THERMAL AND ELECTRIC FIELD EFFECTS ON PEANUT PROTEIN USING MOLECULAR MODELING AND *IN-VITRO* DIGESTIBILITY

BY

SAI KRANTHI KUMAR VANGA

Department of Bioresource Engineering

Faculty of Agriculture and Environmental Sciences

McGill University

Sainte-Anne-de-Bellevue, Quebec, Canada

December 2014

A thesis submitted to the McGill University in partial fulfillment of the requirements of the degree of

Master of Science

In

Bioresource Engineering

ABSTRACT

Food is a complex admixture of various components which are subjected to several processing techniques including thermal, chemical, mechanical, high pressure and irradiation treatments. These techniques are used not only to increase the variety (e.g. Milk – Yogurt, Cheese, Butter, Cream etc.,), but also to improve the shelf life (e.g. pasteurization, boiling, sterilization) by decreasing or inactivating the microbes, endogenous enzymes and toxins in food. Extraction of phytochemicals like antioxidants, and modifications in the component properties like foaming, digestibility etc., can also be obtained by applying various processing methods available today. Proteins are one of the major constituent present in food and are very sensitive to the external stress as caused due to the aforementioned processing methods. Numerous studies have shown that proteins under external stress undergo conformational changes in their structure which also influences their functional properties.

Though extensive studies have been performed for understanding the involvement of proteins in the immunoreactivity causing allergic reactions in humans, the understanding of this concept at an atomic and molecular level is very limited. This is one of the important aspects that has to be addressed, helping the industries to develop processing techniques for engineering food products with minimal reactivity in human body and decreasing the number of people experiencing anaphylaxis and other health issues from food allergies. Thus, comprehending this relationship between the protein functionality and the external stresses is important in bridging the knowledge gap for developing better quality food products. Though various foods are involved in patho-physiology of allergic reactions in humans, peanuts record the highest anaphylactic cases around the world followed by wheat, milk and egg.

In this study, the effects of thermal processing (Hot Air Roasting (HA) and Microwave processing (MW)) and non-thermal processing (High Electric Field processing (HEF)) on the peanut protein conformation have been evaluated using Fourier Transform Infrared Spectroscopy (FT-IR). *In-vitro* Protein Digestibility (IVPD) studies have also been conducted for understanding the effect of aforementioned processing techniques on the digestibility of the protein. The FT-IR studies have shown a significant effect on the conformation of the peanut protein structure with an increasing random coil and α-helix secondary structures when the processing temperature and time are increased. There is also a corresponding increase in the *In-vitro* Protein Digestibility percentage (IVPD %) which suggest that the conformational changes in the secondary structure has resulted in the change of the digestibility of protein.

Molecular dynamics (MD) modeling studies have been performed to visualize the protein behaviour with changing ambient conditions under the influence of temperature, time and application of electric field. Ara h 6 molecule has been used for conducting the MD simulations which is one of the allergen molecule present in peanut. It has all of the peanut secondary structure in the native state. Root Mean Square Deviation (RMSD), Radius of Gyration (Rg), Dipole Moment and Solvent Accessible Surface Area (SASA) have been evaluated to understand the change in the secondary structure and the hydrogen bonding properties during the MD simulations. The results have showed that the HEF treatment has similar effect on the protein structure as of the microwave treatments in spite of being a non-thermal treatment. It also confirms that there are significant changes in the secondary structures resulting from the HEF and microwave processing thus affecting the functional properties of the protein.

RESUMÉ

Les aliments transformés sont composés de divers constituants qui ont été subjugués à différents procédés de transformation comme les traitements thermique, chimique, mécanique, et autres. Ces techniques sont utilisées non seulement pour créer de nouveaux produits (yogourt, fromage, beurre, crème, etc.,), mais aussi pour améliorer la durée de vie utile (pasteurisation, cuisson, ébullition, stérilisation) en diminuant ou en inactivant les microbes, les enzymes et certaines toxines présents dans les produits traités. Ces procédés de transformation peuvent aussi être utilisés pour extraire les composés phytochimiques comme les antioxydants et pour modifier les propriétés physiques ou organoleptiques. Les protéines sont l'un des principaux constituants des aliments et elles sont très sensibles aux contraintes résultant des procédés de transformation. De nombreuses études ont démontré que les protéines soumises à ces procédés subissent des changements conformationnels dans leur structure, ce qui modifie leurs propriétés fonctionnelles.

Bien que des études approfondies ont permis de mieux comprendre l'implication des protéines dans le réaction immunitaire provoquer par les substances allergènes chez les humains, la compréhension de ce phénomène à un niveau atomique et moléculaire est très limitée. Cet aspect important doit être adressée afin d'aider les industries à développer de nouvelles techniques de transformation qui permettront de minimiser les réactions adverses responsable de l'anaphylaxie et d'autres problèmes de santé reliés aux allergies alimentaires. Une meilleure compréhension des effets des procédés de transformation sur la fonctionnalité des protéines devrait permettre le développement de meilleurs produits alimentaires plus sécuritaires. Bien que certains aliments soient impliqués dans la pathophysiologie des réactions allergiques chez l'homme, les arachides sont responsable du plus grand nombre cas d'anaphylaxie dans le monde. Elles sont suivies par le blé, le lait et les œufs.

Dans cette étude, les effets de différents procédés de transformation thermiques (torréfaction par air chaud ou par micro-ondes) et non-thermique (exposition à des champs électriques élevés) sur la conformation de la protéine d'arachide ont été évalués en utilisant la spectroscopie infrarouge à transformée de Fourier et en comparant la digestibilité *in vitro* de ces dernières. Les résultats ont indiqué un effet significatif des traitements sur la conformation de la structure de la protéine d'arachide caractérisée par une augmentation de la formation de spirales aléatoires et de structures secondaires α-hélice lorsque la température et le temps de traitement étaient accrues. De plus, l'augmentation de la température et du temps de traitement ont permis d'accroître la digestibilité *in* vitro des protéines ce qui suggère que les changements de conformation dans la structure secondaire ont entraîné les changements de digestibilité observés.

Une étude de modélisation de la dynamique moléculaire a été effectuée pour visualiser le comportement de la protéine sous l'influence de l'augmentation de la température et de la durée du procédé de transformation ou encore lorsqu'elle est exposée à un champ électrique élevé. Pour cette partie de l'étude, la molécule 'Ara h 6' a été utilisée car elle est l'une des molécules allergènes présente dans l'arachide. De plus, dans sa forme native, cette molécule a toutes les structures secondaires de l'arachide. L'écart quadratique moyen, le rayon de giration, le moment dipolaire et la surface accessible au solvant ont été utilisés pour expliquer les changements dans la structure secondaire et dans les liaisons hydrogène de la protéine. Les résultats ont démontré qu'en dépit d'être un traitement non-thermique, l'exposition aux champs électriques élevés a causé des effets similaires a ceux observés après un traitement thermique par micro-ondes. L'étude de modélisation confirme également que les changements dans les structures secondaires de la protéine ont affecté les propriétés fonctionnelles de cette dernière.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude towards my supervisor Dr. Vijaya Raghavan, for his support and encouragement. He has been a constant source of inspiration with his energy, enthusiasm and scientist intuition. I take a great pride and pleasure in working under his guidance and I am forever indebted to him.

I would also like to thank Dr. Valerie Orsat for her support and for being patient with my problems. Special thanks to Mr. Yvan Gariepy for providing all the technical help for completing the project on time. I am also very grateful to Dr. Darwin Lyew for the support and benevolent help. I gratefully acknowledge the support of the faculty and staff in the Department of Bioresource Engineering and the Macdonald Office of Graduate and Postdoctoral Studies (GaPS).

I gratefully acknowledge the guidance and mentorship of Dr. Ashutosh Singh for clarifying my questions on molecular modeling techniques and the data analysis. Also a special thanks to Dr. Gopu Raveendran Nair for guiding with various technical details.

I also would like to thanks to all the people with whom I have worked with in the lab. All of you have been a great support and motivation for my work. I would also like to thank all my friends here including Brinda, Shravanthi, Divya, Winny, Priyanka, Ademola, Yulin, Zang, Fatih, Sachin, and Naveen for their support.

I am thankful to my mom and all my family members for their prayers and moral support. I would also like to thank my friends back home and elsewhere for all the pleasant memories.

Thesis Format

This thesis is submitted in the format of papers suitable for journal publication. This thesis format has been approved by the Faculty of Graduate and Postdoctoral Studies, McGill University, and follows the conditions outlined in the Guidelines: Concerning Thesis Preparation, which are as follows:

"As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis).

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

The thesis must include the following

(a) A table of contents;

(b) An abstract in English and French;

(c) An introduction which clearly states the rational and objectives of the research;

(d) A comprehensive review of the literature (in addition to that covered in the introduction to each paper);

(e) A final conclusion and summary;

4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers".

Contribution of Authors

The following are the manuscripts prepared for publication:

1. **Vanga, Sai Kranthi Kumar**; Singh, Ashutosh; Raghavan, Vijaya. 2014. "Review of Conventional and Novel food processing methods on Food Protein Allergens" (submitted)
2. **Vanga, Sai Kranthi Kumar**; Singh, Ashutosh; Raghavan, Vijaya. 2014. "Effect of Thermal and Electric Field Treatment on the Conformation of Ara h 6 Peanut Protein Allergen" (Accepted – In press)
3. **Vanga, Sai Kranthi Kumar**; Singh, Ashutosh; Kalkan, Fatih; Gariepy, Yvan; Orsat, Valerie; Raghavan, Vijaya. 2014. "Effect of thermal and high electric field on secondary structure of peanut protein and its digestibility" (Submitted)

The work reported here was performed by Sai Kranthi Kumar Vanga and supervised by Dr. Vijaya Raghavan of the Department of Bioresource Engineering, Macdonald Campus of McGill University, and Montreal. The entire research work was carried out at the Postharvest Technology laboratory, Macdonald Campus of McGill University, Montreal.

Dr. Raghavan has also provided scientific advice and is directly associated with editing and reviewing the manuscript. Dr. Ashutosh Singh has provided the technical help with regard to the molecular modeling software GROMACS and also participated in reviewing the manuscript. Mr. Yvan Gariepy is an academic associate in the Department of Bioresource Engineering and is actively involved in providing the research facilities for conducting the experiments. Dr. Fatih Kalkan has provided technical support for the research work.

TABLE OF CONTENTS

ABSTRACT.....ii

RESUMÉ.....iv

ACKNOWLEDGEMENT.....vi

THESIS FORMAT.....vii

CONTIBUTION OF AUTHORS.....viii

TABLE OF CONTENTS.....ix

LIST OF FIGURES.....xii

LIST OF TABLES.....xiv

CHAPTER 1

INTRODUCTION.....1

1.1 HYPOTHESIS AND IMPLICATIONS.....3
1.2 OBJECTIVES.....3
1.2.1. Overall Objective.....3
1.2.2. Specific Objectives 3

CHAPTER 2

REVIEW OF CONVENTIONAL AND NOVEL FOOD PROCESSING METHODS ON FOOD PROTEIN ALLERGENS.....4

2.1 ABSTRACT.....4
2.2 INTRODUCTION.....5
2.2.1. Food Components.....5
2.2.2. Structure of Protein.....7
2.2.2.1. Primary Structure.....10
2.2.2.2. Secondary Structure.....10
2.2.2.3. Tertiary and Quaternary Structures.....11
2.3 PROTEINS IN HUMAN BODY – ALLERGY.....12
2.3.1. Food Intolerance.....13
2.3.2. Food Allergy.....13
2.4 FOOD PROCESSING.....14
2.4.1. Processing effect on allergen stability.....15
2.4.2. Conventional Food Processing Methods.....17
2.4.2.1. Thermal Processing Methods.....17
2.4.2.1 (A) *Moist Heat*.....20
2.4.2.1 (B) *Dry Heat*.....22
2.4.2.2. Non-Thermal Processing.....24
2.4.2.2 (A) *Proteolysis*.....24

2.4.2.2 (B) *Ultrafiltration*.....26
2.4.2.2 (C) *Fermentation*.....27
2.4.3. Novel Processing Methods.....29
2.4.3.1. Thermal Processing Methods.....29
2.4.3.1 (A) *Microwave heating*.....30
2.4.3.2. Non-Thermal Processing.....34
2.4.3.2 (A) *Gamma-radiation (γ-radiation)*.....34
2.4.3.2 (B) *Pulsed Ultraviolet Light (PUV)* 37
2.4.3.2 (C) *Ultrasound Treatment*.....39
2.4.3.2 (D) *High Hydrostatic Pressure (HHP) / High Pressure (HP)*.....40
2.5 CONCLUDING REMARKS.....44
CONNECTING TEXT.....46

CHAPTER 3

EFFECT OF THERMAL AND ELECTRIC FIELD TREATMENT ON THE CONFORMATION OF ARA H 6 PEANUT PROTEIN ALLERGEN: A MOLECULAR MODELING APPROACH.....47

3.1 ABSTRACT.....47
3.2 INTRODUCTION.....48
3.3 MATERIALS AND METHODS.....51
3.4 RESULTS AND DISCUSSIONS.....53
3.4.1. Secondary Structure Analysis.....53
3.4.2. Root Mean Square Deviation (RMSD).....56
3.4.3. Radius of Gyration (Rg).....59
3.4.4. Dipole Moment.....61
3.4.5. Solvent Accessible Surface Area (SASA).....64
3.5 CONCLUSION.....70
CONNECTING TEXT.....71

CHAPTER 4

EFFECT OF THERMAL AND HIGH ELECTRIC FIELD ON SECONDARY STRUCTURE OF PEANUT PROTEIN AND ITS DIGESTIBILITY 72

4.1 ABSTRACT.....72
4.2 INTRODUCTION.....73
4.3 MATERIALS AND METHODS.....75
4.3.1. Solvents and Reagents.....75
4.3.2. Oven Roasting.....75
4.3.3. Microwave Heating.....75
4.3.4. Electric Field Treatment.....76
4.3.5. FT-IR Spectroscopy.....76
4.3.6. *In Vitro* Protein Digestibility.....77
4.3.7. Statistical Analysis.....77
4.3.8. Notations.....77
4.4 RESULTS AND DISCUSSIONS.....79

4.4.1 FT-IR analysis of Conformational Changes in Peanut Protein……79
4.4.2 Effect of Processing on the protein Digestibility……89
4.5 CONCLUSIONS……92

CHAPTER 5

SUMMARY AND CONCLUSIONS……93

FUTURE WORK……95

REFERENCES……96

LIST OF FIGURES

Figure 2.1 Common Structure of Amino Acid……7

Figure 2.2 Peptide Bond Formation……....9

Figure 2.3 Classification of Conventional Processing Methods……16

Figure 2.4 Classification of Novel Processing Method……17

Figure 2.5 Protein Structure Profile with Temperature……..18

Figure 3.1 The three dimensional structure of the Ara h 6 allergen protein…….53

Figure 3.2 STRIDE analysis showing the evolution of molecule (a) 300K (b) 300K, static electric Field of 0.05V/nm (c) 300K, oscillating electric field of 0.05V/nm at 2450 MHz (d) 425K (e) 425K, static electric Field of 0.05V/nm (f) 425K, oscillating electric field of 0.05V/nm at 2450 MHz. Note: Color code: Magneta denotes α (alpha)- helix, red denotes π (pi) - helix, cyan denotes turn, blue denotes 3–10 helix and white denotes coil. (The X-axis shows the residue numbers and names (from 1 to 127) and the Y- axis shoes the time frames (1 frame = 2 ps) of the simulation……...55

Figure 3.3 RMSD variations observed in Ara h 6 Allergen (a) Heat Treatment (b) Heat Treatment + Static Electric Field (c) Heat treatment + Oscillating Electric Field of 2450 MHz……58

Figure 3.4 Radius of Gyration variations observed in Ara h 6 Allergen (a) Heat Treatment (b) Heat Treatment + Static Electric Field (c) Heat treatment + Oscillating Electric Field of 2450 MHz……...60

Figure 3.5 Dipole Moment Variations in Ara h 6 Allergen (a) Change in Dipole moments with Temperature in Oscillating Electric Field (0.05V/nm) (b) Dipole moment and RMSD variations at 425K and Oscillating electric field (0.05 V/nm)……62

Figure 3.6 Snapshot of surface properties of Ara h 6. (a) 300K (b) molecule at the end of 300K and oscillating electric field (2450Mhz) (c) molecule at the end of 380K and oscillating electric field (2450MHz) (d) Molecule at 425K and oscillating electric field (2450MHz) at 400 ps (e) Molecule at end of 425K and oscillating electric field (2450MHz) Note: non-polar residues (white), basic (Blue), acidic (red) and polar residues (green)……65

Figure 3.7 Solvent Accessible Surface Area (SASA) variation with heat treatments and Electric Fields (Static and Oscillating) of Ara h 6. (a) Hydrophobic surface area with heat treatment, (b) Hydrophobic surface area with heat treatment + static electric field, (c) Hydrophobic surface area with heat treatment + oscillating electric field, (d) Hydrophilic surface area with heat treatment, (e) Hydrophilic surface area with heat treatment + static electric field, (f) Hydrophilic surface area with heat treatment + oscillating electric field……68

Figure 4.1. FT-IR spectrum of raw and microwave processed (100_15) peanut samples……..79

Figure 4.2 Normalized FT-IR spectra of the Amide I region of Hot Air roasted peanut proteins……...80

Figure 4.3 Normalized FT-IR spectra of the Amide I region of Microwave roasted peanut proteins……...81

Figure 4.4 Normalized FT-IR spectra of the Amide I region of High electric field processed peanut proteins……...81

Figure 4.5 Variations in relative areas of the bands fitted to the normalized FT-IT spectra of Amide I region (1700 cm^{-1} – 1600 cm^{-1}) of hot air roasted peanuts……..84

Figure 4.6 Variations in relative areas of the bands fitted to the normalized FT-IT spectra of Amide I region (1700 cm^{-1} – 1600 cm^{-1}) of microwave roasted peanuts……85

Figure 4.7 Variations in relative areas of the bands fitted to the normalized FT-IT spectra of Amide I region (1700 cm^{-1} – 1600 cm^{-1}) of high electric field processed peanuts……86

LIST OF TABLES

Table 1.1 List of few countries whose infant and children suffer from hypersensitivity to the Big 8 allergens……2

Table 2.1 List of all Amino Acids and molecule attached at 'R'……8

Table 2.2 Studies conducted on the moist heat processing……22

Table 2.3 Studies conducted on the dry heat processing……24

Table 2.4 Studies conducted on non – thermal conventional processing methods……29

Table 2.5 Studies conducted on Microwave processing (Novel – thermal) methods……34

Table 2.6 Studies conducted on non-thermal Novel processing methods……44

Table 3.1 RMSD values obtained for Ara h 6……58

Table 3.2 Radius of Gyration values for Ara h 6……61

Table 3.3 Dipole moments……63

Table 3.4 (a) hydrophobic surface areas and (b) hydrophilic surface area……66

Table 4.1 (a) Notations for representing the Hot air roasting of peanut samples……..78

Table 4.1 (b) Notations for representing the Microwave roasting of peanut samples……78

Table 4.1 (c) Notations for representing the High electric field processing of peanut samples…78

Table 4.2. Amide I band frequencies and corresponding Protein Secondary Structure correlations……80

Table 4.3. Positions areas of the bands fitted to the normalized FT-IR spectra of peanut protein (1700 cm^{-1}-1600 cm^{-1}) subjected to different hot air roasting temperatures and treatment time….83

Table 4.4 Positions areas of the bands fitted to the normalized FT-IR spectra of peanut protein (1700 cm^{-1}-1600 cm^{-1}) subjected to different microwave roasting temperatures and treatment time……84

Table 4.5 Positions areas of the bands fitted to the normalized FT-IR spectra of peanut protein (1700 cm^{-1}-1600 cm^{-1}) subjected to different high electric field intensities and treatment time....85

Table 4.6 Effect of hot air roasting on protein digestibility (IVPD %) of peanut protein…….89

Table 4.7 Effect of microwave roasting on protein digestibility (IVPD %) of peanut protein….89

Table 4.8 Effect of high electric field roasting on protein digestibility (IVPD %) of peanut protein……89

CHAPTER 1

INTRODUCTION

The rational for conducting this study lies in the exponential increase in the cases of peanut allergies across the globe in recent years (Helm & Burks, 2000). Various reasons are cited as the probable cause of rise in this number, which include globalization leading to dissipation of wide variety of foods to the world population and genetics of the human body (Arshad et al., 1993; Sampson, 2004; Sampson & McCaskill, 1985). Within North America alone, an estimated 2.1% of the younger population and 1% of adult population suffer hypersensitivity to peanut. The allergen protein when consumed, triggers an Immunoglobulin E (IgE) mediated immune response which may lead to vomiting, rashes, swelling of tongue, anaphylaxis and ultimately death in extreme cases (Dalal et al., 2002; Sampson, 2004; Sicherer, 2002; Skripak et al., 2007). The occurrence of allergy is also particularly dependent on the geographical location which can be influenced by the ancestral eating and living habits (Dalal et al., 2002). Peanut allergy is very much prevalent in the North American and European regions where as very rare in Israel and Japan. This difference is attributed to the early introduction of peanut in the food to infants, the heavy consumption and the overall eating habits (Dalal et al., 2002; Hourihane, 1997).

Though, potentially any food can cause an immune response in human body, eight foods which include peanuts, egg, wheat, milk, soybean, fish, shell fish and tree nuts are responsible for most of the food hypersensitivity cases around the world and are termed as the 'Big 8'(Dalal et al., 2002; Sicherer & Sampson, 2006). The prevalence of the food hypersensitivity is estimated to be around 1-2 % in adult population and 2-8% of the infant and young children in various countries (Bock, 1987; Burks & Sampson, 1993; Sloan & Powers, 1986). However, a survey in Japan reveled that about 12.6% of the children have food hypersensitivity causing an immediate reaction on

consumption of the allergen (Iikura et al., 1999). The following Table 1.1 gives list of countries whose infant population suffer from food allergy.

Table 1.1 List of few countries whose infant and children suffer from hypersensitivity to the Big 8

Adopted from (Brigino & Bahna, 1995; Dalal et al., 2002; Goh et al., 1999; Hill et al., 1986; Hill & Hosking, 2000; Iikura et al., 1999; Rancé et al., 1999; Schloss, 1920; Soller et al., 2012)

Food	Countries
Peanut	USA, Canada, UK, Australia, France, Spain
Egg	USA, Canada, Australia, France, Spain, Israel, Italy, Japan, UK
Milk	USA, Canada, Australia, France, Spain, Israel, Italy, Japan, Singapore
Wheat	USA, Canada, Australia, UK, Japan, UK
Soy	USA, Canada, Australia, Israel
Fish	Australia, Italy, Japan, Singapore, Spain, USA
Shellfish	Japan, Spain, Singapore
Nuts (Tree nuts)	USA, Australia, France (Hazelnut), Israel, Italy, Japan, Spain, Canada.

Various researchers have also showed that the method of processing can also influence the allergenicity of the product obtained. Extensive research work is being done in understanding the effect of specific processing methods and processing parameters on the food protein interactions not only among themselves, but with the other components present in food (Sathe & Sharma, 2009; Sathe et al., 2005). As ways for processing the foods are huge and varied, the effect produced by each of them on the specific type of epitope (conformational and linear epitopes) is very important for analysing the effects on the allergenicity of a certain protein (Davis et al., 2001; Sathe et al., 2005).

1.1 Hypothesis and Implications

Thermal processing has been performed on peanuts for improving the quality and variety in the product for a very long time. But, this thermal and high electric field processing has an effect on the protein structure and thus the functional properties of the protein. Though, researchers have tried to look at these effects on various functional properties, work done in understanding its influence on the protein structure is limited. In this project, we tried to correlate the effect of various processing methods on the conformation of the secondary structure of the peanut protein. Molecular Dynamics (MD) simulations have been introduced as a tool to visualize these changes in the secondary structures.

1.2 Objectives

1.2.1 Overall Objective:

The overall objective of the study is to evaluate the changes in the secondary structure conformations of the peanut protein during thermal and high electric field processing and its effect on the digestibility of the protein. Also to evaluate the MD simulation as a visualization tool for understanding the changes in the protein structure during various process simulations.

1.2.2 Specific Objectives:

1) To investigate the effect of thermal and high electric field processing of peanut on the structural conformation of protein using Fourier transform infrared spectroscopy (FT-IR) technique.

2) To evaluate the applicability of molecular dynamic simulation for studying the effect of processing on food proteins.

CHAPTER II

Review of Conventional and Novel Food Processing Methods on Food Allergens

2.1 Abstract

With the turn of this century, novel food processing techniques have become commercially very important because of their profound advantages over the traditional methods. These novel processing methods tend to preserve the characteristic properties of food including their organoleptic and nutritional qualities better when compared with the conventional food processing methods. During the same period of time, there is a clear rise in the populations suffering from food allergies, especially infants and children. Though, this fact is widely attributed to the changing livelihood of population in both developed and developing nations and to the introduction of new food habits with advent of novel foods and new processing techniques, their complete role is still uncertain. Under the circumstance, it is very important to understand the structural changes in the protein as food is processed to comprehend whether the specific processing technique (conventional and novel) is increasing or mitigating the allergenicity. Various modern means are now being employed to understand the conformational changes in the protein which can affect the allergenicity. In this review, the processing effects on protein structure and allergenicity are discussed along with the insinuations of recent studies and techniques for establishing a platform to investigate future pathway to reduce or eliminate allergenicity in the population.

KEYWORDS: protein structure; microwave processing; food allergenicity; high pressure

2.2 INTRODUCTION:

The human race flourished on the face of earth starting millions of years ago and for all these years food has been the primary source of energy. The source of the food can be plants, animal carcasses or even microbes in many cases. Independent of the source, the main components of food include water, carbohydrates, proteins, lipids, minerals and vitamins along with few minor components such as antioxidants, colorants, flavouring components and any other additives that are added with an intention to impact various qualities and physicochemical properties of food. Depending on the source of food, the quantities of above mentioned components vary giving foods their distinct aroma, colour and palatability. The contents also vary depending on various other factors including species and variety of the animal or plant respectively; time of harvesting for plants and slaughtering for animals; nurturing habits and environment during their life. All these factors affect the inherent quantity and quality of the components of food.

2.2.1 Food Components:

Of the aforementioned elements, water content in various foods can vary widely between 3% (dry fruits, milk powder) to 95% (few fruits and vegetables) (Sikorski, 2007) and this water content determines various physical and chemical properties of food. In 1957, Scott, W.J. (Scott, 1957) became the first researcher to give the relation between the water content and microbial activity in food. His research suggested that the water activity can be correlated with deterioration of food and hence it in turn influences the microbial activity in food (Ayerst, 1969; Rahman, 1995; Scott, 1957). Thus, the shelf life quality of any food is determined by the amount of water present in it. Apart from the shelf life of the product, the water content also determines the crispiness of the few food products including popcorn, potato chips and other cereal based food products (Katz and Labuza, 1981; Roudaut et al., 1998) and also plays a primary role in the amino acid – sugar

browning reactions (Maillard reaction) (Eichner and Karel, 1972). Moreover, the mobility of the components within a food product is also greatly affected by the water content (Karel, 1985). The water content is also an important factor to consider in various food processes such as fermentation (Liu and Tzeng, 1999; Oriol et al., 1988; Zaks and Klibanov, 1988), baking (Sablani et al., 1998; Thorvaldsson and Skjöldebrand, 1998) and microwave heating (Venkatesh and Raghavan, 2004). Other properties like the thermal conductivity, diffusion and specific heat also varies depending upon the moisture content in food thus playing an important role in various food processing aspects (Pomeranz, 1985).

Another component in food of high importance to humans are the carbohydrates or sugars, which act as the primary source of energy to the body (Boyle, 1996). The carbohydrates release about 4 kcal of energy for every gram consumed (Hunt and Stubbs, 1975; Ledikwe et al., 2006; Lee and Putnam, 1973). The amount of the sugars present can vary greatly from 1% (few meat products) to 65% - 70% (cereal grains) (Pomeranz, 1985; Sikorski, 2007). Apart from being the primary energy source, carbohydrates, most importantly the polysaccharides, act as primary structural component in the plant based foods (Carpita and Gibeaut, 1993). Few of the polysaccharides can also be used as food gums, also called the hydrocolloids, which play a major role in determining physical qualities including texture and stability and various other processing parameters (Eliasson, 2006). Apart from the carbohydrates, lipids also act as an important source of energy. They release about 9 kcal of energy per every gram of fat consumed (Bang et al., 1980; Ledikwe et al., 2006). This is the reason fats are called ‘the concentrated source of energy’ as they tend to release more than double the energy on consumption of the same amount of carbohydrate or protein (Boyle, 1996). Fats also play an important role in delivering the body with vital nutrients including Vitamin A, D, E and K (Herrmann, 2011).

Proteins are the next major component present in food apart from carbohydrates and lipids. They play a primary role in cell signaling and immune responses within the human body. They are also responsible for the muscle formation in humans and cell wall structure of the plants. In addition to the wide array of functions of protein, they can also act as a source of energy to the body if required, liberating about 4 kcal of energy per every gram of protein (Ledikwe et al., 2006; Phillips, 2011). Apart from the above, proteins also has wide range of functional, sensorial and nutritional properties in food. And all of the properties are dependent on the structure of the protein (Singh et al., 2013b).

2.2.2 Structure of the protein

'Amino acids' act as the building blocks of proteins and in total there are about 20 amino acids, which have a common structure as shown in Fig 2.1. A molecule of amino acid contains a carboxylic group, primary amine and another molecule (represented in the Figure 1 as R). The molecule attached in place of 'R' differs, forming various amino acids with different characteristic properties (Phillips and Williams; Rahman, 1995).

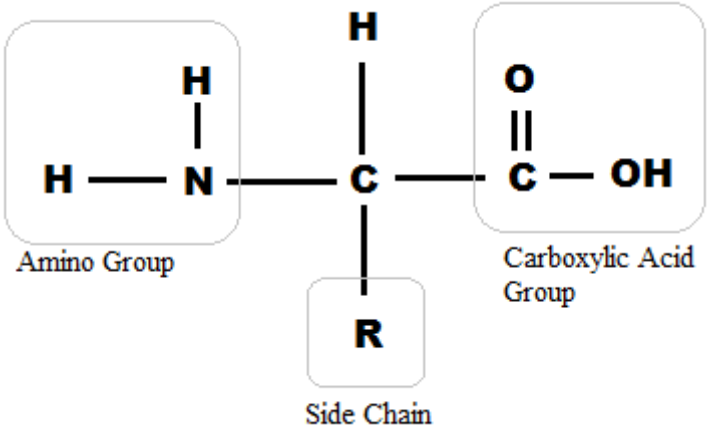
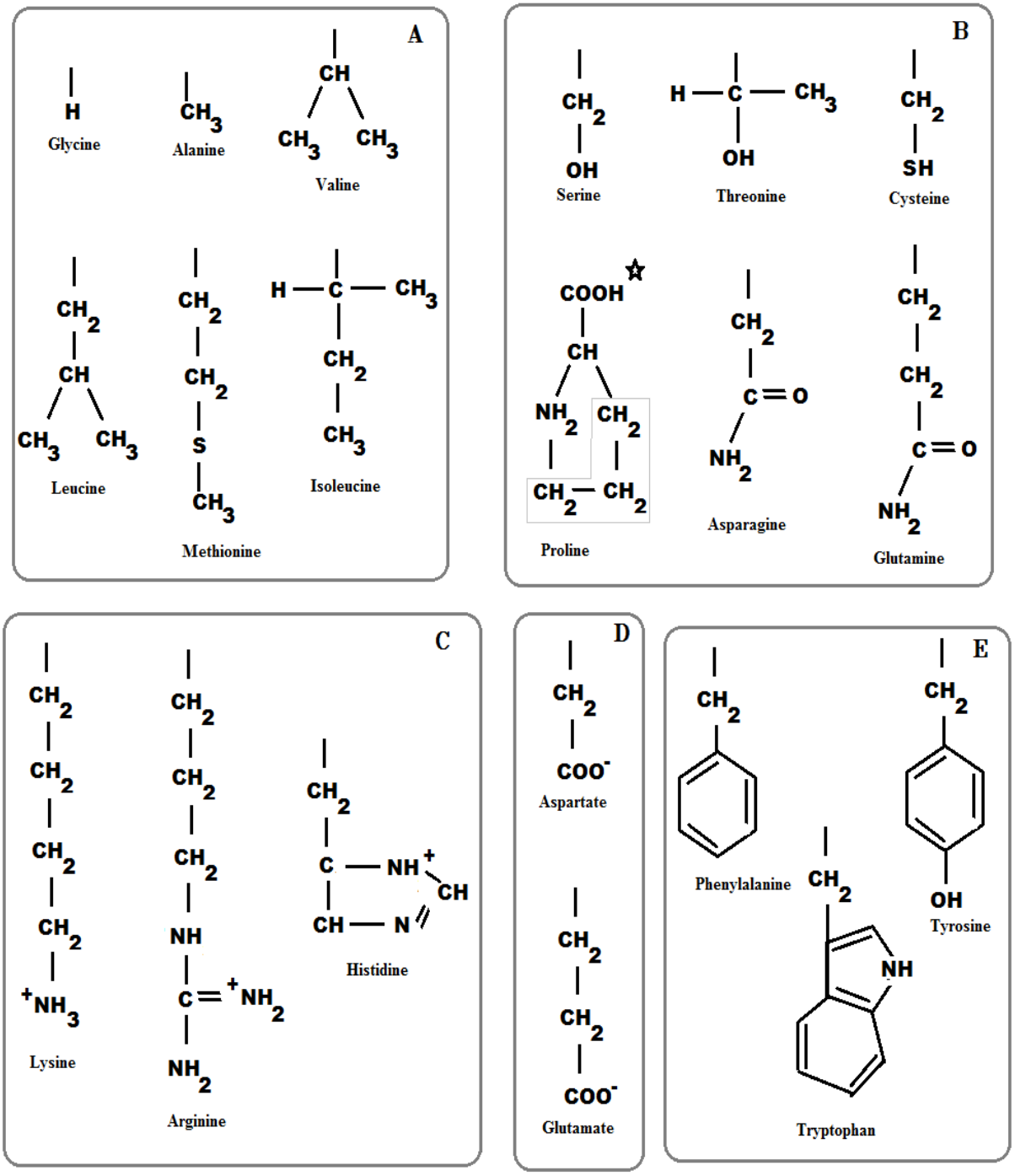


Figure 2.1 Common Structure of Amino Acid

Table 2.1 List of all Amino Acids and molecule attached at 'R'



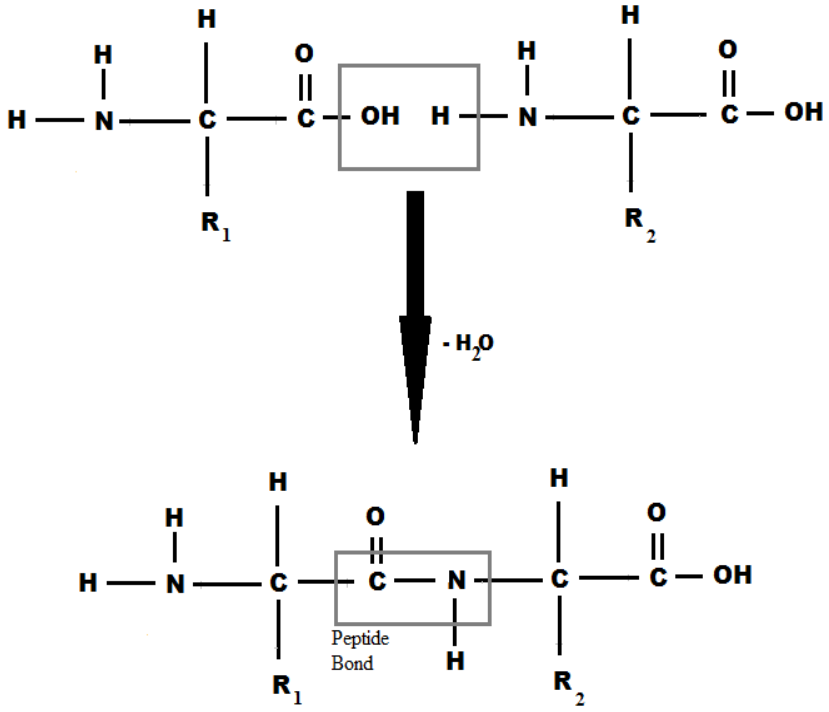


Figure 2.2 Peptide Bond Formation

The amino acids come together forming a peptide bond as shown in Fig 2.2. The peptide bond is formed between the carboxylic group of the first amino acid and amine group of the second amino acid giving out a water molecule (Koshland Jr, 1958; Phillips and Williams; Phillips, 2011). The new terminals are now available for further bonding with other amino acids. The number of amino acids in each protein molecule can vary from 10 to about 30,000 like in titin molecule (Opitz et al., 2003) which is termed as the largest protein. The linear sequence of the amino acids that join together forming a polypeptide chain gives proteins their primary structure (Singh et al., 2013a). Depending on the amino acids in the primary structure, local sub-structures are formed within the protein molecule like alpha helix and beta sheets giving protein its characteristic secondary structure. A single protein molecule can conform to wide range of structures which can be linear or globular which defines the overall structure of the protein (Phillips and Williams). The stability of the protein can vary depending on the hydrogen bond and disulphide bond formations. Few

proteins also have a quaternary structure, where multiple subunits come together to form a complex molecule. Haemoglobin, myoglobin molecules in humans and animals possess a quaternary structure (Cleaves, 2011; Klotz et al., 1970). The hierarchy in structural classification of the proteins into four does not necessarily describe all of the laws, but are made for better understanding of the structural aspects (Gu and Bourne, 2009).

