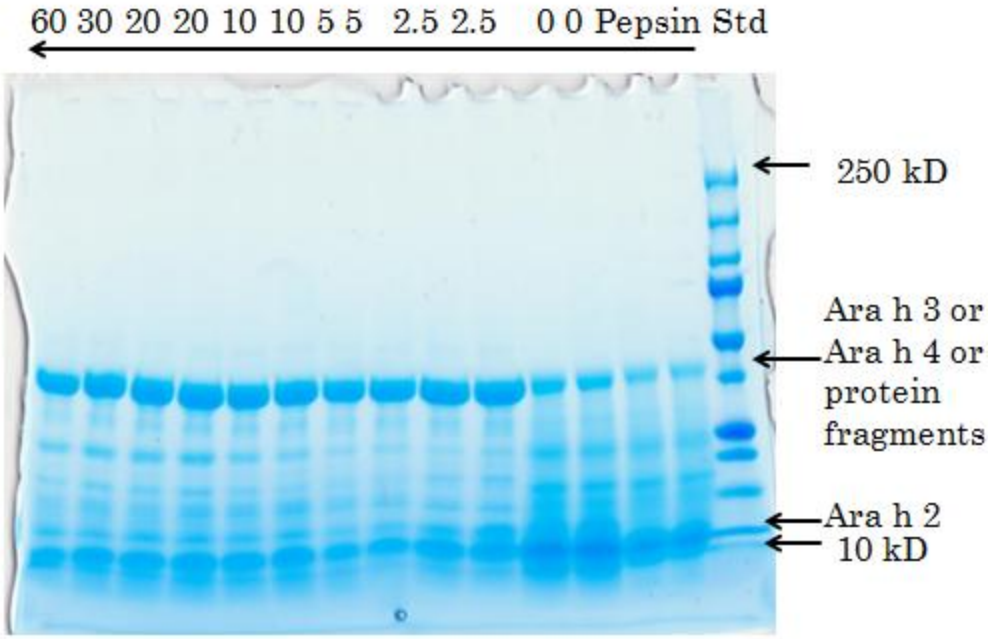
The trypin and α-chymotrypsin proteolysis effects were evaluated for the intestinal digestion. The results in this part revealed that only Ara h 2 and less than 10 kD proteins existed in the raw and roasted peanut samples. For the PL treated samples, there was no specific band, but smears were distributed through the whole gel. This phenomenon has been discussed in Chapter 3 and 4, which was due to the protein

cross-linkage and aggregation. Although Ara h 2 bands were not detected on the SDS-PAGE of PL treated peanut samples, the Western blot showed a strong signal of Ara h 2 and the IgE immunoreactivity of the smear throughout the whole gel at 0 min treatment. After the 0 min treatment, the intestinal digestion started and most of the IgE reactivity of protein fragments and bands disappeared. Sen and others (2002) performed digestion experiments with Ara h 2 using trypsin and found a peptide of Ara h 2 of similar size. Another study conducted in 2010 involved the application of trypsin on purified Ara h 2 digestion and their results matched the study Sen and others (2002) conducted (Koppelman and others 2010). Koppelman and others (2010) observed two bands in the molecular region of 9 kD and 4 kD after Ara h 2 and Ara h 6 were digested. On the SDS-PAGE of raw and roasted peanut samples, the 9 kD bands were observed. However, the Western blot did not show any signals on 9 kD bands. Our results matched the previous studies discussed above. Of course, pepsin has a different specificity to that of trypsin/chymotrypsin and all these results cannot be extropolated easily. There are many cleavage sites in the sequence of Ara h 2 and Ara h 6, but only a few of them are cleaved in real practice. This revealed that the 2S albumin structure may be a more important factor than the primary sequence.

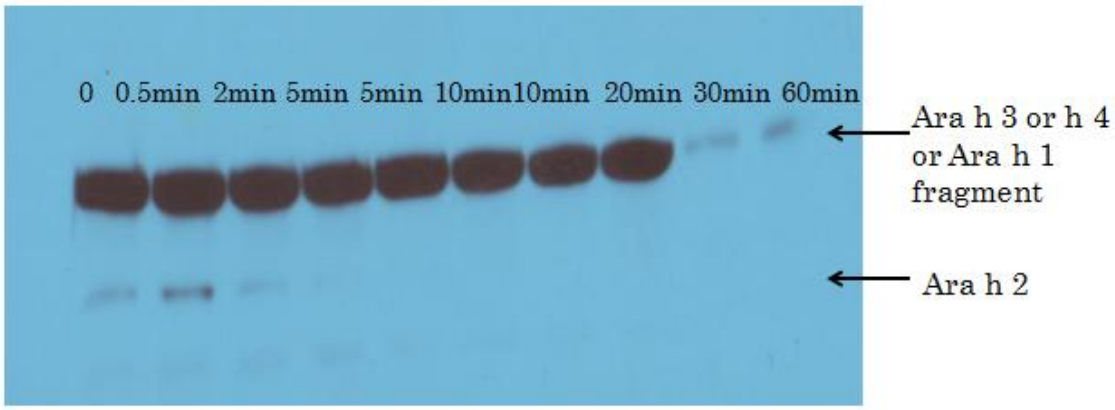
Summary and conclusions

By evaluating the potential gastric and intestinal digestibility of allergenic proteins of PL treated peanuts, it is concluded that Ara h 2 and Ara h 6 are more stable allergens than the other allergenic proteins in raw and roasted peanut extracts. However, all the allergens were reduced in PL treated peanut kernels after 60 min peptic digestion and 5 min intestinal digestion. Digestion-resistant peptides obtained after digestion of Ara h 2 may be responsible for prohibiting the complete digestion of Ara h 2. In the gastric

digestion study, the reduction effect was observed after 30 min digestion. In the intestinal digestion study, the reduction effect was observed after 10 min. Overall, PL reduced allergenic reactivity with human IgE of all the peanut allergens based on the *in vitro* digestion study. So far, all the evidence from Chapter 3 and 4 combined with this chapter have shown the potential of PL on peanut allergens inactivation.

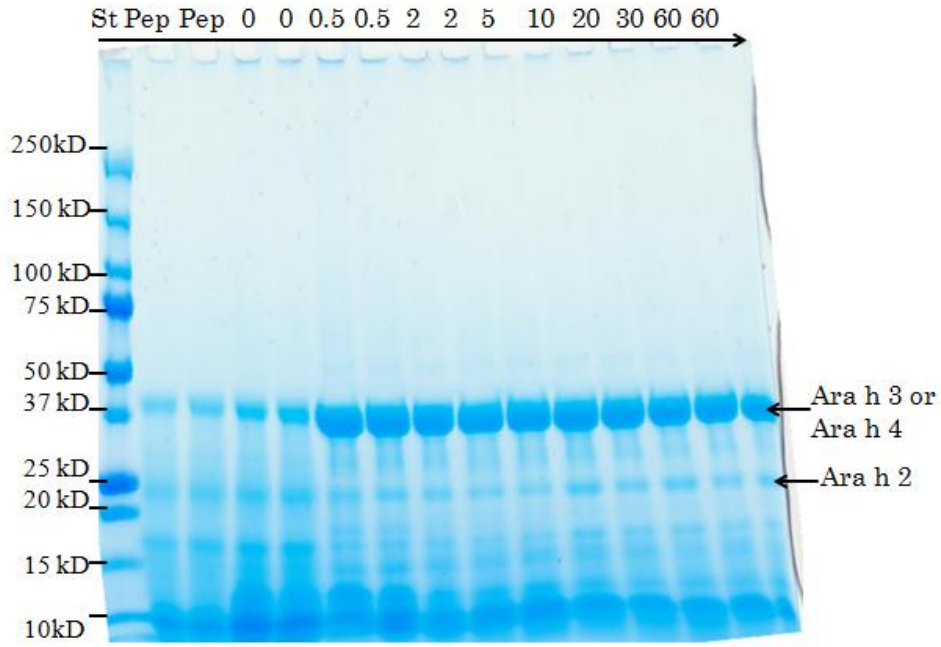


A)

