ABSTRACT

PLUNDRICH, NATHALIE JENNIFER. Novel Hypoallergenic Peanut Protein-Polyphenol Edible Matrices. (Under the direction of Dr. Mary Ann Lila).

Peanuts are widely used in a variety of foods and are a good source of protein, vitamins and minerals. However, they also are among the ‘big eight’ foods that can cause food allergy. Peanut allergy is an immunoglobulin (Ig) E-mediated hypersensitivity disorder towards certain proteins (allergens) in the edible seed. The immune system of a susceptible individual produces, upon initial exposure to peanut allergens, peanut-specific IgE that can cross-link with receptors on mast and basophil cell surfaces (sensitization). Upon re-exposure, certain parts of the allergenic proteins, known as epitopes (binding sites), can bind and cross-link with mast and basophil cell-bound peanut-specific IgE and trigger those cells to release inflammatory mediators responsible for the allergic reaction. Oral immunotherapy (OIT), has extensively been investigated as a means to induce allergen tolerance (desensitization) in food allergic individuals, and can be a successful treatment for individuals to prevent life-threatening consequences upon accidental allergen ingestion. The approach for peanut allergy involves the administration (over months) of increasing small amounts of light roasted 12% fat peanut (PN) flour. However, severe side-effects prevent some patients from participating in this type of therapy. Polyphenolic plant compounds are known for their ability to bind proteins, leading to the formation of soluble and insoluble (precipitated) protein-polyphenol complexes with distinct functionalities. The goal of this research was to create a polyphenol-fortified PN matrix (by complexation of plant polyphenols with PN flour), resulting in a powdered, dry, edible peanut ingredient with
reduced allergenicity, potentially suitable as a safer and possibly more effective OIT ingredient.

In the first study, we investigated the allergenicity of polyphenol-fortified PN matrices (prepared with cranberry, black currant, aronia, muscadine grape (Noble variety), and elderberry juices or cinnamon powder and green tea leaves extracts). PN matrices showed reduced IgE binding to one or more peanut allergens (Ara h 1, Ara h 2, Ara h 3, and Ara h 6) with proanthocyanidin-rich sources (cranberry, cinnamon, green tea, and aronia) generally providing the most efficient reduction in allergenicity. Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) suggested changes in secondary protein structure. Subsequent studies with cranberry-fortified PN matrix revealed that in particular, more highly polymerized procyanidins bound to PN proteins. Follow-up tests with a cranberry-fortified PN matrix demonstrated significantly less basophil degranulation than unmodified PN flour (control) in an ex vivo assay using human blood as well as less mast cell degranulation when used in a peanut oral challenge of peanut-allergic mice. These results suggest that cranberry polyphenol fortification of PN flour resulted in a hypoallergenic PN matrix potentially suitable for OIT.

The second study selected the most promising polyphenol-fortified PN matrices (derived from cranberry, cinnamon, and green tea) and suggested that interactions between polyphenols and proteins are non-covalent in nature (hydrophobic interactions and hydrogen bonding). Solubility behavior of PN protein-polyphenol complexes in simulated gastric fluid (pH 2) used to mimic acidic harsh conditions in vivo showed that, in particular, cranberry and green tea polyphenols rendered PN proteins significantly less soluble (more stable complexes) than compared to unmodified PN proteins (from PN flour). Subjecting whole
polyphenol-fortified PN matrices to a simulated gastric pepsin digestion assay suggested that digestion of PN-allergenic proteins complexed with bioactives from cranberry and green tea was modulated, and resulted in digestive fragments with reduced allergenic potential when screened for their peanut-specific IgE binding capacity.

Collectively, these findings suggest potential applications of hypoallergenic edible PN matrices as second generation peanut ingredients with reduced side effects, which may retain properties integral for modulating the allergic response of patients undergoing OIT.
Novel Hypoallergenic Peanut Protein-Polyphenol Edible Matrices

by
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APPROVED BY:

Dr. Jack P. Davis  Dr. E. Allen Foegeding

Dr. Mary Ann Lila
DEDICATION

I dedicate this work to my wonderful family, friends and professors who supported, encouraged and believed in me every moment of my life
BIOGRAPHY

Nathalie Plundrich was born on May 8\textsuperscript{th}, 1985 in Weingarten i.A., Germany. She is the daughter of Thomas and Gabriele Plundrich. She got her associate degree as a state-approved biological-technical assistant with a major in Biology and Biotechnology in 2007 from the Jörg-Zürn-Gewerbeschule in Überlingen am Bodensee. She continued her education at the Hochschule Furtwangen University, Germany, where she studied Bio-and Process Technologies with a major in Biotechnology. During her undergraduate program she accomplished an internship in Dr. Mary Ann Lila’s lab on the North Carolina Research Campus, Kannapolis, in 2010. She returned to North Carolina in 2011 to work on her Bachelor Thesis project in Dr. Lila’s lab and later that year she graduated with a Bachelor of Science degree. She presented her work at the 6\textsuperscript{th} annual International Workshop on Anthocyanins in 2011. She then moved to Raleigh, North Carolina and became a graduate student at North Carolina State University majoring in Food Science at the Department of Food, Bioprocessing and Nutritional Sciences under the advisory of Dr. Mary Ann Lila. She presented her graduate work at the Berry Health Benefits Symposium in Concord in 2013, at the NCSU Workshop on Allergy and Inflammation in Raleigh in 2013, and at the Experimental Biology meeting in San Diego in 2014.
ACKNOWLEDGMENTS

- Mom and dad for their unconditional love and support all my life, thanks (partially) to my family I have decided to continue on with my PhD.

- My little brother, because he wants to read all my papers, and my thesis (although he doesn’t understand any of it).

- My love, Joel, for his patience and love, being there with me in good and bad times.

- Dr. Mary Ann Lila, my advisor, I truly appreciate our professional relationship and friendship. She gave me unbelievable opportunities to grow and learn so much these past years. Special thanks for her patience while I was trying to write my very first publication. Thanks to her I am a much better writer now. I am grateful to call her my mentor.

- Dr. Jack Davis, for all the peanuts I could possibly eat and for his great support these past two years. Truly has been fun and I am happy to call him a friend.

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- Dr. Brittany White for giving me company in room 226. Thanks for her support and for sharing her knowledge with me to push my work forward.

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• Dr. Mary Grace who has treated me like a daughter and who made me feel at home when I came to the USA the first time in 2010.

• Kartheek Anekella, the very first person I met (online!) intending to participate in the Food Science program in fall 2012, like me. We made it through classes, assignments, posters, publications etc. together, and I will truly miss him when he has finished his PhD.

• Sally Mangum for being such a wonderful, great, loving and caring friend. I truly appreciate our friendship! On a bad day she is always able to cheer me up and her vibrant energy makes me a stronger person.

• Thanks to Josh Kellogg, Ivette Guzman, Isabelle Neutz, Sonja Raithel and Sandra Holzer who I all dearly love and many others for always supporting and believing in me.