2.2.2.1 *Primary structure of protein*

The primary structure is defined by the sequence of the amino acids forming the peptide bonds that gives the protein its unique structure. It was in 1920, Fischer and Hofmeister became the first scientists that proposed the linear structure model for proteins (Fruton, 1972). Later, the works of Sanger (Sanger, 1952) on amino acids have provided enough base that proteins follow the linear sequence and moreover, the proteins can be distinguished based on the sequence of amino acids (Gu and Bourne, 2009). As mentioned earlier, the amino acids have an alpha carbon which is bonded by a Hydrogen molecule, amide molecule (acting as N-terminal) and carboxyl molecule (acting as C-terminal). The molecule that forms the fourth bond with the alpha carbon varies which differentiates the amino acids from one another and further, it also confers each amino acid with specific chemical properties (Gu and Bourne, 2009; Whitford, 2005).

The amino acids in a protein form a covalent bond, which is termed as ‘peptide bond’. The amino acids that form a peptide bond are called ‘residues’ and the atoms that are involved on the formation of peptide bond become the ‘backbone’ of the protein.

2.2.2.2 *Secondary Structure*

Secondary structures are the three-dimensional ordered local features of biopolymers such as proteins. Protein secondary structures can be classified into four major categories: helices, sheets,

loops and turns. Helices are formed when amino acids in the primary structure of the protein form a winding motif, which is stabilized by formation of parallel hydrogen bonds between backbone atoms along the amino acid sequence. There are three types of helices that commonly occur in a protein: α-helices, π-helices and 3_{10}-helices (3/10 helix). These forms of helices differ in their hydrogen bond coordination, i.e. α-helices are formed when residues *i* and *i* + 4 form a hydrogen bond, similarly π-helices and 3_{10}-helices are formed when residues *i* and *i* + 3 and *i* and *i* + 5 form hydrogen bonds between them respectively.

β-sheets are the second most common secondary structure observed in proteins. They consist of β-strands that are connected laterally by hydrogen bonds between backbone atoms. Two forms of β-sheets are commonly observed: parallel and anti-parallel. Out of these two forms anti-parallel β-sheets are more stable due to the well-aligned hydrogen bonds.

Loops and turns are one of the most essential secondary structures observed in a protein. They enable proteins to fold and form compact tertiary structures and play a vital role in determining the flexibility of active regions in the protein responsible for enzymatic reactions and interaction with other macromolecules.

2.2.2.3. *Tertiary and Quaternary Structures*

Proteins tertiary structure represents its three-dimensional configuration in space. It has a single polypeptide chain backbone with one or more secondary structures (figure). Interactions and bonds of side chains within a protein determine the formation of tertiary structure; it is also driven by hydrophobic interactions between secondary structures, which is required to minimize the hydrophobic surface area of the protein that is accessible to water and other solvents. The amounts of hydrophobic residues also determine the stability of the protein tertiary structures.

Quaternary structure of the proteins is formed when a group of proteins interact together and behave in a collective manner. Various bonding interactions including hydrogen bonds, salt bridges and disulphide bonds hold different protein chains into a specific geometry. Two major categories of protein that contain quaternary structures are fibrous and globular proteins. Fibrous proteins include, keratins and collagens which play an important structural role, whereas globular proteins such as insulin, haemoglobin and most enzymes are involved in regulatory roles.

Protein molecules assume a wide array of functions and participate in large amount of reactions in the body. They are able to perform such distinctive functions only because of their versatility and fidelity in the structures. Thus with such large scope of structural conformations, proteins are able to perform a wide array of functions (Gu and Bourne, 2009; Singh et al., 2013b).

2.3 PROTEINS IN HUMAN BODY - ALLERGY

Proteins perform diverse biological functions which can vary from oxygen transfer between various cells (transport proteins), Enzymatic functions (enzymatic proteins), acting as substrates for various biological reactions leading to formation of distinct compounds, defence mechanisms in the body against foreign compounds (defence proteins) and as storage proteins (Whitford, 2005). Though each protein is distinct in its structure, and composition leading to that specific structural conformation, they are formed due to the peptide bonds between the amino acids which are common in all of the proteins. As mentioned earlier, though there are only 20 amino acids, the size and the diversity in the molecular weights of proteins is vast which also affects the functions performed by a protein. The molecular weights of the proteins are represented in Daltons (Da) where 1 Da = 1 amu i.e. atomic mass unit (Thus, 5500 Da = 5.5 kDa) (Whitford, 2005).

The undesirable reactions that happen to human body on consumption of food can be broadly divided into 2 categories:

1. Food Intolerance and 2. Food Allergy/Hypersensitivity

2.3.1 Food Intolerance

'Food Intolerance' may be defined as the adverse reactions caused due to the consumption of food within the human body where the immune system has no involvement in their cause (Andreas, 2009; Lehrer et al., 2002; Lopata and Potter, 2000). Irritable bowel syndrome is commonly associated with food intolerances (Alun Jones et al., 1982; Nanda et al., 1989). Various other symptoms can be similar to that caused by food allergies which can be ranging from respiratory, gastrointestinal, and skin to psychological reactions.

2.3.2 Food Allergy

Food allergy occurs when a food component, mostly an incompletely digested protein is absorbed in the blood stream and elicits an immune response. During digestion food proteins are broken down into individual components i.e. amino acids, which when absorbed in blood do not elicit any immune response, but an undigested protein or partially digested protein is treated as a foreign particle by the immune system, which then activates its IgE-mediated response to neutralize it (Sampson, 2004; Schmitt et al., 2004; Sicherer & Sampson, 2007). In predisposed allergic individuals, the advent of acute allergic reaction is due to engagement of allergen specific IgE antibodies. These antibodies with their high-affinity receptors are expressed on mast cells and basophils. When allergens interact with an antibody the receptor cells determine the release of mediators. The intrinsic biochemical properties of a food allergen also contribute towards its allergenicity. For example, peanut allergen Ara h 1 is very stable and is resistant to heat and digestive enzyme degradation, it also contains a glycan adduct which acts as a TH2 (T helper cell) adjuvant (Shreffler et al., 2006, Koppelman et al., 1999).

Common symptoms of food allergies include skin rashes, abdominal pain, vomiting and diarrhoea. The most dangerous and sometimes fatal response to food allergies is anaphylaxis, which is a whole body reaction leading to difficulty in breathing and a fall in blood pressure potentially causing a shock. At present once an individual is identified allergic to a food after intensive diagnosis using oral food challenges, skin-prick testing and antibody blood testing a complete avoidance of causing food is the only treatment. A complete avoidance of food sometimes leads to nutritional deficiencies and is one of the major driving force for researchers and industries in the field of food processing to come up with alternate novel food sources which can supplement the avoided food products.

2.4 FOOD PROCESSING

Processing of food is not a completely modern concept as archeological proofs suggest that the cavemen and hunting-gathering societies cooked their food after the discovery of fire either by direct heating (in the fire) or by boiling in water. Though preservation of food was not their primary target, they observed that cooking food increased its palatability. With the advent of the agricultural societies, people understood the importance of the storage and preservation of their produce. The processing techniques like sun drying, fermentation to produce alcohol and cereal grinding along with bread making have been developed which spread around the world. The development of the trade routes around the world in the medieval age has led to accelerated spread of knowledge regarding food preservation and processing. The scale of processing and operations have accelerated exponentially as the world population grew during the industrial revolution. The development of electricity led to introduction of electrical appliances in the early twentieth century which increased the speed of processing ten folds. Post world-war societies had growth in the confectionary, ready-to-eat foods and convenience foods (Fellows, 2000; Fellows, 2009). With the

modernization of the society, the quality of the final food product has gained the importance as the consumers became more health conscious which led to the introduction of novel food processing techniques (Fellows, 2000; Fellows, 2009; Jasim et al., 2009).

Today, food is subjected to a wide range of processing treatments and conditions primarily to improve the sensory attributes and/or shelf life of the product. This is either done by removing or inactivating the toxins and microbes present in food and by altering the properties of various components. The large array of processing techniques also contributes in increasing the diversity of food products.

2.4.1. Processing effect on allergen stability

The cause of allergic reaction in the body in most cases would be due to a small linear stretch of amino acids or a specific three dimensional structure which is a part of a much larger protein, which are known as 'epitopes'. A single protein may contain distinct epitopes or just one epitope which repeats itself throughout the structure, but more than one epitope causes the IgE cross-linking. The relationship between the number of epitopes, nature of epitope and the severity of the allergic reaction caused by a certain kind of epitope is still uncertain (Sathe et al., 2005).

The processing of food involves wide array of physical, chemical and biochemical changes which induce alteration of various components including protein and thus the allergenicity of the specific protein epitope. Depending on the processing the epitopes that are present may be destroyed or new epitopes may be formed which is described as 'neoallergen formation' (Sathe et al., 2005; Thomas et al., 2007). These were first reported in 1974 (Spies, 1974) and researchers then believed that the neoallergens might be the cause of allergic reactions in patients who have consumed processed food, but not on consumption of unprocessed food (Sathe et al., 2005). These

neoallergens are also observed in wheat flour (Leduc et al., 2003) and pecans (Malanin et al., 1995). Apart from genesis of neoallergens, processing may also cause an increase in the allergenicity of a certain protein or may cause no change in it. As ways for processing the foods are huge and varied, the effect produced by each of them on the specific type of epitope (conformational and linear epitopes) is very important for analysing the effects on the allergenicity of a certain protein (Davis and Williams, 1998; Davis et al., 2001; Sathe et al., 2005). The food processing methods can be divided as shown in the Figures 2.3 and 2.4 (Andreas, 2009; Sathe et al., 2005).

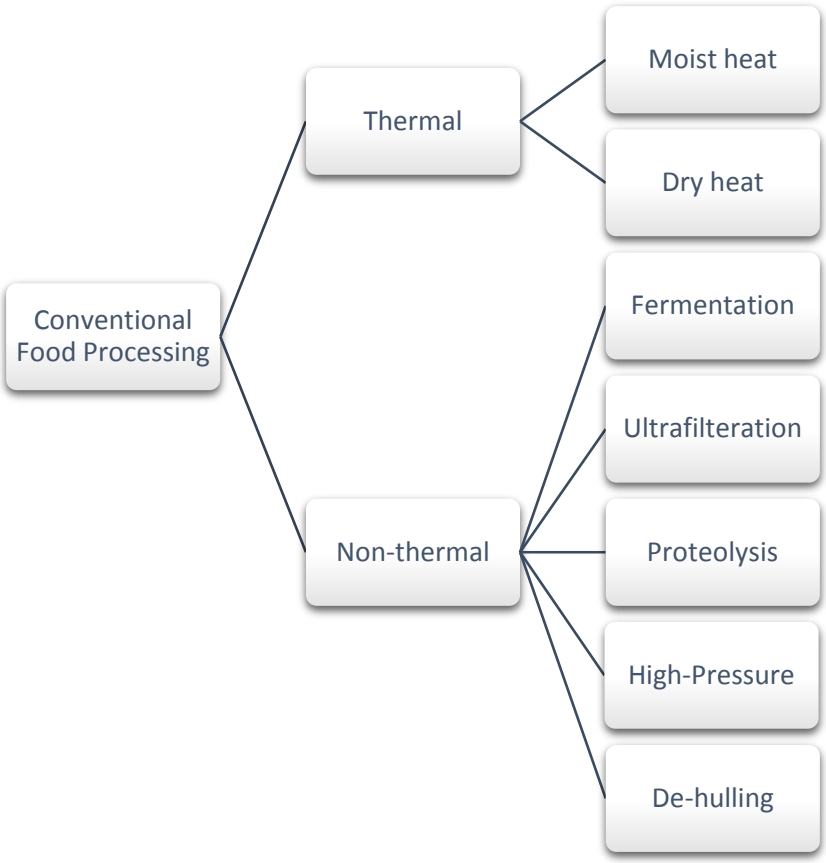


Figure 2.3 Classification of Conventional Processing Methods (Adopted from Lopata, 2009; Andreas, 2009)

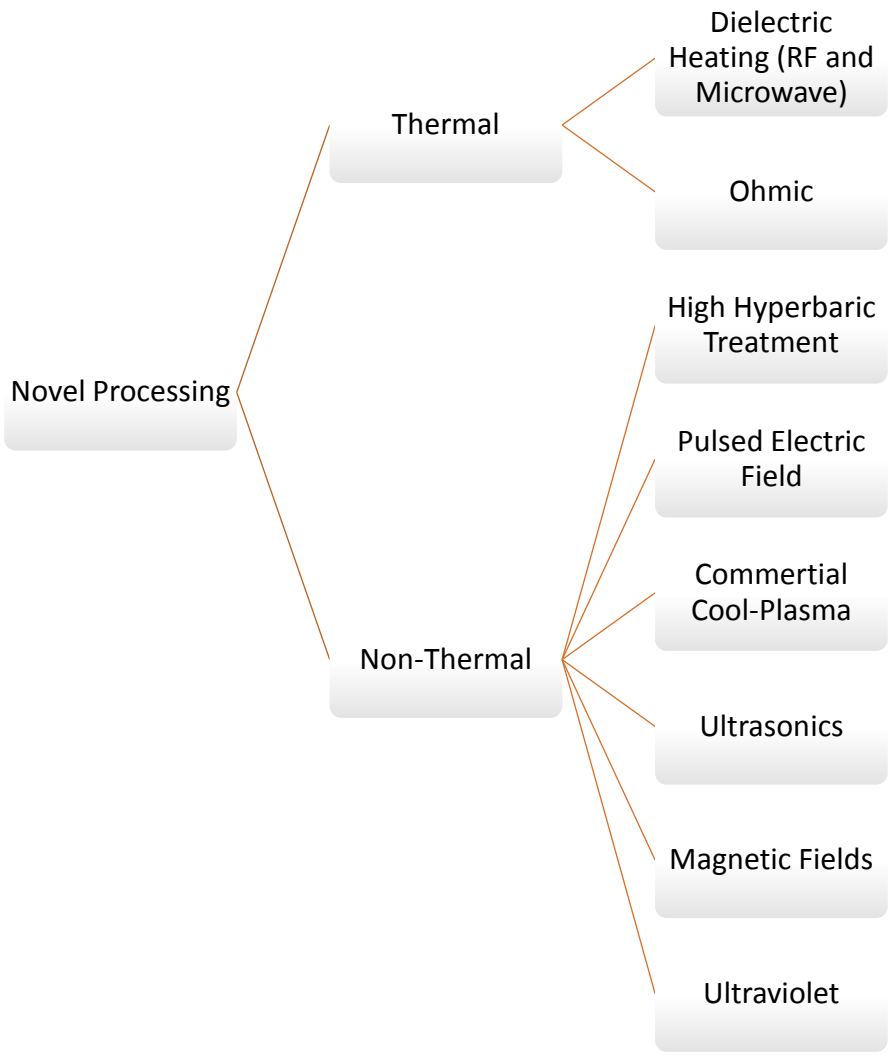


Figure 2.4 Classification of Novel Processing Method

2.4.2 Conventional Food Processing Methods

2.4.2.1 Thermal Processing Methods

The thermal processing is primarily carried out in food for enhancing the microbial safety, texture, digestibility and detoxification. The heating of food is normally done at two temperature ranges which are pasteurization and sterilization. But the most vital factor that has to be considered is what effect heating has on the allergenicity of the allergic component present in certain foods. It can either mitigate the allergenicity or it can also enhance it by changing the native protein

structure as mentioned earlier. Every protein has a native structure which is its most stable form depending on the hydrogen bonds, disulphide bonds, electrostatic and hydrophobic interactions (Boye et al., 1997; Davis and Williams, 1998; Van Holde, 1977). When the heat energy is applied, the protein slowly starts to lose its native structure and higher is the change when the amount of heat applied increases as shown in Figure 2.5.

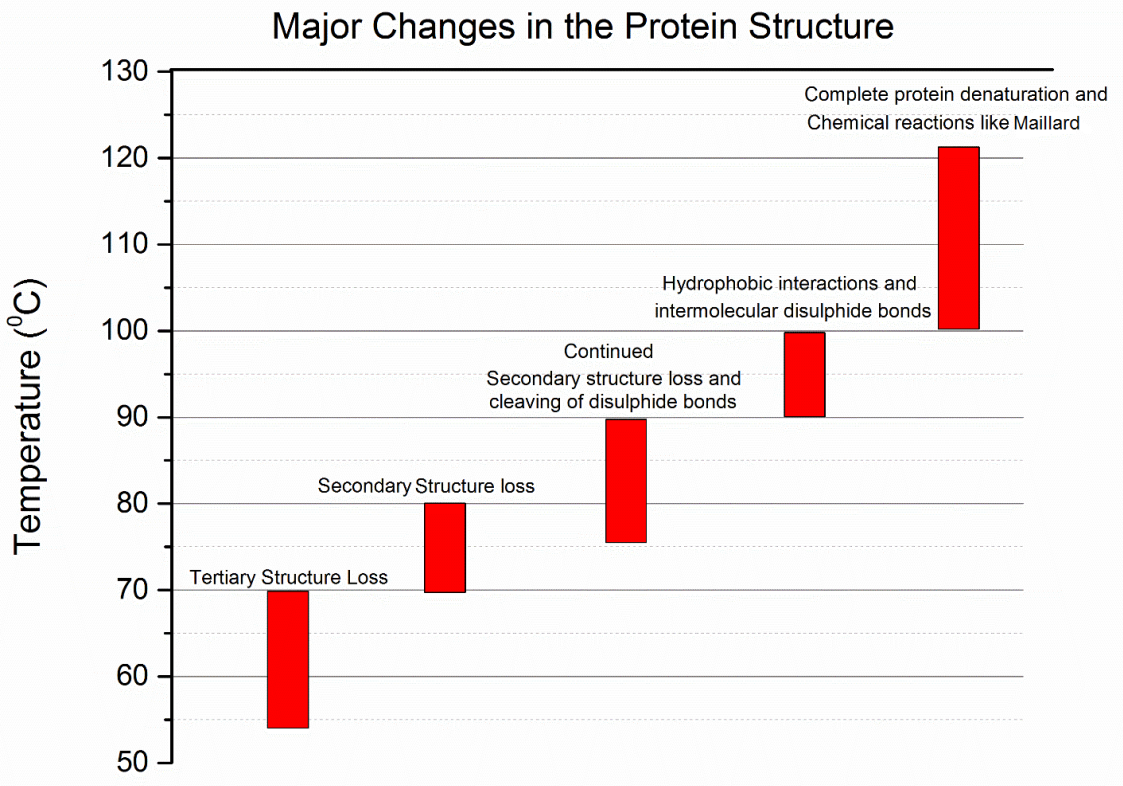


Figure 2.5 Protein Structure Profile with Temperature

As indicated, the molecular bonds that hold the protein allergen structures together tend to modify themselves on application of heat, which shatters the finely poised bonds and adjusts the structure accordingly. The hydrophobic groups or units of the protein structure that are normally inward (in the stable structure), turn in opposite direction (outward) which exposes them to the unfavourable conditions (water present in the protein environment) (Davis and Williams, 1998; Davis et al., 2001). As explained in fig 2.5, the proteins tend to start losing their structure at around 50-60 ^{o}C

depending on their stability, size and molecular weight where loss is in the tertiary structure. And as the temperature is increased, the effect of the heat also grows which leads to a complete denaturation of protein. The temperature can also result in chemical reactions between proteins (amino acids) and other components in the food (Carbohydrates) resulting in undesirable component formation and also discolouration (Example: Maillard reaction) (Davis and Williams, 1998).

Some proteins also possess a unique quality that they tend to fold themselves back to the native structure (not completely) under the absence of external stress primarily due to the interactions between the various amino acids (among themselves) and also between the amino acids and the solvent (Boye et al., 1997; Privalov et al., 1989; Tanford, 1968). But, the changes of protein regaining the original structure are completely dependent on the extent of processing and also on the native protein allergen structure (Tanford, 1968; Thomas et al., 2007). For example, when the protein G-actin was processed to at least 30 ^{o}C above its denaturation temperature, it retained about 60% of the original helical structures (Smith, 1994). Where as in another case, the molecule of β-lactoglobulin when processed, formed a complex with the α-lactoglobulin which is completely denatured and do not possess any significant similarities with the unprocessed native structures (Baer et al., 1976; Davis and Williams, 1998). It can be said that though the heat treatment results in a modification of the structure by partially unfolding or by completely changing to a random coil, there is always a possibility that the protein folds back to its native structure (at least to a certain extent) which can result in retention of allergenicity in the case of an allergen protein molecule which is the biggest hurdle for researchers to tackle.

2.4.2.1 (A) Moist Heat

To process (heat) the food in presence of water can be considered as 'moist heat processing' (eg: boiling, autoclaving). Few protein epitopes (cross-reactive epitopes - 2) that show a change in the conformational structure after moist heating have been reported to be present in cherry and the epitopes are homologous to Bet v 1, a major pollen allergen. An another Bet v 1 homologue from apple which is called Mal d 2 also loses its conformational structure when heated to 363K and regeneration of the unfolded structure is minimal when cooled down to 293K (Andreas, 2009; Marzban et al., 2009). Api g 1 also homologous to Bet v 1, is a major celery allergen was able to retain its allergenicity as it regained most of the initial structure of the molecule (Andreas, 2009; Jankiewicz et al., 1996). Moreover, the thermal stability of the allergen epitopes that belong to the same family also varied widely. For example, consider the Gly m 4 which is also homologous with the Bet v 1 allergen present in soya-bean. It has minimal effects from processing and it tends to retain its allergenicity in lots of high processed soya foods (Kleine-Tebbe et al., 2002; Mittag et al., 2004) suggesting that the environment and the components around the allergen molecule also play a role in the determination of the effects of external stress on the changes occurring in the protein conformational structure. This kind of thermal treatment also showed a reduced IgE reactivity of the Ara h 1 allergen of peanut to be reduced by performing the SDS-Page analysis. They obtained similar results with the Ara h 2 and Ara h 3 allergens in peanuts (Beyer et al., 2001). Also Kiwi fruit sensitivity was shown to be reduced when processed Kiwi (steamed for 5 minutes at 100 ^{o}C) was consumed (Fiocchi et al., 2004). The sensitivity caused due to the β-lactoglobulin present in milk has also been extensively studied (Andreas, 2009; Ehn et al., 2004; Sathe et al., 2005). It was shown that there was a considerable amount of reduction in the IgE sensitivity of the protein as the treatment temperature increased from 74^{o} C to 90^{o} C.

Autoclaving is also a moist heating technique employed for sterilization purposes and changes in the reactivity of various allergens from autoclaving has been evaluated over the past few years. In a study conducted by Venkatachalam *et al.*, cashew nuts were autoclaved at 121^{o} C for 5, 10 20 & 30 minutes and on testing for reactivity, the processing showed very little effect on the protein and they remained stable regardless of the processing parameters used (Venkatachalam et al., 2008). Cuadrado *et al.* (Cuadrado et al., 2009) assessed the effect of boiling (60 minutes) and autoclaving for 30 minutes at 1.2 atm and 2.6 atm on lentil and chickpea proteins. They stated that there was a reduction in the immunoreactivity of the allergens present in them, but only under severe processing conditions. It is to be noted that when processed at such extreme conditions, few highly stable reactive proteins were still present in the legumes which could cause sensitivity in patients on consumption rendering no clinical significance. Lupine flour was also autoclaved (at 138^{o} C and 121^{o} C) to evaluate the effect of this heat treatment on the protein. It was reported that t autoclaving at 138^{o} C for 30 minutes completely removed the potent allergens of 23 kDa and 29 kDa previously known. But, at the same time it also generated a new IgE-binding compound with a molecular weight of about 70 kDa (Álvarez-Álvarez et al., 2005).

The effect of soft-boiling (100^{o} C for 3 minutes) and hard-boiling (100^{o} C for 30 minnutes) of eggs was also evaluated. There was a significant decrease in the ovomucoid and ovalbumin present in the egg, but they observed that it can still cause IgE reactivity on testing (Hoffman, 1983). Change in allergenicity of 10 species of fish has been evaluated after boiling. It was observed that on boiling few proteins were denatured and also new higher molecular weight proteins were formed which were absent in raw fish. Though there was a decrease in the IgE binding ability of the boiled fish proteins, it was not completely destroyed (Bernhisel-Broadbent et al., 1992a; Bernhisel-Broadbent et al., 1992b). Another study by Hansen *et al. (Hansen et al., 1994)* also reported that

the protein from codfish, herring and plaice have a very high stability as the allergens were reactive even after 4 hours of boiling (Hansen et al., 1994). Various studies have also been conducted on the allergenicity of boiled shrimps, but none of them produced any results of clinical importance because of the stability of the allergens (Ayuso et al., 2002; Daul et al., 1988; Daul et al., 1994; Liu et al., 2010; Naqpal et al., 1989; Reese et al., 1999).

Table 2.2 Studies conducted on the moist heat processing

Processing	Food	Studies Conducted
Moist Heating (Boiling, Autoclaving)	**Cherry**	(Andreas, 2009)
	Apple	(Marzban et al., 2009)
	Kiwi	(Fiocchi et al., 2004)
	Celery	(Jankiewicz et al., 1996; Andreas, 2009)
	Peanut	(Beyer et al., 2001)
	Milk	(Andreas, 2009; Ehn et al., 2004; Sathe et al., 2005)
	Soybean	(Kleine-Tebbe et al., 2002; Mittag et al., 2004)
	Cashew nut	(Venkatachalam et al., 2008)
	Lentil	(Cuadrado et al., 2009)
	Chickpea	(Cuadrado et al., 2009)
	Lupine	(Álvarez-Álvarez et al., 2005)
	Eggs	(Hoffman., 1983)
	Fish	(Bernhisel-Broadbent et al., 1992a; Bernhisel-Broadbent et al., 1992b; Hansen et al., 1994)
	Shrimps	(Ayuso et al., 2002; Daul et al., 1988; Daul et al., 1994; Liu et al., 2010; Naqpal et al., 1989; Reese et al., 1999)

2.4.2.1 (B) Dry Heat

Dry heat processing of food involves minimal interaction with water molecules throughout the processing duration. Millard reaction and the enzymatic browning reactions can occur as a result of this kind of thermal processing. The Millard reaction involves a reaction between the free amino acids of the proteins with the reducing sugars (Mottram et al., 2002) (acetones and ketone) present

in food resulting in the formation of aggregates which can affect the allergenicity and the gastric digestibility of the allergen compound or epitope (Thomas et al., 2007). Maleki *et al. (Maleki et al., 2000)* studied the effect of roasting on peanuts and has shown that the Millard reactions have increased the IgE binding capacity of allergens Ara h 1 and Ara h 2 by about 90 times compared to the raw peanuts extracts. Thus, in this case, the Millard reaction resulted in a considerable upsurge in the allergenicity. But, the Millard reactions in the case of Pru av 1 which is a major allergen present in cherries have shown a reduction in the allergenicity (Gruber et al., 2004). In another study on the effect of roasting on hazelnuts conducted by Hansen *et al.* (Hansen et al., 2003) showed that the allergenicity of the birch pollen related allergens of Cor a 1.04 and Cor a 2 to have decreased considerably.

Dry roasting of few varieties of tree nuts have also been widely evaluated. Cashew nut flour proteins have been evaluated for their allergic activity after dry roasting. It was assessed that the allergic compounds present in the flour were relatively unchanged when heated to temperatures of 140^{o} C (20 minutes and 30 minutes), 175^{o} C (15 minutes and 20 minutes) and 200^{o} C (10 minutes and 15 minutes) (Venkatachalam et al., 2008). In a similar study which was conducted on roasted almonds, which were roasted to a temperature of 137.7^{o} C, 148.8^{o} C, 160^{o} C (20 minutes & 30 minutes); 168.3^{o} C and 176.6^{o} C (8 minutes, 10 minutes & 12 minutes) also showed a very high heat stability (Venkatachalam et al., 2002).

It is quite clear that the thermal processing which involves the conventional/traditional heating methods have an impact on the protein structure and thus on the allergenicity. But, there are a few cases that resulted in an increased reactivity of the allergen which is not desirable. So a careful analysis has be conducted on the impact of processing on allergenicity of the allergen compounds. Also the effect of the thermal processing on the type of epitope present in the protein is different

and thus, it should also be considered (Sathe et al., 2005) as another factor in the evaluation which makes the study of the protein structures a critical factor in achieving the desired results.

Table 2.3 Studies conducted on the dry heat processing

Processing	Food	Studies Conducted
Dry Heating (Roasting)	**Cherry**	(Gruber et al., 2004)
	Peanut	(Maleki et al., 2000)
	Hazelnuts	(Hansen et al., 2003)
	Cashew nuts	(Venkatachalam et al., 2008)
	Almonds	(Venkatachalam et al., 2002)

2.4.2.2. Non-Thermal Processing

Non-thermal processing involves wide variety of processing techniques that do not involve heating the food necessarily to impart a certain change in the product. Currently, there are a large number of processes that fall under this category which induce a change in the conformation structure of the protein.

2.4.*2.2 (A) Proteolysis*

Proteolysis or hydrolysis is a reaction that breaks the peptide bond with in the primary structure of the protein between various amino acids, resulting in formation of highly reactive and soluble units of amino acids. This process is also known to produce bitterness which is due to the presence of few specific amino acids formed as a result of hydrolysis; hence not preferred in the processing of foods (Hettiarachchy, 2012). Hydrolysis of proteins can be done either by using acids or enzymes. It was showed that the acid hydrolysis of gluten protein has reduced the foaming ability but had enhanced the emulsifying properties and solubility (Wu et al., 1976). Various enzymes are also used for proteolysis of proteins. This often produces deviations in various protein characteristics which include solubility, foaming property, emulsifying property and water- binding capacity

(Chobert et al., 1988; Diniz and Martin, 1997; Hettiarachchy, 2012; Kuehler and Stine, 1974; Panyam and Kilara, 1996; Qi et al., 1997; Wu et al., 1976).

Thus, we can say that proteolysis potentially disrupts the protein structure which modifies its functional properties and moreover, they can effectively change the structure of both the linear and the conformational epitopes present in food protein. The extent of the processing is highly dependent on the amino acid sequence, secondary structure and also on the modifications in the structure after the action of a specific enzyme (Thomas et al., 2007). In vitro hydrolysis has been performed on the Ara h 2 protein allergen of the peanut which changed the secondary structure of the molecule drastically. But, in the same study, they observed that this change in the secondary structure has not affected the allergenicity of the Ara h 2 allergen because it was able to retain the linear epitopes with in the structure after hydrolysis (Sathe et al., 2005; Sen et al., 2002). Watanabe *et al.* (Watanabe et al., 1990) reported that when rice was treated with the Actinase enzyme, it decomposed the globulin protein responsible for sensitivity in the patients to a great extent. When the treated rice clinically administered to 7 patients who were sensitive to rice, only one showed any allergic reaction. Similar results have been shown with the proteolysis of the soybean (Herman et al., 2003; Thomas et al., 2007; Wilson et al., 2005). In the case of milk, proteolysis is widely associated with the reduction in the number of epitopes which results in the reduction of allergenicity caused due to the milk products particularly infant foods. Protein enzyme treatments are included in production of hypoallergenic infant foods so that the cases of severe allergic reactions can be minimised in infants (Cocco et al., 2003; El-Ghaish et al., 2011a; El-Ghaish et al., 2010a, b; El-Ghaish et al., 2011b; Pescuma et al., 2009; Pescuma et al., 2011).

Hence, hydrolysis of proteins can mitigate the allergic properties of few proteins, but further research has to be done in developing specific enzymes that act only on a particular protein causing

the allergic reaction in the body. Furthermore, care should be taken such that the proteolysis with enzymes do not result in formation of new epitope centres in treated proteins which could cause a detrimental impact on human health.

2.4.*2.2 (B) Ultrafiltration*

Ultrafiltration may be defined as a membrane separation process that is activated by pressures in the range of 2 – 10 atmospheres resulting in removal of high molecular weight solutes from the solutes having lower molecular weight (Sivasankar, 2002). Ultrafiltration finds wide applications in the food industries involving the protein separations. It is used in the Whey protein separation in the cheese manufacturing industries. It is also widely used in the protein concentrate production for the skim milk for the soft cheese manufacturing (Sivasankar, 2002). Ultrafiltration is also widely used in clarification of various fresh fruit juice products (Hsu et al., 1987; Jiraratananon and Chanachai, 1996; Jiraratananon et al., 1997; Kirk et al., 1983; Smith and Charter, 2011; Vladisavljević et al., 2003). A study was conducted by Brenna *et al.* (Brenna et al., 2000) on the peach allergies in juice where they evaluated the effect of ultrafiltration. They have subjected the peach juice to a final ultrafiltration step and they were able to produce a hypoallergenic juice where the ultrafiltration process was able to separate out the epitopes or the proteins containing the epitopes causing allergic reactions. But, they also mentioned that the ultrafiltration step has removed the important components of the juice (like minerals, gums, pectin, sugars) which resulted in a poor quality hypoallergenic juice which lowered sensory attributes (Brenna et al., 2000; Sathe et al., 2005). Moreover, researchers were also able to extract the allergen Sa 1 from the shrimp with the ultrafiltration process in combination with other techniques (Nagpal et al., 1989). Also, ultrafiltration is used in combination with heat denaturation and enzyme proteolysis for production of hypoallergenic infant products (Besler et al., 2001). Olofsson *et al.* (Olofsson et

al., 1981) also said that large peptides that cause allergic reactions can be removed by ultrafiltration process such as fractions of polypeptides having high molecular weights after proteolysis treatment (Adler-Nissen, 1986).