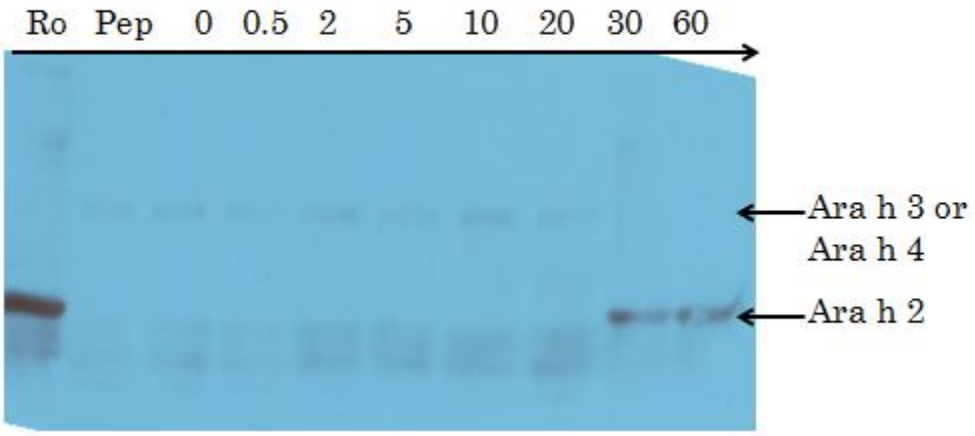


B)

Figure 6-1. Protein profiles and Western blot of pepsin digested raw peanut samples. A) SDS-PAGE of pepsin digested raw peanut samples. B) Western blot of pepsin digested raw peanut samples. Ara h 1 was hardly detected on SDS-PAGE, which was digested by pepsins. Western blot clearly showed that strong signals were detected before 30 min treatment. Ara h 2 reactivity was existed till 5 min treatment.



A)



B)

Figure 6-2. Pepsin digested roasted peanuts were determined by SDS-PAGE and Western blot. A) SDS-PAGE of pepsin digested roasted peanut. B) Western blot of roasted peanut digested by pepsin. This part showed a very strong IgE immunoreactivity signal through 0 min treatment to 60 min treatment. Ara h 2 had an increased IgE reactivity with a longer treatment.

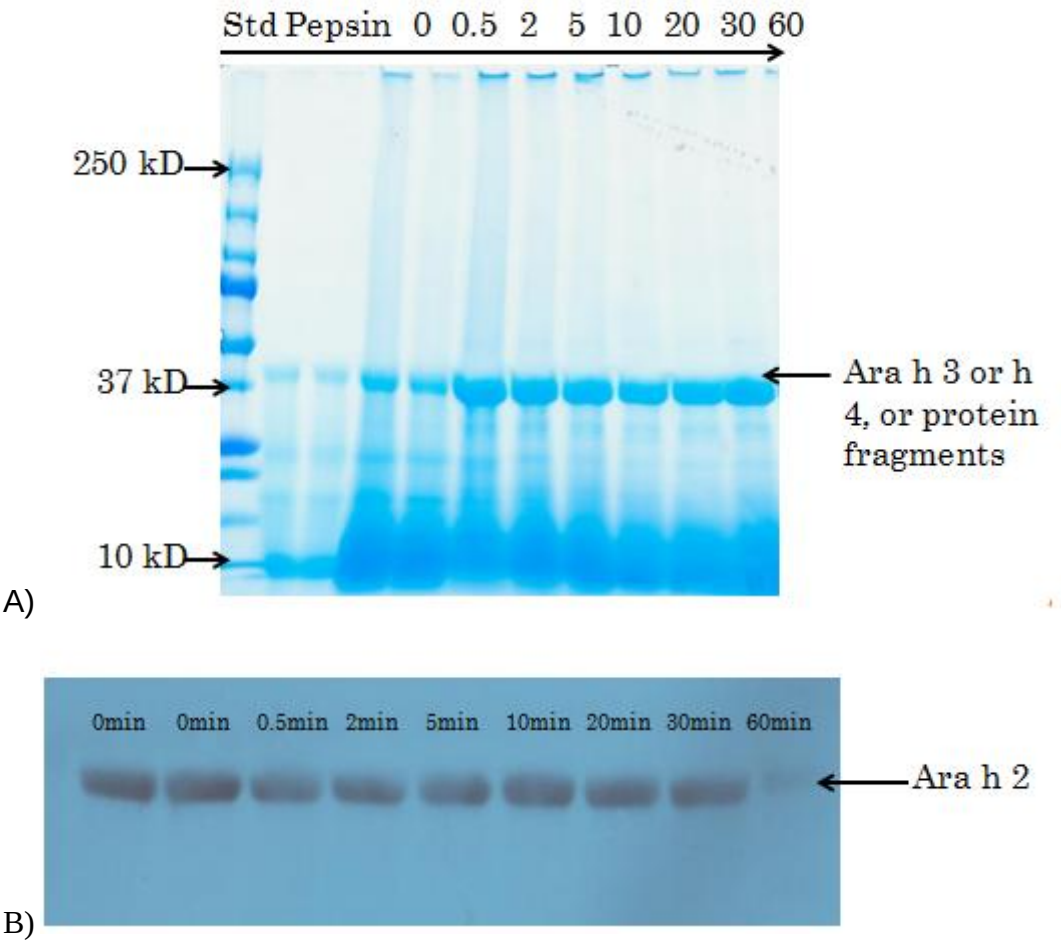
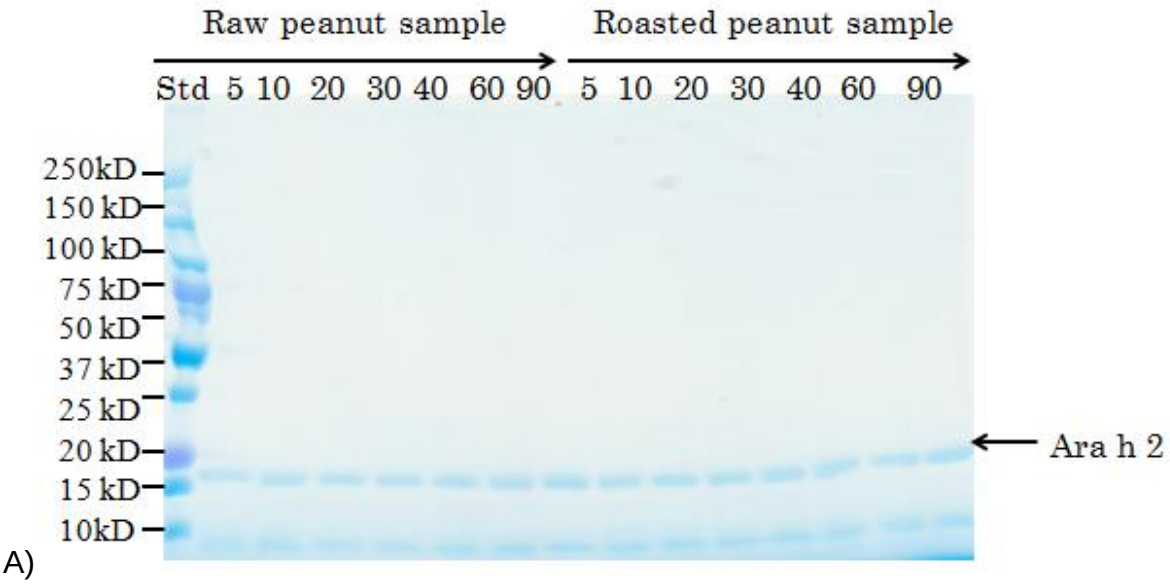
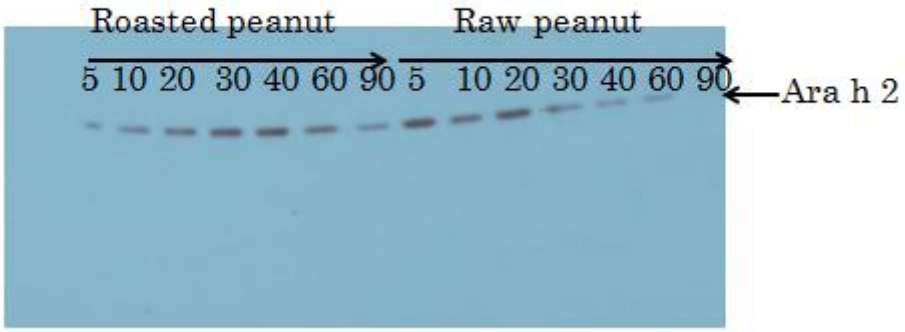


Figure 6-3. Pepsin digestion of PL treated peanut at 12 min. A) SDS-PAGE of pepsin digested PL treated peanut at 12 min. The smears appeared on the SDS-PAGE indicated that protein aggregates were formed during the PL illumination. B) Western blot of pepsin digested PL treated peanut at 12 min. It showed that the IgE immunoreactivity of Ara h 2 was greatly reduced at 60 min digestion.