Thanks to my unconditional curiosity in life and my passion for science, my driving forces
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**LIST OF TERMS**

**allergen** type of antigen that although harmless can trigger an adverse immune response

**allergic reaction** an allergen can cause an allergic reaction (inflammatory response which can range from itching to anaphylactic shock)

**anaphylactic shock or anaphylaxis** severe (often life-threatening) allergic reaction including multiple symptoms

**antacid** a substance that neutralizes stomach acidity

**atopy** a hereditary tendency to develop immediate allergic (hypersensitive) reactions

**basophil** a type of white blood cell that plays a role in mediating hypersensitivity reactions of the immune system

**blanching** a short time cooking process of usually fruits and vegetables (i.e. used to inactivate enzymes, or to remove outer the skin from nuts)

**chymotrypsin** pancreatic protease acting in the upper part of the small intestine

**cohort study** an observational study design during which one or more subjects (called cohorts) are followed for a period of time. Subsequent status evaluations aid in evaluating associations between disease and exposure (i.e. habit of smoking)

**conformation** structural arrangement or form (i.e. of molecule moieties)

**C-terminus** the end of an amino acid chain, terminated by an amino acid with a free carboxyl group (-COOH)

**cutaneous** skin-related

**cysteine** an amino acid

**cytokine** cytokines belong to a group of small proteins that play an important role in cell signaling (mediators)

**dispersion** particles are dispersed in a continuous phase of a different composition (i.e. solid in liquid phase - sugar in water)

**disulfide bond** covalent bond formed by two sulfur atoms derived from two sulphydryl or thiol groups (R-S-S-R)
to emulsify  

to make and emulsion (two otherwise non-mixable liquids can form emulsions through interphase stabilizers). For example milk is a natural emulsion of milk fat and water. Milk proteins act as stabilizers.

enzyme  

enzymes are proteins and catalysts of many biological reactions. Enzymes are usually substrate-specific yielding certain enzymatic products

epidemiology  

science that studies and monitors the patterns, causes, and effects of health and disease conditions in certain populations

epitope  

surface portion of an allergen capable of eliciting an immune response

ex vivo  

in science, experiments conducted with biological material but outside the organism without too much alteration of natural conditions (i.e. use of human blood, or animal tissue material)

fluorescence  

the property of a substance absorbing light (or other electromagnetic radiation) of short wavelength and the immediate emission of longer wavelength light/radiation

glycoprotein  

proteins that contain sugars covalently bound to polypeptide side-chains

homotrimer  

protein which is composed of three identical subunits

hydrogen bond  

is a type of attractive interaction between polar molecules (a hydrogen atom can bind to a highly electronegative atom like nitrogen or oxygen)

hydrolysis  

cleavage of a chemical bond in presence of water

hydrophobic interactions  

interactions between hydrophobic molecules/or moieties (i.e. in an aqueous system hydrophobic molecules will closely come together and separate from the hydrophilic water, which is called hydrophobic effect)

hypersensitivity  

undesirable reactions produced by the normal immune system, including allergies

immunoreactive  

in general: measure of immune response to an antigen; in context: intact and accessible IgE binding epitopes triggering allergic response

in utero  

in the womb, before birth
in vitro in science, experiments performed in test tubes, petri dishes, etc.

in vivo in science, experiments conducted in the living organism

ionic interactions chemical bond between two ions with opposite and attracting charges; typical for salts (i.e. Na\(^+\) Cl\(^-\))

isomer molecules with same molecular formula but different structures/conformations

Maillard reaction non-enzymatic chemical reaction between reducing sugars and amino groups

mast cell immune cell found in various tissues that is involved in regulating immune responses and plays an important role in allergic disease

meta-analysis a study design that uses the compilation and analysis of different but similar studies (of a specific research scope) and evaluates the pooled data for statistical significance

molecular mass synonym for molecular weight, the mass of a molecule

mucosa linings on body cavities that are exposed externally (environment) or internally (organs) such as nose, mouth or stomach

N-terminus refers to the start of an amino acid chain terminated by an amino acid with a free amine group (-NH\(_2\))

pathophysiology describes the functional (physiological) and conditional (pathological) changes associated with a disease or syndrome

pepsin active proteolytic enzyme in the stomach (responsible for breakdown of proteins)

peptide a chain of amino acids forms a peptide

post translational processing also called post translational modifications, is a step in the biosynthesis of proteins. Proteins, translated from mRNA can be modified to increase their functional variety. Modifications include i.e. glycosylation, phosphorylation, or methylation

postnatal after birth

prenatal before birth

proteolysis breakdown of proteins into smaller peptides or amino acids
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
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<tbody>
<tr>
<td>randomized</td>
<td>a study design that randomly assigns subjects into an experimental group or a control group (usually a placebo is used)</td>
</tr>
<tr>
<td>controlled trial</td>
<td></td>
</tr>
<tr>
<td>sensitization</td>
<td>production of allergen-specific IgE after initial exposure to allergen</td>
</tr>
<tr>
<td>T-lymphocyte cell</td>
<td>type of white blood cell that plays a role in cell-mediated immune response (there are many different types of T cells with distinct functions)</td>
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<tr>
<td>transplacental</td>
<td>crossing or going through the placenta (between mother and fetus)</td>
</tr>
<tr>
<td>trypsin</td>
<td>pancreatic protease acting in the upper part of the small intestine</td>
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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ANC</td>
<td>anthocyanin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>Ara h</td>
<td>arachis hypogaea</td>
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<tr>
<td>ATR-FTIR</td>
<td>attenuated total reflectance–Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>a_w</td>
<td>water activity</td>
</tr>
<tr>
<td>DMAC</td>
<td>4-(dimethylamino)cinnamaldehyde</td>
</tr>
<tr>
<td>DP</td>
<td>degree of polymerization</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>GAE</td>
<td>gallic acid equivalents</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HPP</td>
<td>high pressure processing</td>
</tr>
<tr>
<td>IgE</td>
<td>immunoglobulin E (antibody)</td>
</tr>
<tr>
<td>LDS</td>
<td>lithium dodecyl sulfate</td>
</tr>
<tr>
<td>OIT</td>
<td>oral immunotherapy</td>
</tr>
<tr>
<td>PAC</td>
<td>proanthocyanidin</td>
</tr>
<tr>
<td>PEF</td>
<td>pulsed electric field</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>PN</td>
<td>peanut</td>
</tr>
<tr>
<td>PN flour</td>
<td>light roasted 12% fat peanut flour</td>
</tr>
<tr>
<td>SEC</td>
<td>size exclusion chromatography</td>
</tr>
<tr>
<td>SGF</td>
<td>simulated gastric fluid</td>
</tr>
<tr>
<td>SN</td>
<td>soluble nitrogen</td>
</tr>
<tr>
<td>TP</td>
<td>total phenolics</td>
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CHAPTER 1

Peanut Allergy - A Review
Introduction: Food Allergies

Food allergies not only have a detrimental impact on quality of life, but they also can present life-threatening consequences. Various food allergies are responsible for approximately 125,000 emergency room visits and 53,700 episodes of anaphylaxis each year in the USA alone.\(^1\,^2\) The term ‘food allergy’ is defined in the 2010 U.S. National Institutes of Allergy and Infectious Diseases (NIAID)—sponsored guidelines as an ‘adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food’.\(^3\) The allergic reaction or adverse immune response always occurs in response to certain proteins within a food often called allergens or allergenic proteins; these, harmless to most people, are recognized by sensitive individuals as harmful foreign material. It is important to understand that generally the exposure to a food allergen results in a sensitization (towards the allergen) in susceptible individuals involving an immune response cascade with production of certain immune cells which eventually recognize the allergen after re-exposure. This potentially can lead to an allergic reaction (physical symptoms).

Over the last several decades, the prevalence and burden of food allergies has increased and become a worldwide health problem. Approximately 5% of young children and 3% to 4% of adults are affected by food allergy in westernized countries.\(^4\) More than 170 foods have been identified as potential causes for adverse immune responses and associated symptoms and disorders (involving the skin and gastrointestinal and respiratory tracts), however, only a minority of these foods cause the majority of reactions.\(^5\) Over 90% of food allergy is triggered by milk, egg, peanut, tree nuts, shellfish, fish, wheat or soy, yet, common food allergens vary between geographical regions.\(^5\) For example, celery, mustard, sesame,
lupine, and molluscan shellfish have been identified as significant allergens in European countries, while buckwheat is a common allergen in Japan. 6 Table 1 shows estimated rates of food allergies in North America.7-9

Numerous hypotheses have been considered to explain the increased rate and persistence of food allergies. These include 1) the ‘Hygiene Hypothesis’, 2) changes in dietary habits and norms, 3) the more prevalent use of dietary antacids, 4) the sensitizing role of in utero or early oral exposure to allergens, and 5) the ‘Dual-Allergen-Exposure Hypothesis’.