These studies suggest that the ultrafiltration can be effective in separation of allergic compounds from the product when used in combination with other products. But, membranes have to be developed with highly accurate porosity and specificity which could only separate out the specific allergens based on the molecular weight.

2.4.*2.2 (C) Fermentation*

The fermentation is a microbial process where the microbes act on food producing enzymes which impart change in the texture, flavor and various other characteristics such that the final product can be consumed (Bamforth, 2005). Evidences suggest that fermentation was a very old process (Egyptian Civilization) to preserve foods especially milk and fruits which particularly have a very short shelf life when not processed (El-Mansi, 2012). In 1907, Metchnikoff first suggested that the fermented foods are known to have a few health benefits because of the presence of certain kind of microbes (Metchnikoff, 1907). Further research was conducted on fermented foods and it came into light that the presence of Lactic Acid bacteria (LAB) in certain foods and also overall fermentation can promote better health (Anekella and Orsat, 2013; Elmer et al., 1996; Macfarlane and Cummings, 1999; Matsuzaki and Chin, 2000; Salminen et al., 1998).

In todays' society, this process becomes very prominent because of the wide range of products that can be produced from fermenting cereals, legumes, milk and meats which have particularly high content of protein. Thus, changes in the nutritive content especially in the proteins is a very important aspect during the fermentation which has to be further studied. But, the analyses have

indicated that the amount of changes found were minimal (McFeeters, 1988). Studies have also been conducted on assessing the effects of fermentation on the allergens especially soybean products and milk.

Soy products that were analysed included tofu, miso, tempeh, sprouts and mold hydrolyzed soy sauce for finding the effects caused by the fermentation process on the allergenicity. It was reported that these products retained the allergic properties, though the binding capacity of the IgE have diminished considerably (HERIAN et al., 1993). In 2000, techniques like chemical breeding, enzymatic digestion and a physico-chemical treatment were used in the development of the hypoallergenic soybean products. They used processing techniques to mitigate the 3 major allergens in the soybean which are Gly m Bd 60K, Gly m Bd 30K, and Gly m Bd 28K and tested the products using patients allergic to soybean. Their results suggested that about 80% of the patients who are allergic to soybean did not show any adversarial reactions after consumption of the processed hypoallergenic products (Ogawa et al., 2000). In the case of milk, β-lactoglobulin and casein are considered as the most potent allergens (El-Ghaish et al., 2011a). Ehn *et al.* (Ehn et al., 2005) worked on the effects of fermentation and proteolysis on the β-lactoglobulin, and found that both lactobacilli fermentation and proteolysis with trypsin had reduced the IgE binding capacity. But the reduction observed with proteolysis is higher compared to that of the protein denaturation by acid (Ehn et al., 2005; Sathe et al., 2005). Bu *et al.* observed that the allergenicity of β-lactoglobulin was reduced when fermentation began, but as the duration of the fermentation process increased, there was no linear relation between the allergenicity reduction and the amount of proteolysis of the protein (Bu et al., 2010). A 99% reduction was observed in the allergenicity of α-lactalbumin and β-lactoglobulin when sterilized cow's milk was treated with meso- and thermophilic strains or mixed cultures when tested with enzyme-linked immunosorbent assay

ELISA, but there was hardly any effect when skin-prick tests were conducted (Jedrychowski, 1999).

Table 2.4: Studies conducted on non – thermal conventional processing methods

Processing	Food	Studies Conducted
Proteolysis/Hydrolysis	**Rice**	(Watanabe et al., 1990)
	Peanut	(Sen et al., 2002; Sathe et al., 2005)
	Soybean	(Herman et al., 2003; Wilson et al., 2005; Thomas et al., 2007)
	Milk	(Cocco et al., 2003; El-Ghaish et al., 2010a; El-Ghaish et al., 2011a; El-Ghaish et al., 2011b; Pescuma et al., 2009; Pescuma et al., 2011)
Ultrafiltration	**Peach juice**	(Brenna et al., 2000)
	Shrimp	(Nagpal et al., 1989)
	Infant foods	(Besler et al., 2001)
Fermentation	**Soy Products**	(Herian et al., 1993; Ogawa et al., 2000)
	Milk	(Ehn et al., 2005; Bu et al., 2010; Jedrychowski, 1999)

2.4.3 Novel Food Processing Methods

2.4.3.1 Thermal Processing Methods

Increasing concerns about the environment and the emission of Green House Gases (GHG's) led researchers to think about the alternative methods of manufacturing in various processing industries including food industries around the world. This led to the development of energy efficient and green technologies in food processing industries which are the novel technologies. The materialization of the novel technologies not only saves the energy costs and wastage of the natural resources, but it has been shown that the final quality of the product is well preserved compared to the products produced using conventional techniques (Pereira and Vicente, 2010). Thus, the thermal processing using novel technologies has become popular in recent past with their lower production cost advantages and the ability to maintain better quality of the final product.

But, the effects of these processing techniques on the various components is rarely studied especially on the allergens except in processing using microwaves.

2.4.3.1 (A) Microwave Heating

Microwave is an electromagnetic wave within the frequency band of 300 MHz and 300 GHz. These waves find applications in variety of areas which include telecommunications, medical and other scientific fields (Richardson, 2001). In relation to food industry, microwaves are used for thawing of frozen foods, pasteurization, drying and pre-cooking (meats like beef). This is primarily due to the advantages that microwave heating provides over other heating methods which include efficient heating, less time to start, easy for mass commercialization and food with better nutritional and sensory qualities (Decareau and Peterson, 1986; Sun, 2005) . But, microwaves of all the frequencies cannot be used for the food applications. There are two specific frequencies that are dedicated to the industrial applications (food industry) which are 915 MHz and 2.45 GHz (most commercial ovens) (Doona, 2010; Tewari, 2007).

There are two main principles involved in the microwave heating of food. The first and the most important is the 'dipole rotation' and the second one is the 'ionic polarization' or 'ionic conduction'. For the dipole rotation to occur the presence of polar molecules is very important. The most common polar molecules found in food is the water molecule that is randomly oriented all over food. But, in the presence of an electric field (like microwave), the molecule tries to align itself to the orientation of the field appropriately. In a microwave, the field alternates at a very high frequency (2450 MHz) and when the polar molecule is present, after aligning themselves to the field, the resulting interactions between the fast moving (rotating) polar molecules and the other molecules in food produces friction resulting in the heating effect (Doona, 2010; Oliveira and Franca, 2002; Tewari, 2007). The ionic polarization occurs in the presence of high concentrations

of ions in the food. Under the influence of the electric field, the ions in food collide with each other and thus dissipate their kinetic energy which is converted to thermal energy on collision (Doona, 2010; Meda et al., 2005; Oliveira and Franca, 2002; Tewari, 2007). In fact, it is said that the temperature of food can be raised by about 10^{o} C per second with the high intensity of heat produced in microwave processing (Lew et al., 2002; Meda et al., 2005).

Thus, the microwave processing generates heat instantly and the amount of heat generated is highly dependent on the local composition and the overall homogeneity of the food product. Moreover, the heat transfer rate within the food relies on the composition (presence of water) and on the geometry of the food. There are a wide variety of changes caused due to the generation of heat in food during microwave processing. Some of them include gas/water vapor generation (baking), starch gelatinization (corn, potatoes, etc.,), protein denaturation (milk & egg), surface browning (Millard Reaction in baking), caramelization (due to sugar dehydration) and enzyme inactivation. They can also effect the textural and organoleptic properties of the food. This suggests that the microwave processing does have an effect on the components of food (Meda et al., 2005; Mudgett, 1989; Ponne and Bartels, 1995; Schubert, 2005). Pomerai *et al.* (de Pomerai et al., 2003) also studied the effect of the microwaves on the protein conformation without the bulk heating. In this study, they have reported that microwaves not only have a thermal affect as we have seen, but they also have a non-thermal effect on protein conformation especially in biological proteins (de Pomerai et al., 2003). The surface properties of any protein might change on interaction with the microwaves because of variation in bonds with the water molecules and other ions (Meda et al., 2005). Studies have also been conducted in understanding the effect on microwave processing on the allergen proteins present in food.

The microwave processing effect on three important allergens present in celery has been evaluated by Jankiewicz *et al (Jankiewicz et al., 1997)*. The three allergens in celery are Api g 1, celery profilin and multiple allergy bands recognised by the patient's IgE within the mass range of 35 kDa – 90 kDa (called Carbohydrate epitopes). Two kinds of microwave treatments have been conducted on the celery; first at 750 W for 10 minutes where the product temperature was around 100^{o} C and the second at 750 W for 30 minutes where the product temperature was also around 100^{o} C. They have reported that the allergic activity of celery has been reduced under thermal processing. The thermal denaturation (at 100^{o} C for 20-30 minutes) have showed no binding of Api g 1 and Profilin allergens and reduced binding in the Carbohydrate epitopes when tested. The Api g 1 showed reduced to no binding with 10 min processing in the microwave at 100^{o} C whereas profilin had to be processed for 30 minutes to get the same reduction. Carbohydrate epitopes were relatively very stable when processed even for 30 minutes at 100^{o} C. Moreover, they also concluded that further work has to be conducted on the structural changes in the epitopes to understand their modifications under the heat and other external stresses providing more detailed insight into processing effects on them (Jankiewicz et al., 1997).

Gall *et al. (Gall et al., 1994)* evaluated the effect of microwave treatment on the kiwi fruit allergen. They have also reported that the patients with sensitivity towards kiwi fruit allergen have showed high cross reactivity with apple allergen and birch pollen allergen and moderate reactivity towards carrot and avocado. The kiwi fruits were treated in the microwave at 4 levels of temperature (40^{o} C, 60^{o} C, 80^{o} C & 90^{o} C) and they reported that the allergenicity of the kiwi fruit decreased with the increasing temperature which was evaluated by skin prick test (SPT). Microwave processing (700 W for 25 minutes) on the soybean was also evaluated. When enzyme-allergosorbent test

(EAST) were conducted, 9 out of a total of 15 patients showed reactivity towards the microwave processed soybean allergen (Besler et al., 2001; Vieths et al., 1995).

The effect of microwaving on the allergenicity of various nuts have also been evaluated. Wigotzki *et al. (Wigotzki et al., 2000)* evaluated the IgE binding activity of the hazelnut allergens when processed under microwave and also with conventional heating. They processed the nuts until they reached the temperatures of 155^{o} C or more, but the effect on the allergenicity was minimal. They also mentioned that the allergens of lower molecular weight had higher heat stability (at temperatures up to 190^{o} C). In another study, the effect of microwave processing on almonds has been evaluated. They found that the effect of processing on the proteins was minimal but when treated at extreme temperatures (160^{o} C for 20-30 minutes and then 3 minutes of microwave heating) changes the allergenicity. But, under normal processing conditions the allergens in almonds are relatively stable to heat treatments (Venkatachalam et al., 2002). Su *et al. (Su et al., 2004)* also assessed the allergenicity of almonds, cashew nuts and walnuts under microwave processing (500 W for 1 min and 3 min) in combination with γ-irradiation (1–25 kGy) and they reported that the allergens were very stable under the processing conditions (Su et al., 2004). Lupine flour was also reported to be responsible for allergies in few patients and hence the effect of microwave treatment on its flour was evaluated. It was reported that treatments extending up to 30 minutes under microwave had almost no effect on the reactivity of the allergens (Álvarez-Álvarez et al., 2005).

Table 2.5: Studies conducted on Microwave processing (Noval – thermal) methods

Processing	Food	Studies Conducted
Microwave Processing	**Kiwi**	(Gall et al., 1994)
	Soybean	(Besler et al., 2001; Vieths et al., 1995)
	Hazelnuts	(Wigotzki et al., 2000)
	Cashew nuts	(Su et al., 2004)
	Almonds	(Venkatachalam et al., 2002; Su et al., 2004)
	Walnuts	(Su et al., 2004)
	Lupine	(Álvarez-Álvarez et al., 2005)

2.4.3.2 Non-thermal processing methods

Novel non thermal processing methods involve the processing methods that do not heat up the food i.e. the processing is done under sub-lethal temperatures, unless the heat is generated internally (resistive heat). Various non-thermal processes which have been evaluated for processing foods include high hydrostatic pressure processing, gamma-radiation (γ-radiation), pulsed electric field processing, high intensity ultrasound and ultraviolet light processing. The increasing pressure for commercialization of these processes makes it important for researchers to study their effect on the various component of the food, especially the allergens. Though not widely studied, researchers are showing keen interest in recent times as their advantages have been accentuated.

2.4.3.2 (A) Gamma-radiation (γ-radiation)

The process of exposing food to an ionizing radiation is called 'Irradiation'. Though wide range of ionizing radiations are available, the treatment with gamma-radiation is widely employed over the others. These rays are emitted through the radio isotopes Cobalt – 60 and Cesium – 137 (Farkas, 2006). These are primarily used for increasing storage duration by destroying the surface pathogens present on the food. By varying the dosage of radiation, various other desired effects

are achieved which can be extension of shelf life, microbial destruction and reduction in the losses of produce during storage (Farkas, 2006; Lagunas-Solar, 1995). The process of irradiation is also environmentally safe and is an energy efficient process which adds to its marketability (Farkas, 1998; Lagunas-Solar, 1995). The radiation in the ranges of 2-7 kGy (medium dosage level) can eliminate the surface pathogens in the food products without affecting the organoleptic and nutritional qualities of the food (Farkas, 1998). Researchers also mentioned when irradiation is used in combination with other processing treatments, even a low or medium level exposure would actually decrease the chances of the microorganisms to survive in the end product (Farkas, 1998; Farkas, 2006; Lado and Yousef, 2002; Lagunas-Solar, 1995).

With their potential and wide range of applications in disinfection and processing of foods, their effect on the allergenicity of various allergens have also been evaluated, especially in shrimps (Byun et al., 2000; Byun et al., 2002; Sinanoglou et al., 2007; Zhenxing et al., 2007a, b), tree nuts (Su et al., 2004), egg (Byun et al., 2002; Kume and Matsuda, 1995; Seo et al., 2007; Seo et al., 2004) and dairy products (Lee et al., 2001). Byun *et al. (Byun et al., 2000)* studied the effect of gamma radiation on the heat stable proteins present in the Brown shrimp, which were extracted and then irradiated using gamma radiation with dosage levels varying from 0-10 kGy. They reported that a conformational change in the structure of the protein which was deducted by the spectrometric techniques. Also, the IgE binding ability of the heat stable protein allergen was tested using the protein sera from human patients and it was observed that it reduced with the irradiation treatments (Byun et al., 2000). In an another study conducted by Zhenxing *et al.* (Zhenxing et al., 2007b) similar results were observed. They also reported the dependency of the irradiation dosage on the allergenicity of the shrimp protein. As the dosage increased from 0 kGy to 5 kGy, the allergenicity increased, but when the dosage of irradiation increased beyond 10 kGy,

they observed that the allergenicity of shrimp protein reduced significantly as showed by immunoblotting and ELISA tests (Zhenxing et al., 2007b). These researchers also reported that the combination of thermal processing (boiling) after irradiation using gamma radiation showed better results by further decreasing the immunoreactivity of the shrimp protein (Zhenxing et al., 2007a).

Lee *et al.* (Lee et al., 2001) evaluated the effect of gamma radiation treatments on the milk allergens. They reported a reduction in the solubility of the proteins and also a rise in the turbidity which was caused by the agglomeration of the irradiated protein. All of the results suggest that there is a conformational change in the protein structure due to the irradiation treatment on α-casein and β-lactoglobulin (Lee et al., 2001). The effect of irradiation using gamma radiations on Ovalbumin, which is a major protein allergen present in egg white was also evaluated. It was reported that the gamma radiation was able to impart conformational changes in the protein and furthermore, they also specified that the gamma radiation can be used for allergy control specific to Ovalbumin (Lee et al., 2005; Seo et al., 2007; Seo et al., 2004). Su *et al.* (Su et al., 2004) studied the impact of gamma irradiation on the almonds, cashew and walnut protein allergens (tree nuts) and they reported that the irradiation alone or in combination of another heat treatments did not affect the allergenicity of protein allergens in any of the specified nuts.

Food irradiation using gamma radiations seems to be an effective alternative processing technique for coping with the increasing allergy cases. Though, the results seem to indicate that the irradiation can result in producing conformational changes in protein structure, further research has to be conducted on studying their clinical relevancy.

2.4.3.2 (B) Pulsed Ultraviolet Light (PUV)

With growing concerns regarding the excessive use of chemical preservatives in food for extending the shelf life and protecting food from pathogens, alternative preservation techniques have been developed. UV radiations are already being used widely all round the world for the water and air disinfection treatment and decontamination of surfaces in labs. Recently, its use as a replacement of traditional thermal and chemical treatments for shelf life extension in foods is being investigated. These radiations can be used for pasteurization of fresh juices, post-harvest treatments and disinfection of food contact surfaces in processing plants. The wave length of UV rays range from 100 nm to 400 nm. With the increasing demand of using the UV light for various treatments in food industry, the effects of it on the complex food matrix made of distinct components has been investigated widely. Its potential use as an alternative treatment for mitigating allergens in food is also explored (Koutchma, 2009).

Chung *et al. (Chung et al., 2008)* evaluated the effect of PUV treatment on peanut extract and liquid peanut butter and reported a marked reduction in the IgE binding capacity of the allergen present in them. It was observed that the allergen with molecular weight 63 kDa displayed a reduced solubility suggesting a change in its structural conformation. But, on the other hand, the allergen protein of molecular weight 20 kDa showed no change (Chung et al., 2008). Yang *et al.* (Yang et al., 2012) also conducted similar studies on peanut allergens by using PUV. They also reported a reduction in the IgE binding capacity after the treatment of Ara h 1, Ara h 2 and Ara h 3 allergens which are the major peanut allergens. Similar studies were also conducted on the soybean allergen by Yang *et al. (Yang et al., 2010)* where the effect of PUV treatment has been investigated. They also reported a reduction in the soybean allergenicity by up to 50% when treated for about 6 minutes. They concluded mentioning that PUV treatment can be used in production of

lower allergenic soy products, but relevant clinical analysis have to be conducted (Yang et al., 2010). Shriver *et al.* (Shriver et al., 2011) also conducted studies to evaluate the IgE binding capacity of the shrimps when treated with PUV. This study also concluded that treating tropomyosin which is the major shrimp allergen with PUV for about 4 minutes reduced its IgE binding capacity. Li *et al. (Li et al., 2013)* investigated the effect of PUV on the allergenicity of almond protein extracts. The protein extracts of almond were exposed to PUV from 0.5 min to 10 min and their IgE binding capacity was evaluated using the Electrophoresis, Western Blot and ELISA. They reported that PUV to have reduced the IgE binding capacity in the almond extracts better than boiling treatment.

Manzocco *et al. (Manzocco et al., 2012)* studied the ultraviolet processing on the egg white and they reported no change in the immunoreactivity even though it was sensitive to the UV radiation. The effect of UV light (specifically UV-C) has been evaluated by Tammineedi *et al. (Tammineedi et al., 2013)* on allergenicity of casein and whey proteins (α-lactalbumin and β-lactoglobulin). In this study the major protein allergens extracted from milk were treated for 15 minutes resulting in significant reduction of allergenicity. The electrophoresis tests also revealed that the three protein bands tested had showed a reduced intensity after the treatment with UV radiation.

The use of UV rays for producing hypo allergic food products is possible as most of the work suggests that this treatment had reduced the allergenicity of the allergens. But, clinical tests and in vivo studies have to be conducted for determining the applicability of this technology on a large scale.

2.4.3.2 (C) Ultrasound Treatment

Ultrasound treatments use mechanical waves of frequencies varying between 20 kHz – 100 kHz (Feng et al., 2011) which results in agitation and formation of bubbles in the food system due to compression and rarefaction and these bubbles formed collapse eventually when they reach their critical size. When these bubbles blast, the temperatures and pressure can go up to 5000 K and 1000 atm in the local region. These extreme parameters would result in conformational changes in the structure of the protein allergens (Shriver and Yang, 2011). It was also reported by Sonia *et al. (Soria and Villamiel, 2010)* that physical effects on the components in food can also be caused due to the high shear stress and velocity gradients resulting in micro streams and moreover, the protein modification can also be a result of the radicals generated from water (Shriver and Yang, 2011; Soria and Villamiel, 2010). The work done on the effect of ultrasonic treatment on allergens is very limited to date.

Zhenxing *et al. (Zhenxing et al., 2006)* assessed the effect of high intensity ultrasound on the structures of protein extract from shrimps. He also compared the results with the effect on boiling of shrimp by studying protein by Electrophoresis and ELISA tests. They treated the protein extract with high intensity ultrasonic waves of 30 Hz and 800 W for about 1.5 hours with temperatures ranging from 0^{o} C to 50^{o} C. This study reported that the ultrasonic treatment has reduced the allergenicity of the shrimp protein extract especially the ultrasonic treatment at 50^{o} C. Another study was also conducted which evaluated the effect of ultrasonic treatments on tropomyosin allergen present in the shrimp protein by Li *et al. (Li et al., 2006).* They also reported a reduction in the IgE reactivity after high intensity ultrasonic treatment of tropomyosin. Moreover, they also observed a rise in the formation of lower molecular weight protein fragments with prolonged

treatment times which suggests that the ultrasonic treatment has an effect on the protein conformation (Li et al., 2006).

Stanic-Vucinic *et al*. (Stanic-Vucinic et al., 2012) investigated the effect of high intensity unltrasonification on the milk allergenicity especially the β-lactoglobulin. They reported that the ultrasonic treatment has resulted in a very minor changes in the IgE binding capacity of the β-lactoglobulin allergen even though a considerable amount of structural deviations were observed. They concluded that though sonication treatment resulted in conformational changes in the structure of proteins and allergens, there is no significant effect on the allergenicity of the milk proteins (Stanic-Vucinic et al., 2012). Tammineedi *et al. (Tammineedi et al., 2013)* have also reported similar results where they evaluated the effect of high intensity ultrasound treatment on whey proteins and casein which are major milk allergens.

2.4.3.2 (D) High Hydrostatic Pressure (HHP) / High Pressure (HP)

The use of hydrostatic pressure in the field of food processing was initiated for inactivation of the micro-organisms for preservation of fruits and vegetables (Hite et al., 1914) and milk (Hite, 1899). This led to the expansion of new microbial inactivation technique which was also widely studied for understanding its effectiveness on bacteria and viruses (Atanasiu et al., 1951; Basset et al., 1956; Basset and Macheboeuf, 1933; Basset and Macheboeuf, 1932; Basset et al., 1935a; Basset et al., 1935b; Basset et al., 1933; Vignais et al., 1952). Later on, researchers have observed the potential of the high pressure or high hydrostatic pressure treatments in food processing and evaluated the effect on various food components like enzymes, proteins and various other bio molecules (Knorr, 1993; Morild, 1981; Popper and Knorr, 1990). Researchers understood that the conformational structure can be changed by using pressure as a factor (Rivalain et al., 2010). Moreover, studies conducted by Zhang *et al. (Zhang, 1995)* and various other researchers revealed

that the conformational changes in the protein caused by the high pressure treatment can be different from those changes induced by the heat denaturation and can also result in preservation of few specific structures of the biomolecule (Smeller, 2002; Winter et al., 2005; Zhang, 1995). Thus, the effects of high hydrostatic pressure on allergen proteins have been widely studied especially the milk allergens.

The use of HHP systems in combination with proteolysis treatments have been widely looked on for producing hypoallergenic foods especially whey protein hydrolysates. Chicón *et al.* (Chicón et al., 2008a) concluded that the HP treatments (200 MPa to 400 MPa) performed on whey protein isolate resulted in no change of the IgE binding capacity compared to the untreated sample. They mentioned that the HP treatments of 400 MPa had conformational changes in the protein structure and digestibility, but did not affect the allergenicity. In another study conducted by Chicón *et al. (Chicón et al., 2008b)* the effect of HP treatment on the enzymatic proteolysis and in turn on the IgE binding capacity were evaluated. Their results show that the high pressure treatment has resulted in a reduction of time required for the proteolysis from about 48 hours (when done at atmospheric pressure) to within 20 minutes (for trypsin). But, they also reported that the hydrolysates produced through this accelerated process showed IgE reactivity when tested. Thus, it was observed that even though the HP treatment resulted in accelerating the process incredibly, it was unable to completely produce a hypoallergenic product. Further, they also mentioned that it is possible to use HP treatment in accelerated proteolysis treatment with modified parameters which can result in production of hypoallergenic products (Chicón et al., 2008b). Peñas *et al.* (Peñas et al., 2006b) further analysed the relation between the proteolysis and HP treatments. They reported that the combination of treatments can not only accelerate the hydrolysis, but allergenicity can also be reduced depending on the type of enzyme used. The enzymes Corolase PN-L and

Neutrase showed a considereable reduction in the IgE binding when treated at 300 MPa for about 15 minutes during proteolysis (Peñas et al., 2006b). They also reported in another study that the enzymes like pepsin, trypsin and chymotrypsin have a better effect on the allergenicity of β-lactoglobulin when treated under HP (Peñas et al., 2006a) and same results were also obtained by Bonomi *et al.(Bonomi et al., 2003).* They reported that the reduction in the epitope binding sites might be caused by the unfolding of the hydrophobic core of the β-lactoglobulin protein (Bonomi et al., 2003). Zeece *et al. (Zeece et al., 2008)* and López-Expósito *et al. (López-Expósito et al., 2012)* also investigated the effect of HP processing on the β-lactoglobulin using an *in vitro* pepsin digestion and electrophoresis. They reported that the HP treatments have increased the digestibility of proteins when treated at 400 MPa for only about 10 minutes and this treatment has a potential application in manufacturing of hypoallergenic products. Zhong *et al.* (Zhong et al., 2011) reported data on the effects of dynamic HP treatments in combinations with temperatures ranging from 70^{o} C to 90^{o} C. Their data suggested that the temperature has enhanced the effect of high pressure processing in reducing the allergenicity when treated above 160 MPa. They also reported that the treatments below the pressure of 80 MPa led to an increase in the allergenicity of the β-lactoglobulin, but it reduced when parameters were above 80 MPa. But, on the contrary, study conducted by Kleber *et al. (Kleber et al., 2007)* revealed that HHP treatments at 200 MPa, 400 MPa and 600 MPa at temperatures between 30^{o} C to 68^{o} C (treatment times: 0, 10 and 30 minutes) resulted in an increased allergic responses when tested using ELISA. But, the treatments at temperatures 60^{o} C and 68^{o} C did show a reduction in allergenicity, but it was not very significant.

Apart from the milk allergens, effects of HP treatments have also been evaluated in various other products. Hydrostatic pressure effect on soybean allergens have been evaluated by Peñas *et al.* (Peñas et al., 2011) and Li *et al.* (Li et al., 2012). Peñas *et al.* (Peñas et al., 2011) reported that only

the HHP process does affect the allergenicity of the soybean. When HHP treated seeds were sprouted, they had a huge reduction in the allergenicity compared to sprouts that were not treated with HHP (prior to sprouting). Thus, this study showed that HHP treatment prior to sprouting would be a great approach in producing hypoallergenic soybean products. Li *et al. (Li et al., 2012)* reported that the HHP treatments had significant effect on the secondary structures of the soy allergens and this treatment had a great potential in reducing the allergenicity helping in manufacture of hypoallergenic soy based infant formula.

Few other researchers also showed that HHP treatments reduced the allergenicity of the shrimp heat stable proteins (Kim et al., 2006). Kato *et al. (Kato et al., 2000)* worked on HHP treatments on rice and they reported that on treating rice at 300 MPa for about 120 minutes resulted in reduced allergenicity. This was due to the cell damage caused by the high pressure which resulted in solubilisation and a better extraction of the allergens into the solvent. But, compatibility of the specific solvent and also the solubility of the allergens in that specific solvent will play a major role in using this method. HHP effect on the almond allergenicity was also studied, but this treatment showed no change in the allergenicity compared to the untreated almonds (Li et al., 2013). Houska *et al. (Houska et al., 2009)* evaluated the effect of HP on apple juice which contains the major allergen Mal d 1. They reported that treatment (parameters followed: 450 MPa – 550 MPa for 3 – 10 minutes) showed no effect on the allergenicity of the molecule.

Thus, the use of HP treatments in production of hypoallergenic foods is being widely evaluated because of the effectiveness in most allergen cases. It has been observed that HP treatments are particularly more effective when used in combination with other processing treatments like proteolysis or heat treatment (boiling). But, it is important to take a note that clinical studies have

to be further conducted to analyze the relevance of using these treatments for producing the hypoallergenic foods.

Table 2.6: Studies conducted on non-thermal Noval processing methods

Processing	Food	Studies Conducted
Gamma Radiation	**Shrimp**	Byun et al., 2000; Byun et al., 2002; Sinanoglou et al., 2007; Zhenxing et al., 2007a, b)
	Egg	Byun et al., 2002; Kume and Matsuda, 1995; Seo et al., 2007; Seo et al., 2004; Lee et al., 2005)
	Milk	(Lee et al., 2001)
	Cashew nuts	(Su et al., 2004)
	Walnut	(Su et al., 2004)
	Almonds	(Su et al., 2004)
Ultraviolet Light	**Peanut**	(Chung et al., 2008; Yang et al., 2012)
	Soybean	(Yang et al., 2010)
	Shrimp	(Shriver et al., 2011)
	Almonds	(Li et al., 2013)
	Egg	(Manzocco et al., 2012)
	Milk	(Tammineedi et al., 2013)
Ultrasound	**Shrimp**	(Zhenxing et al., 2006; Li et al., 2006)
	Milk	(Stanic-Vucinic et al., 2012; Tammineedi et al., 2013)
High Pressure/ High Hydrostatic Pressure	**Milk**	(Chicón et al., 2008a; Chicón et al., 2008b; Peñas et al., 2006a; Peñas et al., 2006b; Bonomi et al., 2003; Zeece et al., 2008; López-Expósito et al., 2012; Zhong et al., 2011; Kleber et al., 2007)
	Soybean	(Peñas et al., 2011; Li et al., 2012)
	Shrimp	(Kim et al., 2006)
	Rice	(Kato et al., 2000)
	Almond	(Li et al., 2013)
	Apple	(Houska et al., 2009)

2.5 CONCLUDING REMARKS

In conclusion, it is very important for us to characterize the allergies at the molecular level which will help us in understanding them and their reactions with our immune system leading to development of techniques for their mitigation. Most of the food processes have a definite effect

on the conformational structure of the protein and thus also on the allergenicity. Few of them have shown a promise for the future in developing the hypoallergenic foods by reduction or by mitigation of the reactivity on processing, but few others showed an increased reactivity and formation of epitope centres resulting in new reactive sites. Thus, further careful evaluation has to be conducted for determining the influence of specific process on the allergens.

CONNECTING TEXT

In the review, we have seen that various processing methods had effect on the allergenicity of a food product. This changes in most cases were due to the conformational changes in the secondary structures of the allergen protein. The next part of the thesis deals with application of molecular dynamic (MD) simulation technique to study the effect of external electric field stress on the conformation and surface properties of proteins. Since protein's functional properties are dependent on its structure, the effect of the thermal stress and an external electric field using MD on Ara h 6 peanut protein allergen could help understand the implications. The results obtained for MD simulation of Ara h 6 allergen could help in validating the results of FT-IR study on the effect of thermal and electric field stresses on peanut protein.

CHAPTER III

Effect of Thermal and Electric Field Treatment on the Conformation of Ara h 6 Peanut Protein Allergen

3.1 ABSTRACT

The study uses molecular dynamic simulation to evaluate the effect of static and oscillating electric field (2450 MHz) of intensity 0.05V/nm at different temperatures (300K, 380K and 425K) on structural conformation of Ara h 6 peanut protein allergen. The conformational changes in the protein were studied with respect to root mean square deviation, radius of gyration, dipole moment and solvent accessible surface area. The increase in temperature and application of external electric fields, both static and oscillating fields had significant effect on the conformation of Ara h 6, specifically the helical secondary structures. It was observed that the root mean square deviation increased with a rise in temperature and application of external electric fields had no significant effect on it at any given temperatures. This study also demonstrated that exposure to external stresses including thermal and electric fields induces conformational changes in the protein, which may impact its physico-chemical properties.

3.2 INTRODUCTION

In today's world, globalization has led to an increase in cultural and social exchanges among various communities of the world. This inter-exchange confers dissipation of various forms of cuisines increasing the diversity in food available for consumption. This exposure to numerous forms of food has been indicated as one of the reasons for an increase in population that suffer from food allergies (Helm & Burks, 2000). Food allergy is defined as an immune response towards a particular protein or a group of proteins present in a food. Although, any food may cause an immune response, peanuts, tree nuts, milk, soybean, wheat, egg, fish and shellfish are responsible for most of the food allergies around the world (Sicherer & Sampson, 2006). It has been estimated that approximately 3% of the adult population and 3-8% of the infants are allergic to one or more foods and this number is growing (Rona et al., 2007; Venter et al., 2008; Zuidmeer et al., 2008). Several researchers have suggested that genetic predisposition (Arshad et al., 1993; Sampson, 2004) could also be another major issue. Moreover, hygiene hypothesis which states that excessive cleanliness around an individual interrupts with the normal development of immune system because of lack of external environmental triggers may also lead to an increase in susceptibility to allergens related to environment and food (Guarner et al., 2006; Helm & Burks, 2000; Yazdanbakhsh et al., 2002).

In North America, peanuts are one of the major source of food allergy. Sicherer, *et al*. (Sicherer et al., 2010) estimated that about 1% of the population in United States of America is sensitive to peanuts. They also suggested that over the years there has been an overwhelming increase from 0.6 % in 1999 to 2.1% in 2008 in the younger population (< 18 years) who are affected by sensitivity towards peanuts (Sicherer et al., 2010). Within Peanuts, there are about 13 protein allergens recognized by The International Union of Immunological Societies

Nomenclature subcommittee**,** (*Arachis hypogaea* – Ara h) Ara h 1 to Ara h 13, of which Ara h 1, 2 and 3 are major peanut allergens (Breiteneder & Radauer, 2004; Burks et al., 1992; Sampson, 2004; Schmitt et al., 2004; Sicherer & Sampson, 2007). These allergens of peanuts cause IgE-mediated immune responses. The portion of the allergen, which is recognised by IgE is called an epitope. These epitopes consist of linear contiguous stretch of amino acids or three dimensional structural motif that cross link with IgE triggering an allergenic response in a sensitized individual (Sathe & Sharma, 2009).

The allergenicity of a specific compound can be defined as its ability to introduce allergic response and it may vary with varying environmental conditions**.** In food, proteins are found within a complex matrix where its interaction with other food components such as carbohydrates may alter its functionality and make it allergic. Processing of food also impact proteins and alter their digestibility and conformation, which alters their ability to induce allergenic responses (Sicherer & Sampson, 2010). In recent years several studies have revealed that food processing can also play an alternative role in moderating the allergic response of food proteins (Clare Mills et al., 2009; Cucu et al., 2012; Thomas et al., 2007). In 2012, Cucu *et al.* (Cucu et al., 2012) studied the impact of thermal processing and glycation on the basophil activation by hazelnut proteins. They reported that thermal processing of hazelnut protein in the presence or absence of wheat protein had no significant effect on the stimulatory activity of basophil for patients with systemic allergic reaction (SR) or oral allergy syndrome (OAS). They also observed that incubation of hazelnut protein with glucose completely suppressed the stimulatory activity of basophil in OAS patients. They concluded that SR patients were more susceptible to allergic reaction for both processed and unprocessed hazelnut protein as compared to OAS patients. In another study Yang *et al*.(Yang et al., 2012) applied pulsed ultraviolet light on raw and roasted peanuts and peanut buttery slurry to

reduce peanut allergenicity. Their SDS-PAGE and ELISA analysis revealed that with increase in treatment time the allergenicity of peanut protein reduced compared to control. Hence it can be assumed that by introducing novel food processing techniques allergenicity of food proteins can be altered and reduced to a significant level.