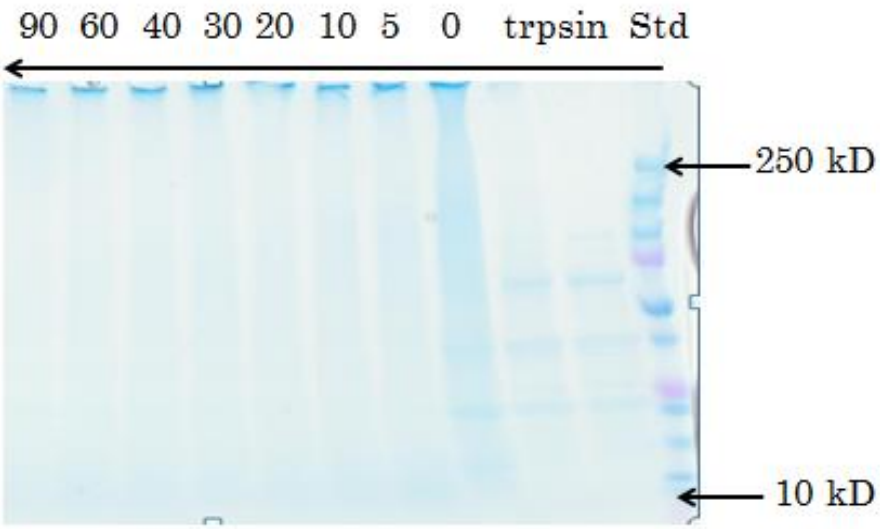


A)

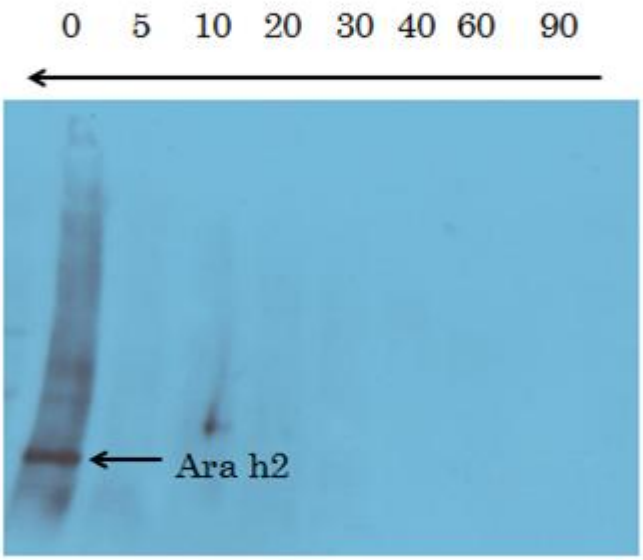


B)

Figure 6-4. SDS-PAGE and Western blot of mimic intestinal-digestion of raw and roasted peanuts. A) SDS-PAGE results. B) Western blot results. Most protein bands were not detected except 17 kD (Ara h 2). The western blot results indicated that the immunoreactivity of Ara h 2 was still strong in both raw and roasted peanut before 90 min treatment. 90 min treatment group has a reduction of IgE binding of Ara h 2.



A)



B)

Figure 6-5. SDS-PAGE and Western blot of mimic intestinal- digestion of PL treated peanut at 12 min. A) SDS-PAGE results. The smear appearing on SDS-PAGE at 0 min treatment was observed. With enzymatic digestions, the smears were gradually disappeared. B) Western Blot results. Western blot showed that the smears had the IgE reactivity through the whole gel. After 20 min treatment, the overall signals were hardly detected.

CHAPTER 7
EXAMINATION OF QUALITY CHANGES (TEXTURE, COLOR, ANTIOXIDANT CAPACITY, LIPID OXIDATION) OF THE WHOLE PEANUTS BEFORE AND AFTER PL TREATMENT

Chapter 7 examines the quality of PL treated peanut kernels. The texture, color changes, moisture, total phenolics, and lipid oxidation of treated kernels were determined. The Chapter 7 aims at evaluating the edibility and nutrients of PL treated peanuts, which are important for potential future production of allergen-reduced or hypoallergenic peanuts.

Excessive moisture loss was observed in previous treated protein extracts of peanut and shrimp during PL treatment. Because of the infrared light portion of PL, the temperature will increase during PL treatment, even for a short time, such as 3 min (Yang and others 2012). As we observed when the kernels were treated in Chapter 1, the surface temperature readings could reach as high as 120 °C. The moisture loss and temperature increase are the reasons for texture change after PL treatment.

UV light makes 54% of pulsed light (Krishnamurthy and others 2007). Both UV and ultrasound have been demonstrated that they can increase the concentrations of antioxidants and polyphenolic compounds (Sales and Resurreccion 2010). The reason for the polyphenols increase is because of increased enzymes that are responsible for the biosynthesis of secondary metabolites, such as flavonoids (Cantos and others 2000). In recent years, phenolic phytochemicals in foods have received increased interests from consumers and researchers because of their antioxidant activity and health benefits. In addition to being important from a nutritional perspective, the antioxidant capacity is also important for food stability, as oxidation often leads

unacceptable flavors, colors, or loss of nutrients (StAngelo 1996; O'Keefe and Wang 2006).

Peanuts are rich in energy and are high in oil and protein content, but with a low percentage of carbohydrates and ash (Stangelo and others 1979). Peanut contains around 50-55% oil, 30-35% linoleic acid, and 45-50% oleic acid (Coupland and McClements 1996). Lipid oxidation occurs during storage of peanut products and contributes to undesirable flavors in peanuts. The oxidation reactions could produce numerous aliphatic aldehydes, ketones, and alcohols (Bett and Boylston 1992). Finally, off-flavors such as oxidized, cardboard, and a painty taste will increase in peanut products (Gills and Resurreccion 2000; Grosso and Resurreccion 2002). This process can be accelerated at higher temperatures, such as deep-fat frying, with increases in free fatty acid and polar matter contents, foaming, color, and viscosity (Coupland and McClements 1996). As PL technology increases the surface temperature of treated food samples, we speculate that PL may increase the lipid oxidation of treated peanut kernels.

Chapter 7 will focus on the color, texture, total phenolics, and lipid oxidation for PL treated peanut kernels.

Material and Method

Sample preparation

The PL treatment on the peanut kernels was the same as the Chapter 5.

Texture Analysis

The conventionally roasted peanut kernels, which were used as control, were manually split into halves. Both the PL and conventionally roasted half peanut kernels were subjected to a TA.XT Plus Texture Analyzer from Stable Micro System

(Godalming, UK) with a 2 mm diameter probe of T – 52 model. This instrument was equipped with a 50-kg load cell. The data were recorded and analyzed by the Exponent Stable Micro System TEE 32v 4.0.8.0 software. Each half peanut kernel was oriented with the germ side down. The probe was placed in the center of the peanut kernel. The probe was set up to travel 2 mm distance into the kernel at the test speed of 2 mm / s. Maximum force during penetration and withdrawal of the needle from the peanut kernel was measured to evaluate the hardness of the peanut kernel. Each peanut sample had 15 kernels. A deformation curve was obtained from the analysis, which showed a major, sharp peak that produced the maximum force for each peanut kernel. The characteristic of hardness (kg) was compared between the PL roasted peanut and control using the maximum force data.