The ‘Hygiene Hypothesis’ suggests that modern reductions in early childhood exposures to infectious agents such as microbes or parasites increases susceptibility to develop allergic diseases, by suppressing natural immune system development.11 This hypothesis originally proposed by Strachan in 198912 has been supported by several epidemiological studies. For example, results from the Swiss SCARPOL study showed a strong relationship between growing up on a farm with continuous exposure to environmental microbial components and a lower risk to develop allergic disease.13 A

<table>
<thead>
<tr>
<th>food source</th>
<th>infant or child</th>
<th>adult</th>
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<tbody>
<tr>
<td>milk</td>
<td>2.5</td>
<td>0.3</td>
</tr>
<tr>
<td>egg</td>
<td>1.5</td>
<td>0.2</td>
</tr>
<tr>
<td>peanut</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>tree nuts</td>
<td>0.5</td>
<td>0.6</td>
</tr>
<tr>
<td>fish</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>shellfish</td>
<td>0.1</td>
<td>2.0</td>
</tr>
<tr>
<td>wheat, soy</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>sesame</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>overall</td>
<td>5.0</td>
<td>3.0 – 4.0</td>
</tr>
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similar, cross-sectional survey study evaluated almost 1,000 children in traditional alpine farming areas in Germany, Austria and Switzerland and further revealed that environmental exposure during pregnancy (mothers continued to work in stables) or infancy (first year of life) led to a strong protection from allergic disorders, atopy (a hereditary tendency to develop immediate allergic reactions), wheeze, and asthma.\textsuperscript{14}

However, little work has been done in respect to food allergy. Several studies investigated the effect of infant delivery mode on the development of food allergies. A recent meta-analysis evaluated the relationship between cesarean delivery and allergy outcome.\textsuperscript{15} Six studies showed some effect of cesarean delivery on the increase of food allergy or food atopy risk. It is suggested that early colonization (i.e. thought to more likely occur during natural delivery through contact with maternal faecal and vaginal microorganisms\textsuperscript{16}) of the infant by colonic microflora protects against the development of allergic disease. However, other explanations are possible and controversial. The question remains still unanswered as to if and to what degree the ‘Hygiene Hypothesis’ can be taken accountable for increased food allergy rates.

Another intriguing theory proposes that changes in micronutrient (i.e. vitamins) and macronutrient (i.e. fat) dietary composition coincident with modern dietary trends and increased reliance on processed and fortified foods over the past decades has led to increased prevalence of food allergies.\textsuperscript{11} For example, excessive levels of vitamin D (now routinely fortified into milk and other modern products, and recommended as a supplement) have been epidemiologically shown to increase allergy occurrence. Two separate cohort studies showed that infants who received vitamin D supplementation were more susceptible to food
allergies.\textsuperscript{17,18} The noticeable move from animal to plant-based dietary fats (like vegetable oil or margarine) is also believed to be part of the explanation for increased food allergy rates. Defenders of this hypothesis argue that the more prevalent consumption of omega-6-polyunsaturated fatty acids (found in plants) and a simultaneous decrease in omega-3-polyunsaturated fatty acids (found i.e. in oily fish) may be provoking increased incidence of allergies. Omega-6-type fatty acids have been shown to increase the production of certain immunological mediators, while omega-3-type fatty acids have been shown to prevent or decrease the formation of those mediators (anti-inflammatory effect).\textsuperscript{19} This is noteworthy, since these mediators ultimately can lead to an increased production of immune cells involved in allergic disease.

It has also been suggested that the more common use of \textit{antacid medications} (i.e. acid-suppression agents for impaired digestion disorders such as stomach ulcers or even just heartburn and indigestion) may prevent or impair complete destruction of potential allergens during the digestive process and potentially render food allergens immunoreactive.\textsuperscript{20}

Another highly controversial hypothesis discusses the tendency towards \textit{sensitization} to allergens \textit{prenatally} (\textit{in utero}, during pregnancy) \textbf{or} \textit{postnatally} (after birth). The general consensus has always been that early exposure to food allergens during pregnancy or lactation (breast-feeding) could lead to food allergies.\textsuperscript{11,21} Studies suggest \textit{in utero} sensitization based primarily on the demonstration in cord blood T cells (a type of immune cell) that respond to allergens \textit{in vitro}.\textsuperscript{22-24} However, controversy remains, whether sensitization to allergens is based on transplacental mechanisms or happens exclusively postnatal.\textsuperscript{25}
Until recently, families with an infant at elevated risk of developing allergic disorders (based on family history) were recommended by the American Academy of Pediatrics to avoid the common dietary food allergens milk, egg, fish and tree nuts in the first, second and third year of life, respectively. Peanut was recommended to be avoided in an infant’s diet during the first three years of life and women were even recommended to avoid peanuts or peanut products in their diet if they were expecting a child or if they were breast-feeding their infant. Avoidance, and other investigated intervention strategies such as pre- and postnatal dietary probiotic or omega-3-polyunsaturated fatty acid supplementation (possible protective effect), should aim to eliminate allergenic food proteins during pregnancy, breast-feeding, and early childhood. However, it has also been observed that the strict avoidance intervention approach can actually increase risk of allergic disease or allergic sensitization in the offspring as shown in a trial investigating strict egg and cow's milk avoidance in the last trimester of pregnancy.

In fact, the long-kept belief that allergic sensitization to food allergens exclusively happens through the oral route and prevention of food allergies is most effectively accomplished by elimination of the food target from the diet has been challenged by the ‘Dual-Allergen-Exposure Hypothesis’. This theory proposes that a minimal dose of a food can cutaneously (through skin contact) sensitize an infant whereas early consumption of food proteins can induce oral tolerance and that timing and balance of skin and oral exposure determine whether a child will have allergy or tolerance. A cohort study by Du et al. supports this hypothesis. This study showed that the prevalence of peanut allergy in school-aged Israeli Jewish children was only 0.17%, compared to 1.85% in Jewish children in the
UK. The Israeli children consumed on average approximately 7.1 g of peanut at an early age (8-14 months) whereas the children in the UK had 0% exposure.\textsuperscript{29} However, randomized controlled trials are needed to confirm the hypothesis that earlier ingestion of peanut is protective against later allergy development.

To date, the timing of allergen sensitization is controversial and questions remain as to whether prenatal intervention methods have a protective effect against the development of food allergies or not, and when, in what quantities and at which frequency allergenic foods should be introduced. In general, various factors, including genetic (family history) and environmental factors (such as being, or not being exposed to infectious agents) likely play a role as to whether a child develops food allergy or not.

**Peanut Allergy**

Peanut (\textit{Arachis hypogaea} L.) allergy, one of the ‘big eight’ food allergies known, is also one of the most severe, estimated to affect about 1% of infants and children and about 0.6% of adults living in the USA with increasing prevalence particularly in young adolescents.\textsuperscript{8,30} Peanut allergy is typically considered a persistent allergy and unlike most food allergies, only about 20% of children allergic to peanuts outgrow this disorder.\textsuperscript{31} A study by Fleischer \textit{et al.}, following children with resolved peanut allergy (they initially survived or passed a peanut oral food challenge) observed a recurrence of the allergy; 8% of the children appearing to have outgrown the disorder (tolerating peanut) experienced recurrence. Typically, children who had avoided or limited their peanut intake after they
passed food challenge were the ones who experienced recurrence. Usually, patients who consumed concentrated forms of peanut frequently had a much lower risk of recurrence.\textsuperscript{10}

The majority of fatal food allergic reactions reported in the USA are attributable to peanuts. In a 2001 report, Bock \textit{et al.} evaluated 32 fatalities recorded from 1994 to 1999 in a national registry kept by members of the American Academy of Allergy, Asthma & Immunology and The Food Allergy and Anaphylaxis Network.\textsuperscript{32} In this 2001 report, the 32 individuals were divided into two groups. Group 1 had enough data to conclusively determine that peanut was the food responsible for symptoms in 14 (67\%) of 21 subjects who died from anaphylactic reactions. For the 11 subjects in group 2 (with less information available), in 5 cases peanut was judged to be the most likely culprit. The same investigators later evaluated further fatalities which have been filed between 2001 and 2006 (31 subjects).\textsuperscript{33} This second report showed that 17 of the 31 fatalities were attributable to peanut.