It is well known that during processing food components undergo several chemical and physical changes; hence protein subjected to external stresses such as thermal, chemical and electrical stresses may lead to conformational changes to the molecular structure (Singh et al., 2013b). The functional properties of proteins depend on their structure and any change in it may render them non-functional (Singh et al., 2013a). In recent years several studies have been conducted to understand the impact of processing on protein conformation and its relation to the functional properties (Clare Mills et al., 2009; Davis et al., 2001; Mondoulet et al., 2005; Singh et al., 2013a; Singh et al., 2012; Singh et al., 2013b). Several techniques including Fourier Transformation Infrared Spectroscopy (FT-IR), Nuclear Magnetic Resonance Imaging (NMR), X-ray diffraction can be used to study the conformational changes in protein during processing, but these techniques are expensive and can only relate the structure of proteins before and after processing. To overcome these shortcomings Singh et al. (Singh et al., 2013b) applied molecular dynamic (MD) simulation technique to gain insight into protein dynamics under the influence of external static electric field. MD simulation technique is widely used to study the structural and dynamic properties of biomolecules and has been applied in the field of pharmacology and molecular biology for development of accurate and novel drug systems.

In this study we evaluated the conformational change in Ara h 6 peanut protein allergen under the influence of external stresses including thermal and static/oscillating electric field. We selected Ara h 6 as the model protein for this study because it is an important allergen in peanuts and is

approximately 59% homologues with the Ara h 2, which is regarded as a major peanut allergen. Several researchers have reported that Ara h 2 and Ara h 6 allergens are harder to digest in humans compared to the Ara h 1 or Ara h 3 (Koppelman et al., 2005; Koppelman et al., 2010). Moreover, recent studies have also suggested that allergic reactivity to various isoforms of Ara h 6 can be observed in the growing allergic population (Bernard et al., 2007b; Flinterman et al., 2007; Peeters et al., 2007). The isoforms are the same protein but with a slight disparity in the sequence i.e., a very similar duplicate. In 2007, Bernard *et al.,* (Bernard et al., 2007a) found an isoform of Ara h 6 allergen and its derivative compound from peanut protein that were as reactive or more reactive than that of Ara h 2. All the aforementioned findings suggested that studying the behaviour of Ara h 6 under the influence of external stresses might provide an insight on how various food processing techniques might affect their allergenic properties of peanut proteins.

3.3 MATERIALS AND METHODS

MD simulation on Ara h 6 was performed using a classical MD algorithm as implemented in Groningen machine for chemical simulations (GROMACS) software package, version 4.5.5 from the Stockholm Center for Biomembrane Research, Stockholm, Sweden (Hess et al., 2008). Peanut allergen Ara h 6 consists of 127 amino acid residues, where more than 40% of the secondary structure consists mainly of helices (6 helices) and one 3/10 helix. Ara h 6 starting configuration with the PDB (Berman et al., 2000) accession code 1W2Q (Lehmann et al., 2006a) was used for this study. All atom CHARMM27 force field was used to describe the potential energy and provide functions and parameters for every type of atom in the system (Astrakas et al., 2012). The protein configuration was enclosed in a periodic cubic water box of dimensions 10.215 X 10.215 X 10.215 (nm) containing 34838 water molecules to satisfy the minimum image convention. The water model selected for this study was TIP3P and three sodium ions were added to neutralize the system.

The neutral solvated protein system was first energy minimized with converging criterion of maximum force value of 10 kJ/nm/mol using steepest descent for 20000 steps and equilibrated to constant volume (NVT) and temperature (NPT) for 200 ps.

During the MD simulation the temperature was maintained using Berendsen thermostat and pressure was set at 1 bar. The constant temperature and pressure coupling was 0.1 ps and 2 ps respectively and to limit the short-range non-bonded interactions, van der Waals interaction and long-range electrostatic interactions, a cut-off of 1 nm was used. PME algorithm was used with grid spacing of 0.16 nm and the time step during the simulation was 2 ps. A total of nine MD simulations were run to evaluate the effect of temperature (300 K, 380 K and 425 K), static electric field of intensity 0.05 V/nm and oscillating electric field of intensity 0.05 V/nm and frequency of 2.45 GHz. All the electric fields were applied at the *x* axis of the equilibrated solvated protein system. The thermal treatment conditions were chosen to repeat the conditions of study performed by Beyer et al. (Beyer et al., 2001). The electric field conditions were selected to simulate microwave assisted processing conditions. All simulations were run for 1 ns and the results obtained were sufficiently distinct to conclude from the observation.

The effect of temperature and external electric field on secondary structure of the Ara h 6 protein was characterized using STRIDE algorithm implemented in visual molecular dynamics (VMD) (Humphrey et al., 1996). Root mean square deviation (RMSD) of backbone atoms was calculated with starting structure as a reference. Also radius of gyration (Rg), which is defined as the root mean square distance from each atom of the protein to their centroid was calculated as indication of conformational changes in the protein. Surface hydrophobicity and hydrophilicity of the system were also estimated to evaluate the effect of temperature and external electric field on

the surface properties of the protein. Changes in these parameters were studied using GROMACS analyzing tools. The snapshots of protein conformational change were taken using VMD.

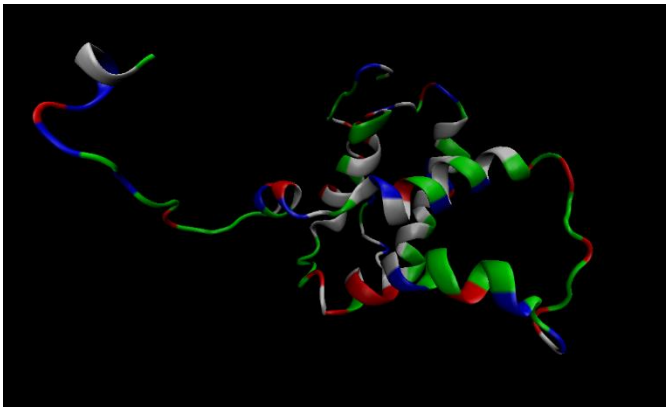


Figure 3.1. The three dimensional structure of the Ara h 6 allergen protein

3.4 RESULTS AND DISCUSSIONS

The main purpose of this study was to examine the effect of thermal treatments at 300 K, 380 K and 450 K with or without the application of static or oscillating electrical fields on conformation of Ara h 6 protein (Fig. 3.1). The aforementioned temperatures were selected to compare the results with those obtained by Beyer et al. (Beyer et al., 2001). They reported that boiling peanuts in water at 100 ^{0}C for 20 minutes or frying them in vegetable oil for about 5-10 minutes moderated the IgE binding intensity of peanut protein allergens as compared to roasting them at 170 ^{0}C for about 20 minutes. The modifications to the IgE binding properties of the peanut caused during the processing may be due to the conformational deviations in the structure.

3.4.1 *Secondary structure analysis*

VMD STRIDE algorithm was used to study the effect of temperature and external static and oscillating electric field on secondary structure of Ara h 6 (Fig.3.2). Ara h 6 allergen is a fraction of a large protein present in peanuts called the 2S Albumin. Apart from the Ara h 6, Ara h 2 and

Ara h 7 are also present in the seed storage proteins of the peanuts and are members of the 2S Albumin family (Lehmann et al., 2006b). All these allergens belong to prolamine protein superfamily (Kreis et al., 1985) which also includes α-amylase/trypsin inhibitors, nsLTP's (non-specific Lipid Transfer Proteins) (Shewry et al., 2002). 2S albumin is a protein with its molecular mass ranging from 12-15 kDa (Lehmann et al., 2006b). The Ara h 6 has a molecular weight calculated to be about 14.5 kDa (Koppelman et al., 2005; Koppelman et al., 2003) and comprises of 127 amino acids, where more than 44% of the secondary structure consisting of helices (five α-helixes and one 3/10 helix) (Fig 3.2 (a)). The five α-helixes are formed between the residues 16-20 (CYS-VAL), 26-33 (LYS-MET), 53-66 (SER-GLU), 74-84 (MET-ASN) and 95-109 (VAL-ASN) residues of the allergen molecule and the 3/10 helix is between 35-37 (ARG-MET) residues.

The STRIDE analysis is done to understand the changes that take place within the protein structure during the period of simulation. This analysis provides the secondary structural variations in the protein subjected to stress corresponding to the time period for which the simulation is run in the form of a chart (X axis: the residues and Y axis: Frames/Time). When these charts are observed as in Fig 3.2, it can see that as the simulation time progresses representing the increasing stress on the protein secondary structures, changes in the color are observed which represent the variations in the specific secondary structure. Through STRIDE analysis not only do we observe the change, but also the residues involved in the secondary structure variations and the exact time when the change was initiated can also be pointed out. Moreover, the comparison between the initial structural representations and the final will provide us with the secondary structures that deviated the most during the course of simulation with application of known external stresses.

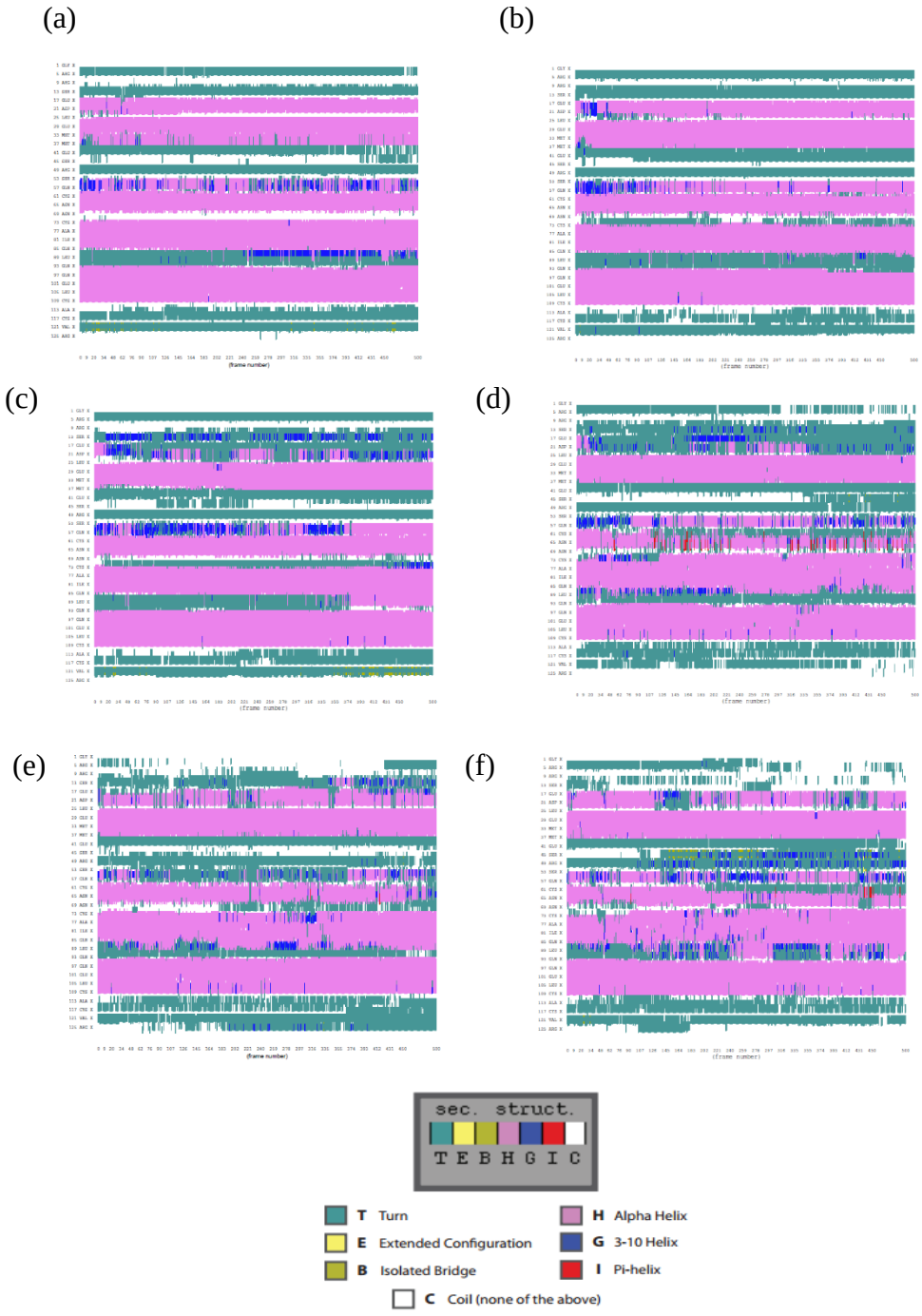


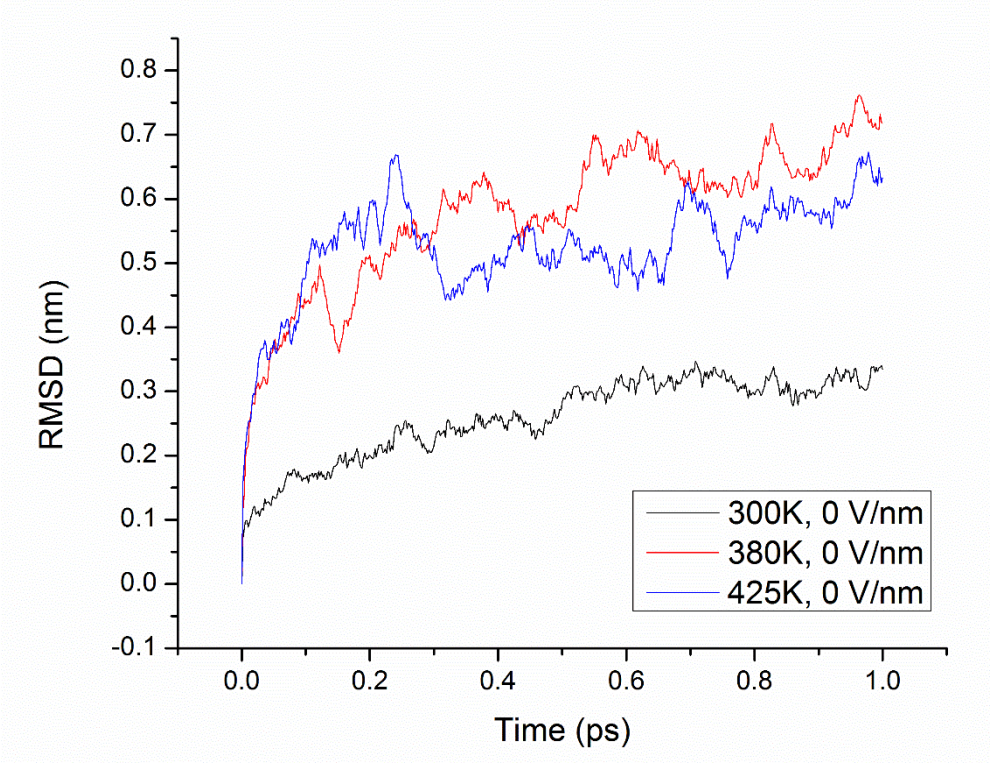
Figure 3.2. STRIDE analysis showing the evolution of molecule (a) 300K (b) 300K, static electric Field of 0.05V/nm (c) 300K, oscillating electric field of 0.05V/nm at 2450 MHz (d) 425K (e) 425K, static electric Field of 0.05V/nm (f) 425K, oscillating electric field of 0.05V/nm at 2450 MHz. Note: Color code: Magneta denotes α (alpha)- helix, red denotes π (pi) - helix, cyan denotes turn, blue denotes 3–10 helix and white denotes coil. (The X-axis shows the residue numbers and names (from 1 to 127) and the Y- axis shoes the time frames (1 frame = 2 ps) of the simulation.

It can be observed from the Fig 3.2 (a) and (b) that temperature of 300K had no significant effect on the secondary structure of the protein with or without the application of static electric field (0.05V/nm), but application of oscillating electric field of 2.45 GHz, 0.05V/nm (Fig 3.2 (c)), resulted in conversion of α-helix (CYS (residue: 16)-VAL (residue: 20)) into 3/10 helix and turns. As the temperature was increased to 425K from 300K in the absence of external electric field, significant changes in the secondary structure of the protein were observed (Fig 3.2. (d)).The most significant change was the transformation of the α-helix (SER (residue: 53)-GLU (residue: 66)) into a π-helix apart from the turns and 3/10 helixes for a brief period of time during the simulation. Similar results were obtained when external static and oscillating electric field were applied at 425K, the α-helix (SER (residue: 53)-GLU (residue: 66)) transformed into 3/10 helixes and turns. Thus, it is clear that the stresses caused by the increasing temperature and electric field (static and oscillating) have an effect on the secondary structure of the Ara h 6 allergen molecule.

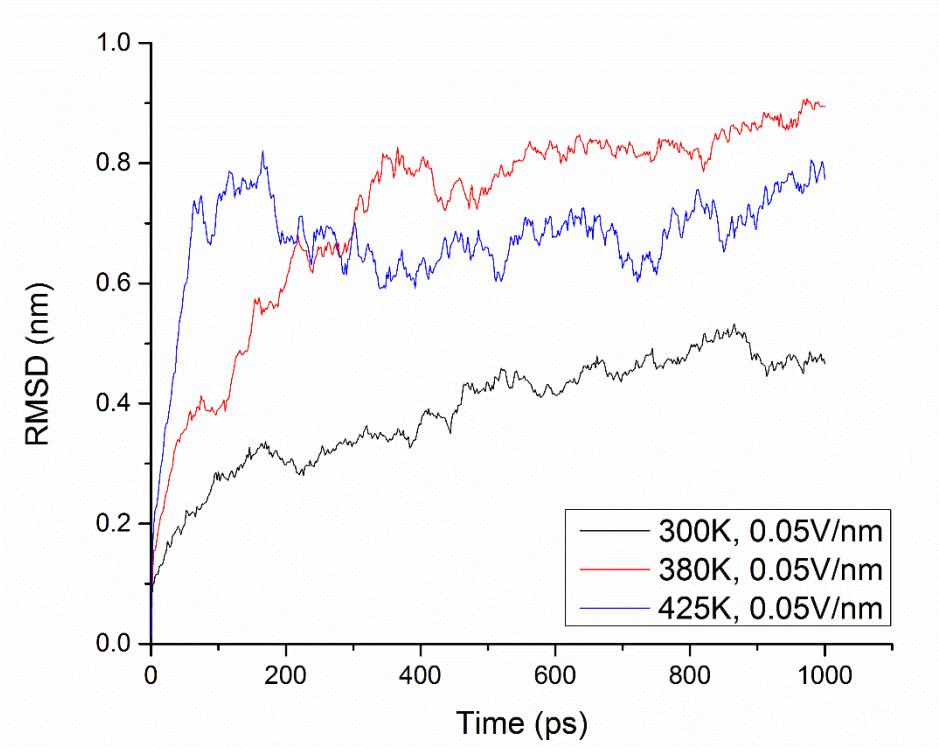
3.4.2 *Root Mean Square Deviation (RMSD)*

The Root Mean Square Deviation provides the arithmetical value of the deviations in the structure of the molecule. The RMSD is calculated by evaluating the changes in the structure with a reference molecule that is normally the control (not under any external stress) (Budi et al., 2004). It was observed that the application of temperature had a significant effect on the RMSD value of the Ara h 6 allergen. There was a significant difference in values of RMSD obtained as the temperature increased from 300K to 380K (Fig 3.3(a)). When the temperature was further raised to 425K, it was observed that the RMSD values obtained were similar to that of 380K.

(a)


RMSD (nm)
Time (ps)
300K, 0 V/nm
380K, 0 V/nm
425K, 0 V/nm

(b)


RMSD (nm)
Time (ps)
300K, 0.05V/nm
380K, 0.05V/nm
425K, 0.05V/nm

(c)

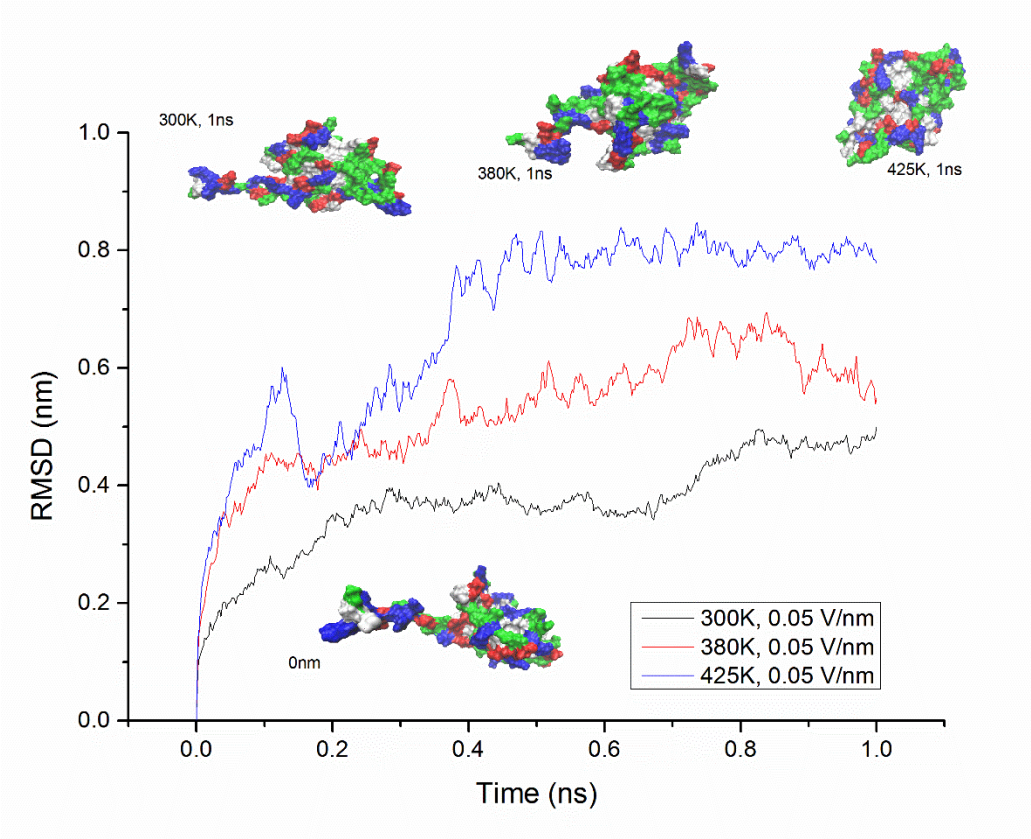


Figure 3.3. RMSD variations observed in Ara h 6 Allergen (a) Heat Treatment (b) Heat Treatment + Static Electric Field (c) Heat treatment + Oscillating Electric Field of 2450 MHz

Table 3.1: RMSD values obtained for Ara h 6.

Temperatures **(K)**	No Electric Field **(nm)**	Static Electric Field (0.05V/nm) **(nm)**	Oscillating Electric Field (0.05V/nm , 2450MHz) **(nm)**
300K	7.281 ± 2.420	7.895 ± 3.750	6.808 ± 2.428
380K	10.557 ± 4.300	10.771 ± 4.755	10.976 ± 4.644
425K	10.492 ± 4.113	11.391 ± 3.393	11. 309 ± 3.849

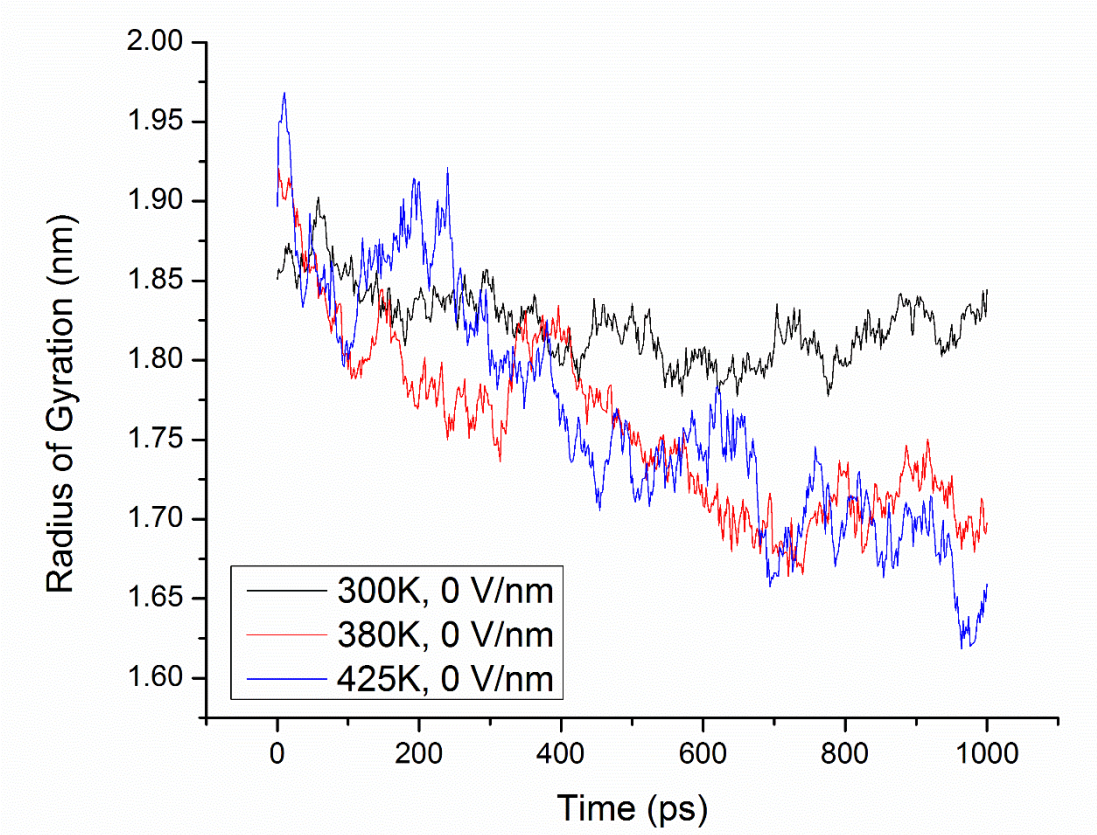
From Table 3.1 it can be concluded that application of static and oscillating electric field had no significant effect on the RMSD values at 300 and 380K when compared to control. At 425K static and oscillating electric field had higher RMSD values compared to the control (Fig 3.3(b-c)).

These results conform the STRIDE analysis of secondary structure in which it was observed that secondary structures changed as the temperature rose from 300K to 425K under both the static and oscillating electric fields.

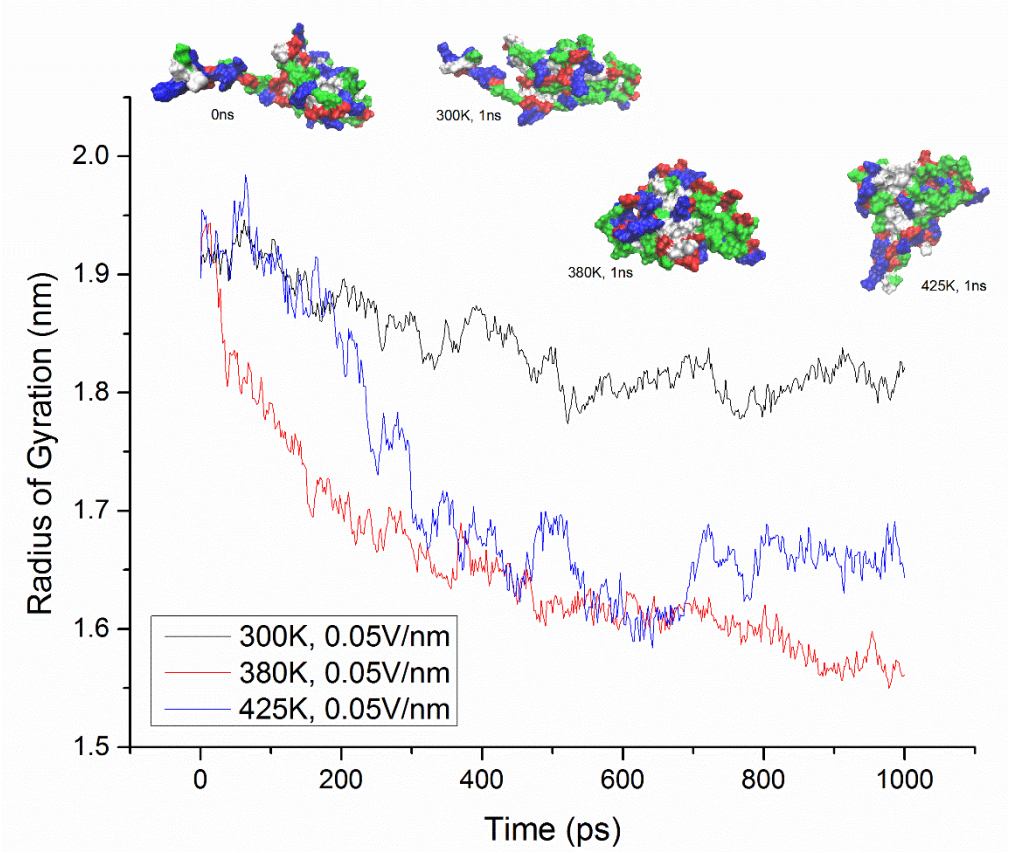
3.4.3 *Radius of Gyration (Rg)*

Radius of Gyration is defined as the measurement of the overall spread of the atoms in a given molecule with respect to its centre of mass (Budi et al., 2004; Singh et al., 2013b). It was observed that there was a significant reduction in radius of gyration of the Ara h 6 molecule at temperatures 380K and 425K compared to the control temperature of 300K. Application of external electric field provided similar observations (Fig 3.4).

(a)



(b)



(c)

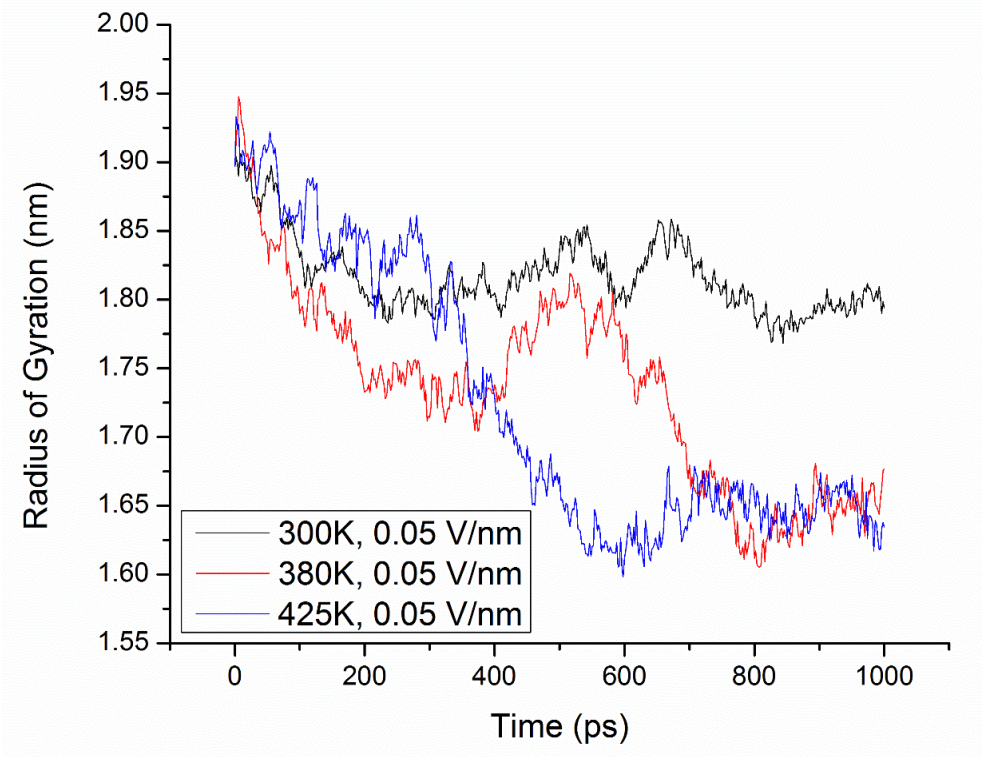


Figure 3.4. Radius of Gyration variations observed in Ara h 6 Allergen (a) Heat Treatment (b) Heat Treatment + Static Electric Field (c) Heat treatment + Oscillating Electric Field of 2450 MHz.

Table 3.2: Radius of Gyration values for Ara h 6.

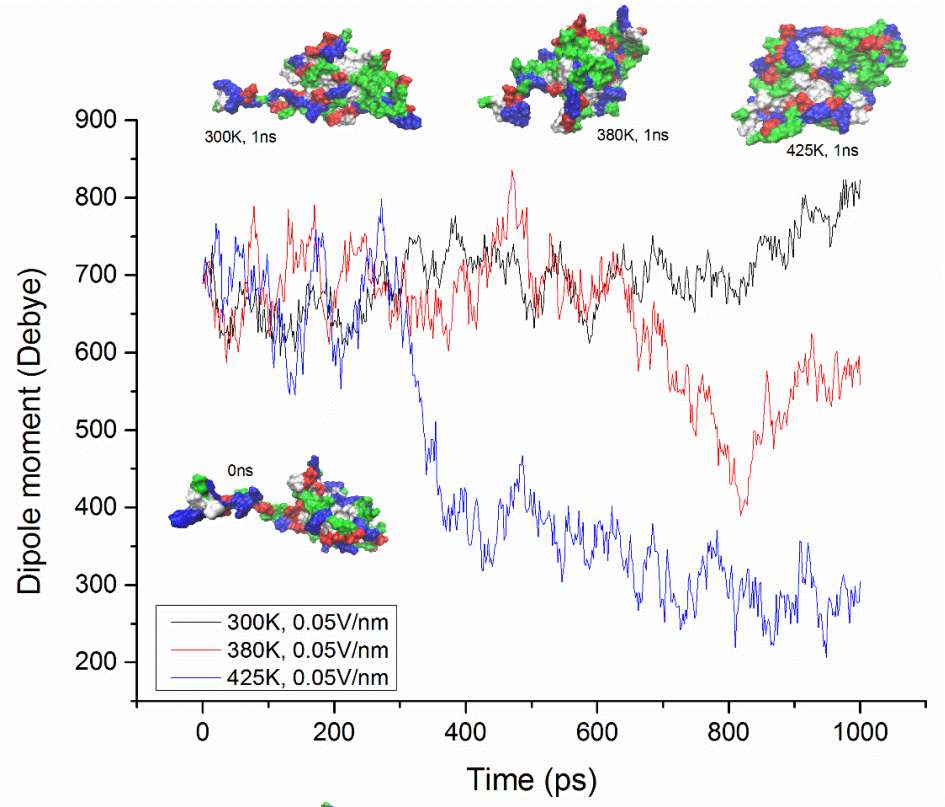
Temperatures **(K)**	No Electric Field **(nm)**	Static Electric Field (0.05V/nm) **(nm)**	Oscillating Electric Field (0.05V/nm , 2450MHz) **(nm)**
300K	1.823 ± 0.023	1.840 ± 0.041	1.817 ± 0.027
380K	1.757 ± 0.056	1.655 ± 0.081	1.735 ± 0.071
425K	1.765 ± 0.076	1.719 ± 0.105	1.724 ± 0.098

Lobanov et al., (Lobanov et al., 2008) suggested that radius of gyration for a protein can act as an indicator for the compactness of atomic packing. They reported that lower the radius of gyration of the molecule the more packed is the structure. In 2012, Liu et al., (Liu et al., 2012) indicated that the peanut protein isolate (PPI) had compacted on dry-heating. In their work, they suggested that the mixture and conjugates of PPI and Dextran when subjected to dry-heat had more compaction and that they had exhibited better functional properties. In the current case of Ara h 6 allergen, with the application of temperature and external electric fields, a compaction in the molecule was observed. The effects of this compaction on the functional properties and their clinical usability have to be studied further.