Color Analysis

To quantify the color changes, a machine vision system consisting of a Nikon D 200 digital camera housed inside a light box [42.5cm (W) x 61.0cm (L) x 78.1 cm (H)] (Wallat and others 2002) was used to measure the color of peanut kernels with a D65 (daylight) lamp and 10° observer angle. Each image was calibrated against a yellow color reference tile (L=87.09, a=7.71, b=70.75) that was obtained from the Lab Sphere X-Rite Company (North Sutton, NH). The software LensEyeSK® v10.0.0 (Engineering and Cyber Solutions, Inc., FL) was applied to collect and analyze the images. To obtain precise calibration, the initial image was obtained using the yellow-round reference tile that was then converted to a rectangular (~150 x 95 pixels) processed image that was without interference from the black border of the yellow tile. Then, the processed images were subjected to background corrections to produce the corrected images. The final corrected images were calibrated with a standard yellow color (Lref, aref, bref). The

L, a, and b values from the Hunter color system were recorded individually for each peanut sample. The color analysis was conducted in triplicate for each sample. The Hunter L, a, and b color space is organized in a cube form. The maximum L is 100 (white), the minimum L is 0 (black). Positive “a” stands for red. Negative “a” stands for green. Positive b stands for yellow, negative b stands for blue. The coordinates of three color parameters were expressed using C* (chroma), h° (hue angle), and total color difference ΔE using Eqs. 7-1, 7-2 and 7-3.

$$C^* = \sqrt{(a^2 + b^2)} \quad \text{.....(7-1)}$$

$$h^\circ = \tan^{-1}\left(\frac{b}{a}\right) \quad \text{.....(7-2)}$$

$$\Delta E = \sqrt{\left(L_{ref} - L_{sample}\right)^2 + \left(a_{ref} - a_{sample}\right)^2 + \left(b_{ref} - b_{sample}\right)^2} \quad \text{.....(7-3)}$$

Total Antioxidants Capacity Evaluation

Extraction of total antioxidants

The methods for the extraction of hydrophilic and lipophilic antioxidants were followed by (Prior and others 2003) and (Wu and others 2004). The ratio of sample to solvent was 1:20 and three extractions were conducted to facilitate extraction of lipids from peanuts by mixing 0.125 g of grounded peanuts with 2.5 ml hexane/dichloromethane purchased from Fisher Scientific 1:1 v/v (Fair lawn, NJ, USA) in a 50 ml glass tube (Fisher Scientific, Fair Lawn, NJ). The mixture was vortexed for 1 min and sonicated for 10 min. Samples were centrifuged at 24,000g for 15 min at 4 °C to obtain a stable residue. All hexane extracted was combined in another glass tube and then evaporated under nitrogen in a dry block heater at 30 °C. The dried hexane extract was used for L-ORAC assay. The residue was evaporated under vacuum conditons to

remove hexane and then 5 ml of acetone-water-acetic acid (AWA) solution containing 70 ml acetone, 29.5 ml water and 0.5 ml acetic acid was added. The mixture was vortexed for 2 min. The samples were held at room temperature for 10 min with occasional shaking and then centrifuged as described above. The AWA extract supernatant was transferred to a test tube and used in the H-ORAC assay. This assay was done under yellow light.

Hydrophilic-oxygen radical absorbance capacity (H-ORAC) assay

The H-ORAC procedure was followed by Prior and others (2003). Assays were prepared in Costar polystyrene flatbottom black 96 microwell plates (Corning; Acton, Massachusetts).

A sodium salt solution of Fluorescein (Reidel-deHaen; Seelze, Germany) was prepared freshly at a final concentration of 70 nM in 75 mM phosphate buffer. Trolox (Aldrich; Milwaukee, WI) standards were prepared from 50 to 3.12 µM in phosphate buffer. 2,20-azobis (2-amidino-propane) dihydrochloride (AAPH) (Wako; Richmond, VA) was prepared freshly at a final concentration of 153 mM in phosphate buffer before the actual usage. Fluorescence was measured using Spectra Max Gemini XPS microplate reader (Molecular Devices, Sunnyvale, CA). The fluorescence was monitored at 485 nm excitation and 530 nm missions for 40 min at 1 min intervals.

The reaction was conducted in 75 mM phosphate buffer at pH 7.4 with a final reaction volume of 250 µl. Hydrophilic extracts were diluted using phosphate buffer and fell within the linear region of the Trolox standard curve. Diluted sample extracts and standards, both at 50 µl, was added to the wells followed by 100 µl of the fluorescein solution. Each sample was subjected to triplicate runs. The solution was rapidly added with a multi-channel pipette. The plate containing only the samples and standards +

fluorescein was incubated in a plate reader for 10 min at 37 °C. 50 µl of the AAPH solution was then rapidly added via a multi-channel pipette during the incubation. Prior to the first measurement, samples were shaken for 5 s using a medium orbital intensity. The antioxidant capacities of the extracts were expressed as mmol TE/L of peanut samples.

Lipophilic-oxygen radical absorbance capacity (L-ORAC) assay

The L-ORAC procedure was adapted from (Prior and others 2003). Solutions of 7% pharmaceutical grade randomly methylated beta cyclodextrin (RMCD) purchased from Trappsol®; CTD, Inc. (High Springs, FL, USA) was prepared in 1:1 acetone/water (7% RMCD). Trolox standards were prepared from 200 uM to 1.56 µM in 7% RMCD. Lipophilic extracts were diluted using 7% RMCD (1:25) to fall within the linear region of the Trolox standard curve. Fluorescein was prepared at a final concentration of 21.5 nM in 75 mM phosphate buffer. AAPH was prepared freshly with a final concentration of 77 mM in phosphate buffer immediately prior to usage. 25 µl of diluted sample extracts and standards were added to the wells in triplicate followed by 120 µl of the fluorescein solution as quickly as possible. Then, the mixture was incubated in the plate reader for 15 min at 37 °C. 80 µl of the AAPH solution was rapidly added by using a multi-channel pipette. Prior to the first measurement, samples were shaken for 5 s using a medium orbital intensity. The antioxidant capacities of the extracts were expressed as mmol TE/L of peanut samples.

Lipid Oxidation

For this part, fresh raw runner type peanuts were directly harvested from the field and stored at 4°C and 10% moisture. These fresh raw peanuts were considered to be absent with lipid oxidation occurrence, but the conventionally roasted peanut had a high

percentage of lipid oxidation (Win and others 2011). These peanut samples were provided by Dr. Barry Tillman in the Agronomy Department, University of Florida.

Peroxide value

2 g of raw peanut samples or PL treated peanut samples were mixed with 6 ml of chloroform methanol (1:1 v/v) solution. The mixture was homogenized using a homogenizer from the ESGE Ltd. (Mettlen, Switzerland). Each sample was duplicated at this time. Then, 7 ml of NaCl (0.5%) was added and vortexed for 30s and followed by centrifugation at 2000 rpm for 10 min in cold room (4°C). After centrifugation, 2 ml of the chloroform layer was taken by a syringe (Sigma, St. Louis, MO). Fresh ferrous solution was prepared by adding 0.2 g barium chloride in 25 ml 0.4 N HCl (kept cold and stable). At the same time, 0.1 g ferrous sulphate hepta hydrate was dissolved in 10 ml distilled water. Next, 3 ml of each solution was taken using a pipette and centrifuged for 3min. The supernatant was taken immediately for use. The next step was to add 50 µl of ferrous solution and ammonium thiocyanate solution (7.5 g ammonium thiocyanate in 25 ml distilled water). Finally, the mixture was incubated for 10 min at RT. The color was developed and recorded by 415 Spectro Master Spectrophotomete produced by Fisher scientific (Drive Hudson, NH, USA) at 500 nm.