Globally, the incidence of peanut allergy is also a concern. Between 1989 and 1995, the prevalence of sensitization to peanuts of 3-year old children in the UK increased from 1.3\% to 3.2\%.\textsuperscript{34} In France, the prevalence of peanut allergy is 0.2\% - 0.4\% and over 1\% of children have peanut allergy in Canada.\textsuperscript{35,36} Few epidemiological studies on peanut allergy prevalence have been performed in Asia, however, a study from 2010 suggests that approximately 0.5\% of 14-16-year old local Singapore schoolchildren and approximately 0.4\% of 14-16-year old Filipino schoolchildren are sensitive to peanuts.\textsuperscript{37} Nevertheless, peanut allergy to date seems to be most prevalent and increasing in westernized countries.
Pathophysiology of Peanut Allergy

The allergic reaction to peanuts is provoked by various proteins in the edible seed and, like other food allergies, belongs to the immunoglobulin (Ig) E antibody type I immediate hypersensitivity reactions. (There are also type II, III and IV hypersensitivity reactions that won’t be discussed further here).

In common with other food allergens, the mucosal surface of the gastrointestinal tract is generally the place where, after peanut ingestion, proteins are introduced to the system. Once proteins have penetrated mucosal barriers, they are captured and processed by specific cells such as dendritic cells (antigen-presenting cells). Subsequently, proteins are presented on their cell surfaces to other immunological cells and ultimately decomposed into smaller peptides. The composition of cells of the immune system and their functions are shown in Figure 1.38
Figure 1. Types of immune cells and their functions. Cells highly relevant to peanut allergy are marked in red (adapted from Casiday and Frey, Washington University, online lab tutorial, 2001).39

These peptides are then transferred to naïve helper T-lymphocyte cells (short T helper cells) which have never been exposed to the antigen, leading to the activation of the latter. It is generally thought that, through this oral route in susceptible individuals (who usually have a genetic predisposition to allergic disease), activated T helper cells lead to the secretion of various cytokines (mediators) and a subsequent activation of peanut-specific IgE antibody-producing B cells (B lymphocytes). Subsequently, peanut-specific IgE antibodies are bound to high affinity IgE receptors on mast cells or basophils (both cell types contain granules containing inflammatory mediators such as histamine). Upon re-exposure to peanut proteins,
segments of the allergenic proteins known as epitopes or antigenic determinants (surface portion of the allergen capable of eliciting an immune response) bind and cross-link with the cell-bound peanut specific IgE eventually resulting in a downstream cascade, releasing various inflammatory mediators (such as histamine or prostaglandins) responsible for the allergic immune response (Figure 2).40

Figure 2. Sensitization and allergic reaction to peanut (adapted from Burks et al., 2008)40
Peanut Allergens

Thirteen peanut allergens (Ara h 1 – Ara h 13) have been recognized by the Allergen Nomenclature Sub-Committee of the International Union of Immunological Societies. Among these, Ara h 1, Ara h 2, Ara h 3 and Ara h 6 are major allergens of concern. Ara h 1 and Ara h 2 are recognized from more than 90% of peanut-allergic patients. Studies suggested that Ara h 1 is the major peanut allergen of concern and it was believed that Ara h 1 was more important than Ara h 2. However, recent studies have shown that Ara h 2 has a greater ability to trigger release of histamine from human basophils containing peanut-specific IgE and has a higher reactivity in skin tests in peanut-sensitive individuals compared to Ara h 1. In vitro and in vivo Ara h 3 released less histamine compared to Ara h 2 and Ara h 6, however, it was similar to that of Ara h 1. Ara h 3 has been categorized as both a major and minor allergen, as it was recognized from about 44, 53 and 77% of peanut-sensitive patients, respectively. Results may have differed due to a difference in study populations. IgE binding and histamine release capacities of Ara h 6 are described to be similar to that of Ara h 2.

Ara h 1

Approximately 12–16% of the total peanut protein consists of Ara h 1, a glycoprotein, which belongs to the vicilin (7S globulin) family (Figure 3). Burks et al. initially described Ara h 1 as a 63.5 kDa protein, however, subsequent trials by other groups reported the molecular weight of this allergen to fall within a range between 63 and 68 kDa. The WHO/IUIS Allergen Nomenclature Subcommittee finally accepted and listed Ara h 1 as a 64
13 kDa protein. Ara h 1 was reported to be able to form, due to hydrophobic and ionic interactions, a highly stable homotrimer structure of about 180 kDa, with its 23 individual IgE binding epitopes located in monomer-monomer contact regions. An oligomeric form of the allergen has also been identified using techniques such as preparative SEC or nano-LC-MS. Ara h 1 seems to be naturally occurring in oligomeric form, however, a fluorescence spectroscopy analysis showed that extraction and separation techniques disrupt the native form of Ara h 1.

Figure 3. The core fragment of Ara h 1 (left) and Ara h 1 trimer in ribbon representation (right). Numbers indicate the ends of fragments that could be traced (from Chruszcz et al., 2011).
**Ara h 2**

Ara h 2, a glycoprotein, belongs to the conglutin family that is related to the 2S albumin family and was reported to make up about 5.9 – 9.3% of the total protein content in the seed (Figure 4). Ara h 2 consists of two isomers, Ara h 2.0101 and Ara h 2.0201, with molecular masses of 16.7 and 18 kDa running as a doublet on PAGE. The allergen contains 10 IgE binding epitopes that are distributed along the linear structure.

![Ribbon diagram of the peanut allergen Ara h 2](from Barre et al., 2005)

**Ara h 3**

Ara h 3 is part of the 11S legumin family and a peanut glycinin (Figure 5). Although the gene encoding Ara h 3 results in the translation of a 60 kDa protein, it has been observed
that the protein undergoes posttranslational processing ultimately leading to the formation of an about 400 kDa protein consisting of six basic and acid subunits. However, under denaturing conditions, the native protein is decomposed into peptides with masses of 14, 16, 25, 28, 42, and 45 kDa.\textsuperscript{69} MALDI-TOF-MS and various molecular models based on the amino acid sequence of Ara h 3 revealed eight different IgE binding epitopes.\textsuperscript{70}

Figure 5. Peanut allergen Ara h 3 is represented with each of the monomers shown in a different color. The gray monomer is marked for linear epitopes 1, 2, and 3 displayed in red, green, and blue, respectively (from Jin \textit{et al.}, 2009).\textsuperscript{71}
**Ara h 6**

Like Ara h 2, the peanut allergen Ara h 6 (Figure 6) belongs to the conglutin family (2S albumin). Ara h 6 has a molecular weight of about 15 kDa.\(^{52,72}\) Ara h 2 and Ara h 6 are to 59% homologous in respect to their amino acid sequence, yet, they are two independent peanut allergens. Conformational modeling indicated high similarity between tertiary structures of the two allergens.\(^{72}\) Therefore, it is possible that conserved parts of the two proteins could lead to IgE cross-reactivity between Ara h 2 and Ara h 6, which in fact has been shown by Koppelman *et al.*\(^{52}\)

![Figure 6. Peanut allergen Ara h 6. Here, protein chains are colored using a rainbow color gradient to show direction from N-terminus to C-terminus (entry 1W2Q from Protein Data Bank).\(^{73}\)](image)
Intervention Strategies

Before discussing and comparing some of the approaches currently taken to intervene in or alleviate peanut allergy, some characteristics about food allergens, in particular peanut allergens, need to be discussed. Allergenic food proteins, compared to non-allergenic food proteins, are generally very resistant to heat and gastric digestion. They can withstand acidic gastric conditions (found in the stomach) and are relatively stable to proteolytic (enzyme) digestion in the gastrointestinal tract. Pepsin has shown to be the active proteolytic enzyme involved in the degradation of peanut allergens as observed by Kopper et al. in their work with Ara h 1. Due to the protein’s ability to form highly stable homotrimer structures, it is, together with its IgE binding epitopes, protected from digestion. In regards to Ara h 2, proteolysis is impaired by the highly compact and small size of this protein strengthened by disulfide bonds between its 8 cysteine moieties. Extensive digestion of peanut allergens by gastrointestinal enzymes can result in the survival or formation of peptide fragments that remain immunoreactive (intact and accessible IgE binding epitopes) as previously shown.