3.4.4 *Dipole Moment*

The Dipole moment of a protein molecule occurs because of its secondary structure conformations. With an application of external electric field, the protein tries to align itself in the direction of the applied field (Singh et al., 2013b).

(a)



(b)

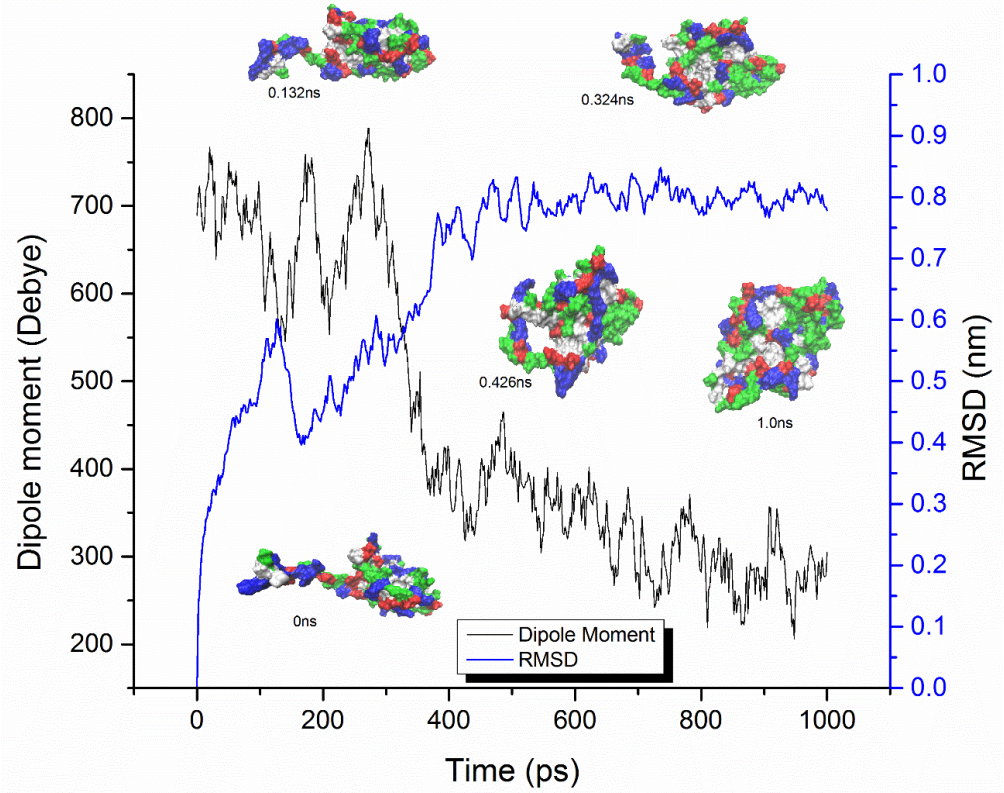


Figure 3.5. Dipole Moment Variations in Ara h 6 Allergen (a) Change in Dipole moments with Temperature in Oscillating Electric Field (0.05V/nm) (b) Dipole moment and RMSD variations at 425K and Oscillating electric field (0.05 V/nm).

Table 3.3: Dipole moments

Temperatures **(K)**	Oscillating Electric Field (0.05V/nm , 2450MHz) **(nm)**
300K	698.03 ± 46.06
380K	639.97 ± 86.42
425K	445.94 ± 167.33

From Fig 3.5 (a) it can be observed that the dipole moment of the Ara h 6 protein has reduced significantly with the electric field. Previous research has shown that the helical structures in the protein have their own dipole moment which contribute to the overall dipole moment of the protein molecule (Budi et al., 2004). In the case of Ara h 6 allergen, the STRIDE analysis performed has clearly shown that the α-helices present were converted to 3/10 helices and turns with the application of temperature (425K) in the simulation as shown in Fig 3.1 . The deviations in helical structures would result in a change in their individual dipole moments in return causing an alteration in the total dipole moment of the molecule.

Fig 3.5 (b) shows the relation between the RMSD and the dipole moment of the Ara h 6 molecule at a temperature of 425K in an oscillating field of 0.05V/nm. It can be observed that the molecule underwent a significant change in terms of the RMSD and total dipole moment in the time period of 0.13 ns to 0.34 ns (Fig 3.5 (b)). The snapshots of molecule of Ara h 6 has shown a considerable amount of change and thus the variation in both the RMSD and Dipole moments are to be observed in the figure. The RMSD values increased from about 0.43 nm to 0.75 nm and remained mostly constant after that for the rest of simulation, whereas the dipole moment of the molecule decreased from 762 to 394 Debye's. When the same time interval is observed in Fig 3.2 (f) (between frames 100 and 200), it can be clearly observed to note a lot of deviation in the residues 43-51 and α-helix

between 74-84 residues. From Fig 3.4 (c) it can be clearly observed that the radius of gyration also decreased significantly in this time interval.

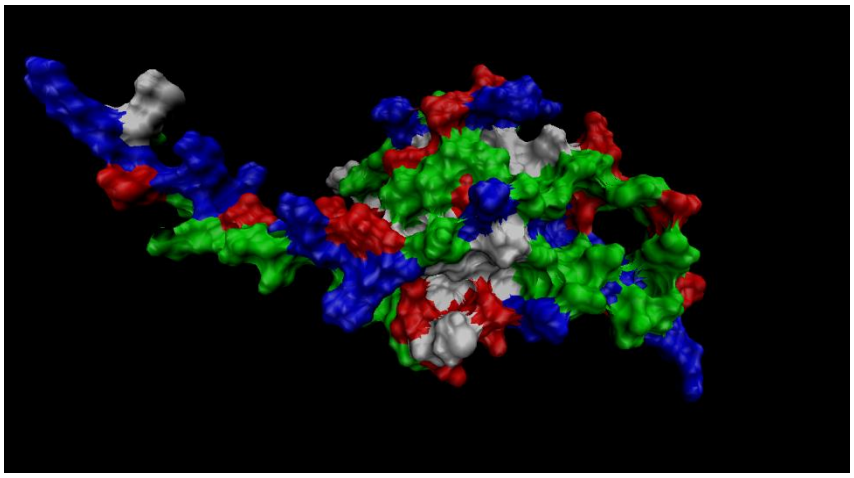
3.4.5 Solvent Accessible Surface Area (SASA)

The surface properties play a vital role in determining various functional characteristics of the protein (Fig 3.6). But, these surface properties are highly dependent on the secondary structures and thus change in these structure can result in alteration of the surface properties which in turn cause functional property variation. The surface properties of the protein also play a critical role in determining the molecular interactions. Thus, a change in the secondary structure can result in modifications of the molecular interaction (interactions with various solvents and enzymes) property of the protein (Singh et al., 2013a; Singh et al., 2013b; Wellner et al., 2005).

The hydrophobic and hydrophilic properties of the surface of the protein play an important role in determining the interaction properties. The application of the static and oscillating electric field to the molecule resulted in surface area reduction similar to that with the application of only heat. In comparison, the oscillating fields had a little more pronounced effect on the molecule to the static fields [Fig 3.7 (b), (c), (e) and (f)]. Thus, effect of the overall reduction in the SASA must be further analysed to determine the changes in the molecular interactive properties. In this study, it has been observed that the heat treatment resulted in a change of the hydrophobic and hydrophilic surface areas as in Table 3.4. There is a considerable amount of reduction in the surface areas, especially the hydrophilic compared to the hydrophobic with an increase in temperature from control of 300K to 380K and then to 425K as shown in Fig 3.7 (a) and (d). The simulations in this study were run for 1 ns due to computational limitations. But, the results observed clearly showed that there is an effect of the applied external stresses on the surface area of the molecule. Further

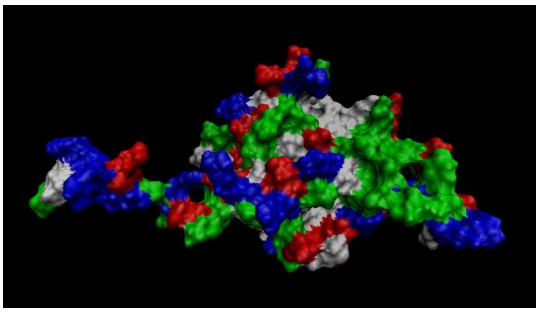
research is required to extensively analyze the aforementioned effect using simulations ran for longer time periods such as 10 -100 ns.

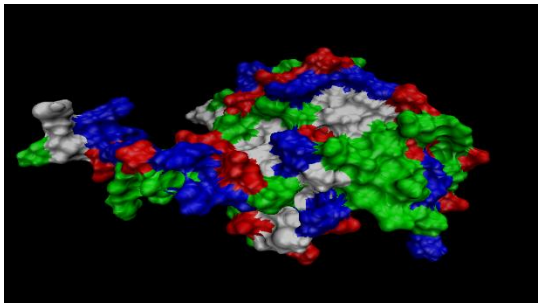
(a)



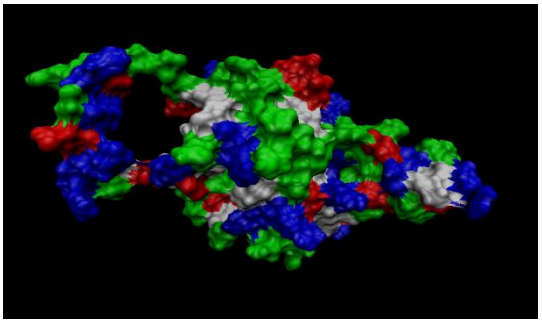
(b)

(c)





(d) (e)



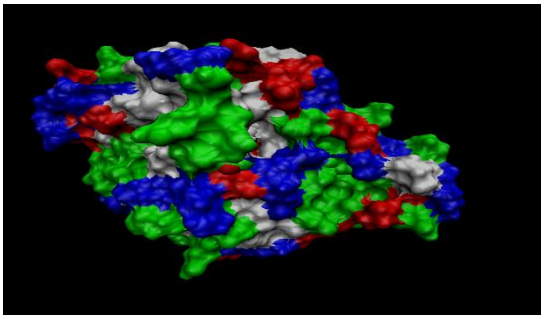


Figure 3.6. Snapshot of surface properties of Ara h 6. (a) 300K (b) molecule at the end of 300K and oscillating electric field (2450Mhz) (c) molecule at the end of 380K and oscillating electric field (2450MHz) (d) Molecule at 425K and oscillating electric field (2450MHz) at 400 ps (e) Molecule at end of 425K and oscillating electric field (2450MHz) Note: non-polar residues (white), basic (Blue), acidic (red) and polar residues (green).

Table 3.4: (a) hydrophobic surface areas and (b) hydrophilic surface area

(a)

Temperatures **(K)**	No Electric Field **(nm)**	Static Electric Field (0.05V/nm) **(nm)**	Oscillating Electric Field (0.05V/nm , 2450MHz) **(nm)**
300K	36.934 ± 0.754	36.028 ± 0.848	36.693 ± 1.157
380K	34.659 ± 1.232	35.725 ± 1.242	33.694 ± 1.751
425K	33.644 ± 1.751	34.340 ± 1.803	33.331 ± 2.042

(b)

Temperatures **(K)**	No Electric Field **(nm)**	Static Electric Field (0.05V/nm) **(nm)**	Oscillating Electric Field (0.05V/nm , 2450MHz) **(nm)**
300K	75.836 ± 2.409	76.095 ± 1.335	75.164 ± 2.689
380K	69.956 ± 3.356	71.367 ± 3.215	70.584 ± 3.311
425K	69.373 ± 3.317	68.423 ± 4.026	68.561 ± 3.254

From Fig 3.6 (a) it can be observed that there is a protrusion that lies out of the core structure of the Ara h 6 molecule to which α-helix (CYS (residue: 16)-VAL (residue: 20)) almost acts as the connecting point. In the case of application of temperatures 300K and 380K, the rapid vibrations of the protruded part must have caused a strain on the secondary structure as suggested in the Fig 3.2 (a), (b) and (c) causing deviations resulting in formation of 3/10 helices and turns in α-helix (CYS (residue: 16)-VAL (residue: 20)). But when the 425K temperature is applied along with an electric field, both static and oscillating, the vibrations were so high that the protruded part folded back into the core molecule and hence putting lower amount of strain on the α-helix (CYS (residue: 16)-VAL (residue: 20)) of the allergen Fig 3.2 (e) and (f). But, this folding had visually aided in showing the compaction of the molecule with the application of external thermal and electric field stress. Thus structural deviations can be achieved with the application of external stresses on a

molecule, but the effect that these changes in the Ara h 6 molecule on the functional properties have to be further studied.

The figure 3.6 (a) shows the structure of the Ara h 6 molecule with no external stress application. With the increase in the temperature from 300 K to 380 K shown in figure 3.6(b) & (c), the overall structure of the molecule has a very slight change in the structure as the molecule compacted. But, when the temperature increased to 425K and oscillating electric field is applied, after 400 ps (Fig 3.6(d)) into the simulation, we have observed that the residue 1 which is actually protruded out of the molecule reverted back and joined in with the core of the molecule. By the end of simulation, the protrusion of the molecule almost joined into the core structure resulting in change of secondary structure of the protein.

Moreover, the hydrophobic dipole moment also contributes to the overall dipole moment of the molecule. The hydrophobic dipole moment is the measure of the irregularities in the surface between the hydrophobic and hydrophilic ends of the protein. When any protein has a high value of hydrophobic dipole moment it suggests that the structure of the protein it asymmetrical, i.e. one end of the protein has a high amount of hydrophobic surface while the other end of the protein has a higher amount of hydrophilic surface contributing to the dipole moment of the whole molecule. In the case of globular proteins, this is a little different because the dipole moments are directed towards the centre of the molecule. Rose *et al*. (Rose et al., 1985) have showed that the surfaces cluster around a centre and this also contributes to the folding of the molecule.

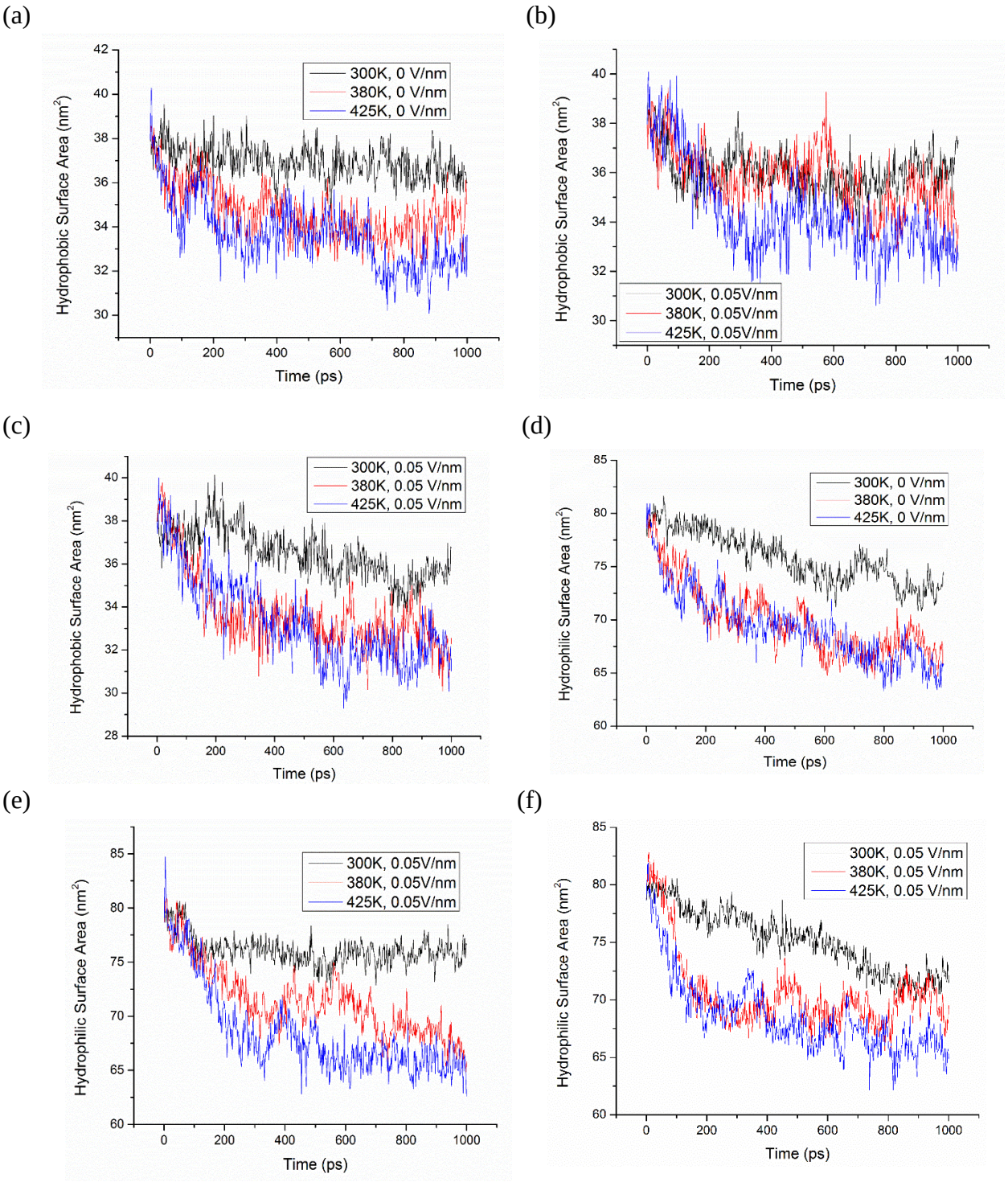


Figure 3.7. Solvent Accessible Surface Area (SASA) variation with heat treatments and Electric Fields (Static and Oscillating) of Ara h 6. (a) Hydrophobic surface area with heat treatment, (b) Hydrophobic surface area with heat treatment + static electric field, (c) Hydrophobic surface area with heat treatment + oscillating electric field, (d) Hydrophilic surface area with heat treatment, (e) Hydrophilic surface area with heat treatment + static electric field, (f) Hydrophilic surface area with heat treatment + oscillating electric field.

The Ara h 6 molecule initially has an extended structure as shown in Fig 3.1 at 300 K. But, with an increase in temperature to 425K with an oscillating field of 0.05 V/nm, the structure of the molecule compacted leading to rearrangement of the surface resulting in change of the hydrophobic and hydrophilic surfaces. This overall reduction in the surface area of the Ara h 6 molecule leading to this compaction resulted in a structure similar to globular protein at the end of 1 ns. This change in the overall structure should have resulted in the reduction of the hydrophobic dipole moment also resulting in the overall dipole moment reduction.

When an external stress is applied on a protein it is bound to change its conformation to obtain a more stable state and that the change in the secondary structure can directly affect the functional properties. Previously, the exact relation between the protein structure and the protein functions was quite unclear. Fischer (Fischer, 1894; Lemieux & Spohr, 1994) first proposed the "lock and key" mechanism in 1894. In 1931, Wang (Wu, 1931) gave the first reference relating the protein functions to its structure when he gave the first theory of protein denaturation which was later also documented by Edsall (Edsall, 1995). As mentioned earlier, Liu et al., (Liu et al., 2012) reported a compaction in the PPI and dextran mixture on dry heating and he also mentioned that this had a considerable effect on the functional properties of the protein. They observed that the solubility of the PPI dextran mixture was higher compared to that of the PPI alone. Emulsifying and Foaming properties were also tested and it was found out that the PPI dextran mixture were better emulsifying and foaming agents than the PPI alone. They have attributed this change to the predicted compaction in the molecule on dry heating the mixture of PPI and Dextran. This shows that the change in the secondary structure of the protein can result in a change in the functional properties of the molecule.

3.5 CONCLUSION

It is clear from the above observations that the secondary structure of Ara h 6 allergen can be altered under applied external stresses including thermal and electric fields. The results observed suggest that the effect of heat on the protein molecule is much more compared to that of the electric field, both static and oscillating. But, the effect of these changes in the secondary structure of the molecule leading to further changes in the functional properties have to be studied. In the case of Ara h 6, further tests have to be conducted to verify if the variations caused in the secondary structure and in turn in the SASA would have significant effect on the allergenicity of the protein in peanuts.

CONNECTING TEXT

Since MD studies are conducted within the limitation of computation dynamics, it is important to validate and compare their results with experimental ones. Hence in the following chapter an experimental study on effect of application of the thermal and external electric fields on conformation of peanut protein was carried out using Fourier Transform Infrared Spectroscopy (FT-IR). The results obtained from FT-IR analysis will be compared with observations made through MD technique for validation. *In vitro* protein digestibility (IVPD) studies have also been conducted to the processed peanut protein to further support the data.

CHAPTER 4

EFFECT OF THERMAL AND HIGH ELECTRIC FIELD ON SECONDARY STRUCTURE OF PEANUT PROTEIN AND ITS DIGESTIBILITY

4.1 ABSTRACT:

Effect of the thermal and high electric field application on the secondary structure conformation of peanut protein has been evaluated using the FT-IR spectroscopy. The spectra of the treated and non-treated samples have revealed conformational changes in the protein structure. The Amide I region between the wavelengths 1700 – 1600 cm^{-1} of the spectra have been studied. Within thermal treatments, both hot air roasting and microwave processing treatments have been evaluated. Hot air roasting was performed at temperatures 50 ^{o}C, 75 ^{o}C and 100 ^{o}C form 15, 30 and 45 minutes while the microwave treatments were conducted at the same temperatures, but for 5, 10,15 and 20 minutes. Three experimental conditions of the high electric field intensity of 10 kV, 15 kV and 20 kV for 60, 120 and 180 minutes were also evaluated. Changes have been observed at 1654 – 1650 cm^{-1}, indicating conformational changes in the α-helix secondary structure. Similar changes were also observed at various other wavelengths indicating changes in the 3/10 helix, β-sheets and random coils present in the protein. With an increase in the treatment time, the secondary structure reorganizations have increased with creation of new random coils and aggregated strands. Curve-fitting using Gaussian band shapes have further supported the observations. *In vitro* protein digestibility (IVPD %) studies have also been performed whose results also support the observations from the spectra.

Keywords: Peanut protein, FT-IR spectroscopy, *in vitro* protein digestibility, protein secondary structure.

4.2 INTRODUCTION

Peanuts are primarily consumed all over the world and have advantages of being energy dense and also is a rich source of proteins and fibre (Burton-Freeman, 2000; Holt et al., 1995; Kirkmeyer & Mattes, 2000; Maleki & Hurlburt, 2004; Putnam & Allshouse, 1994). But, with the turn of the century, peanut allergy has become one of the most important health concern in various developed countries primarily due to globalization and gene related factors (Arshad et al., 1993; Ortolani & Pastorello, 2006). Apart from peanut, wheat, tree nuts, fish, egg, soy and shell fish account for most of the allergies around the world (Dalal et al., 2002; Sampson, 1994; Sampson & McCaskill, 1985). Allergic reactions have also been observed with consumption of few fruits and vegetables like apple (Bucher et al., 2004; Hansen et al., 2004; Zuercher et al., 2010), cherry (Gruber et al., 2004; Scheurer et al., 1999; Schweimer et al., 1999) and celery (Ballmer-Weber et al., 2002; Ortolani et al., 1993; Silverstein et al., 1986). Currently, about 2 % - 8% of the world's infants and children suffer from allergies and this is on an alarming rise and has to be addressed quickly (Bock, 1987; Burks & Sampson, 1993; Burks et al., 1998). For the food industries, this is an important step from food safety perspective to keep the allergy causing components at bay from processing. There should be development of new products and substitutes which cater the needs of the population that are sensitive to the allergens; the trust therefore should be towards commercialization of new safe products.

Various physico-chemical properties are governed by the protein structure and various recent studies have provided insight in understanding the effect of specific changes in the protein secondary structures and their effect on the functional and chemical properties (Anderson et al., 1996; Deshpande & Damodaran, 1989; Gross & Jaenicke, 1994; Mozhaev et al., 1996; Nakai, 1983; Sorgentini et al., 1995a; Sorgentini et al., 1995b; Tatham & Shewry, 1985; Whisstock &

Lesk, 2003). Though various methods have been used to study the changes in the protein structures, use of Fourier Transform Infrared Spectroscopy (FT-IR) is widely adopted and accepted in the scientific community. The measurement of the intensity and the wavelength of Infrared radiation (IR) for a selected sample is through the FT-IR spectroscopy. The repeating units in the protein normally give rise to 9 characteristic IR absorption bands which are called A, B and I – VII. The most vital for studying the protein backbone among the above mentioned bands are I & II vibrational bands (Kong & Yu, 2007; Surewicz & Mantsch, 1988; Susi & Byler, 1986). Among the two, the amide band I is the most sensitive ranging between the wavelengths 1700 cm^{-1} to 1600 cm^{-1} that accounts for about 80% of the peptide linkages i.e. C = O stretch. Thus, the frequency of the Amide I region is highly associated to the secondary structures of the protein (Haris & Severcan, 1999; Kong & Yu, 2007). Depending on the force fields, hydrogen bonds and the other vibrational bands are very complex and have little use in the protein conformation studies except for the amide II band. This band deals primarily with the NH bending and CN stretching vibrations and is less sensitive when compared to the Amide I region (Krimm & Bandekar, 1986; Lee et al., 1990).

Koppelman et al., (Koppelman et al., 1999) isolated and studied the effects of thermal processing on the secondary, tertiary and quaternary structures of Ara h 1 which is a major peanut allergen. This study revealed that processing purified Ara h 1 to temperatures of 80 ^{o}C – 90 ^{o}C resulted in irreversible changes in the secondary structures. But, it was also shown that the thermal processing did not have any effect on the IgE binding capacity and thus the allergenicity of the compound was similar to that of the native Ara h 1 allergen. Thus, though there were considerable changes in the structural conformation, the allergic character of the Ara h 1 is very stable. Digestion studies were performed on Ara h 1 by Maleki et al,. (Maleki et al., 2000b) which showed that the surface

accessible hydrophobic amino acid residues are very stable and protected, thus remain unaffected by the processing.

Thus, in this study FT-IR is used to understand the changes in the secondary structure of the overall protein and *In vitro* protein digestibility have been employed to understand the structure and functional relation of various thermal and high electric field processing.

4.3 MATERIALS AND METHODS

Peanuts (Valencia variety) have been bought from Kernal Peanuts, Ontario which had an initial moisture content of 9.3% on wet basis. The moisture was determined by drying the peanut powder in an oven at 105^{o}C for 24 hours, after which the weight became constant. Fresh peanut meal was prepared before subjecting it to thermal and high electric field treatments.

4.3.1 Solvents and Reagents

All reagents and solvents used were of HPLC grade from Fisher Scientific (Ottawa, ON, Canada). The enzymes employed for the IVPD % determination were purchased from Sigma Aldrich (Oakville, Ontario Canada).

4.3.2. Oven Roasting

For oven heating, the peanut flour was evenly spread on Aluminum dishes, and were placed in an oven which are pre-set to the processing temperatures of 50 ^{o}C, 75 ^{o}C, and 100 ^{o}C. The roasting was done for 15, 30 and 45 minutes in the oven. After cooling to the room temperatures, the samples were stored at 4°C until further analysis was conducted (Alonso et al., 2000; Koppelman et al., 1999). All the experiments were conducted in triplicates.

4.3.3. Microwave Heating

The peanut flour was placed in glass beakers and exposed to microwaves at a power density of 2 W/g for 5, 10, 15 and 20 minutes in a conventional microwave oven. The processed samples were then taken out and were cooled to room temperature. These were stored at 4°C in air-tight containers for further analysis.

4.3.4. Electric Field Treatment

The apparatus used consists two parallel electrodes that are connected to a General Electric Ignition Transformer and a Superior Electric Co. Powerstat adjustable transformer. The required electric fields of 10 kV, 15 kV and 20 kV between the two parallel electrodes was adjusted using the powerstat transformer which modulated the input AC voltage of the ignition transformer. A solid copper wire of diameter 0.05 cm was used as the top electrode and a copper plate was used as the ground electrode. A sample of about 5 g was placed in between the electrodes using specially designed cubicles to hold the sample. These samples were exposed to the electric field strengths of 10 kV, 15 kV and 20 kV for about 60, 120 and 180 minutes. The distance between the electrodes was fixed at one centimeter. The wire electrode was placed about 0.8 cm from the surface of the sample. The samples were subjected to the electric field and they were further analysed.

4.3.5. FT-IR Spectroscopy

All the FT-IR spectral analysis of the peanut samples was conducted using the Nicolet iS5 ATR-FT-IR spectrometer. For all the samples, 32 spectra at 4 cm^{-1} resolution were averaged to give the final spectrogram. The spectra of an empty ATR diamond acted as the background reference. The spectra was analyzed using OMNIC software (version 8, Thermo Nicolet Instrument Corp., Madison, WI) and OriginPro (Version 9, OriginLab Corporation, Northampton, MA, USA) (Georget and Belton, 2006).

4.3.6. *In vitro* Protein Digestibility (Multi Enzyme Method)

The *In vitro* Protein Digestibility (IVPD) of the peanut protein was evaluated using the three enzyme method (Astwood et al., 1996; Bodwell et al., 1980; Hsu et al., 1977). A multi-enzyme mixture was prepared, containing 1.6 mg/ml trypsin, 3.6 mg/ml chymotrypsin, and 1.3 mg/ml peptidase and its pH was adjusted to 8.0. This mixture was placed in an ice bath and was continuously stirred. A working protein suspension was prepared by dissolving samples to yield about 312.5 mg of protein in 50 ml of distilled water whose pH was also adjusted to 8.0. The pH adjustments were done using 0.1N NaOH and 0.1N HCl. 5 ml of the multi-enzyme solution was added to the samples which was maintained at 37 ^{o}C in a water bath for digestion with continuous stirring. The pH was measured after about 10 minutes of digestion and the *In vitro* Protein Digestibility (IVPD) was calculated (Hsu et al., 1977).

$$IVPD\ \% = 210.46 - (18.10 \times pH_{10min}) \quad \text{(Equation 4.1)}$$

4.3.7 Statistical Analysis

The analysis of variance (ANOVA) of the samples were performed using JMP software (ver. 11, SAS Institute Inc., Cary, NC, USA). The fitness of each model for full factorial design was carried out using Tukey HSD method at 95% confidence ($p \leq 0.05$) level. The data is tabulated in accordance with Kalkan et al., (Kalkan et al., 2014).

4.3.8 Notations

Specific notations have been used in this manuscript to indicate various processing methods. These notations have been tabulated below in Tables 4.1 (a), (b) and (c) and the same format has been followed throughout the script. (Example: 50-15 represents processing if the sample at 50 ^{o}C for 15 minutes)

Table 4.1 (a) Notations for representing the Hot air roasting of peanut samples

	Temperature (°C)		
	50	**75**	**100**
Time (min)			
15	50-15	75-15	100-15
30	50-30	75-30	100-30
45	50-45	75-45	100-45

Table 4.1 (b) Notations for representing the Microwave roasting of peanut samples

	Temperature (°C)		
	50	**75**	**100**
Time (min)			
5	50-5	75-5	100-5
10	50-10	75-10	100-10
15	50-15	75-15	100-15
20	50-20	75-20	100-20

Table 4.1 (c) Notations for representing the High electric field processing of peanut samples

	Voltage (kV)		
	10	**15**	**20**
Time (min)			
60	10-60	15-60	20-60
120	10-120	15-120	20-120
180	10-180	15-180	20-180

4.4. Results and Discussions

4.4.1 FT-IR analysis of Conformational Changes in Peanut protein

Fourier Transform infrared spectroscopy was used to study the effect of thermal and high electric field processing on the peanut protein conformation. In this study, the Amide I region of the peanut protein is studied which is one of the most sensitive and most useful for the interpretation of the secondary structures of the protein (Kong & Yu, 2007). The Figure 4.1 represents the FT-IR spectrum obtained for the raw and microwave processed peanut samples between the regions of 4000 cm^{-1} to 500 cm^{-1}.

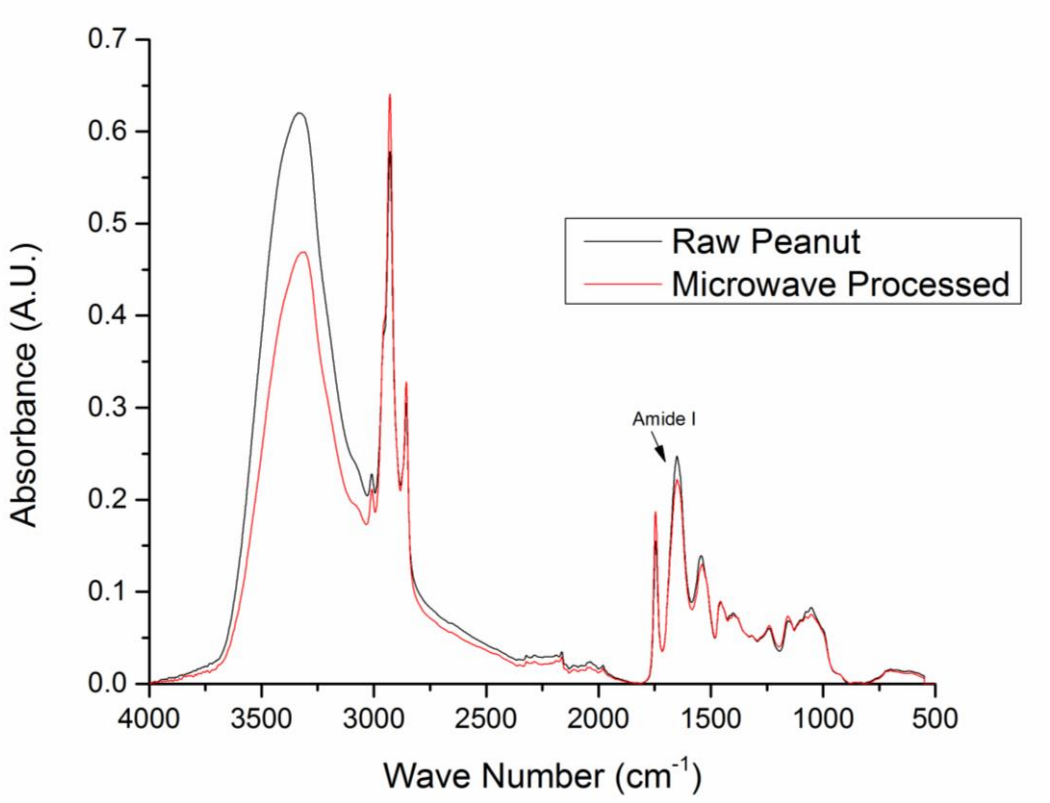


Figure 4.1 FT-IR spectrum of raw and microwave processed (100-15) peanut samples.