Thiobarbituric acid reactive substances (TBARS) assay

TCA extracting solution was prepared with 75 g trichloroacetic acid, 1 g propyl gallate, and 1g EDTA in 1 liter of distilled water. 2 g of peanut samples were mixed with 5 ml TCA extraction solution. Each peanut sample was triplicate at this step. Then, the mixture was homogenized using a homogenizerfrom the ESGE Ltd. (Mettlen, Switzerland) for 30 s. The mixture was then filtered by Whatman #1 filter paper. At the same time, TBA solution was prepared using 2.89 g of thiobarbituric acid in 1L distilled

water. Another glass tube with 2 ml TBA solution was prepared and 2 ml of filtrate was vortexed for 10 s. The tube was incubated in a water bath at 100 °C for 40 min. The data were recorded at 532 nm using a 415 Spectro Master Spectrophotomete manufactured by Fisher Scientific (Drive Hudson, NH, USA).

Results and Discussion

Texture Analysis

The mean maximum force (hardness) recorded during the compression tests of both PL-treated and dry roasted samples are shown in Table 7-1. The roasted peanuts were used as control over raw peanuts, since the PL-treated peanuts were also roasted due to the photo-thermal effect of PL and may serve as the starting material to manufacture peanut butter like the dry roasted peanuts. For the dry roasted peanuts, the mean maximum force value was 1.76 ± 0.38 kg.

At 10 cm distance from lamp, the peanuts PL treated for 5 min, 6 min, and 7 min exhibited a significant difference ($p < 0.05$) in hardness compared to control, with the maximum force values of the former higher than that of the latter. However, the 8-min PL treated peanut had a similar hardness to the roasted peanut ($p > 0.05$). The hardness of PL-treated peanut sample at 8.5 min was also not statistically different from that of the roasted peanut ($p < 0.05$), although the former showed a higher compression force value (i.e., 1.83 kg vs. 1.76 kg). The hardness value (1.52 kg) at 9.5 min duration, which was representative of nearly burnt peanut kernels, was statistically different from that of the roasted peanut (1.76 kg), because the burnt peanut kernels tended to soften its texture (Kita et al., 2006). For the data in Table 7-1, it can be seen that for shorter PL durations (e.g., 5, 6 and 7 min vs. 8 and 8.5 min), the peanuts were not roasted enough yet by PL as evident by the significant difference ($p < 0.05$) in their hardness compared

to control, because under-roasting would generate a harder texture, while well-roasted peanuts exhibit softer texture (Kita and others 2006).

At 7 cm distance from lamp, both PL durations of 5 min and 6 min showed a significantly ($p < 0.05$) higher hardness value (2.2 kg and above) than that of the roasted peanut (1.76 kg). At the 6.5 min mark, the texture was shown to be statistically similar to that of the roasted peanut ($p < 0.05$). Longer treatment times beyond 6.5 min significantly reduced the hardness of PL treated peanuts (e.g., 1.40 kg), as shown in Table 7-1. Similarly, the significantly lower hardness value at 7 min corresponded to a texture of nearly burnt peanut kernels that tended to be softened as mentioned earlier.

Temperature and Moisture Changes During PL Illumination

During roasting, proper moisture reduction helps produce a crispy texture. If the nuts are heavily roasted, the sugars in the nuts can decrease with an increased temperature. Therefore, higher temperatures and longer periods of roasting can decrease the quality of the nuts (Ozdemir and Devres 1999). A study conducted by Kita and others (2006) indicated that decrease in moisture can directly affect the mechanical properties (e.g., hardness) of roasted nuts. Roasting at higher temperatures for the same time could reduce the hardness of walnuts, but extended roasting time did not show any significant effect on their hardness (Kita and Figiel 2006). Demir and others (2004) found that both temperature and time had strong effects on hazelnut texture.

Due to the photothermal effect of PL illumination, which can cause a pronounced instantaneous temperature rise (Li and others 2011) and can also be observed in Table 7-2, a similar roasting effect on the peanut kernels to that of the conventional dry roasting was noticeable after PL treatments. The surface temperature of PL treated peanuts after 5 min exposure at both 10 cm and 7 cm distances from lamp was 35.1 ±

1.8°C and 50.3 ± 1.1°C, respectively (Table 7-2). The surface temperature for the maximum exposure times, 9.5 min for 10 cm distance to lamp and 7 min for 7 cm distance to lamp, were 116.0 ± 2.1°C and 108.6 ± 4.6°C, respectively (Table 7-2). Comparison of the temperature rises in Table 7-2 between 7 cm and 10 cm distances to lamp showed that shorter distance generated great photo-thermal impact during illumination given the same duration. Previous studies by Krishnamurthy and others (2008) have also shown that reduction of microorganisms is proportional to treatment time, but it is inversely proportional to the sample's distance from lamp.

It is worth mentioning that the temperatures in Table 7-2 were detected with a 5 - 10 s delay due to the time required to remove the sample from the chamber before temperature measurement by the handheld infrared thermometer. So, the instantaneous temperature of the sample during the PL treatment could be higher than the surface temperatures recorded. This inference was supported by the nearly burnt appearance of the peanuts after 9.5 min PL treatment at 10 cm distance to lamp or after 7 min exposure at 7 cm distance from lamp and their surface temperatures of around 110°C as shown in Table 7-2. The temperature 110°C was below the smoke point of peanut oil (188°C to 198°C) (Morgan, 1942) and should also be lower than the smoke point of the whole peanut kernels, so this temperature was not sufficient to burn the peanut kernels. It is thus reasonable to believe that the instantaneous temperature of the peanuts after 9.5 min PL illumination at 10 cm distance to lamp or 7 min exposure at 7 cm distance from lamp must have exceeded 188°C.

Moisture loss percentages during PL treatments are also shown in Table 7-2. Traditional dry roasted peanuts gave a moisture loss around 3% with uniform heating at

165°C for 15 min. The PL-treated peanuts at 10 cm distance to lamp resulted in a moisture loss ranging from 1.4% - 5.6% for 5 – 9.5 min PL durations. The samples at 7 cm distance to lamp caused a moisture loss ranging from 2.0% - 8.8% for 5 – 7 min Pl durations. The higher moisture loss encountered in the samples at 7 cm distance to lamp, given the same time, was probably due to higher instantaneous temperatures incurred in the peanut kernels.

Color Analysis

The values for the color dimensions, i.e., L, a, b, h°, ΔE, and C*, for the PL-treated and dry-roasted peanuts are shown in Table 7-3. Since most of the area of the burnt peanuts was charred, the samples related to 9.5 min in the 10 cm group and 7 min in the 7 cm group were not entered for color analysis. The ANOVA was carried out to determine the statistical difference of each color dimension, and Duncan's multiple range tests at 95% significance level was used for separating the means.

The L* values indicate the lightness of the processed peanut samples. Statistical analysis showed that the dry roasted peanuts had a similar L value ($p>0.05$) to that of 7 min and 8 min PL samples at 10 cm distance to lamp (Table 7-3). Below 7 min, the PL-treated peanuts exhibited lighter color than the dry roasted peanuts, while above 8 min, the PL-treated peanuts exhibited darker color than the dry roasted peanuts. However, for the 7 cm distance group, PL treated peanuts at 5-6.8 min all showed a significantly ($p<0.05$) darker color than the dry roasted peanuts.

The a* value parameter, which indicates the rate of red color of processed peanuts, was found to be statistically similar between the PL-treated peanuts for 8.5 min at 10 cm distance to lamp and the dry roasted peanuts ($p>0.05$). For all other PL durations, the a* value of PL-treated peanuts was significantly different ($p<0.05$)

compared to that of the dry roasted peanuts. In general, extended exposure and shorter distance to lamp produced a more red color on the sample.