Numerous processing and non-processing strategies to try to alleviate peanut allergy have been investigated including 1) thermal and non-thermal food processing techniques, 2) enzymatic hydrolysis, 3) controlled Maillard modifications, 4) genetic engineering/biotechnological methods, 5) non-allergen-specific immunomodulation by dietary phytochemicals, and 6) allergen-specific immunotherapy approaches.

Food processing techniques, under certain conditions, can modulate allergenicity by altering IgE binding epitopes. As mentioned, in order to cause a type I hypersensitivity
reaction, epitopes on food allergens need to bind and cross-link with allergen-specific IgE located on mast cells or basophils. Generally, two types of epitopes can exist on allergens: linear and conformational epitopes. Linear epitopes (recognizable by their linear continuous amino acid sequences or primary structures) contain critical amino acid residues, which determine whether IgE can bind to the allergen or not. Any modification of critical amino acid residues in linear epitopes can interrupt IgE binding and possibly reduce or eliminate allergenicity. Modulation of conformational allergen epitopes (which consist of discontinuous amino acids that can be recognized by the immune system through the protein’s three-dimensional tertiary conformation), on the other hand, can potentially affect IgE binding capacity. In both cases, epitopes can potentially be altered by thermal and non-thermal food processing techniques, leading to reduced or increased reactivity of a food allergen.

Thermal processing techniques can affect changes in the reactivity of peanut allergens. Since most peanut allergens are soluble in aqueous solutions, the majority of studies have been done on extractable/soluble proteins from peanuts which underwent various thermal processing treatments and compared with raw peanuts. The allergenic potential of insoluble protein complexes such as present in various processed food matrices has been relatively understudied, however, one study evaluated the insoluble portion after a protein extraction from roasted peanuts and found that the presence of insolubilized peanut proteins may potentially have influence on their continued presence in the digestive tract. Dry roasting is the probably most common thermal processing technique applied to peanuts. Although it has been shown that roasting decreases the solubility of peanut proteins, others
have shown that, despite this decrease, paradoxically more IgE binds to proteins from roasted peanuts than to those from raw peanuts.\textsuperscript{79} It has been shown, in particular for Ara h 1, that roasting did not affect IgE binding epitopes and the allergen remained immunoreactive.\textsuperscript{80} Results in a study by Pomés \textit{et al.} showed that roasted peanuts (10 to 15 min) were found to have 22-fold higher extractable Ara h 1 compared with raw peanuts. However, longer roasting times resulted in a lower level of protein solubility.\textsuperscript{81} Roasting peanuts has also been shown to increase IgE binding capacity when compared with boiling or frying.\textsuperscript{82} Beyer \textit{et al.} reported reduced IgE binding to Ara h 1, Ara h 2 and Ara h 3 in fried (5 or 10 min in vegetable oil) and boiled (20 min at 100 °C) peanuts than as compared to roasted (20 min at 170 °C) peanuts.\textsuperscript{83} A similar study by Mondoulet \textit{et al.} attributed the decrease in IgE reactivity in boiled peanuts to the loss of soluble proteins in the water used for boiling. Proteins recovered from the cooking water remained immunoreactive, as verified by Western blotting.\textsuperscript{57} In fact, during the roasting process (using dry heat), a non-enzymatic chemical process, called Maillard reaction, leads to the glycosylation of amino groups with reducing sugars (which either contain an aldehyde group or can form one in solution through isomerism) naturally present in peanuts. The resulting advanced glycation end-products are highly stable.\textsuperscript{84} These studies indicate that both the type of thermal processing and the allergen molecule are important when assessing the effects of thermal processing on peanut allergenicity.

Novel \textbf{non-thermal processing techniques} such as high pressure processing (HPP) and pulsed electric field (PEF) treatments have been investigated to alleviate the need for thermal processing in order to secure food safety. HPP (400 – 700 MPa) and PEF (20 – 40
kV/cm and energy input of 40 – 1000 kJ/kg) at ambient temperature can pasteurize foods while HPP combined with elevated temperatures (>80 °C and >600 MPa) enables sterilization of foods. During PEF high-voltage pulses are applied to electro conductive foods which are placed between two electrodes causing electroporation (permeabilization) of cell membranes through the induced movement of ions by the electric field.\textsuperscript{85}

Since both non-thermal applications have the potential to modify protein structure, the allergenicity of a given food allergen may also be altered. Studies have shown that HPP has denaturing effects as well as effects on emulsifying properties of whey proteins, while studies using PEF also showed interesting effects on the denaturation, aggregation and structure of soybean protein isolates, horseradish peroxidase and pectin esterase.\textsuperscript{86-89} A study by Husband \textit{et al.} showed that a combination of HPP and thermal processing is an effective technique to reduce the allergenicity of apple allergens Mal d 1, Mal d 3 and celeriac allergen Api g in plant tissues.\textsuperscript{90} However, a related study by Johnson \textit{et al.} investigating the effect of high temperature, HPP and PEF on purified Mal d 1, Mal d 3 and on purified peanut allergens Ara h 2 and Ara h 6 showed little effect on allergen structure.\textsuperscript{85}

\textbf{Enzymatic hydrolysis} is another attractive approach to reduce allergenicity of some foods. Several successful trials using various enzymes for allergen removal have been performed. For example, actinase AS (a microbial protease preparation used in food processing) was used in a two-stage process in order to produce hypoallergenic rice grains.\textsuperscript{91} Another study used enzymes such as trypsin, chymotrypsin (pancreatic proteases acting in the upper part of the small intestine), and pancreatin (a mixture of pancreatic enzymes derived from certain animals which is used to treat various digestive conditions) to provide a
substantially allergen-free whey protein hydrolyzate in a multi-step process. Studies using peanuts or peanut extracts have shown variable outcomes. A study by Yu et al. examined the use of chymotrypsin and trypsin under various processing conditions to reduce peanut allergens in whole roasted peanut kernels. Briefly, peanut kernels were blanched or not prior to incubation in different enzyme solutions, drying and subsequent protein extraction from ground kernels. Results showed that Ara h 1 and Ara h 2 (used as markers for effectiveness) were substantially reduced in a Tris–HCl buffer (pH 8.3) extractable fraction by 100% and 98%, when optimized process conditions were applied, respectively. Blanching of roasted peanuts even showed an enhancing effect on enzyme treatment. However, while this approach was able to degrade allergens into smaller peptides (as observed by SDS-PAGE) researchers also reported that these may or may not retain their IgE binding capacity in the soluble fractions of treated whole roasted peanuts. Despite some success, many studies have shown that food allergens are commonly resistant to gastric digestion. Peanut allergens, in particular Ara h 2 (as mentioned before) but also Ara h 6 are highly stable to denaturation and proteolysis. In addition, Ara h 2 can act as a trypsin inhibitor and a study by Maleki et al. showed that roasting promoted this function. Another study working on aqueous soluble fractions of light roast peanut flour used the enzymes alcalase (a protease derived from certain bacteria), pepsin, and flavourzyme (a fungal protease from Aspergillus oryzae) to degrade proteins in solution. Western blotting, inhibition ELISA and basophil activation tests were used to examine IgE reactivity and results indicated that peanut-specific IgE binding capacity was decreased through different enzyme treatments (ELISA) while a
subsequent test showed that IgE cross-linking capacity was retained, hence suggesting that prepared hydrolyzates were not hypoallergenic (basophil activation test).\(^6\)

As mentioned, heat treatment is a widely used industrial food processing technique applied to peanuts and other foods and leads to advanced glycation end-products if amino groups and reducing sugars are present in the food matrix. Thus, another approach to reduce food allergenicity is **controlled Maillard-type modification**. Several studies evaluated the IgE binding capacity of allergens modified by Maillard reactions and reported that glycation either increased (using peanut and scallop allergens)\(^7,8\), decreased (using cherry and squid allergens)\(^9,10\), or had no effect on IgE binding (using cherry and peanut allergens)\(^5,7,9\).