The amide I band lies between the wavelengths 1700 cm^{-1} – 1600 cm^{-1} and it includes component overlapping bands of various secondary structures including α-helices, β-sheets, turns and randomly coiled conformations (Ahmed et al., 2007; Kong & Yu, 2007). This is mainly related to

the C=O stretching vibrations coupled with the in-plane NH bending. This region also influences the hydrogen bond patterns and thus primarily determines the backbone secondary structure of the protein. Previous researchers have obtained the correlations between the Amide I frequency and the secondary structures as shown in Table 4.2.

Table 4.2. Amide I band frequencies and corresponding Protein Secondary Structure correlations (Adapted from (Carbonaro et al., 2008; Georget & Belton, 2006; Jackson & Mantsch, 1995; Kong & Yu, 2007; Menéndez et al., 1995).

S. No	Structure	Amide I frequency (cm^{-1})
1	Aggregated Strands (Intra molecular)	1610-1628
2	α helix turns	1630
3	β sheets	1640-1626, 1670-1690
4	α helix	1660-1648
5	3/10 helix (3_{10} helix)	1663-1666
6	Antiparallel β sheets / aggregated strands (Inter molecular)	1675-1695
7	Random Coils/ Unordered	1640-1650
8	Turns	1662-1672, 1673

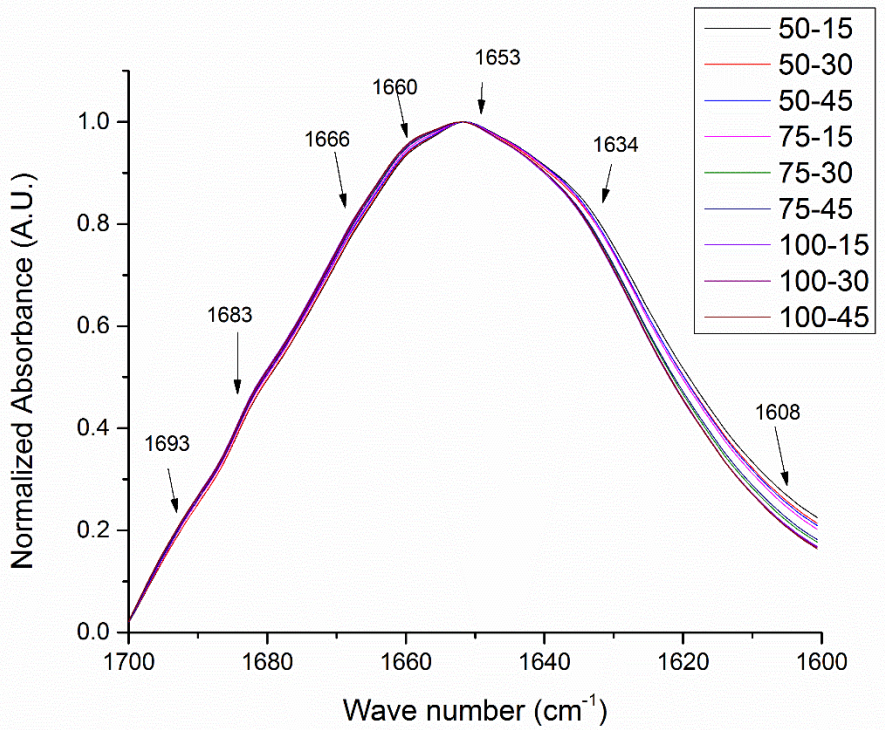


Figure 4.2 Normalized FT-IR spectra of the Amide I region of Hot Air roasted peanut proteins.

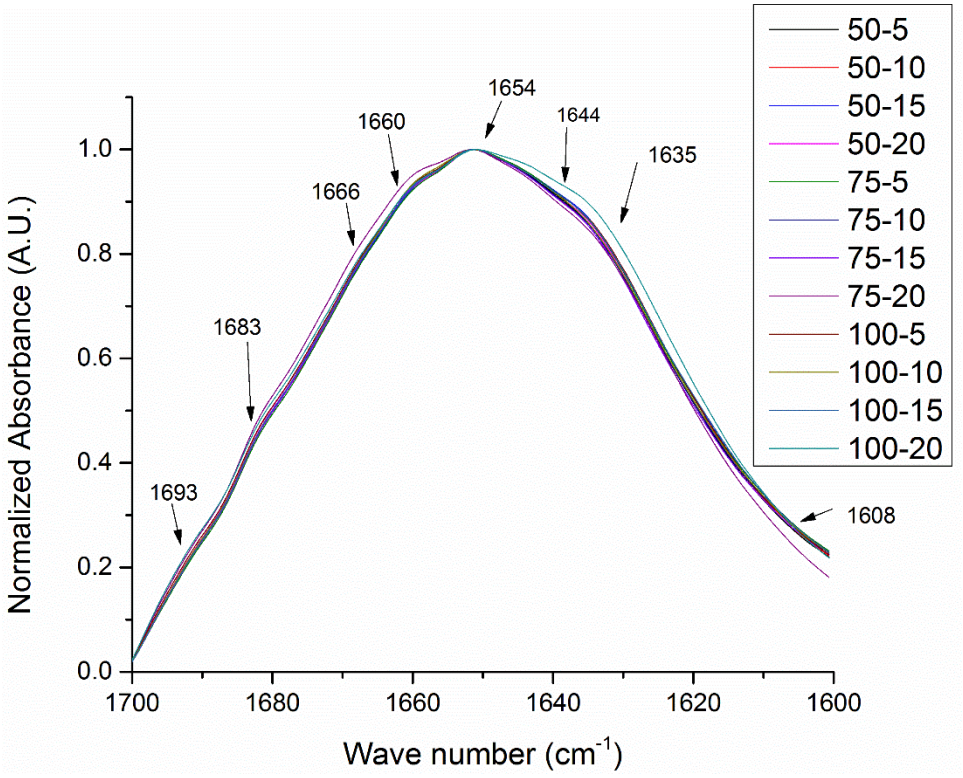


Figure 4.3 Normalized FT-IR spectra of the Amide I region of Microwave roasted peanut proteins.

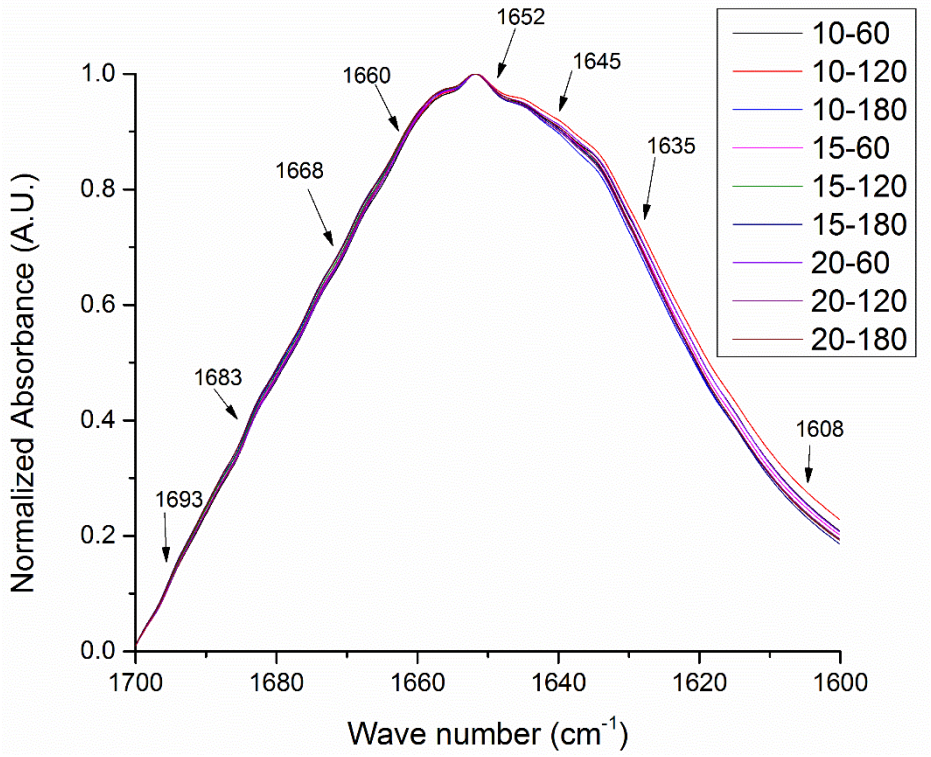


Figure 4.4 Normalized FT-IR spectra of the Amide I region of High electric field processed peanut proteins.

Figure 4.2, 4.3 and 4.4 show the normalized spectra of hot air, microwave and electric field processed samples of peanut protein Amide I region respectively. Distinct peaks were observed in the regions of 1633 - 1640 cm^{-1} (β-sheets), 1645-1647 cm^{-1} (Random coils), 1652-1657 cm^{-1} (α helix), 1660 cm^{-1} (α helix), 1664-1667 cm^{-1} (turns), 1682 - 1696 cm^{-1} (anti parallel β-sheets). The bands at 1608 cm^{-1} have been ignored from our study to improve the clarity of the results as their absorbance band typically is 1595 cm^{-1} which falls outside the Amide I region. The relocations of the structure at this band have increased as the samples were processed for a longer time or at higher temperature. The data analysis showed that the maximum relocation in the secondary structure conformation at different wavelengths was observed in the samples that were subjected to longer processing time.

In order to understand the effects of various processing methods on the protein secondary structure conformation, a curve fitting procedure was applied using OriginPro (Version 9, Origin Lab Corporation, Northampton, MA, USA). The software is used to minimize the sum of squares of the differences between various experimental spectra and the calculated spectra created by the summation of the component curves. The second derivative spectra is created by the software which is used in identification of the component bands which give the results in terms of the peak areas at corresponding wave lengths. Both the normalization and the curve fitting for finding the specific peaks was done separately for each of the processing methods. Table 4.3 shows the changes in the peak areas as the processing time increases for 15 min, 30 min and 45 min at three different temperatures. Table 4.4 shows the peak areas for microwave processing samples at 50 ^{o}C, 75 ^{o}C and 100 ^{o}C at four levels of temperatures; 5 min, 10 min, 15 min and 20 min. The Table 4.5 shows data of the peak areas for high voltage electric field processing at 10 kV, 15 kV and 20 kV for processing time if 60 min, 120 min and 180 min.

Table 4.3. Position areas of the bands fitted to the normalized FT-IR spectra of peanut protein (1700 cm^{-1}-1600 cm^{-1}) subjected to different hot air roasting temperatures and treatment time.

Experimental Conditions					
50-15		**50-30**		**50-45**	
Wavelength (cm^{-1})	**Area (%)**	**Wavelength (cm^{-1})**	**Area (%)**	**Wavelength (cm^{-1})**	**Area (%)**
1615	4.18	1615	3.25	1609	0.68
1634	33.64	1634	33.30	1617	4.04
1652	21.41	1653	24.43	1634	33.96
1660	0.21	1660	0.15	1645	1.16
1666	31.0	1666	29.51	1653	20.29
1683	7.74	1683	7.55	1660	0.52
1693	1.82	1693	1.81	1666	29.68
				1683	7.85
				1693	1.82
75-15		**75-30**		**75-45**	
Wavelength (cm^{-1})	**Area (%)**	**Wavelength (cm^{-1})**	**Area (%)**	**Wavelength (cm^{-1})**	**Area (%)**
1609	0.62	1609	0.59	1608	0.51
1617	3.99	1617	3.36	1616	3.82
1634	34.04	1635	33.31	1634	33.35
1645	3.39	1645	4.90	1645	7.17
1654	15.11	1654	13.61	1654	10.44
1660	0.87	1660	1.49	1660	2.28
1666	32.11	1668	32.25	1667	32.34
1683	8.04	1683	8.32	1684	8.21
1693	1.83	1693	1.84	1693	1.88
100-15		**100-30**		**100-45**	
Wavelength (cm^{-1})	**Area (%)**	**Wavelength (cm^{-1})**	**Area (%)**	**Wavelength (cm^{-1})**	**Area (%)**
1609	0.59	1608	0.55	1608	0.56
1617	3.50	1616	3.54	1616	3.43
1635	33.08	1635	33.52	1634	33.52
1645	4.68	1645	6.64	1644	7.69
1654	14.33	1654	11.22	1652	9.84
1660	1.06	1660	2.04	1660	2.73
1668	32.64	1667	32.33	1666	32.14
1683	8.21	1684	8.24	1683	8.16
1693	1.91	1693	1.92	1693	1.93

Table 4.4 Position areas of the bands fitted to the normalized FT-IR spectra of peanut protein (1700 cm^{-1}-1600 cm^{-1}) subjected to different microwave roasting temperatures and treatment time.

Experimental Conditions							
50-5		**50-10**		**50-15**		**50-20**	
Wavelength (cm^{-1})	**Area (%)**	**Wavelength (cm^{-1})**	**Area (%)**	**Wavelength (cm^{-1})**	**Area (%)**	**Wavelength (cm^{-1})**	**Area (%)**
1615	3.24	1615	3.17	1615	3.17	1615	3.13
1634	37.55	1634	34.64	1635	35.06	1634	34.55
1652	16.24	1653	20.03	1652	20.03	1645	0.79
1660	1.08	1660	0.52	1660	0.53	1653	23.10
1666	32.35	1666	32.12	1666	31.86	1660	0.36
1683	7.81	1683	7.78	1683	7.66	1666	28.23
1693	1.73	1693	1.74	1693	1.69	1683	7.94
						1693	1.90
75-5		**75-10**		**75-15**		**75-20**	
Wavelength (cm^{-1})	**Area (%)**	**Wavelength (cm^{-1})**	**Area (%)**	**Wavelength (cm^{-1})**	**Area (%)**	**Wavelength (cm^{-1})**	**Area (%)**
1615	3.05	1616	2.85	1615	2.94	1608	0.79
1635	35.33	1635	35.12	1635	34.5	1617	4.39
1645	0.42	1645	0.95	1645	1.73	1635	31.4
1653	20.1	1654	22.51	1653	22.85	1645	2.39
1660	0.47	1660	0.38	1660	0.37	1653	21.77
1666	31.29	1666	28.67	1666	27.98	1660	1.53
1684	7.62	1683	7.76	1684	7.84	1666	27.69
1693	1.72	1693	1.79	1693	1.9	1684	8.14
						1693	1.9
100-5		**100-10**		**100-15**		**100-20**	
Wavelength (cm^{-1})	**Area (%)**	**Wavelength (cm^{-1})**	**Area (%)**	**Wavelength (cm^{-1})**	**Area (%)**	**Wavelength (cm^{-1})**	**Area (%)**
1608	0.68	1616	4.25	1609	0.65	1609	0.79
1617	4.15	1634	31.5	1616	4.45	1616	4.46
1635	31.85	1644	5.02	1635	31.71	1635	30.16
1645	0.34	1652	22.19	1645	6.06	1645	7.03
1653	22.83	1660	0.2	1652	21.72	1652	19.66
1660	2.57	1666	27.5	1660	0.44	1660	0.94
1666	27.89	1683	7.49	1666	25.26	1666	27.28
1684	7.78	1693	1.85	1683	7.95	1683	7.69
1693	1.91			1693	1.76	1693	1.99

Table 4.5 Position areas of the bands fitted to the normalized FT-IR spectra of peanut protein (1700 cm^{-1}-1600 cm^{-1}) subjected to different high electric field intensities and treatment time.

Experimental Conditions					
10-60		**10-120**		**10-180**	
Wavelength (cm^{-1})	**Area (%)**	**Wavelength (cm^{-1})**	**Area (%)**	**Wavelength (cm^{-1})**	**Area (%)**
1615	3.37	1615	3.13	1615	3.54
1634	35.63	1634	34.55	1634	34.32
1652	20.33	1645	0.45	1645	0.57
1660	0.55	1653	23.1	1653	22.46
1666	30.62	1660	0.36	1660	0.75
1683	7.65	1666	29.38	1666	28.75
1693	1.85	1683	7.2	1683	8.22
		1693	1.83	1693	1.82
15-60		**15-120**		**15-180**	
Wavelength (cm^{-1})	**Area (%)**	**Wavelength (cm^{-1})**	**Area (%)**	**Wavelength (cm^{-1})**	**Area (%)**
1615	3.41	1615	3.68	1608	0.76
1634	34.33	1634	35.01	1615	3.87
1645	0.26	1645	0.6	1634	33.4
1654	55.46	1653	27.86	1645	0.75
1660	0.75	1660	0.42	1654	20.62
1666	28.75	1666	28.76	1660	0.35
1684	8.22	1683	8.01	1666	28.23
1693	1.82	1693	1.66	1684	7.96
				1693	1.92
20-60		**20-120**		**20-180**	
Wavelength (cm^{-1})	**Area (%)**	**Wavelength (cm^{-1})**	**Area (%)**	**Wavelength (cm^{-1})**	**Area (%)**
1617	7.39	1608	0.52	1608	0.82
1635	33.4	1616	4.26	1616	4.46
1645	1.73	1635	34.99	1635	32.52
1654	22.03	1645	2.73	1645	4.56
1660	0.73	1654	21.15	1654	19.86
1666	27.69	1660	0.33	1660	0.94
1683	8.13	1666	26.68	1666	26.28
1693	1.9	1683	7.49	1683	8.53
		1693	1.85	1693	2.03

From the data presented in Table 4.3 and Fig 4.5, it is observed that for almost all of the experiments the β sheets and the α helix are major secondary structures accounting for about 50 % - 55% of the total secondary structure. The turns and the 3/10 helices together account to about another 30% which have an overlapping wavelenghts between 1662 – 1672 cm^{-1} (as shown in Table 4.2).

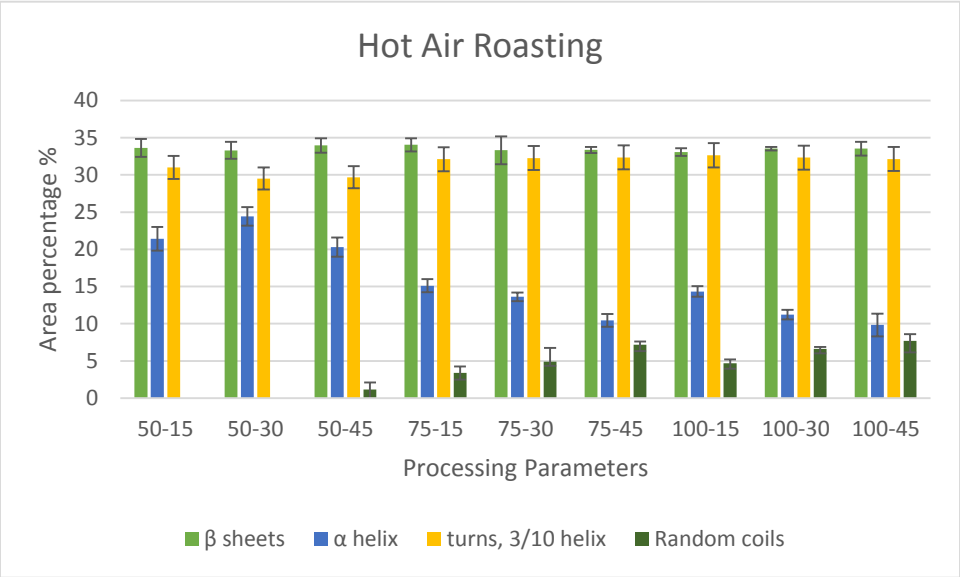


Figure 4.5 Variations in relative areas of the bands fitted to the normalized FT-IR spectra of Amide I region (1700 cm^{-1} – 1600 cm^{-1}) of hot air roasted peanuts

But as the processing time is incresed from 15 min to 45 min, there is a relocation in the α helix structures with an exception of 50-30 where there was a slight rise in the area of the peaks assigned to α helices. This reduction resulted in a growth in random coil secondary structures, which were absent initially in the 50-15 and 50-30 processing, but later appeared as the temperature and the time of processing were increased. There was also a slight relocations in the anti parallel β sheets (not shown in Fig 4.5), which suggests that there was formation of protein aggregates with an increasing temperature. Similar results were observed in wheat gliadin protein when processed at various temperatures (Mangavel et al., 2001).

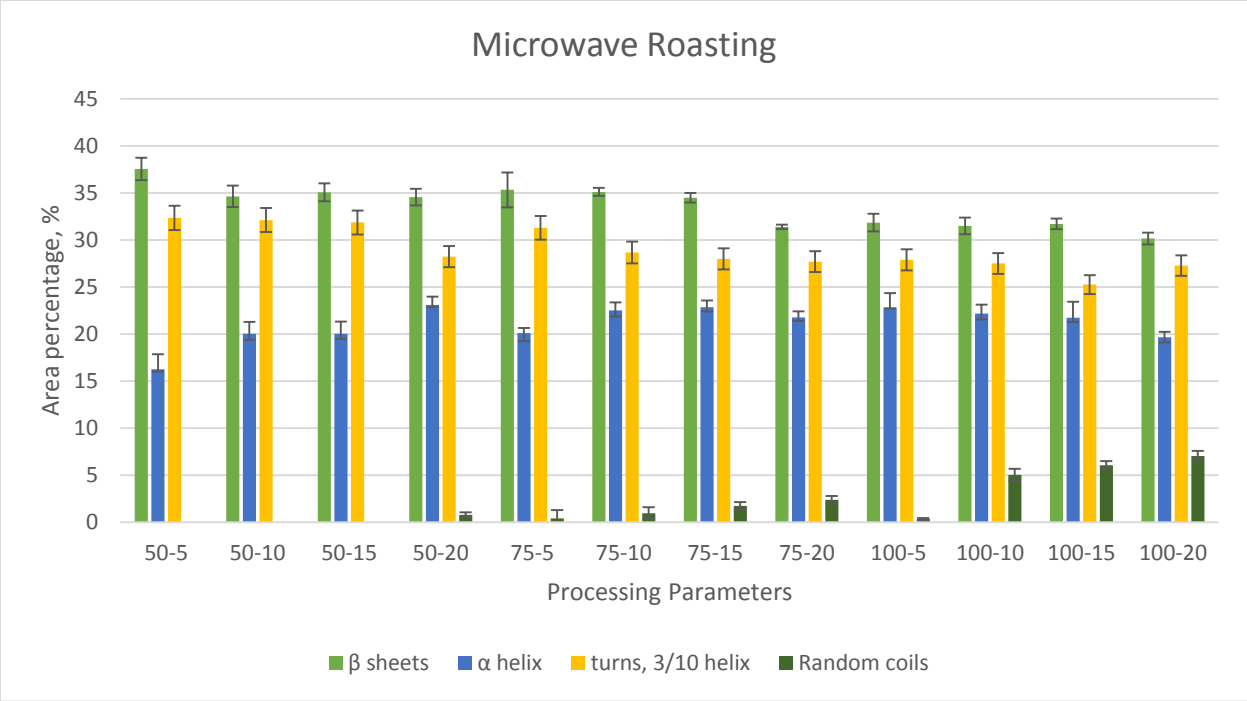


Figure 4.6 Variations in relative areas of the bands fitted to the normalized FT-IR spectra of Amide I region (1700 cm^{-1} – 1600 cm^{-1}) of microwave roasted peanuts

The data corresponding to the microwave processing of nuts in Table 4.4 and Fig 4.6 also show that the major secondary structures in the peanut protein are β sheets and α helix accounting to a sum of 55 %. But unlike the hot air roasting, microwave roasted peanut protein showed no particular trend in the areas of α helix peaks. When processing time was increased from 5 to 20 min at 50 ^{o}C there was an increase in the α helix from about 16.24 % to 23.1 % which is similar to that of the hot air roasting between 15 min and 30 min roasting at 50 ^{o}C which showed a rise in the α helix from 21.41 % to 24.43 %. On processing peanuts at 100 ^{o}C, there was again a relocation in α helix peaks resulting in their peak areas falling from 22.83 % to 19.86 %. It can also be observed in Fig.4.6 that microwave processing also resulted in increasing random coils in the protein secondary structure similar to hot air roasting. This result was first observed in 50-20 samples, but with an increase in the processing time and temperature, there was a gradual rise

observed in the random coils from 0.42 % to 2.39 % for samples processed at 75 ^{o}C and from 0.34 % to 7.03% for processing at 100 ^{o}C in microwave. This is due to relocations in the β sheet and 3/10 helix secondary structures.

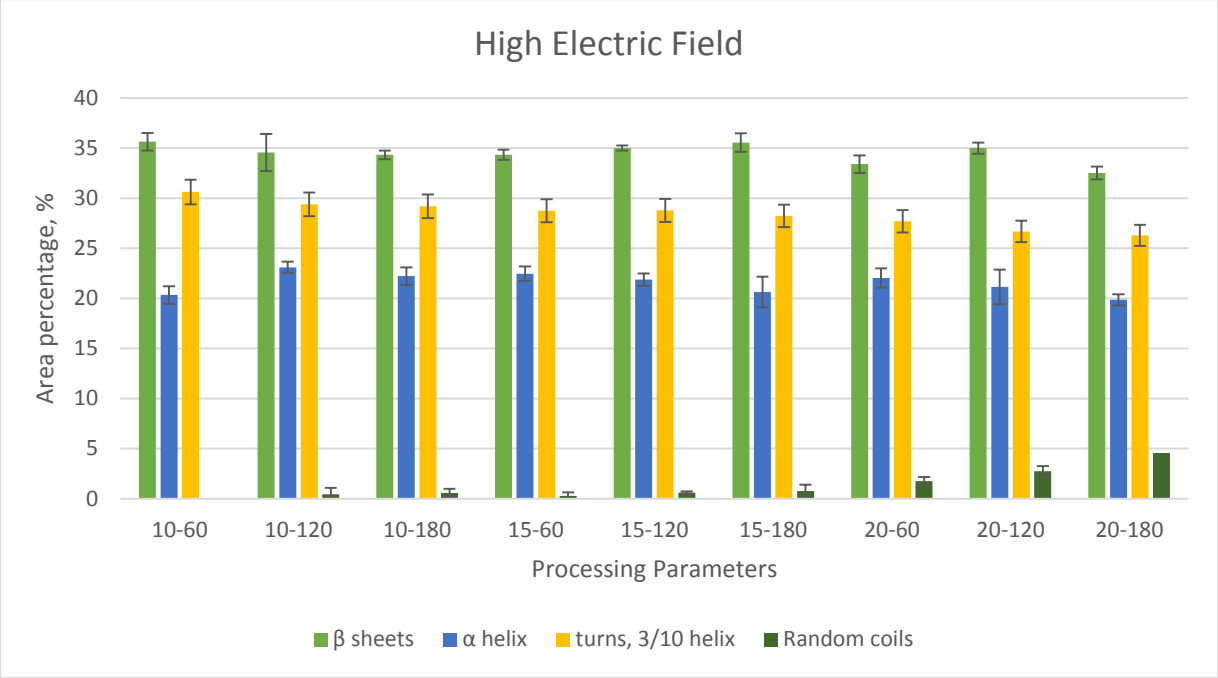


Figure 4.7 Variations in relative areas of the bands fitted to the normalized FT-IR spectra of Amide I region (1700 cm^{-1} – 1600 cm^{-1}) of high electric field processed peanuts

In the high voltage electric processing, a corona wind is generated by passing high voltage through a very thin electrode. This corona wind bombards charged particles on to the food which results in changes in the secondary structure (Singh et al., 2013; Singh et al., 2012). This corona wind resulted in formation of random coils in the protein similar to microwave and hot air processing of peanut protein. In the case of α helix, there is no particular trend in the case of 10 kV, but for 15 kV and 20 kV processing, it was observed that there is a slight decrease in the peak areas. Similar trend was also observed for turns and 3/10 helix at 10 kV, 15 kV and 20 KV as shown in Fig 4.7.

Table 4.6 Effect of hot air roasting on protein digestibility (IVPD %) of peanut protein

	Temperature (ºC)			
	50	**75**	**100**	**Mean**
Time (min)				
15	83.58 e	87.84 bcd	90.73 a	87.38 b
30	86.11 d	88.47 bc	91.00 a	88.52 a
45	87.02 cd	89.46 ab	91.36 a	89.28 a
Mean	85.57 c	88.59 b	91.03 a	

Common letters types indicate the lack of a significant difference ($P > 0.05$) based on ANOVA and Tukey's HSD. The 'Mean' represent the mean data independent of the other parmeter.

Table 4.7 Effect of microwave roasting on protein digestibility (IVPD %) of peanut protein

	Temperature (ºC)			
	50	**75**	**100**	**Mean**
Time (min)				
5	85.84 h	88.10 ef	89 cd	87.71 d
10	87.02 g	89.28 bcd	89.91 abcd	88.74 c
15	87.56 gf	89.91 abcd	90.28 ab	89.25 b
20	89.10 de	90.19 abc	90.82 a	90.03 a
Mean	87.38 c	89.37 b	90.05 a	

Common letters types indicate the lack of a significant difference ($P > 0.05$) based on ANOVA and Tukey's HSD. The 'Mean' represent the mean data independent of the other parmeter.

Table 4.8 Effect of high electric field roasting on protein digestibility (IVPD %) of peanut protein

	Voltage (kV)			
	10	**15**	**20**	**Mean**
Time (min)				
60	86.56 e	87.56 d	88.47 c	87.53 c
120	88.38 cd	88.47 c	90.10 b	88.97 b
180	89.10 c	90.73 ab	91.54 a	90.46 a
Mean	88.01 c	88.91 b	90.03 a	

Common letters types indicate the lack of a significant difference ($P > 0.05$) based on ANOVA and Tukey's HSD. The 'Mean' represent the mean data independent of the other parmeter.

4.4.2 Effect of Processing on the protein quality

The effect of various processing methods on the protein digestability has been summarized in the Tables 4.6, 4.7 and 4.8. The digestability of the raw peanut protein (unprocessed peanut protein) was found to be around 81.22 ± 0.23 % which is significantly lesser than all of the processed peanut protein samples. Similar values were reported by Bodwell et al., (Bodwell et al., 1980) on

their analysis using the 3 enzyme method. In the case of hot air roasting of peanuts (Table 4.6), the protein digestability has incresed significantly with an increase in treatment temperature. With an increse in treatment time, there is a significant increase from 15 minutes to 30 minutes, but not from 30 minutes to 45 minutes. When the combination of the parmeters is considered, there is a significant rise in the protein digestability as processing time incresed from 15 minutes to 45 minutes at 50 ^{o}C. But no significant growth was observed as the processing time increased from 15 minutes to 45 minutes in the case of 75 ^{o}C and 100 ^{o}C.

The *in vitro* protein digestability (IVPD %) of the microwave roasted peanuts is also summarized in Table 4.7. Results were similar to that of the hot air roasted peanuts. There was an effect of time and temperature on the digestibilty regradless of each other. In combination, significant difference was observed in the case of 50 ^{o}C with an increase in processing time. For the peanut samples that are processed at 75 ^{o}C and 100 ^{o}C, significant difference was only observed between the 5 minute processing and 20 minutes processing. The other levels showed no significant difference. In the case of estimting the protein digestability of samples treated with high electric field, there is a significant increase in the digestability with temperature and time regardless to each other. There is also a significant differecnce observed in digestability with increasing processing time at 10 kV, 15 kV and 20 kV voltages.

Thus, increasing digestibility clearly suggests that there are structural rearrngements taking place with in protein conformation leading to change in the funcional properties (Duodu et al., 2003). Similar studies were conducted to understand the structure and digestability relation in dry beans (*Phaseolus vulgaris*) and green peas (*Pisum sativum*) (Deshpande & Damodaran, 1989), sorghum

(*Sorghum bicolor*) and maize (*Zea mays*) (Duodu et al., 2001; Duodu et al., 2003) and rice (Sagum & Arcot, 2000). Duodu et al., (Duodu et al., 2001) reported that there was an increase antiparallel, intermolecular β-sheets which resulted from reorganization of the α-helical structures which could have revealed the binding sites for the enzymes to act on. But the same study also revealed that secondary structure changes can be less relevant compared to the invaginated structure of the protein body. It was proposed that this provides the enzymes a better access for binding with the core of the protein.

The protein digestability has been directly related to the allergenicity and the solubility of the proteins (Bannon et al., 2003; Sen et al., 2002). Thus an increase in the protein digestability should directly reduce the allergenicity. But, in the case of peanuts various studies conducted have showed that their allergenicity has increased with an increase in roasting time and temperature (Beyer et al., 2001; Burks et al., 1998; Maleki et al., 2000b). Davis et al., (Davis & Williams, 1998) and Beyer et al., (Beyer et al., 2001) in their studies have reported that treating proteins over the temperature of 80 ^{o}C has resulted in loss of secondary and tertiary structures and fromation of random coils with fully unfolded configuration. In our study on peanut protein, FT-IR analysis has also revelaed the similar results. With an increase in the treatment time in all the cases (hot air roasting, microwave and high electric field treatments) there is an increase in the random coils and turns as more stable 3/10 helices and α helices have opened up and relocated thermselves. The random coils observed at 1645 cm^{-1} have increased from 0 % - 7.69 % of the whole secondary structure for hot air treatments 50-15 to 100-45. For microwave treatments, this was between 0 % - 7.03 % between 50-5 to 100-20 and for high electric field processing it rose from 0% at 10-60 to 4.56% at 20-180. The increasing random coil structures in the protein conformation may have

opened up new binding sites, resulting in an increased allergenicity of the protein as reported in various other studies (Beyer et al., 2001; Maleki et al., 2000a; Maleki et al., 2000b).

4.5 CONCLUSION

The study conducted on the peanut protein clearly showed that the external processing factors which include hot air roasting, microwave roasting and high electric field processing can influence the conformational secondary structure. The gaussian analysis of the FT-IR spectra and the *In vitro* Protein digestabillity (IVPD %) have been conducted on the processed protein which showed that there are conformed changes in the protein structures as predicted by previous other studies. It can also be observed that the high electric field processing at 20 kV has similar conformational changes as of thermal processing methods like hot air roasting and microwave processing. Further studies need to be conducted at higher electric field intensities of 50 kV – 70 kV which are highly used in various food processing techniques like pulse electric field processing.

CHAPTER V

SUMMARY AND CONCLUSION

Proteins are a major nutritional and energy source for the human population around the world. But, there are few cases where people are allergic to specific sections of proteins called ‘epitopes’ which cause various health complications including anaphylaxis and even death in few cases. Though any food can trigger an allergic reaction, eight of them are responsible for more than 90% of the cases. They are peanut, milk, wheat, egg, fish, shell fish and tree nuts. Currently about 8% of world’s infant and children, 2 % of the adult population suffer from hypersensitivity to at least one kind of food and this number is on the rise making it one of the most important health issue that has to be addressed soon. Many researchers have shown that various functional properties are directly dependent on the structural conformation of the protein and changes in the structure can result in deviations in the physico-chemical functionality of the protein. Several studies have been conducted in understanding the influence of the structural conformation on the allergenicity. When foods are processed using thermal, chemical, non- thermal methods, there are secondary structural changes which can either mitigate or aggravate the allergenicity of the allergen considered.

In the review the questions of how processing methods have affected the allergenicity of various foods is addressed; processing methods included both conventional and novel methods, including thermal and non-thermal. Apart from the big 8 as mentioned above, processing effects on various other allergic foods like fruits and vegetables have also been delineated. Though major part of the literature showed that the processing has an effect on the secondary structure, it always did not result in mitigation or alleviation of allergic properties. Moreover, there were cases when processing has actually resulted in increased allergic reactivity. But of various food products,

peanuts were found to be a common cause of anaphylactic reactions around the world making it the centre of our study.