The b* value parameter indicates the rate of yellowness of the sample. The dry roasted peanuts had a similar ($p>0.05$) yellow color to that of the PL-treated peanuts for 8.5 min at 10 cm distance to lamp. Other PL-treated samples in the 10 cm group were significantly different ($p <0.05$) compared to the dry roasted peanuts. Overall, less exposure time and longer distance produced less yellow color and vice versa. For 7 cm distance to lamp, the yellowness of dry roasted peanuts was statistically similar ($p>0.05$) to that of PL-treated peanuts at 6 and 6.8 min, but statistically different ($p<0.05$) from that of all other PL treatments. However, for the yellowness, the difference between the roasted and PL-treated samples was not large in general.

The C* and ΔE values measure the saturation and total color differences from the yellow color reference, respectively. Hue angle ho was used to express the true color of processed peanut samples. Results showed that the total color difference (ΔE) of the PL-treated peanuts for 8 min at 10 cm distance to lamp was similar ($p>0.05$) to that of the dry roasted peanuts. However, none of the PL samples at 7 cm distance to lamp had similar ΔE to dry roasted peanuts, with the former appearing to be slightly lighter than the latter. Most hue angle h_o values of the PL-treated peanuts at both distances to lamp were similar to that of the dry roasted peanuts ($p>0.05$), except for 5 min at 10 cm distance to lamp. The C* value of the dry roasted peanuts was similar ($p>0.05$) to that of the 8.5 min PL-treated peanuts at 10 cm distance to lamp and also similar to that of 6 min, 6.5 min and 6.8 min PL-treated samples at 7 cm distance to lamp.

Overall, the color results suggested that the PL treatment as a potential roasting technique was capable of producing similar color to that of dry roasted peanuts if we set an appropriate combination of PL duration ranging from 5 min to 8.5 min and sample distance to lamp ranging from 7 cm to 10 cm inclusive. The color development of dry roasted peanuts is primarily a result of the Maillard reaction, which is an important non-enzymatic browning reaction in the development of flavor and color in peanuts. This Maillard reaction was also reported to take place during the PL processing of foods (Maleki and others 2000a).

Antioxidant Capacity

H-ORAC results were shown in Figure 7-1. Roasted and raw peanuts were considered as controls. The mean separations were analyzed by tukey HSD. The results indicated that roasted peanut and 10 min treatment groups were not significantly different ($p > 0.05$). Roasted peanuts contained 24888 ± 3513 μmoles trolox/100g and the 10 min treatment group had 24864 ± 339 μmoles trolox / 100g. Raw peanuts were significantly lower ($p < 0.05$) in H-ORAC than other samples. The TE value of raw peanut was 8870 ± 5230 μmoles trolox / 100g and three times lower than the roasted peanuts. The 12 min treated peanut was significantly higher ($p < 0.05$) than the roasted peanut. The TE value of 12 min treated peanut was 63830 ± 5841 μmoles tolox / 100g and it was 2.6 times higher than the roasted one. Although the 15 min treated peanut was also significantly higher ($p < 0.05$) than both the roasted peanut and 12 min treated peanut samples, the 15 min was not edible and the color had turned black. Overall, hydrophilic antioxidants capacity was significantly increased ($p < 0.05$) after PL treatment.

L-ORAC results described lipophilic antioxidant capacity in raw/roasted peanuts and PL treated peanuts (Figure 7-2). Tukey HSD was applied for statistical analysis. Raw peanut samples had 50393 ± 10 μmoles trolox / 100g, which was significantly lower than other peanut samples. The 12 min treatment group contained 80593 ± 14 μmoles trolox / 100g and it was significantly higher than the raw peanut samples, 10 min, and 11 min treatment groups.

Overall, the L-ORAC and H-ORAC results of raw and roasted peanuts matched the results that were conducted by Davis and others (2010). Another study also demonstrated that UV light could increase total antioxidant capacity in treated peanut about three times more than the raw peanuts (Sales and Resurreccion 2010). The UV light effect on increasing antioxidant capacity was also observed in blueberries with a dose of 2.15 and 4.30 kJ/m^2 (Wang and others 2009). Conventional roasted peanuts also has increased total antioxidant capacity (Davis and others 2010), which agrees with our results.

Lipid Oxidation

The peroxide value of PL treated peanut samples and the control (raw) is presented in Figure 7-3. The data was plotted from 0 day to 10 days, to partially simulate shelf life conditions, at room temperature. Cumene hydroperoxide (CPO) was used as a standard to calculate the treated samples. The results were analyzed by tukey HSD using JMP 10.0. The shelf life of 10 days clearly showed a significant increased peroxide value ($p < 0.05$) of all the samples. Raw peanut was the lowest one since we used fresh samples. The 15 min sample was the lowest one among the PL treated peanuts, but could not be accounted for accurate evaluation because the

primary oxidation step had passed. The 12 min sample was significantly more oxidized ($p < 0.05$) than 9 min treatment.

TBARS results are presented in Figure 7-4. In this figure, secondary oxidation trends of each sample were described. Tukey HSD was used for statistical analysis. All the PL treated peanuts showed a linear increase from 0 day to 10 days. The treatment at 15 min had the largest significant oxidation degree ($p < 0.05$) than other samples after the samples were collection following PL illumination. Both the peroxide value and the TBARS results demonstrated that a longer PL treatment resulted in higher oxidation values.

The peroxide value is an indicator of the initial stages of oxidative changes. This method could evaluate the total hydroperoxide content and is one of the most common quality indicators of fats and oil during the storage (Shahidi and Zhong 2010). During the lipid oxidation, malonaldehyde (MDA), a minor component of fatty acids, was produced. TBARS was used to evaluate the secondary lipid oxidation process (Ke and others 1984). Lipid oxidation produces the oxygen containing compounds, such as aliphatic aldehydes, ketones, and alcohols (Coleman and others 1994). In addition, free radicals, hydroperoxides, and secondary products formed during lipid oxidation can interact with nitrogen containing compounds, such as proteins and pyrazines, and modify the precursors for the Maillard reaction (Williams and others 2006). Lipids can be oxidized by enzymatic (lipoxygenase) and non enzymatic reactions, such as oxygen, light, metal ions (Kanner 1994). In peanuts, lipoxygenase acts as a catalyst to oxidize polyunsaturated fatty acids to form hydroperoxides, which then produce secondary products (Fennema 1996). Several non-thermal processing methods have been

explored on lipid oxidation of peanuts. Gamma radiation and pulsed electric field can also induce the lipid oxidation of peanut seeds, which shortens the shelf life of peanut oil and changes the quality of peanut seeds (Zeng and others 2010; de Camargo and others 2012). Although the 10-day study on the lipid oxidation was not long enough to warrant a bigger picture on the variation of the lipid oxidation in the PL treated peanut kernels, the current date from this study seemed to suggest that the peanuts roasted by the PL technique were prone to lipid oxidation, possibly due in part to more cell rupture occuring under the high instantaneous temperature during PL roasting. For future work, gas chromatography should be conducted for the profile analysis of oxidized lipids. A longer storage period is required to obtain the whole picture of lipid oxidation in the PL treated peanut kernels by both peroxide value and TBARS methods.

Table 7-1. Texture comparison between the dry roasted and PL treated peanuts

	Treatment	Hardness (kg)
	Dry roasted peanut (Control)	1.76±0.38[a]
PL treatment (10 cm)	5min	2.04±0.20[b]
	6min	2.06±0.15[b]
	7min	1.93±0.24[b]
	8min	1.74±0.30[a]
	8.5min	1.83±0.40[ab]
	9.5min	1.52±0.24[c]
PL treatment (7 cm)	5min	2.48±0.31[b]
	6min	2.20±0.38[b]
	6.5min	1.64±0.24[a]
	6.8min	1.40±0.12[c]
	7min	1.11±0.31[d]

Data are expressed as mean ± standard deviation (n=46). Means in the same column not followed by the same letter are significantly different at $p \leq 0.5$.

Table 7-2. Surface temperature and moisture loss of dry roasted and PL treated peanuts.