Observations did not appear to follow a general effect but rather depended on the type of allergen and sugar. A study by Taheri-Kafrani *et al.* evaluated the effects of heating and glycation of β-lactoglobulin (a major milk allergen) on its recognition by IgE of sera from cow milk allergic patients and found that a low or moderate glycation degree (%) of β-lactoglobulin with various reducing sugars did not decrease IgE binding while a high glycation degree was associated with decreased recognition of β-lactoglobulin by IgE from patient sera. Researchers suggested a ‘masking’ effect due to sugars. Modifications of β-lactoglobulin due to Maillard reactions occur mainly on lysyl (amino acid lysine) residues which are part of IgE binding epitopes found on β-lactoglobulin. Hence, modification of these residues can weaken or prevent IgE binding. However, the studies on record used different *in vitro* approaches to evaluate allergic potential of food allergens that underwent Maillard reactions, thus, the research outcomes remain to be carefully evaluated *in vivo*. 
**Biotechnological or genetic modification approaches** offer further opportunities to reduce or eliminate food allergens, and have intensively been investigated. The primary focus of these approaches has been modification of the allergen-bearing plant. Transgenic plants (with genetically modified DNA) have increasingly been developed and are generally considered safe.\(^\text{101}\) Genetic engineering, including methods such as gene silencing (which ‘blocks’ genes encoding for allergenic proteins leading to a plant that no longer expresses these proteins) or modification of the primary amino acid sequence of genes encoding allergens, has great potential to eliminate allergenic portions, thus could facilitate production of hypoallergenic or allergen-free foods.\(^\text{102-104}\) With respect to peanut allergens, Chu *et al.* described gene silencing of Ara h 2 and Ara h 6, producing peanut plants with suppressed expression of Ara h 2 and Ara h 6.\(^\text{105}\) While it is possible that suppression of one allergen could lead to an up-regulation of another allergen, this study showed that blocking of Ara h 2 and Ara h 6 left expression of Ara h 1 and Ara h 3 unaffected. Another report by Dodo *et al.* observed that transgenic peanut seeds created through gene silencing did not contain Ara h 2 and showed reduced IgE binding capacity.\(^\text{106}\) However, when they evaluated individual seeds of the first transgenic generation they found that not necessarily every seed in a pod (usually containing two) lacked Ara h 2. Therapy with engineered (mutated) recombinant proteins provides another option to alleviate peanut allergy. Li *et al.* showed that heat-killed *Escherichia coli* producing engineered (mutated) Ara h 1, Ara h 2, and Ara h 3 administered to peanut allergic mice had a long-term protective effect against peanut hypersensitivity.\(^\text{107}\)

Despite these promising examples, genetic engineering is currently limited by multiple factors such as the potential risk for newly overexpressed proteins, consumer
acceptance of genetically modified plants, microorganisms or proteins, regulatory difficulties and costs.

A more natural and consumer-approved way to intervene with food allergy may be the strategic application of plant-based bioactive compounds - phytochemicals. Many studies have investigated dietary phytochemicals for their non-allergen-specific anti-allergic potential and immunomodulatory characteristics. Polyphenols, including anthocyanins (red, purple or blue plant pigments) and proanthocyanidins (responsible for astringency) from fruits, vegetables, and other edible plant sources, are known for their anti-inflammatory properties. They may modulate pathways and immune cell functions in an allergic immune response, potentially leading to an alleviation of allergy symptoms. Qu et al. created an herbal ingredient consisting of 9 different herbs, called Food Allergy Herbal Formula 2 (FAHF 2), which was able to entirely suppress anaphylactic symptoms in peanut allergic mice challenged monthly with peanut for up to 6 months. When protective effects vanished and the formula was administered to mice again, full protection returned. In addition, clinical phase I trials in humans have shown great promise.\\(^{108-110}\)

Another noteworthy property that certain plant compounds such as phenolic acids, procyanidins or condensed tannins are known for is their natural binding affinity to proteins. The binding of polyphenolic compounds to proteins can lead to the formation of protein-polyphenol complexes which often remain insoluble (precipitated).\\(^{111}\) This characteristic has been used as a strategy to make allergens unavailable for triggering an allergic immune response. Several studies were conducted in which phenolic acids were bound to peanut proteins to form insoluble complexes, which rendered the remaining soluble portion of
peanut extracts less allergenic. For example, both raw and roasted peanut extracts treated with caffeic acid, showed decreased IgE binding capacity when evaluated by inhibition ELISA and showed reduced levels of Ara h 1 and Ara h 2, attributable to the formation of insoluble high molecular weight compounds.\textsuperscript{112}

Compared to non-allergen-specific therapy approaches, \textbf{allergen-specific guided immunotherapy} provides a chance to directly address a specific allergen. It refers to the administration of increasing amounts of an allergen to sensitive patients in order to desensitize them and build up tolerance against the food, ultimately preventing severe reactions upon subsequent encounters. The traditional method of treating patients with a specific allergen, subcutaneous immunotherapy, was shown to be unsafe in food allergy (but has shown to be useful in inhalant allergen sensitivity such as asthma).\textsuperscript{113-115} However, two other promising approaches have recently intensively been explored, sublingual immunotherapy (SLIT) and oral immunotherapy (OIT). In SLIT, a small amount of the allergen (usually a protein solution) is placed underneath a patient’s tongue in order to trigger the allergen’s transportation to Langerhans cells (dendritic cells located in skin and mucosa) in the oral mucosa. The allergen uptake by these cells is thought to be tolerated and leads to a down-regulation of the allergic response. A few studies have been conducted using SLIT, mainly related to hazelnut\textsuperscript{116}, kiwi\textsuperscript{117}, or peach allergy\textsuperscript{118}. Recently, SLIT has also been tested in a peanut allergy randomized-controlled clinical study by Kim and coworkers.\textsuperscript{119} Generally, allergen-specific immunotherapy starts with an initial escalation day (determining the starting allergen dose), followed by a build-up phase (increasing dose over several months), and a maintenance phase (holding a specific dose), prior to a final oral food challenge (to evaluate
therapy efficacy). In this report by Kim et al., young children with a median age of 5 years were treated by SLIT using a peanut extract for 12 months before they subsequently were orally challenged to evaluate their build-up tolerance. Results showed that subjects treated by SLIT tolerated about a 20-fold higher dose than compared with placebo subjects (when medians were compared). While it should be noted, that not all subjects in the active group were completely desensitized to peanut (since some patients did not tolerate a higher dose than patients in the placebo group), results have been encouraging seeing the effects of peanut SLIT after only 12 months. OIT, on the other hand, is based on ingestion of increasing amounts of the allergenic food. After an initial open-label study for egg allergy in children\textsuperscript{120}, a trial with peanut allergic patients followed soon after.\textsuperscript{121} Here, the (standard) active ingredient used was light roast 12\% fat peanut flour. Open-label peanut OIT, applied to a small number of patients (1 to 16 years old, 28 subjects), was shown to be safe when conducted in a highly regulated clinical setting with trained staff. The majority of subjects were successfully desensitized after treatment for 8 months.\textsuperscript{122} A follow-up randomized, double-blind, placebo-controlled study in peanut-allergic children to evaluate the safety and effectiveness of peanut OIT was conducted by Varshney et al.\textsuperscript{123} Initial escalation, build-up, and maintenance phases were followed by an oral food challenge after about 1 year. Results revealed that OIT treated subjects were able to ingest the maximum cumulative amount of 5000 mg peanut flour without adverse reaction, whereas placebo subjects were only able to ingest a median of 280 mg. Several immunological tests confirmed that peanut OIT induced desensitization and concurrent immune modulation.
Long-term immune tolerance remains to be evaluated. When comparing peanut SLIT with peanut OIT researchers found that OIT appears to be more effective than SLIT. However, since only a low allergen dose is required for SLIT (2 mg) compared with OIT (300 to 4,000 mg), SLIT may be safer and more effective for patients who cannot tolerate OIT. While both therapy approaches are highly promising, feasible, likely cost-effective and are thought to be adopted as widely applicable future food allergy treatments, it should be recognized that a current rapid dissemination beyond highly controlled clinical settings is limited. More studies with bigger subject groups need to be performed, showing that both methods can safely and effectively be applied to a broader range of patients. In addition, some individuals are often not able to complete the initial phase of the therapy since they suffer from (sometimes severe) associated side effects such as gastrointestinal problems or even anaphylactic shock.\textsuperscript{121,123,124}

**Research Objectives**

In our novel research approach we hypothesized that since medium-polarity phytochemicals (like anthocyanins and proanthocyanidins) naturally bind to proteins and form stable complexes, it may be possible to efficiently modify/mask epitopes on allergenic peanut proteins in a food-grade manner via natural stable complexation of peanut product with plant bioactives. Previously, various processing (thermal and non-thermal) intervention studies have focused on peanut extracts and (sometimes isolated) peanut allergens. Allergens have been extracted from raw, roasted, boiled or fried peanuts, and also from peanut products like peanut butter. These strategies have involved removal of the allergens by complex-
formation through bound phytochemicals. However, to our knowledge, a practical means for efficient modification of allergenic peanut proteins (in whole roasted peanut flour) with natural plant-derived polyphenols to form a dry, powdered, hypoallergenic peanut ingredient (flour) suitable for OIT has not previously been accomplished. Hence, we explored the potential for deliberately complexing allergenic peanut epitopes with natural dietary antioxidant compounds (specifically, polyphenolic phytochemicals) to create a hypoallergenic chimeric protein-polyphenol complex, which potentially could facilitate in safer OIT treatments with reduced side effects or complications.