The primary objective of this study was to evaluate the effect of thermal processing and high electric fields on the secondary structure conformation of the peanut protein using FT-IR spectroscopy. FT-IR is one of the oldest and widely used experimental technique to study the secondary structure deviations in the protein. With this analysis, we have clearly observed that there are conformed irreversible conformational changes in the protein secondary structure. Also, as the intensity of the thermal processing increased, there were secondary structure relocations observed, especially α-helix and β-sheets, resulting in formation of random coils. Thus, predictions of other researchers about the increased allergenicity and other functional properties have been further observed with this work. Also, it showed that the hot air roasting and microwave processing, both being thermal do not have the same effect on the protein conformation changes. This work also provided a base showing the effect of high electric field intensity on the protein secondary structure for further research.

The second objective was to evaluate the applicability of molecular dynamics simulations for studying the effects of processing parameters on food proteins. In this thesis, the analysis of Ara h 6 peanut allergen was performed using GROMACS under conditions like thermal processing, static electric field and oscillating electric fields. This study has also showed that the processing has a conformational effect on the secondary structure. In particular, the simulation data has showed that the extended structures have folded back into the core of the molecule, which has revealed new binding sites for enzymes to act on. Radius of Gyration, Root Mean Square Deviation (RMSD), Dipole moment and solvent accessible surface area (SASA) have been evaluated for the Ara h 6 molecule. The particular secondary structure conformation revealed by the molecular

dynamic simulations has a reduction in the solvent accessible surface area. Thus, there would be deviations observed in the surface bonding patterns with water and other molecules enzymes. Further analysis of the structural dependency of the functional properties in particular with Ara h 6 have to be conducted in understanding the implications of the variations in surface hydrophobicity and hydrophilicity. This study has showed that the molecular modeling can be applied to study the protein molecules.

FUTURE WORK

1) Application of higher voltages (50kV – 70 kV) to study the protein secondary structures which are comparable to various food processing techniques including ohmic heating and pulse electric field processing.

2) Simulation studies can be conducted for extended periods of time like 10 ns – 50 ns for more extensive data on the various parameters.

3) More biological samples have to be studied for better evaluation of different types of proteins.

4) Further analysis using SDS-PAGE, ELISA and immunoblot have to be performed for analysing the complete effect on the allergenicity of various protein molecules.

REFERENCES

Adler-Nissen, J. (1986). Enzymic hydrolysis of food proteins. Elsevier Applied Science Publishers ; Sole distributor in the USA and Canada : Elsevier Science Pub. Co., London; New York; New York, NY, USA.

Ahmed, J., Ramaswamy, H.S., Ayad, A., Alli, I., Alvarez, P. 2007. Effect of high-pressure treatment on rheological, thermal and structural changes in Basmati rice flour slurry. *Journal of Cereal Science*, **46**(2), 148-156.

Alonso, R., Aguirre, A., Marzo, F. 2000. Effects of extrusion and traditional processing methods on antinutrients and in vitro digestibility of protein and starch in faba and kidney beans. *Food Chemistry*, **68**(2), 159-165.

Alun Jones, V., Shorthouse, M., McLaughlan, P., Workman, E., and Hunter, J. (1982). Food intolerance: a major factor in the pathogenesis of irritable bowel syndrome. *The Lancet*. **320**: 1115-1117.

Álvarez-Álvarez, J., Guillamón, E., Crespo, J. F., Cuadrado, C., Burbano, C., Rodríguez, J., Fernández, C., and Muzquiz, M. (2005). Effects of extrusion, boiling, autoclaving, and microwave heating on lupine allergenicity. *Journal of Agricultural and Food Chemistry*. **53**: 1294-1298.

Anderson, O.D., Békés, F., Gras, P.W., Kuhl, J., Tam, A. 1996. HMW glutenins: Structure function relationships step by step. *Proc. 6 Intern. Workshop on Gluten Proteinsth*, 195-198.

Andreas, L. (2009). Allergenicity of Food and Impact of Processing. **In:** Novel Food Processing, pp. 459-478. CRC Press.

Anekella, K., and Orsat, V. (2013). Optimization of microencapsulation of probiotics in raspberry juice by spray drying. *LWT - Food Science and Technology*. **50**: 17-24.

Arshad, S.H., Stevens, M., Hide, D.W. 1993. The effect of genetic and environmental factors on the prevalence of allergic disorders at the age of two years. *Clinical & Experimental Allergy*, **23**(6), 504-511.

Astrakas, L.G., Gousias, C., Tzaphlidou, M. 2012. Structural destabilization of chignolin under the influence of oscillating electric fields. *Journal of Applied Physics*, **111**(7), 074702-074702-8.

Astwood, J.D., Leach, J.N., Fuchs, R.L. 1996. Stability of food allergens to digestion in vitro. *Nature biotechnology*, **14**(10), 1269-1273.

Atanasiu, P., Barbu, E., and Basset, J. (1951). Effect of very high pressure on Newcastle virus. I. Dissociation of infectious power and of hemagglutination. *Action des pressions très élevées sur le virus de Newcastle. I. Dissociation du pouvoir infectieux et de l'hémagglutination.* **81**: 340-343.

Ayerst, G. (1969). The effects of moisture and temperature on growth and spore germination in some fungi. *Journal of Stored Products Research*. **5**: 127-141.

Ayuso, R., Lehrer, S. B., and Reese, G. (2002). Identification of continuous, allergenic regions of the major shrimp allergen pen a 1 (Tropomyosin). *International Archives of Allergy and Immunology*. **127**: 27-37.

Baer, A., Oroz, M., and Blanc, B. (1976). Serological studies on heat-induced interactions of α-lactalbumin and milk proteins. *Journal of Dairy Research*. **43**: 419-432.

Ballmer-Weber, B.K., Hoffmann, A., Wüthrich, B., Lüttkopf, D., Pompei, C., Wangorsch, A., Kästner, M., Vieths, S. 2002. Influence of food processing on the allergenicity of celery:

DBPCFC with celery spice and cooked celery in patients with celery allergy. *Allergy: European Journal of Allergy and Clinical Immunology*, **57**(3), 228-235.

Bamforth, C. W. (2005). Food, fermentation, and micro-organisms. Blackwell Science, Oxford; Ames, Iowa.

Bang, H., Dyerberg, J., and Sinclair, H. M. (1980). The composition of the Eskimo food in north western Greenland. *The American journal of clinical nutrition*. **33**: 2657-2661.

Bannon, G., Fu, T.-J., Kimber, I., Hinton, D.M. 2003. Protein digestibility and relevance to allergenicity. *Environmental health perspectives*, **111**(8), 1122.

Basset, J., and Macheboeuf, M. (1933). Etudes sur les effets biologiques des ultra-pressions. Etudes de l'immunité: Influence des pressions très élevées sur certains antigènes et anticorps. *C. R. Acad. Sci*. **196**: 67-69.

Basset, J., and Macheboeuf, M. A. (1932). Etude sur les effets biologiques des ultrapressions: Résistance des bactéries, des diastases et des toxines aux pressions très élevées. *C. R. Acad. Sci*. **195**: 1431-1433.

Basset, J., Lépine, P., and Chaumont, L. (1956). Effects of high pressures on the poliomyelitis virus (Lansing strain). *Ann Inst Pasteur*. **90**: 575-596.

Basset, J., Nicolaus, S., and Macheboeuf, M. A. (1935a). L'action de l'ultrapression sur l'activité pathogène de quelques virus. *CR Acad Sc*. **200**: 1882-1884.

Basset, J., Wòllman, E., Macheboeuf, M. A., and Bardach, M. (1933). Études sur les effets biologiques des ultrapressions: Action des pressions très élevées sur des bactériophages et sur un virus invisible (virus vaccinal). *C R Acad Sc*. **196**: 1138-1139.

Basset, J., Wollman, E., Macheboeuf, M. A., and Bardach, M. (1935b). Etudes sur les effets biologiques des ultra-pressions: action des pressions élevées sur les tumeurs. *CR Hebd Acad Sci*. **200**: 200.

Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., Shindyalov, I.N., Bourne, P.E. 2000. The Protein Data Bank. *Nucleic Acids Research*, **28**(1), 235-242.

Bernard, H., Mondoulet, L., Drumare, M., Paty, E., Scheinmann, P., Thaï, R., Wal, J. 2007a. Identification of a new natural Ara h 6 isoform and of its proteolytic product as major allergens in peanut. *Journal of agricultural and food chemistry*, **55**(23), 9663-9669.

Bernhisel-Broadbent, J., Scanlon, S. M., and Sampson, H. A. (1992a). Fish hypersensitivity. I. In vitro and oral challenge results in fish- allergic patients. *Journal of Allergy and Clinical Immunology*. **89**: 730-737.

Bernhisel-Broadbent, J., Strause, D., and Sampson, H. A. (1992b). Fish hypersensitivity. II: Clinical relevance of altered fish allergenicity caused by various preparation methods. *Journal of Allergy and Clinical Immunology*. **90**: 622-629.

Besler, M., Steinhart, H., and Paschke, A. (2001). Stability of food allergens and allergenicity of processed foods. *Journal of Chromatography B: Biomedical Sciences and Applications*. **756**: 207-228.

Beyer, K., Morrow, E., Li, X. M., Bardina, L., Bannon, G. A., Burks, A. W., and Sampson, H. A. (2001). Effects of cooking methods on peanut allergenicity. *Journal of Allergy and Clinical Immunology*. **107**: 1077-1081.

Bock, S.A. 1987. Prospective appraisal of complaints of adverse reactions to foods in children during the first 3 years of life. *Pediatrics*, **79**(5), 683-688.

Bodwell, C., Satterlee, L., Hackler, L. 1980. Protein digestibility of the same protein preparations by human and rat assays and by in vitro enzymic digestion methods. *The American journal of clinical nutrition*, **33**(3), 677-686.

Bonomi, F., Fiocchi, A., Frøkiær, H., Gaiaschi, A., Iametti, S., Poiesi, C., Rasmussen, P., Restani, P., and Rovere, P. (2003). Reduction of immunoreactivity of bovine β-lactoglobulin upon combined physical and proteolytic treatment. *Journal of Dairy Research*. **70**: 51-59.

Boye, J., Ma, C., and Harwalkar, V. (1997). Thermal denaturation and coagulation of proteins. *FOOD SCIENCE AND TECHNOLOGY-NEW YORK-MARCEL DEKKER-*: 25-56.

Boyle, M. A. Z. G. (1996). Personal nutrition. West Pub. Co., Minneapolis.

Breiteneder, H., Radauer, C. 2004. A classification of plant food allergens. *Journal of Allergy and Clinical Immunology*, **113**(5), 821-830.

Brenna, O., Pompei, C., Ortolani, C., Pravettoni, V., Farioli, L., and Pastorello, E. A. (2000). Technological processes to decrease the allergenicity of peach juice and nectar. *Journal of Agricultural and Food Chemistry*. **48**: 493-497.

Brigino, E., Bahna, S.L. 1995. Clinical features of food allergy in infants. *Clinical Reviews in Allergy & Immunology*, **13**(4), 329-345.

Bu, G., Luo, Y., Zhang, Y., and Chen, F. (2010). Effects of fermentation by lactic acid bacteria on the antigenicity of bovine whey proteins. *Journal of the Science of Food and Agriculture*. **90**: 2015-2020.

Bucher, X., Fichier, W.J., Dahinden, C.A., Helbling, A. 2004. Effect of tree pollen specific, subcutaneous immunotherapy on the oral allergy syndrome to apple and hazelnut. *Allergy: European Journal of Allergy and Clinical Immunology*, **59**(12), 1272-1276.

Budi, A., Legge, S., Treutlein, H., Yarovsky, I. 2004. Effect of external stresses on protein conformation: a computer modelling study. *European Biophysics Journal*, **33**(2), 121-129.

Burks, A.W., Sampson, H. 1993. Food allergies in children. *Journal of Combinatorial Theory, Series A*, **23**(6), 230-252.

Burks, A.W., Williams, L.W., Connaughton, C., Cockrell, G., O'Brien, T.J., Helm, R.M. 1992. Identification and characterization of a second major peanut allergen, Ara h II, with use of the sera of patients with atopic dermatitis and positive peanut challenge. *Journal of Allergy and Clinical Immunology*, **90**(6 I), 962-969.

Burks, W., Sampson, H.A., Bannon, G.A. 1998. Peanut allergens. *Allergy: European Journal of Allergy and Clinical Immunology*, **53**(8), 725-730.

Burton-Freeman, B. 2000. Dietary fiber and energy regulation. *Journal of Nutrition*, **130**(2 SUPPL.), 272S-275S.

Byun, M.-W., Kim, J.-H., Lee, J.-W., Park, J.-W., Hong, C.-S., and Kang, I.-J. (2000). Effects of Gamma Radiation on the Conformational and Antigenic Properties of a Heat-Stable Major Allergen in Brown Shrimp. *Journal of Food Protection*. **63**: 940-944.

Carbonaro, M., Maselli, P., Dore, P., Nucara, A. 2008. Application of Fourier transform infrared spectroscopy to legume seed flour analysis. *Food Chemistry*, **108**(1), 361-368.

Carpita, N. C., and Gibeaut, D. M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal*. **3**: 1-30.

Chicón, R., Belloque, J., Alonso, E., and López-Fandiño, R. (2008a). Immunoreactivity and digestibility of high-pressure-treated whey proteins. *International Dairy Journal*. **18**: 367-376.

Chicón, R., Belloque, J., Alonso, E., Martín-Álvarez, P. J., and López-Fandiño, R. (2008b). Hydrolysis under High Hydrostatic Pressure as a Means To Reduce the Binding of β-Lactoglobulin to Immunoglobulin E from Human Sera. *Journal of Food Protection*. **71**: 1453-1459.

Chobert, J. M., Bertrand-Harb, C., and Nicolas, M. G. (1988). Solubility and emulsifying properties of caseins and whey proteins modified enzymically by trypsin. *Journal of Agricultural and Food Chemistry*. **36**: 883-892.

Chung, S. Y., Yang, W., and Krishnamurthy, K. (2008). Effects of Pulsed UV-Light on Peanut Allergens in Extracts and Liquid Peanut Butter. *Journal of Food Science*. **73**: C400-C404.

Clare Mills, E.N., Sancho, A.I., Rigby, N.M., Jenkins, J.A., Mackie, A.R. 2009. Impact of food processing on the structural and allergenic properties of food allergens. *Molecular Nutrition and Food Research*, **53**(8), 963-969.

Cleaves, H. J. (2011). Quaternary Structure (Protein). *Encyclopedia of Astrobiology*: 1396-1397.

Cocco, R. R., Järvinen, K. M., Sampson, H. A., and Beyer, K. (2003). Mutational analysis of major, sequential IgE-binding epitopes in αs1-casein, a major cow's milk allergen. *Journal of Allergy and Clinical Immunology*. **112**: 433-437.

Cuadrado, C., Cabanillas, B., Pedrosa, M. M., Varela, A., Guillamón, E., Muzquiz, M., Crespo, J. F., Rodriguez, J., and Burbano, C. (2009). Influence of thermal processing on IgE reactivity to lentil and chickpea proteins. *Molecular nutrition & food research*. **53**: 1462-1468.

Cucu, T., De Meulenaer, B., Bridts, C., Devreese, B., Ebo, D. 2012. Impact of thermal processing and the Maillard reaction on the basophil activation of hazelnut allergic patients. *Food and Chemical Toxicology*, **50**(5), 1722-1728.

Dalal, I., Binson, I., Reifen, R., Amitai, Z., Shohat, T., Rahmani, S., Levine, A., Ballin, A., Somekh, E. 2002. Food allergy is a matter of geography after all: Sesame as a major cause of severe IgE-mediated food allergic reactions among infants and young children in Israel. *Allergy: European Journal of Allergy and Clinical Immunology*, **57**(4), 362-365.

Daul, C. B., Morgan, J. E., Hughes, J., and Lehrer, S. B. (1988). Provocation-challenge studies in shrimp-sensitive individuals. *Journal of Allergy and Clinical Immunology*. **81**: 1180-1186.

Daul, C. B., Slattery, M., Reese, G., and Lehrer, S. B. (1994). Identification of the major brown shrimp (Penaeus aztecus) allergen as the muscle protein tropomyosin. *International Archives of Allergy and Immunology*. **105**: 49-55.

Davis, P. J., Smales, C. M., and James, D. C. (2001). How can thermal processing modify the antigenicity of proteins? *Allergy: European Journal of Allergy and Clinical Immunology, Supplement*. **56**: 56-60.

Davis, P., and Williams, S. (1998). Protein modification by thermal processing. *Allergy*. **53**: 102-105.

de Pomerai, D. I., Smith, B., Dawe, A., North, K., Smith, T., Archer, D. B., Duce, I. R., Jones, D., and Candido, E. P. M. (2003). Microwave radiation can alter protein conformation without bulk heating. *FEBS letters*. **543**: 93-97.

Decareau, R. V., and Peterson, R. A. (1986). Microwave processing and engineering.

Deshpande, S.S., Damodaran, S. 1989. Structure-Digestibility Relationship of Legume 7S Proteins. *Journal of Food Science*, **54**(1), 108-113.

Diniz, F., and Martin, A. (1997). Effects of the extent of enzymatic hydrolysis on functional properties of shark protein hydrolysate. *LWT-Food Science and Technology*. **30**: 266-272.

Doona, C. J. K. K. F. F. E. (2010). Case studies in novel food processing technologies innovations in processing, packaging and predictive modelling. Woodhead Publishing, Oxford.

Duodu, K.G., Tang, H., Grant, A., Wellner, N., Belton, P.S., Taylor, J.R.N. 2001. FTIR and Solid State13C NMR Spectroscopy of Proteins of Wet Cooked and Popped Sorghum and Maize. *Journal of Cereal Science*, **33**(3), 261-269.

Duodu, K.G., Taylor, J.R.N., Belton, P.S., Hamaker, B.R. 2003. Factors affecting sorghum protein digestibility. *Journal of Cereal Science*, **38**(2), 117-131.

Edsall, J.T. 1995. Hsien Wu and the First Theory of Protein Denaturation (1931). in: *Advances in Protein Chemistry*, (Eds.) F.M.R.J.T.E. C.B. Anfinsen, S.E. David, Vol. Volume 46, Academic Press, pp. 1-5.

Ehn, B. M., Allmere, T., Telemo, E., Bengtsson, U., and Ekstrand, B. O. (2005). Modification of IgE binding to β-lactoglobulin by fermentation and Proteolysis of cow's milk. *Journal of Agricultural and Food Chemistry*. **53**: 3743-3748.

Ehn, B. M., Ekstrand, B., Bengtsson, U., and Ahlstedt, S. (2004). Modification of IgE Binding during Heat Processing of the Cow's Milk Allergen β-Lactoglobulin. *Journal of Agricultural and Food Chemistry*. **52**: 1398-1403.

Eichner, K., and Karel, M. (1972). Influence of water content and water activity on the sugar-amino browning reaction in model systems under various conditions. *Journal of Agricultural and Food Chemistry*. **20**: 218-223.

El-Ghaish, S., Ahmadova, A., Hadji-Sfaxi, I., El Mecherfi, K. E., Bazukyan, I., Choiset, Y., Rabesona, H., Sitohy, M., Popov, Y. G., Kuliev, A. A., Mozzi, F., Chobert, J.-M., and Haertlé, T. (2011a). Potential use of lactic acid bacteria for reduction of allergenicity and for longer conservation of fermented foods. *Trends in food science & technology*. **22**: 509-516.

El-Ghaish, S., Dalgalarrondo, M., Choiset, Y., Sitohy, M., Ivanova, I., Haertlé, T., and Chobert, J. M. (2010a). Characterization of a new isolate of Lactobacillus fermentum IFO 3956 from Egyptian Ras cheese with proteolytic activity. *European Food Research and Technology*. **230**: 635-643.

El-Ghaish, S., Dalgalarrondo, M., Choiset, Y., Sitohy, M., Ivanova, I., Haertlé, T., and Chobert, J. M. (2010b). Screening of strains of Lactococci isolated from Egyptian dairy products for their proteolytic activity. *Food Chemistry*. **120**: 758-764.

El-Ghaish, S., Rabesona, H., Choiset, Y., Sitohy, M., Haertlé, T., and Chobert, J. M. (2011b). Proteolysis by Lactobacillus fermentum IFO3956 isolated from Egyptian milk products decreases immuno-reactivity of α s1-casein. *Journal of Dairy Research*. **78**: 203-210.

Eliasson, A.-C. (2006). Carbohydrates in food. CRC/Taylor & Francis, Boca Raton, FL.

El-Mansi, M. (2012). Fermentation microbiology and biotechnology. CRC Press, Boca Raton, FL.

Elmer, G. W., Surawicz, C. M., and McFarland, L. V. (1996). Biotherapeutic agents: A neglected modality for the treatment and prevention of selected intestinal and vaginal infections. *Journal of the American Medical Association*. **275**: 870-876.

Farkas, J. (1998). Irradiation as a method for decontaminating food: a review. *International Journal of Food Microbiology*. **44**: 189-204.

Farkas, J. (2006). Irradiation for better foods. *Trends in food science & technology*. **17**: 148-152.

Fellows, P. (2000). Food processing technology principles and practice. CRC Press ; Woodhead Pub., Boca Raton, Fla.; Cambridge, England.

Fellows, P. J. (2009). Food processing technology: principles and practice. Elsevier.

Feng, H., Barbosa-Canovas, G., and Weiss, J. (2011). *Ultrasound technologies for food and bioprocessing food engineering series*.

Fiocchi, A., Restani, P., Bernardo, L., Martelli, A., Ballabio, C., D'Auria, E., and Riva, E. (2004). Tolerance of heat-treated kiwi by children with kiwifruit allergy. *Pediatric Allergy and Immunology*. **15**: 454-458.

Fischer, E. 1894. Einfluss der Configuration auf die Wirkung der Enzyme. *Berichte der deutschen chemischen Gesellschaft*, **27**(3), 2985-2993.

Flinterman, A.E., Van Hoffen, E., Den Hartog Jager, C.F., Koppelman, S., Pasmans, S.G., Hoekstra, M.O., Bruijnzeel-Koomen, C.A., Knulst, A.C., Knol, E.F. 2007. Children with peanut allergy recognize predominantly Ara h2 and Ara h6, which remains stable over time. *Clinical and Experimental Allergy*, **37**(8), 1221-1228.

Fruton, J. S. (1972). Molecules and life.

Gall, H., Kalveram, K.-J., Forck, G., and Sterry, W. (1994). Kiwi fruit allergy: a new birch pollen–associated food allergy. *Journal of Allergy and Clinical Immunology*. **94**: 70-76.

Georget, D.M., Belton, P.S. 2006. Effects of temperature and water content on the secondary structure of wheat gluten studied by FTIR spectroscopy. *Biomacromolecules*, **7**(2), 469-475.

Goh, D., Lau, Y., Chew, F., Shek, L., Lee, B. 1999. Pattern of food-induced anaphylaxis in children of an Asian community. *Allergy*, **54**(1), 84-86.

Gross, M., Jaenicke, R. 1994. Proteins under pressure. The influence of high hydrostatic pressure on structure, function and assembly of proteins and protein complexes. *European Journal of Biochemistry*, **221**(2), 617-630.

Gruber, P., Vieths, S., Wangorsch, A., Nerkamp, J., and Hofmann, T. (2004). Maillard reaction and enzymatic browning affect the allergenicity of Pru av 1, the major allergen from cherry (Prunus avium). *Journal of Agricultural and Food Chemistry*. **52**: 4002-4007.

Gu, J., and Bourne, P. E. (2009). Structural bioinformatics, Vol 44. John Wiley & Sons.

Guarner, F., Bourdet-Sicard, R., Brandtzaeg, P., Gill, H.S., McGuirk, P., van Eden, W., Versalovic, J., Weinstock, J.V., Rook, G.A.W. 2006. Mechanisms of disease: The hygiene hypothesis revisited. *Nature Clinical Practice Gastroenterology and Hepatology*, **3**(5), 275-284.

Hansen, K. S., Ballmer-Weber, B. K., Lüttkopf, D., Skov, P. S., Wüthrich, B., Bindslev-Jensen, C., Vieths, S., and Poulsen, L. K. (2003). Roasted hazelnuts - Allergenic activity evaluated by double-blind, placebo-controlled food challenge. *Allergy: European Journal of Allergy and Clinical Immunology*. **58**: 132-138.

Hansen, K.S., Khinchi, M.S., Skov, P.S., Bindslev-Jensen, C., Poulsen, L.K., Malling, H.J. 2004. Food allergy to apple and specific immunotherapy with birch pollen. *Molecular Nutrition and Food Research*, **48**(6), 441-448.

Hansen, T. K., Stahl Skov, P., Poulsen, L. K., and Bindslev-Jensen, C. (1994). Allergenic activity of processed fish. *Allergy Clin Immunol News*. **2**.

Haris, P.I., Severcan, F. 1999. FTIR spectroscopic characterization of protein structure in aqueous and non-aqueous media. *Journal of Molecular Catalysis - B Enzymatic*, **7**(1-4), 207-221.

Helm, R.M., Burks, A.W. 2000. Mechanisms of food allergy. *Current Opinion in Immunology*, **12**(6), 647-653.

HERIAN, A. M., TAYLOR, S. L., and BUSH, R. K. (1993). Allergenic reactivity of various soybean products as determined by RAST inhibition. *Journal of Food Science*. **58**: 385-388.

Herman, E. M., Helm, R. M., Jung, R., and Kinney, A. J. (2003). Genetic modification removes an immunodominant allergen from soybean. *Plant Physiology*. **132**: 36-43.

Herrmann, W. O. R. (2011). Vitamins in the prevention of human diseases. De Gruyter, Berlin; New York.

Hess, B., Kutzner, C., Van Der Spoel, D., Lindahl, E. 2008. GROMACS 4: Algorithms for highly efficient, load-balanced, and scalable molecular simulation. *Journal of chemical theory and computation*, **4**(3), 435-447.

Hettiarachchy, N. S. S. K. M. M. R. K. A. (2012). Food proteins and peptides chemistry, functionality, interactions, and commercialization. Taylor & Francis, Boca Raton, FL.

Hill, D.J., Firer, M.A., Shelton, M.J., Hosking, C.S. 1986. Manifestations of milk allergy in infancy: Clinical and immunologic findings. *Journal of Pediatrics*, **109**(2), 270-276.

Hill, D.J., Hosking, C.S. 2000. Infantile colic and food hypersensitivity. *Journal of Pediatric Gastroenterology and Nutrition*, **30**(SUPPL. 1), S67-S76.

Hite, B. H. (1899). The effect of pressure in the preservation of milk. *Bull. West Virginia Univ. Agric. Exp. Stn.* **58**: 15-35.

Hite, B. H., Giddings, N. J., and Weakly, C. E. (1914). The effect of pressure on certain microorganisms encountered in the preservation of fruits and vegetables. *Bull. West Virginia Univ. Agric. Exp. Stn.* **146**: 1-67.

Hoffman, D. R. (1983). Immunochemical identification of the allergens in egg white. *Journal of Allergy and Clinical Immunology*. **71**: 481-486.

Holt, S.H.A., Brand Miller, J.C., Petocz, P., Farmakalidis, E. 1995. A satiety index of common foods. *European Journal of Clinical Nutrition*, **49**(9), 675-690.

Hourihane, J.O.B. 1997. Peanut allergy — current status and future challenges. *Clinical & Experimental Allergy*, **27**(11), 1240-1246.

Houska, M., Heroldova, M., Vavrova, H., Kucera, P., Setinova, I., Havranova, M., Honzova, S., Strohalm, J., Kminkova, M., Proskova, A., and Novotna, P. (2009). Is high-pressure treatment able to modify the allergenicity of the main apple juice allergen, Mal d1? *High Pressure Research*. **29**: 14-22.

Hsu, H., Vavak, D., Satterlee, L., Miller, G. 1977. A multienzyme technique for estimating protein digestibility. *Journal of Food Science*, **42**(5), 1269-1273.

Hsu, J., Heatherbell, D., Flores, J., and Watson, B. (1987). Heat-unstable proteins in grape juice and wine. II. Characterization and removal by ultrafiltration. *American Journal of Enology and Viticulture*. **38**: 17-22.

Humphrey, W., Dalke, A., Schulten, K. 1996. VMD: visual molecular dynamics. *Journal of molecular graphics*, **14**(1), 33-38.

Hunt, J., and Stubbs, D. (1975). The volume and energy content of meals as determinants of gastric emptying. *The Journal of physiology*. **245**: 209-225.

Iikura, Y., Imai, Y., Imai, T., Akasawa, A., Fujita, K., Hoshiyama, K., Nakura, H., Kohno, Y., Koike, K., Okudaira, H., Iwasaki, E. 1999. Frequency of Immediate–Type Food Allergy in Children in Japan. *International Archives of Allergy and Immunology*, **118**(2-4), 251-252.

Jackson, M., Mantsch, H.H. 1995. The use and misuse of FTIR spectroscopy in the determination of protein structure. *Critical Reviews in Biochemistry and Molecular Biology*, **30**(2), 95-120.

Jankiewicz, A., Aulepp, H., Baltes, W., Bögl, K. W., Dehne, L. I., Zuberbier, T., and Vieths, S. (1996). Allergic Sensitization to Native and Heated Celery Root in Pollen-Sensitive Patients Investigated by Skin Test and IgE Binding. *International Archives of Allergy and Immunology*. **111**: 268-278.

Jankiewicz, A., Baltes, W., Bögl, K. W., Dehne, L. I., Jamin, A., Hoffmann, A., Haustein, D., and Vieths, S. (1997). Influence of food processing on the immunochemical stability of celery allergens. *Journal of the Science of Food and Agriculture*. **75**: 359-370.

Jasim, A., Hosahalli, S. R., Stefan, K., and Joyce, I. B. (2009). From Odors to Behaviors in Caenorhabditis elegans. **In:** Novel Food Processing, pp. 1-6. CRC Press.

Jedrychowski, L. (1999). Reduction of the Antigenicity of Whey Proteins by Lactic Acid Fermentation. *Food and Agricultural Immunology*. **11**: 91-99.

Jiraratananon, R., and Chanachai, A. (1996). A study of fouling in the ultrafiltration of passion fruit juice. *Journal of Membrane Science*. **111**: 39-48.

Jiraratananon, R., Uttapap, D., and Tangamornsuksun, C. (1997). Self-forming dynamic membrane for ultrafiltration of pineapple juice. *Journal of Membrane Science*. **129**: 135-143.

Kalkan, F., Singh, A., Vanga, S.K.K., Raghavan, V., Kara, M. 2014. Effects of Tempering Moisture and Roll Differential on Wheat Size Reduction Process during the First Break Rollers. *Transactions of the ASABE (American Society of Agricultural and Biological Engineers)*, **57**(5), 1425-1429.

Karel, M. (1985). Effects of Water Activity and Water Content on Mobility of Food Components, and their Effects on Phase Transitions in Food Systems. **In:** Properties of Water in Foods, pp. 153-169. Simatos, D., and Multon, J. L. (Eds.), Springer Netherlands.

Kato, T., Katayama, E., Matsubara, S., Omi, Y., and Matsuda, T. (2000). Release of Allergenic Proteins from Rice Grains Induced by High Hydrostatic Pressure. *Journal of Agricultural and Food Chemistry*. **48**: 3124-3129.

Katz, E. E., and Labuza, T. P. (1981). Effect of Water Activity on the Sensory Crispness and Mechanical Deformation of Snack Food Products. *Journal of Food Science*. **46**: 403-409.

Kim, S.-M., Park, J.-G., Kim, K., Lee, J., Byun, M., Park, S., and Ahn, D. (2006). Study on the Changes in Allergen Allergenicity Originated from Shrimp by Physical Treatments. *Journal of the Korean society of Food Science and Nutrition*. **35**.

Kirk, D., Montgomery, M., and Kortekaas, M. (1983). Clarification of pear juice by hollow fiber ultrafiltration. *Journal of Food Science*. **48**: 1663-1667.

Kirkmeyer, S.V., Mattes, R.D. 2000. Effects of food attributes on hunger and food intake. *International Journal of Obesity*, **24**(9), 1167-1175.

Kleber, N., Maier, S., and Hinrichs, J. (2007). Antigenic response of bovine β-lactoglobulin influenced by ultra-high pressure treatment and temperature. *Innovative Food Science and Emerging Technologies*. **8**: 39-45.

Kleine-Tebbe, J., Wangorsch, A., Vogel, L., Crowell, D. N., Haustein, U. F., and Vieths, S. (2002). Severe oral allergy syndrome and anaphylactic reactions caused by a Bet v 1-related PR-10 protein in soybean, SAM22. *Journal of Allergy and Clinical Immunology*. **110**: 797-804.

Klotz, I. M., Langebman, N., and Dahnall, D. (1970). Quaternary structure of proteins. *Annual review of biochemistry*. **39**: 25-62.

Knorr, D. (1993). Effects of high-hydrostatic-pressure processes on food safety and quality. *Food Technology*. **47**: 156-161.

Kong, J., Yu, S. 2007. Fourier transform infrared spectroscopic analysis of protein secondary structures. *Acta Biochimica et Biophysica Sinica*, **39**(8), 549-559.

Koppelman, S.J., Bruijnzeel-Koomen, C.A.F.M., Hessing, M., De Jongh, H.H.J. 1999. Heat-induced conformational changes of Ara h 1, a major peanut allergen, do not affect its allergenic properties. *Journal of Biological Chemistry*, **274**(8), 4770-4777.

Koppelman, S.J., De Jong, G.A.H., Laaper-Ertmann, M., Peeters, K.A.B.M., Knulst, A.C., Hefle, S.L., Knol, E.F. 2005. Purification and immunoglobulin E-binding properties of peanut

allergen Ara h 6: Evidence for cross-reactivity with Ara h 2. *Clinical and Experimental Allergy*, **35**(4), 490-497.

Koppelman, S.J., Hefle, S.L., Taylor, S.L., De Jong, G.A.H. 2010. Digestion of peanut allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6: A comparative in vitro study and partial characterization of digestion-resistant peptides. *Molecular Nutrition and Food Research*, **54**(12), 1711-1721.

Koppelman, S.J., Knol, E.F., Vlooswijk, R.A.A., Wensing, M., Knulst, A.C., Hefle, S.L., Gruppen, H., Piersma, S. 2003. Peanut allergen Ara h 3: Isolation from peanuts and biochemical characterization. *Allergy: European Journal of Allergy and Clinical Immunology*, **58**(11), 1144-1151.

Koshland Jr, D. (1958). Application of a theory of enzyme specificity to protein synthesis. *Proceedings of the National Academy of Sciences of the United States of America*. **44**: 98.

Koutchma, T. N. F. L. J. M. C. I. (2009). Ultraviolet light in food technology principles and applications. CRC Press, Boca Raton.

Kreis, M., Forde, B.G., Rahman, S., Miflin, B.J., Shewry, P.R. 1985. Molecular evolution of the seed storage proteins of barley, rye and wheat. *Journal of Molecular Biology*, **183**(3), 499-502.

Krimm, S., Bandekar, J. 1986. Vibrational spectroscopy and conformation of peptides, polypeptides, and proteins. *Advances in Protein Chemistry*, **38**, 181-364.

Kuehler, C., and Stine, C. (1974). Effect of enzymatic hydrolysis on some functional properties of whey protein. *Journal of Food Science*. **39**: 379-382.