	Duration	Surface temperature (°C)	Moisture loss (%)
Dry roasted (Control)		160.0±2.1°C	3.0±0.5%
PL Treatment (10 cm)	5min	35.1±1.8°C	1.4±0.2%
	6min	48.7±2.3°C	2.6±0.3%
	7min	65.3±2.1°C	3.2±0.3%
	8min	98.5±4.5°C	4.4±0.4%
	8.5min	107.0± 3.7°C	4.6±0.5%
	9.5min	116.0±2.1°C	5.6±0.5%
PL Treatment (7 cm)	5min	50.3±1.1°C	2.0±0.2%
	6min	61.2±2.4°C	2.8±0.3%
	6.5min	88.3±2.8°C	4.0±0.2%
	6.8min	98.5±3.2°C	5.5±0.3%
	7min	108.6±4.6°C	8.8±0.4%

Peanut samples before treatment were at room temperature (23.1±0.3° C).

Table 7-3. Comparison of color between dry roasted and PL treated peanuts.

	Duration	Color Analysis					
		L*	a*	b*	ΔE	h°	C*
Control	Dry Roasted	64.57 ±2.57[a]	12.43 ±2.44[a]	35.39 ±0.83[a]	42.31 ±0.96[a]	1.23 ±0.05[a]	37.51 ±1.59[a]
PL (10 cm)	5min	74.00 ±1.08[d]	0.05 ±0.23[e]	19.78 ±0.90[d]	53.19 ±0.77[d]	0.00 ±1.71[b]	19.78 ±0.90[e]
	6min	68.04 ±1.08[bc]	8.20 ±0.98[d]	29.73 ±1.29[bc]	45.26 ±0.74[bc]	1.30 ±0.03[a]	30.84 ±1.38[cd]
	7min	66.26 ±0.83[ab]	7.65 ±0.89[d]	28.56 ±0.67[c]	47.07 ±0.54[c]	1.31 ±0.02[a]	29.56 ±0.83[d]
	8min	66.94 ±2.53[ab]	8.38 ±0.67[d]	31.69 ±1.31[b]	44.45 ±0.59[abc]	1.31 ±0.01[a]	32.78 ±1.40[bc]
	8.5min	59.52 ±0.87[ef]	14.38 ±1.71[ab]	35.24 ±1.76[a]	45.51 ±0.69[bc]	1.18 ±0.03[a]	38.05 ±2.24[a]
	9.5min						
PL (7 cm)	5min	69.91 ±2.16[c]	8.03 ±0.30[d]	28.42 ±1.58[c]	45.65 ±1.20[bc]	1.29 ±0.01[a]	29.53 ±1.55[d]
	6min	59.53 ±3.18[ef]	15.12 ±2.90[b]	33.74 ±2.60[a]	46.92 ±1.12[c]	1.15 ±0.05[a]	36.97 ±3.02[a]
	6.5min	58.20 ±2.61[ef]	14.65 ±14.08[b]	31.89 ±1.46[bc]	49.04 ±1.24[bc]	1.13 ±0.08[a]	35.09 ±1.11[a]
	6.8min	57.41 ±0.82[f]	15.91 ±1.78[bc]	35.12 ±2.98[a]	47.14 ±2.48[c]	1.15 ±0.01[a]	38.56 ±3.44[a]
	7min						

Data are expressed as mean ± standard deviation (n=46). Means in the same column not followed by the same letter are significantly different at $p \leq 0.05$.

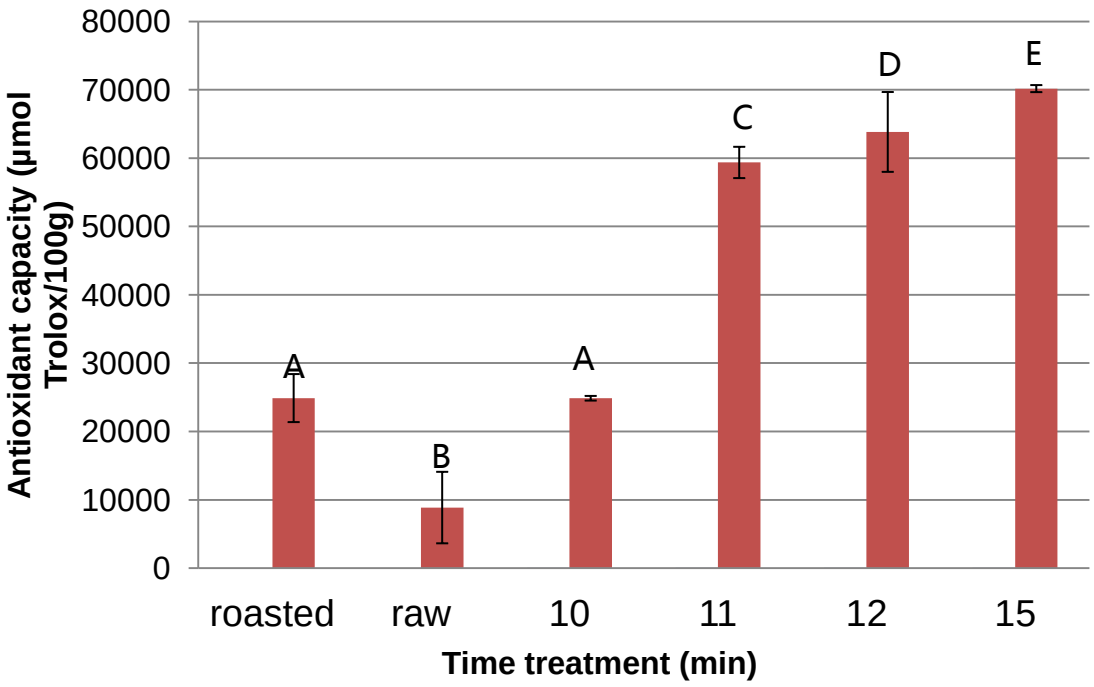


Figure 7-1. H-ORAC of PL treated peanuts. Roasted and raw peanuts were considered as controls. Tukey HSD was applied to analyze the means separation.

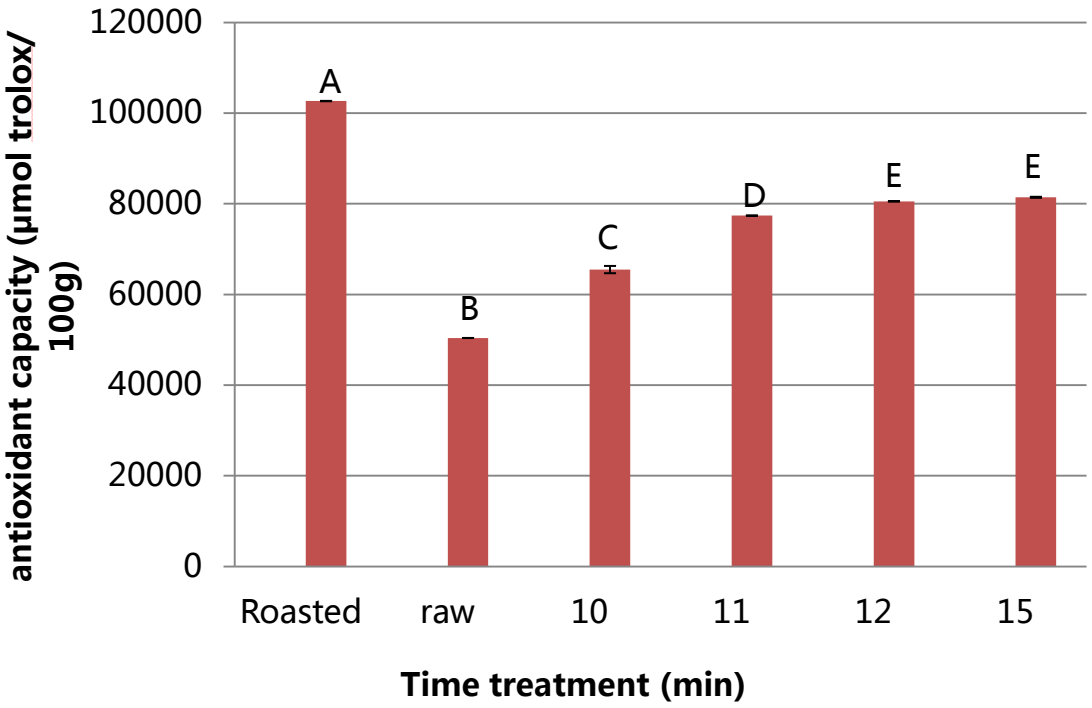


Figure 7-2. L-ORAC of PL treated peanuts. Roasted and raw peanuts were considered as controls. Tukey HSD was applied to analyze the means separation.