Polyphenol-fortified peanut matrices were a) screened for qualitative changes in IgE binding capacity, secondary protein structure, basophil activation capability (ex vivo), and mast cell degranulation (in vivo), to gauge the potential for various polyphenol moieties to modulate allergenicity, and b) evaluated for their protein-polyphenol interaction and stability in solutions as well as during gastric digestion (in vitro) as an initial attempt to understand possible in vivo mechanisms for reduced allergenicity of certain polyphenol-fortified peanut matrices.
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CHAPTER 2

Novel Strategy to Create Hypoallergenic Peanut Protein-Polyphenol Edible Matrices for Oral Immunotherapy

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Novel Strategy To Create Hypoallergenic Peanut Protein—Polyphenol Edible Matrices for Oral Immunotherapy

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§Market Quality and Handling Research Unit, ARS, U.S. Department of Agriculture, Raleigh, North Carolina 27695, United States

ABSTRACT: Peanut allergy is an IgE-mediated hypersensitivity. Upon peanut consumption by an allergic individual, epitopes on peanut proteins bind and cross-link peanut-specific IgE on mast cell and basophil surfaces triggering the cells to release inflammatory mediators responsible for allergic reactions. Polyphenolic phytochemicals have high affinity to bind proteins and form soluble and insoluble complexes with unique functionality. This study investigated the allergenicity of polyphenol-fortified peanut matrices prepared by completing various polyphenol-rich plant juices and extracts with peanut flour. Polyphenol-fortified peanut matrices reduced IgE binding to one or more peanut allergens (Ara h 1, Ara h 2, Ara h 3, and Ara h 6). Attenuated total refection—Fourier transform infrared spectroscopy (ATR-FTIR) suggested changes in secondary protein structure. Peanut protein—cranberry polyphenol fortified matrices triggered significantly less basophil degranulation than unmodified flour in an ex vivo assay using human blood and less mast cell degranulation when used to orally challenge peanut-allergic mice. Polyphenol fortification of peanut flour resulted in a hypoallergenic matrix with reduced IgE binding and degranulation capacity, likely due to changes in protein secondary structure or masking of epitopes, suggesting potential applications for oral immunotherapy.

KEYWORDS: basophil, cranberry, mouse model, peanut allergy, polyphenols

INTRODUCTION

Food allergies not only have a detrimental impact on quality of life but they also can present life-threatening consequences and are responsible for approximately 125,000 emergency room visits and 53,700 episodes of anaphylaxis each year in the United States alone.1,2 More than 170 foods have been identified as potential causes for adverse immune responses and associated symptoms and disorders (involving the skin and gastrointestinal and respiratory tracts), but only a minority of these foods cause the majority of reactions.6,7 >90% of food allergy is triggered by milk, egg, peanut, tree nuts, shellfish, fish, wheat, or soy.7 Peanut allergy is considered one of the most severe food allergies, with the majority of fatal food allergic reactions reported in the United States attributable to peanuts.5 Furthermore, unlike most food allergies, only about 20% of children allergic to peanuts outgrow this disorder.4 Peanut allergic reactions involve an IgE-mediated immunological response to various proteins within the edible seed. Upon peanut consumption by an allergic individual, segments of the allergenic proteins known as epitopes bind and cross-link peanut-specific IgE on mast cell and basophil surfaces, which ultimately results in downstream cascades responsible for the allergic response.5,8 Of the 12 recognized peanut allergens, Ara h 1, Ara h 2, Ara h 3, and Ara h 6 are the major concern.6,9

Because peanut allergy can present life-threatening consequences, there is intense interest in developing therapeutic strategies that could reduce the danger and severity of the allergic reaction to peanuts in sensitive patients. Oral immunotherapy (OIT), which involves highly regulated administration, is a clinical setting, of very small doses of milled roasted peanut flour, is a strategy that has recently shown promise for desensitizing some allergic patients, to attenuate a potentially life-threatening reaction to a chance ingestion of a peanut product.10-12 However, OIT carries significant risks of side effects, including gastrointestinal problems, wheezing, and even anaphylactic shock; these barriers currently preclude rapid dissemination of the therapeutic approach beyond highly controlled clinical settings. As with other forms of immunotherapy, some individuals are unable to endure associated side effects and are not able to complete the initial phase of the OIT.10-12 To enable provision of this promising therapy to a broader spectrum of patients, new ingredients or technologies are sought, which reduce the allergic potential of peanut proteins and associated side effects without eliminating their properties integral for desensitization.

Dietary polyphenols have independently been investigated for their antiallergenic potential. Polyphenols, including anthocyanins (ANC) and proanthocyanidins (PAC) from fruits, vegetables, and other edible plant sources, contribute biological functionality and health-promoting benefits mainly linked to their antioxidant and anti-inflammatory capacities.13,14 Plant polyphenols may modulate biological pathways and immune

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cell functions in an allergic immune response, leading to an alleviation of allergy symptoms. Polyphenols are also known to have significant binding affinity for proteins, which can lead to the formation of soluble and insoluble (precipitated) protein–polyphenol complexes. Protein–polyphenol interactions can either be through hydrogen bonding or hydrophobic interactions, or polyphenols may cross-link between proteins. Several studies were conducted in which phenolic acids such as caffeic, chlorogenic, fericac, gallic, and recently tannic acid were bound to peanut proteins to form insoluble complexes, which rendered the remaining soluble portion of peanut and peanut butter extracts less allergic. However, a practical means for efficient modification of allergenic peanut proteins with natural plant-derived polyphenols to form a hypoallergenic peanut ingredient suitable for OIT has not previously been accomplished.

Recently, we described a novel tactic for stabilizing binding plant polyphenols to high-protein flour or protein isolates (soy, hemp, peanut, or pea) to create low-calcium, dry, health-protective matrices (granular or powdered ingredients) with improved bioavailability, efficacy, and shelf stability of the fruit-derived components. In the present work, we describe a novel approach to bind selected polyphenols to peanut flour, the current active ingredient for OIT, with the goal of masking IgE binding epitopes and attenuating allergenicity. A series of fruit juices and plant extracts with different characteristic polyphenol profiles were stabilly complexed with powdered protein, and the modified peanut protein–polyphenol chimeric matrices were screened for qualitative changes in IgE binding capacity, secondary protein structure, basophil activation capacity (in vitro), and mast cell degranulation (in vivo), to gauge the potential for various polyphenol moieties to modulate allergenicity. The ultimate goal of this research is to create dry, edible peanut matrices (peanut flour stably complexed with polyphenols) with reduced allergic potential and associated side effects that retain properties integral for modulating the allergic response of patients as a highly controlled, hypoallergenic edible OIT ingredient.