Kume, T., and Matsuda, T. (1995). Changes in structural and antigenic properties of proteins by radiation. *Radiation Physics and Chemistry*. **46**: 225-231.

Lado, B. H., and Yousef, A. E. (2002). Alternative food-preservation technologies: efficacy and mechanisms. *Microbes and Infection*. **4**: 433-440.

Lagunas-Solar, M. C. (1995). Radiation processing of foods: An overview of scientific principles and current status. *Journal of Food Protection®*. **58**: 186-192.

Ledikwe, J. H., Blanck, H. M., Khan, L. K., Serdula, M. K., Seymour, J. D., Tohill, B. C., and Rolls, B. J. (2006). Dietary energy density is associated with energy intake and weight status in US adults. *The American journal of clinical nutrition*. **83**: 1362-1368.

Leduc, V., Moneret-Vautrin, D. A., Guerin, L., Morisset, M., and Kanny, G. (2003). Anaphylaxis to wheat isolates: Immunochemical study of a case proved by means of double-blind, placebo-controlled food challenge [1]. *Journal of Allergy and Clinical Immunology*. **111**: 897-899.

Lee, D., and Putnam, G. (1973). The response of rainbow trout to varying protein/energy ratios in a test diet. *The Journal of Nutrition*. **103**: 916-922.

Lee, D.C., Haris, P.I., Chapman, D., Mitchell, R.C. 1990. Determination of protein secondary structure using factor analysis of infrared spectra. *Biochemistry®*, **29**(39), 9185-9193.

Lee, J. W., Seo, J. H., Kim, J. H., Lee, S. Y., Kim, K. S., and Byun, M. W. (2005). Changes of the antigenic and allergenic properties of a hen's egg albumin in a cake with gamma-irradiated egg white. *Radiation Physics and Chemistry*. **72**: 645-650.

Lee, J.-W., Kim, J.-H., Yook, H.-S., Kang, K.-O., Lee, S.-Y., Hwang, H.-J., and Byun, M.-W. (2001). Effects of Gamma Radiation on the Allergenic and Antigenic Properties of Milk Proteins. *Journal of Food Protection*. **64**: 272-276.

Lehmann, K., Schweimer, K., Reese, G., Randow, S., Suhr, M., Becker, W.M., Vieths, S., Rosch, P. 2006a. Structure and stability of 2S albumin-type peanut allergens: implications for the severity of peanut allergic reactions. *Biochem J*, **395**(3), 463-72.

Lehmann, K., Schweimer, K., Reese, G., Randow, S., Suhr, M., Becker, W.M., Vieths, S., Rösch, P. 2006b. Structure and stability of 2S albumin-type peanut allergens: Implications for the severity of peanut allergic reactions. *Biochemical Journal*, **395**(3), 463-472.

Lehrer, S. B., Ayuso, R., and Reese, G. (2002). Current understanding of food allergens. *Annals of the New York Academy of Sciences*. **964**: 69-85.

Lemieux, R.U., Spohr, U. 1994. How Emil Fischer was led to the lock and key concept for enzyme specificity. *Advances in carbohydrate chemistry and biochemistry*, **50**, 1-20.

Lew, A., Krutzik, P. O., Hart, M. E., and Chamberlin, A. R. (2002). Increasing rates of reaction: microwave-assisted organic synthesis for combinatorial chemistry. *Journal of combinatorial chemistry*. **4**: 95-105.

Li, H., Zhu, K., Zhou, H., and Peng, W. (2012). Effects of high hydrostatic pressure treatment on allergenicity and structural properties of soybean protein isolate for infant formula. *Food Chemistry*. **132**: 808-814.

Li, Y., Yang, W., Chung, S.-Y., Chen, H., Ye, M., Teixeira, A. A., Gregory, J. F., Welt, B. A., and Shriver, S. (2013). Effect of pulsed ultraviolet light and high hydrostatic pressure on the antigenicity of almond protein extracts. *Food and Bioprocess Technology*. **6**: 431-440.

Li, Z. X., Lin, H., Cao, L. M., and Jameel, K. (2006). Effect of high intensity ultrasound on the allergenicity of shrimp. *Journal of Zhejiang University. Science. B.* **7**: 251-256.

Liu, B.-L., and Tzeng, Y.-M. (1999). Water content and water activity for the production of cyclodepsipeptides in solid-state fermentation by Metarhizium anisopliae. *Biotechnology letters*. **21**: 657-661.

Liu, G. M., Cheng, H., Nesbit, J. B., Su, W. J., Cao, M. J., and Maleki, S. J. (2010). Effects of Boiling on the IgE-Binding Properties of Tropomyosin of Shrimp (Litopenaeus vannamei). *Journal of Food Science*. **75**: T1-T5.

Liu, Y., Zhao, G., Zhao, M., Ren, J., Yang, B. 2012. Improvement of functional properties of peanut protein isolate by conjugation with dextran through Maillard reaction. *Food Chemistry*, **131**(3), 901-906.

Lobanov, M.Y., Bogatyreva, N., Galzitskaya, O. 2008. Radius of gyration as an indicator of protein structure compactness. *Molecular Biology*, **42**(4), 623-628.

Lopata, A., and Potter, P. (2000). Allergy and other adverse reactions to seafood. *Allergy Clin Immunol Int*. **12**: 271-281.

López-Expósito, I., Chicón, R., Belloque, J., López-Fandiño, R., and Berin, M. C. (2012). In vivo methods for testing allergenicity show that high hydrostatic pressure hydrolysates of β-lactoglobulin are immunologically inert. *Journal of Dairy Science*. **95**: 541-548.

Macfarlane, G. T., and Cummings, J. H. (1999). Probiotics and prebiotics: Can regulating the activities of intestinal bacteria benefit health? *British Medical Journal*. **318**: 999-1003.

Malanin, K., Lundberg, M., and Johansson, S. G. O. (1995). Anaphylactic reaction caused by neoallergens in heated pecan nut. *Allergy: European Journal of Allergy and Clinical Immunology*. **50**: 988-991.

Maleki, S.J., Chung, S.Y., Champagne, E.T., Raufman, J.P. 2000a. The effects of roasting on the allergenic properties of peanut proteins. *Journal of Allergy and Clinical Immunology*, **106**(4), 763-768.

Maleki, S.J., Hurlburt, B.K. 2004. Structural and functional alterations in major peanut allergens caused by thermal processing. *Journal of AOAC International*, **87**(6), 1475-1479.

Maleki, S.J., Kopper, R.A., Shin, D.S., Park, C.W., Compadre, C.M., Sampson, H., Burks, A.W., Bannon, G.A. 2000b. Structure of the major peanut allergen Ara h 1 may protect IgE-binding epitopes from degradation. *Journal of Immunology*, **164**(11), 5844-5849.

Mangavel, C., Barbot, J., Popineau, Y., Guéguen, J. 2001. Evolution of wheat gliadins conformation during film formation: A Fourier transform infrared study. *Journal of Agricultural and Food Chemistry*, **49**(2), 867-872.

Manzocco, L., Panozzo, A., and Nicoli, M. C. (2012). Effect of ultraviolet processing on selected properties of egg white. *Food Chemistry*. **135**: 522-527.

Marzban, G., Herndl, A., Pietrozotto, S., Banerjee, S., Obinger, C., Maghuly, F., Hahn, R., Boscia, D., Katinger, H., and Laimer, M. (2009). Conformational changes of Mal d 2, a thaumatin-like apple allergen, induced by food processing. *Food Chemistry*. **112**: 803-811.

Matsuzaki, T., and Chin, J. (2000). Modulating immune responses with probiotic bacteria. *Immunology and Cell Biology*. **78**: 67-73.

McFeeters, R. (1988). Effects of Fermentation on the Nutritional Properties of Food. **In:** Nutritional Evaluation of Food Processing, pp. 423-446. Karmas, E., and Harris, R. (Eds.), Springer Netherlands.

Meda, V., Orsat, V., Raghavan, V., Schubert, H., and Regier, M. (2005). Microwave heating and the dielectric properties of foods. *The microwave processing of foods*: 61-75.

Menéndez, M., Gasset, M., Laynez, J., López-Zumel, C., Usobiaga, P., Töpper-Petersen, E., Calvete, J.J. 1995. Analysis of the Structural Organization and Thermal Stability of two Spermadhesins. *European Journal of Biochemistry*, **234**(3), 887-896.

Metchnikoff, E. (1907). The prolongation of life. Optimistic studies. *The Prolongation of Life: Optimistic Studies*.

Mittag, D., Vieths, S., Vogel, L., Becker, W. M., Rihs, H. P., Helbling, A., Wüthrich, B., and Ballmer-Weber, B. K. (2004). Soybean allergy in patients allergic to birch pollen: Clinical investigation and molecular characterization of allergens. *Journal of Allergy and Clinical Immunology*. **113**: 148-154.

Mondoulet, L., Paty, E., Drumare, M.F., Ah-Leung, S., Scheinmann, P., Willemot, R.M., Wal, J.M., Bernard, H. 2005. Influence of thermal processing on the allergenicity of peanut proteins. *Journal of Agricultural and Food Chemistry*, **53**(11), 4547-4553.

Morild, E. (1981). The Theory of Pressure Effects on Enzymes. *Advances in protein chemistry*. **34**: 93-166.

Mottram, D. S., Wedzicha, B. L., and Dodson, A. T. (2002). Food chemistry: acrylamide is formed in the Maillard reaction. *Nature*. **419**: 448-449.

Mozhaev, V.V., Heremans, K., Frank, J., Masson, P., Balny, C. 1996. High pressure effects on protein structure and function. *Proteins: Structure, Function and Genetics*, **24**(1), 81-91.

Mudgett, R. (1989). Microwave food processing. *Food technology (USA)*.

Nagpal, S., Rajappa, L., Metcalfe, D. D., and Subba Rao, P. V. (1989). Isolation and characterization of heat-stable allergens from shrimp (Penaeus indicus). *Journal of Allergy and Clinical Immunology*. **83**: 26-36.

Nakai, S. 1983. Structure-function relationships of food proteins with an emphasis on the importance of protein hydrophobicity. *Journal of Agricultural and Food Chemistry*, **31**(4), 676-683.

Nanda, R., James, R., Smith, H., Dudley, C., and Jewell, D. (1989). Food intolerance and the irritable bowel syndrome. *Gut*. **30**: 1099-1104.

Naqpal, S., Rajappa, L., Metcalfe, D. D., and Rao, P. V. (1989). Isolation and characterization of heat-stable allergens from shrimp (Penaeus indicus). *J. Allergy Clin. Immunol*. **83**: 26-36.

Ogawa, A., Samoto, M., and Takahashi, K. (2000). Soybean allergens and hypoallergenic soybean products. *Journal of nutritional science and vitaminology*. **46**: 271-279.

Oliveira, M. E. C., and Franca, A. S. (2002). Microwave heating of foodstuffs. *Journal of Food Engineering*. **53**: 347-359.

Olofsson, M., Buhler, M., and Wood, R. (1981). Process for the preparation of a purified protein hydrolysate. Google Patents.

Opitz, C. A., Kulke, M., Leake, M. C., Neagoe, C., Hinssen, H., Hajjar, R. J., and Linke, W. A. (2003). Damped elastic recoil of the titin spring in myofibrils of human myocardium. *Proceedings of the National Academy of Sciences*. **100**: 12688-12693.

Oriol, E., Raimbault, M., Roussos, S., and Viniegra-Gonzales, G. (1988). Water and water activity in the solid state fermentation of cassava starch by Aspergillus niger. *Applied Microbiology and Biotechnology*. **27**: 498-503.

Ortolani, C., Pastorello, E.A. 2006. Food allergies and food intolerances. *Best Practice and Research: Clinical Gastroenterology*, **20**(3), 467-483.

Ortolani, C., Pastorello, E.A., Farioli, L., Ispano, M., Pravettoni, V., Berti, C., Incorvaia, C., Zanussi, C. 1993. IgE-mediated allergy from vegetable allergens. *Annals of Allergy*, **71**(5), 470-476.

Panyam, D., and Kilara, A. (1996). Enhancing the functionality of food proteins by enzymatic modification. *Trends in food science & technology*. **7**: 120-125.

Peeters, K.A.B.M., Koppelman, S.J., Van Hoffen, E., Van Der Tas, C.W.H., Den Hartog Jager, C.F., Penninks, A.H., Hefle, S.L., Bruijnzeel-Koomen, C.A.F.M., Knol, E.F., Knulst, A.C. 2007. Does skin prick test reactivity to purified allergens correlate with clinical severity of peanut allergy? *Clinical and Experimental Allergy*, **37**(1), 108-115.

Peñas, E., Gomez, R., Frias, J., Baeza, M. L., and Vidal-Valverde, C. (2011). High hydrostatic pressure effects on immunoreactivity and nutritional quality of soybean products. *Food Chemistry*. **125**: 423-429.

Peñas, E., Préstamo, G., Luisa Baeza, M., Martínez-Molero, M. I., and Gomez, R. (2006a). Effects of combined high pressure and enzymatic treatments on the hydrolysis and immunoreactivity of dairy whey proteins. *International Dairy Journal*. **16**: 831-839.

Peñas, E., Snel, H., Floris, R., Préstamo, G., and Gomez, R. (2006b). High pressure can reduce the antigenicity of bovine whey protein hydrolysates. *International Dairy Journal*. **16**: 969-975.

Pereira, R. N., and Vicente, A. A. (2010). Environmental impact of novel thermal and non-thermal technologies in food processing. *Food Research International*. **43**: 1936-1943.

Pescuma, M., Hébert, E. M., Dalgalarrondo, M., Haertlé, T., Mozzi, F., Chobert, J. M., and De Valdez, G. F. (2009). Effect of exopolysaccharides on the hydrolysis of ß-lactoglobulin by lactobacillus acidophilus CRL 636 in an in vitro gastric/pancreatic system. *Journal of Agricultural and Food Chemistry*. **57**: 5571-5577.

Pescuma, M., Hébert, E. M., Rabesona, H., Drouet, M., Choiset, Y., Haertlé, T., Mozzi, F., De Valdez, G. F., and Chobert, J. M. (2011). Proteolytic action of Lactobacillus delbrueckii subsp. bulgaricus CRL 656 reduces antigenic response to bovine β-lactoglobulin. *Food Chemistry*. **127**: 487-492.

Phillips, G. O. W. P. A. (2011). Handbook of food proteins. Woodhead Publishing, Cambridge, UK; Philadelphia.

Pomeranz, Y. (1985). Functional properties of food components. Academic Press, Orlando, Fla.

Ponne, C. T., and Bartels, P. V. (1995). Interaction of electromagnetic energy with biological material — relation to food processing. *Radiation Physics and Chemistry*. **45**: 591-607.

Popper, L., and Knorr, D. (1990). Applications of high-pressure homogenization for food preservation. *Food Technol.* **44**: 84-89.

Privalov, P., Tiktopulo, E., Venyaminov, S. Y., Griko, Y. V., Makhatadze, G., and Khechinashvili, N. (1989). Heat capacity and conformation of proteins in the denatured state. *Journal of Molecular Biology*. **205**: 737-750.

Putnam, J.J., Allshouse, J.E. 1994. *Food Consumption, Prices, and Expenditures, 1970-93*. US Department of Agriculture, Economic Research Service.

Qi, M., Hettiarachchy, N., and Kalapathy, U. (1997). Solubility and emulsifying properties of soy protein isolates modified by pancreatin. *Journal of Food Science*. **62**: 1110-1115.

Rahman, S. (1995). Food properties handbook. CRC Press, Boca Raton, FL.

Rancé, F., Kanny, G., Dutau, G., Moneret-Vautrin, D.A. 1999. Food hypersensitivity in children: Clinical aspects and distribution of allergens. *Pediatric Allergy and Immunology*, **10**(1), 33-38.

Reese, G., Ayuso, R., Carle, T., and Lehrer, S. B. (1999). IgE-binding epitopes of shrimp tropomyosin, the major allergen Pen a 1. *International Archives of Allergy and Immunology*. **118**: 300-301.

Richardson, P. (2001). Thermal technologies in food processing. CRC Press ; Woodhead Pub., Boca Raton, Fla.; Cambridge, England.

Rivalain, N., Roquain, J., and Demazeau, G. (2010). Development of high hydrostatic pressure in biosciences: Pressure effect on biological structures and potential applications in Biotechnologies. *Biotechnology Advances*. **28**: 659-672.

Rona, R.J., Keil, T., Summers, C., Gislason, D., Zuidmeer, L., Sodergren, E., Sigurdardottir, S.T., Lindner, T., Goldhahn, K., Dahlstrom, J., McBride, D., Madsen, C. 2007. The prevalence of food allergy: A meta-analysis. *Journal of Allergy and Clinical Immunology*, **120**(3), 638-646.

Rose, G., Geselowitz, A., Lesser, G., Lee, R., Zehfus, M. 1985. Hydrophobicity of amino acid residues in globular proteins. *Science*, **229**(4716), 834-838.

Roudaut, G., Dacremont, C., and Meste, M. L. (1998). INFLUENCE OF WATER ON THE CRISPNESS OF CEREAL-BASED FOODS: ACOUSTIC, MECHANICAL, AND SENSORY STUDIES. *Journal of Texture Studies*. **29**: 199-213.

Sablani, S., Marcotte, M., Baik, O., and Castaigne, F. (1998). Modeling of simultaneous heat and water transport in the baking process. *LWT-Food Science and Technology*. **31**: 201-209.

Sagum, R., Arcot, J. 2000. Effect of domestic processing methods on the starch, non-starch polysaccharides and in vitro starch and protein digestibility of three varieties of rice with varying levels of amylose. *Food Chemistry*, **70**(1), 107-111.

Salminen, S., Ouwehand, A. C., and Isolauri, E. (1998). Clinical applications of probiotic bacteria. *International Dairy Journal*. **8**: 563-572.

Sampson, H.A. 1994. Food allergies. *Principles and Practice of Pediatrics*, 227-232.

Sampson, H.A. 2004. Update on food allergy. *Journal of Allergy and Clinical Immunology*, **113**(5), 805-819.

Sampson, H.A., McCaskill, C.C. 1985. Food hypersensitivity and atopic dermatitis: Evaluation of 113 patients. *Journal of Pediatrics*, **107**(5), 669-675.

Sanger, F. (1952). The arrangement of amino acids in proteins. *Advances in protein chemistry*. **7**: 1.

Sathe, S.K., Sharma, G.M. 2009. Effects of food processing on food allergens. *Molecular nutrition & food research*, **53**(8), 970-978.

Sathe, S.K., Teuber, S.S., Roux, K.H. 2005. Effects of food processing on the stability of food allergens. *Biotechnology Advances*, **23**(6), 423-429.

Scheurer, S., Son, D.Y., Boehm, M., Karamloo, F., Franke, S., Hoffmann, A., Haustein, D., Vieths, S. 1999. Cross-reactivity and epitope analysis of Pru a 1, the major cherry allergen. *Molecular Immunology*, **36**(3), 155-167.

Schloss, O.M. 1920. Allergy in infants and children. *Am J Dis Child*, **19**, 433-437.

Schmitt, D.A., Cheng, H., Maleki, S.J., Burks, A.W. 2004. Competitive inhibition ELISA for quantification of Ara h 1 and Ara h 2, the major allergens of peanuts. *Journal of AOAC International*, **87**(6), 1492-1497.

Schubert, H. R. M. (2005). The microwave processing of foods. Woodhead, Cambridge.

Schweimer, K., Sticht, H., Nerkamp, J., Boehm, M., Breitenbach, M., Vieths, S., Rösch, P. 1999. NMR spectroscopy reveals common structural features of the birch pollen allergen Bet v 1 and the cherry allergen Pru a 1. *Applied Magnetic Resonance*, **17**(2-3), 449-464.

Scott, W. (1957). Water relations of food spoilage microorganisms. *Adv. Food Res*. **7**: 168.

Sen, M., Kopper, R., Pons, L., Abraham, E. C., Burks, A. W., and Bannon, G. A. (2002). Protein structure plays a critical role in peanut allergen stability and may determine immunodominant IgE-binding epitopes. *Journal of Immunology*. **169**: 882-887.

Seo, J.-H., Kim, J.-H., Lee, J.-W., Yoo, Y.-C., Kim, M. R., Park, K.-S., and Byun, M.-W. (2007). Ovalbumin modified by gamma irradiation alters its immunological functions and allergic responses. *International Immunopharmacology*. **7**: 464-472.

Seo, J.-H., Lee, J.-W., Lee, Y.-S., Lee, S.-Y., Kim, M.-R., Yook, H.-S., and Byun, M.-W. (2004). Change of an egg allergen in a white layer cake containing gamma-irradiated egg white. *Journal of Food Protection*®. **67**: 1725-1730.

Shewry, P.R., Beaudoin, F., Jenkins, J., Griffiths-Jones, S., Mills, E.N.C. 2002. Plant protein families and their relationships to food allergy. *Biochemical Society Transactions*, **30**(6), 906-910.

Shreffler, W.G., Castro, R.R., Kucuk, Z.Y., Charlop-Powers, Z., Grishina, G., Yoo, S., Burks, A.W., Sampson, H.A. 2006. The major glycoprotein allergen from Arachis hypogaea, Ara h 1, is a ligand of dendritic cell-specific ICAM-grabbing nonintegrin and acts as a Th2 adjuvant in vitro. *Journal of Immunology*, **177**(6), 3677-3685.

Shriver, S. K., and Yang, W. W. (2011). Thermal and nonthermal methods for food allergen control. *Food Engineering Reviews*. **3**: 26-43.

Shriver, S., Yang, W., Chung, S.-Y., and Percival, S. (2011). Pulsed ultraviolet light reduces immunoglobulin E binding to Atlantic white shrimp (Litopenaeus setiferus) extract. *International journal of environmental research and public health*. **8**: 2569-2583.

Sicherer, S.H. 2002. Clinical update on peanut allergy. *Annals of Allergy, Asthma and Immunology*, **88**(4), 350-361.

Sicherer, S.H., Muñoz-Furlong, A., Godbold, J.H., Sampson, H.A. 2010. US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. *Journal of Allergy and Clinical Immunology*, **125**(6), 1322-1326.

Sicherer, S.H., Sampson, H.A. 2006. 9. Food allergy. *Journal of Allergy and Clinical Immunology*, **117**(2, Supplement 2), S470-S475.

Sicherer, S.H., Sampson, H.A. 2007. Peanut allergy: Emerging concepts and approaches for an apparent epidemic. *Journal of Allergy and Clinical Immunology*, **120**(3), 491-503.

Sicherer, S.H., Sampson, H.A. 2010. Food allergy. *Journal of Allergy and Clinical Immunology*, **125**(2 SUPPL. 2), S116-S125.

Sikorski, Z. E. (2007). Chemical and functional properties of food components. CRC Press, Boca Raton, FL.

Silverstein, S.R., Frommer, D.A., Dobozin, B., Rosen, P. 1986. Celery-dependent exercise-induced anaphylaxis. *Journal of Emergency Medicine*, **4**(3), 195-199.

Sinanoglou, V. J., Batrinou, A., Konteles, S., and Sflomos, K. (2007). Microbial Population, Physicochemical Quality, and Allergenicity of Molluscs and Shrimp Treated with Cobalt-60 Gamma Radiation. *Journal of Food Protection*. **70**: 958-966.

Singh, A., Munshi, S., and Raghavan, V. (2013a). Effect of External Electric Field Stress on Gliadin Protein Conformation. *Proteomes*. **1**: 25-39.

Singh, A., Nair, G.R., Rahimi, J., Gariepy, Y., Raghavan, V. 2013. Effect of Static High Electric Field Pre-Treatment on Microwave-Assisted Drying of Potato Slices. *Drying Technology*, **31**(16), 1960-1968.

Singh, A., Orsat, V., and Raghavan, V. (2013b). Soybean Hydrophobic Protein Response to External Electric Field: A Molecular Modeling Approach. *Biomolecules*. **3**: 168-179.

Singh, A., Orsat, V., Raghavan, V. 2012. A Comprehensive Review on Electrohydrodynamic Drying and High-Voltage Electric Field in the Context of Food and Bioprocessing. *Drying Technology*, **30**(16), 1812-1820.

Singh, A., Orsat, V., Raghavan, V. 2013b. Soybean Hydrophobic Protein Response to External Electric Field: A Molecular Modeling Approach. *Biomolecules*, **3**(1), 168-179.

Sivasankar, B. (2002). Food processing and preservation. PHI Learning Pvt. Ltd.

Skripak, J.M., Matsui, E.C., Mudd, K., Wood, R.A. 2007. The natural history of IgE-mediated cow's milk allergy. *Journal of Allergy and Clinical Immunology*, **120**(5), 1172-1177.

Sloan, A.E., Powers, M.E. 1986. A perpective on popular perceptions of adverse reactions to foods. *Journal of Allergy and Clinical Immunology*, **78**(1 II), 127-133.

Smeller, L. (2002). Pressure-temperature phase diagrams of biomolecules. *Biochimica et Biophysica Acta - Protein Structure and Molecular Enzymology*. **1595**: 11-29.

Smith, D. M. (1994). Protein interactions in gels: protein-protein interactions. Marcel Dekker, New York.

Smith, J., and Charter, E. (2011). Functional food product development. John Wiley & Sons.

Soller, L., Ben-Shoshan, M., Harrington, D.W., Fragapane, J., Joseph, L., St Pierre, Y., Godefroy, S.B., La Vieille, S., Elliott, S.J., Clarke, A.E. 2012. Overall prevalence of self-reported food allergy in Canada. *Journal of Allergy and Clinical Immunology*, **130**(4), 986-988.

Sorgentini, D.A., Wagner, J.R., Añón, M.C. 1995a. Effects of thermal treatment of soy protein isolate on the characteristics and structure-function relationship of soluble and insoluble fractions. *Journal of Agricultural and Food Chemistry*, **43**(9), 2471-2479.

Sorgentini, D.A., Wagner, J.R., Añón, M.C. 1995b. Effects of thermal treatment of soy protein isolate on the characteristics and structure-function relationship of soluble and insoluble fractions. *Journal of Agricultural and Food Chemistry*, **43**(9), 2471-2479.

Soria, A. C., and Villamiel, M. (2010). Effect of ultrasound on the technological properties and bioactivity of food: A review. *Trends in Food Science and Technology*. **21**: 323-331.

Spies, J. R. (1974). Allergens. *Journal of Agricultural and Food Chemistry*. **22**: 30-36.

Stanic-Vucinic, D., Stojadinovic, M., Atanaskovic-Markovic, M., Ognjenovic, J., Grönlund, H., van Hage, M., Lantto, R., Sancho, A. I., and Velickovic, T. C. (2012). Structural changes and allergenic properties of β-lactoglobulin upon exposure to high-intensity ultrasound. *Molecular nutrition & food research*. **56**: 1894-1905.

Su, M., Venkatachalam, M., Teuber, S. S., Roux, K. H., and Sathe, S. K. (2004). Impact of γ-irradiation and thermal processing on the antigenicity of almond, cashew nut and walnut proteins. *Journal of the Science of Food and Agriculture*. **84**: 1119-1125.

Sun, D.-W. (2005). Emerging technologies for food processing. Elsevier Academic Press, San Diego, Calif.

Surewicz, W.K., Mantsch, H.H. 1988. New insight into protein secondary structure from resolution-enhanced infrared spectra. *Biochimica et Biophysica Acta (BBA)/Protein Structure and Molecular*, **952**(C), 115-130.

Susi, H., Byler, D.M. 1986. [13] Resolution-enhanced fourier transform infrared spectroscopy of enzymes. in: *Methods in Enzymology*, Vol. 130, pp. 290-311.

Tammineedi, C. V. R. K., Choudhary, R., Perez-Alvarado, G. C., and Watson, D. G. (2013). Determining the effect of UV-C, high intensity ultrasound and nonthermal atmospheric plasma treatments on reducing the allergenicity of α-casein and whey proteins. *LWT - Food Science and Technology*. **54**: 35-41.

Tanford, C. (1968). Protein denaturation. *Adv. Protein Chem*. **23**: 282.

Tatham, A.S., Shewry, P.R. 1985. The conformation of wheat gluten proteins. The secondary structures and thermal stabilities of α-, β-, γ- and ω-Gliadins. *Journal of Cereal Science*, **3**(2), 103-113.

Tewari, G. J. V. K. (2007). Advances in thermal and non-thermal food preservation. Blackwell Pub., Ames, Iowa.

Thomas, K., Herouet-Guicheney, C., Ladics, G., Bannon, G., Cockburn, A., Crevel, R., Fitzpatrick, J., Mills, C., Privalle, L., and Vieths, S. (2007). Evaluating the effect of food processing on the potential human allergenicity of novel proteins: International workshop report. *Food and Chemical Toxicology*. **45**: 1116-1122.

Thorvaldsson, K., and Skjöldebrand, C. (1998). Water diffusion in bread during baking. *LWT-Food Science and Technology*. **31**: 658-663.

Van Holde, K. (1977). Effects of amino acid composition and microenvironment on protein structure.

Venkatachalam, M., Monaghan, E. K., Kshirsagar, H. H., Robotham, J. M., O'Donnell, S. E., Gerber, M. S., Roux, K. H., and Sathe, S. K. (2008). Effects of Processing on Immunoreactivity of Cashew Nut (Anacardium occidentale L.) Seed Flour Proteins. *Journal of Agricultural and Food Chemistry*. **56**: 8998-9005.

Venkatachalam, M., Teuber, S. S., Roux, K. H., and Sathe, S. K. (2002). Effects of roasting, blanching, autoclaving, and microwave heating on antigenicity of almond (Prunus dulcis L.) proteins. *Journal of Agricultural and Food Chemistry*. **50**: 3544-3548.

Venkatesh, M., and Raghavan, G. (2004). An overview of microwave processing and dielectric properties of agri-food materials. *Biosystems Engineering*. **88**: 1-18.

Venter, C., Pereira, B., Voigt, K., Grundy, J., Clayton, C.B., Higgins, B., Arshad, S.H., Dean, T. 2008. Prevalence and cumulative incidence of food hypersensitivity in the first 3 years of life. *Allergy: European Journal of Allergy and Clinical Immunology*, **63**(3), 354-359.

Vieths, S., Zagon, J., Fischer, K., Dehne, L., and Bögl, K. (1995). Verarbeitungsbedingte Einfluesse auf das allergene Potential von Lebensmitteln. Die Sojaallergie als Modellbeispiel. *Lebensmittelchemie*. **49**.

Vignais, P., Barbu, E., Macheboeuf, M., and Basset, M. (1952). Antigenic and vaccinal power of bacteria killed by pressure. *Pouvoir antigène et vaccinant de bactéries tuées par pression.* **34**: 43-46.

Vladisavljević, G., Vukosavljević, P., and Bukvić, B. (2003). Permeate flux and fouling resistance in ultrafiltration of depectinized apple juice using ceramic membranes. *Journal of Food Engineering*. **60**: 241-247.

Watanabe, M., Miyakawa, J., Ikezawa, Z., Suzuki, Y., Hirao, T., Yoshizawa, T., and Arai, S. (1990). Production of Hypoallergenic Rice by Enzymatic Decomposition of Constituent Proteins. *Journal of Food Science*. **55**: 781-783.

Wellner, N., Mills, E.C., Brownsey, G., Wilson, R.H., Brown, N., Freeman, J., Halford, N.G., Shewry, P.R., Belton, P.S. 2005. Changes in protein secondary structure during gluten deformation studied by dynamic Fourier transform infrared spectroscopy. *Biomacromolecules*, **6**(1), 255-261.

Whisstock, J.C., Lesk, A.M. 2003. Prediction of protein function from protein sequence and structure. *Quarterly reviews of biophysics*, **36**(03), 307-340.

Whitford, D. (2005). Proteins : structure and function. J. Wiley & Sons, Hoboken, NJ.

Wigotzki, M., Steinhart, H., and Paschke, A. (2000). Influence of varieties, storage and heat treatment on IgE-binding proteins in hazelnuts (Corylus avellana). *Food and Agricultural Immunology*. **12**: 217-229.

Wilson, S., Blaschek, K., and de Mejia, E. G. (2005). Allergenic Proteins in Soybean: Processing and Reduction of P34 Allergenicity. *Nutrition Reviews*. **63**: 47-58.

Winter, R., Dzwolak, W., Wolynes, P. G., Dobson, C. M., and Saykally, R. J. (2005). Exploring the temperature-pressure configurational landscape of biomolecules: From lipid membranes to proteins. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences*. **363**: 537-563.

Wu, C. H., Nakai, S., and Powrie, W. D. (1976). Preparation and properties of acid-solubilized gluten. *Journal of Agricultural and Food Chemistry*. **24**: 504-510.

Wu, H. 1931. Studies on denaturation of proteins. XIII. A theory of denaturation. *Chin J Physiol*, **5**(4), 321-344.

Yang, W. W., Chung, S.-Y., Ajayi, O., Krishnamurthy, K., Konan, K., and Goodrich-Schneider, R. (2010). Use of pulsed ultraviolet light to reduce the allergenic potency of soybean extracts. *International Journal of Food Engineering*. **6**.

Yang, W., Mwakatage, N., Goodrich-Schneider, R., Krishnamurthy, K., and Rababah, T. (2012). Mitigation of Major Peanut Allergens by Pulsed Ultraviolet Light. *Food and Bioprocess Technology*. **5**: 2728-2738.

Yazdanbakhsh, M., Kremsner, P.G., van Ree, R. 2002. Allergy, parasites, and the hygiene hypothesis. *Science*, **296**(5567), 490-494.

Zaks, A., and Klibanov, A. M. (1988). The effect of water on enzyme action in organic media. *Journal of Biological Chemistry*. **263**: 8017-8021.

Zeece, M., Huppertz, T., and Kelly, A. (2008). Effect of high-pressure treatment on in-vitro digestibility of β-lactoglobulin. *Innovative Food Science and Emerging Technologies*. **9**: 62-69.

Zhang, J. (1995). NMR study of the cold, heat, and pressure unfolding of ribonuclease A. *Biochemistry®*. **34**: 8631-8641.

Zhenxing, L., Caolimin, L., and Jamil, K. (2006). Reduction of allergenic properties of shrimp (Penaeus Vannamei) allergens by high intensity ultrasound. *European Food Research and Technology*. **223**: 639-644.

Zhenxing, L., Hong, L., Limin, C., and Jamil, K. (2007a). Impact of irradiation and thermal processing on the immunoreactivity of shrimp (Penaeus vannamei) proteins. *Journal of the Science of Food and Agriculture*. **87**: 951-956.

Zhenxing, L., Hong, L., Limin, C., and Jamil, K. (2007b). The influence of gamma irradiation on the allergenicity of shrimp (Penaeus vannamei). *Journal of Food Engineering*. **79**: 945-949.

Zhong, J., Liu, C., Liu, W., Cai, X., Tu, Z., and Wan, J. (2011). Effect of dynamic high-pressure microfluidization at different temperatures on the antigenic response of bovine β-lactoglobulin. *European Food Research and Technology*. **233**: 95-102.

Zuercher, A.W., Holvoet, S., Weiss, M., Mercenier, A. 2010. Polyphenol-enriched apple extract attenuates food allergy in mice. *Clinical and Experimental Allergy*, **40**(6), 942-950.

Zuidmeer, L., Goldhahn, K., Rona, R.J., Gislason, D., Madsen, C., Summers, C., Sodergren, E., Dahlstrom, J., Lindner, T., Sigurdardottir, S.T., McBride, D., Keil, T. 2008. The prevalence of plant food allergies: A systematic review. *Journal of Allergy and Clinical Immunology*, **121**(5), 1210-1218.e4.