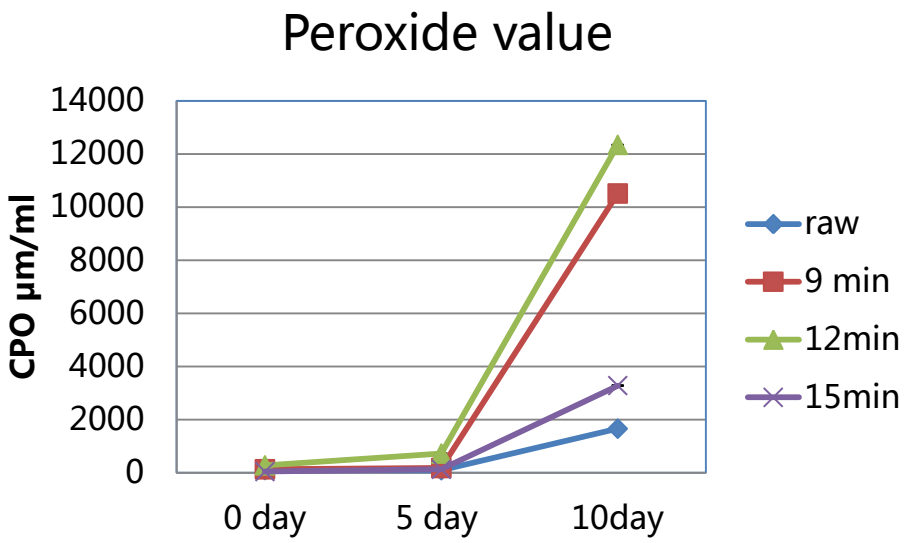


Figure 7-3. Peroxide value of raw peanut, PL treated peanuts at 9 min, 12 min, 15 min on 0 day, 5 days, and 10 days shelf life at room temperature. Y axis stands for CPO concentration. X axis stands for shelf life.

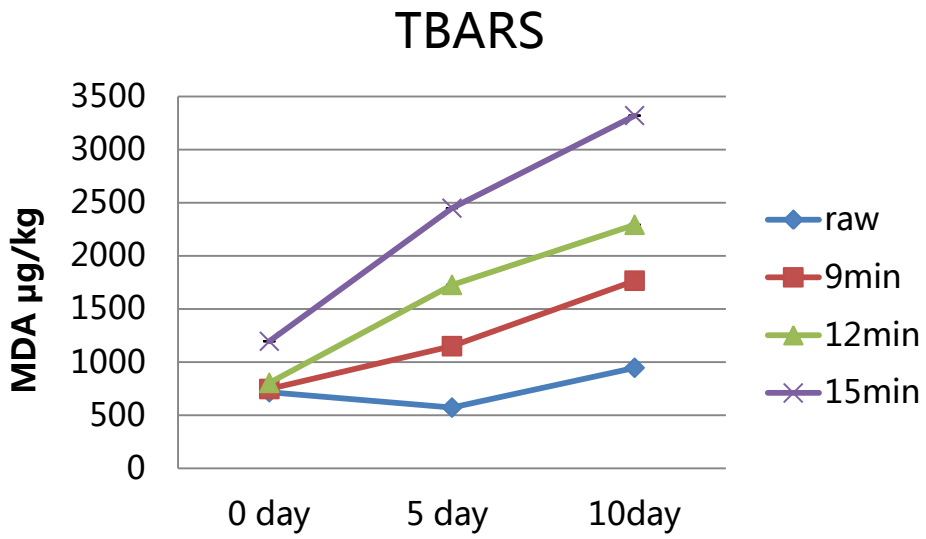


Figure 7-4. TBARS of raw peanut, PL treated peanuts at 9 min, 12 min, 15 min on 0 day, 5 days, and 10 days shelf life at room temperature. Y axis stands for MDA concentration. X axis stands for shelf life.

CHAPTER 8
OVERALL CONCLUSIONS

PL is considered a non-thermal technology that is widely used in food preservation. Our study has demonstrated that the extended treatment for allergen reduction could significantly increase the surface temperature of peanut kernels. Due to this phenomenon, this technology is not considered non-thermal technology, especially in extended treatments that last several minutes. The initial results demonstrated that PL was capable of reducing all major allergens of whole peanut kernels, including the polypeptide fragments degraded from Ara h 1- h 4 during PL treatments, to an undetectable level.

Due to the thermal effect of PL on the peanut kernels, the initial SDS-PAGE results clearly showed that protein macromolecules were formed during this process. To validate PL technology, insoluble fractions were examined using the Novex NuPAGE gel system. The Western blot of the denatured system showed that treatment below 5.5 min at 10 cm had the strongest IgE binding signals in the 250 kD to 460 kD or larger range. The Western blot results of the non-denaturing gel had similar results in that the IgE binding signal of 6.5 min treatment was removed completely, but before 6.5 min treatment, the immunoreactivity of IgE was clearly observed on the top area of the membrane. The overall IgE reactivity of the total protein extract of PL processed peanuts was shown with indirect ELISA. The signals of all PL treated samples were significantly lower than the raw samples. So far, all the results illustrate that PL treatment may reduce allergens in peanuts via *in vitro* immunoassay tests.

The PL treatment times, distance from the light source (also relates to the PL intensity), number of pulses, thickness of the sample, and duration of illumination are

considered to be critical parameters for the optimization of a PL system. Duration and distance are two major factors in this system. Factorial analysis demonstrated that these two factors interact. To optimize this equipment and obtain constant treatments, a distance of 10 cm was selected. Multivariate ANOVA analysis showed that the 12 min treatment was the best treatment time for generating edible roasted peanuts with lowIgE reactivity.

After the peanuts are swallowed and digested, the proteins undergo different enzymatic reactions and enter different pH environments. The structures of proteins will be affected. The *in vitro* digestion study that included pepsin, trypsin, and α-chymotrypsin revealed that PL treated peanut protein extracts have IgE reactivity throughout the whole gel under a pH of 2.0. Gastric digestion removed the immunoreacivity of IgE after a 90 min digestion of PL treated peanuts. For intestinal digestion, IgE reactivity throughout the whole gel under a pH of 8.3 was also observed. Moreover, the 10 min intestinal digesion showed that the IgE reactivity was completely removed from the PL treated peanuts. This study revealed that Ara h 2 and Ara h 6 were more stable allergens than other allergenic proteins in raw and roasted peanut extracts.

During the PL illumination, moisture loss had a positive correlation with temperature and a hard texture was produced during this illumination. Overall antioxidant capacities were significantly higher in 12 min treated peanuts than the raw, roasted, 10min, and 11min treatment groups. Also, PL could initiate the lipid oxidation occurrence.

In summary, PL can notably reduce the IgE reactivity to peanut allergenic proteins based on *in vitro* testing. To obtain final validation of this technology, a clinical trial is still required. The 12 min treatment group at 10 cm provided the best quality as compared to conventional roasted peanuts. This provides insight for commercial production of hypoallergenic peanuts and peanut butter.

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BIOGRAPHICAL SKETCH

Xingyu Zhao was born in Shijiazhuang, Hebei, China. She obtained her bachelor's degree of biotechnology in Department of Biological Science, North China Coal Medical College in 2008. She started doctoral program in Department of Food Science and Human Nutrition at University of Florida in June 2010 and graduated in December 2013.