### MATERIALS AND METHODS

**Materials and Reagents.** Lipophilic Sephadex LH-20, Amberlite XAD-7, Folacin–Coccolites reagent (FCR), 4-dimethylaminophenol-cinnamaldehyde (DMAC), catechin, echinacea, gallic acid, and tannin from porcine gastric mucosa (3502 U mg⁻¹ protein, 92% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Procyanidin A2 and procyanidin B1 were obtained from Chromadex (Irvine, CA, USA). Procyanidin trimers (epi-(4′→6) × 2′→4′; 2′→O→7′)-epi-(4′→8, 2′→O→7′)-cat (1:1:1) and tetramer (epi-(4′→6) × 2′→4′; 2′→O→7′)-epi-(4′→8, 2′→O→7′)-epi-(4′→6) × 2′→4′; 2′→O→7′)-cat were obtained from Plantaq (Downers Grove, IL, USA). NiPAC LDS sample buffer (6×) and Triton X-100/SDS running buffer were purchased from Invitrogen (Carlsbad, CA, USA). Novex Tricine gel, Novex Tricine SDS running buffer (16×), Novex Tricine SDS running buffer (2×), NiPAC reducing agent (10×), ScieBlue Plus2 prestained protein standard, Magic Mark XP Western protein standard, SimplyBlue SafeStain, and bovine serum albumin (BSA) were purchased from Thermo Scientific (Rockford, IL, USA). Fluorescein-illustrated cell sorting (FACS) lysing solution was obtained from BD Biosciences (San Jose, CA, USA). Ethylendiaminetetraacetic acid (EDTA) and 2% sterile bovine serum albumin. Human rIL-3 was obtained from Millipore (Billerica, MA, USA). Chlorella tonsis was purchased from List Biological Laboratories, Inc. (Campbell, CA, USA).

**Plant Materials.** A series of fruit juice concentrates and plant extracts, representing different characteristic phytochemical profiles, was chosen to evaluate the potential utility of using characteristic plant components to modulate the allergenicity of peanut flour. Elderberry (Sambucus nigra L.) and aeroast (Arum melanocarpa) juice concentrate (65 “Brix) was obtained from Muscades Products Corp. (Wayne, PA, USA), and black currant (Ribes nigrum) juice concentrate (65 % Brix) was provided by a commercial supplier (36). Mentha piperita (Oatway, IN, USA), Muscades grape (Vitis rotundifolia) juice concentrate from the Noble variety (65 “Brix) was obtained from Muscades Products Corp. (Wayne, PA, USA), and black currant (Ribes nigrum) juice concentrate (45 Brix) was provided by a commercial supplier (37). The following 3%, 20 g juice extract was obtained from Denver Bio-Corporate Ltd. (Colesville, MD, USA). Cinnamon powder (Cinnamomum verum) and green tea leaves (Camellia sinensis) (from Sri Lanka were provided by the Department of Food Science and Human Nutrition, University of Vermont, VT, USA). Lactobacillus 12% fat peanut (PN) flour (Arachis hypogaea L.) was obtained from Golden Peanut Co. LLC (Alpharetta, GA, USA).

**Preparation of Juices and Extracts.** Dried green tea leaves were ground into a fine powder prior to extraction. Cinnamon and green tea powders were extracted with 50% ethanol (1:3, v/v). The mixtures were heated in a water bath at 60°C for 1 h. Because the cinnamon powder extract was very viscous, it was subsequently diluted 1:1 (v/v) with 50% ethanol to obtain a final 1:10 (v/v) dilution. The solutions were centrifuged at 7000 rpm for 20 min (Sorvall RC-5B refrigerated centrifuge, DuPont Instruments, Wilmington, DE, USA) and filtered, and the ethanol was evaporated. The extracts were then reconstituted with water to original volume. Each precipitated material was separated by centrifugation, and the supernatant was filtered before mixing with PN flour. Juice concentrate was filtered via dilution prior to mixing with PN flour because high viscosity otherwise interfered with effective mixing and separation of the flour. Arum, Noble muscadine grape, and elderberry juice concentrates were diluted 3 times (1:1, v/v) with water, Black currant juice concentrate was diluted 4 times with water (1:1.5, v/v). Cranberry juice concentrate was diluted to both 2 times (1:1, v/v) and 4 times (1:1.5, v/v) with water. Degrees Brix was determined by measuring refractive index using an Abbemat 550 refractometer to estimate total sugar content (Anton Paar, Ashland, VA, USA).

**Quantification of Polyphenols.** Polyphenols were quantified in the original juices and extracts (Table 1). Total phenolics (TP) were determined using the Folin–Ciocalteu assay according to the method of Eraldi et al.21 Results were expressed as milligrams per milliliter gallic acid equivalents (GAE) based on a gallic acid standard curve. Total proanthocyanidins (PAC) were determined using the 4-dimethylaminophenol-cinnamaldehyde (DMAC) assay with modifications. Briefly, samples were diluted in 80% ethanol (v/v) when necessary. Fifty microliters of diluted or undiluted sample, blank (80% ethanol), or standard (procyanidin A2) was added to a 96-well plate. Subsequently, 150 μL of a 0.1% DMAC solution dissolved in acetic acid (ethanol, water, HCl; 75:12.5:12.5, v/v/v) was added to the plate and mixed using a multichannel pipet. Absorbance was measured using a Tecan® plate reader (Tecan Group Ltd., The Newcastle, Switzerland) at 640 nm for 30 min in 1 min intervals at 25 °C. Quantification of total PAC was performed from the maximum absorbance observed and expressed as micrograms per milliliter procyanidin A2 equivalents. HPLC analysis of total monomeric anthocyanins (ANC) was performed using an Agilent Technologies 1200 series HPLC (Santa Clara, CA, USA) with a photodiode array (PDA) detector. Samples were filtered through 0.2 μm cellulose syringe filters, and 5 μL was injected onto a Supelcosil LC-18 column (250 mm × 4.6 mm × 5 μm, 7011

Table 1. Total Phenolics (TP), Total Proanthocyanidins (PAC), and Total Monomeric Anthocyanins (ANC) Concentrations and Brix Values in Juices and Extracts

<table>
<thead>
<tr>
<th>Source</th>
<th>TP (mg mL⁻¹)</th>
<th>PAC (mg mL⁻¹)</th>
<th>ANC (mg mL⁻¹)</th>
<th>Brix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cranberry juice</td>
<td>5.0</td>
<td>1.6</td>
<td>1.1</td>
<td>27.5</td>
</tr>
<tr>
<td>Juice</td>
<td>2.1</td>
<td>0.7</td>
<td>0.5</td>
<td>14.1</td>
</tr>
<tr>
<td>Red cabbage</td>
<td>0.9</td>
<td>0.4</td>
<td>NA</td>
<td>2.5</td>
</tr>
<tr>
<td>Green tea leaves</td>
<td>3.2</td>
<td>0.6</td>
<td>NA</td>
<td>6.5</td>
</tr>
<tr>
<td>Green tea leaves</td>
<td>3.17</td>
<td>0.5</td>
<td>NA</td>
<td>6.5</td>
</tr>
<tr>
<td>Green tea leaves</td>
<td>3.17</td>
<td>0.5</td>
<td>NA</td>
<td>6.5</td>
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<td>Green tea leaves</td>
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<tr>
<td>Green tea leaves</td>
<td>3.17</td>
<td>0.5</td>
<td>NA</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Values in each column with different letters are significantly different at p < 0.05. Determined by Folin–Ciocalteu assay and expressed as mg mL⁻¹ gallic acid equivalents. Determined by DPPH assay as mg mL⁻¹ procyanidin A2 equivalents. Determined by HPLC as mg mL⁻¹ cyanidin-3-O-glucoside equivalents. NA, not applicable.

Sapiello, Lorentorf, PA, USA) column, which was maintained at 30 °C. The mobile phase consisted of 5% formic acid in distilled H₂O (A) and 100% MeOH (B). Flow rate was set at 1 mL min⁻¹ with a gradient as follows: 0–5 min, 10% B; 5–15 min, 15% B; 15–20 min, 20% B; 20–25 min, 25% B; 25–35 min, 30% B; 35–45 min, 35% B; 45–60 min, 100% B. The injection volume of 10 μL was used as an external standard. Samples were detected at 250 nm and expressed as cyanidin-3-O-glucoside equivalents.

Preparation of Polyphenol-Rich-Petrolatum Matrices. White flour was combined with diluted juice concentrates or extracts (30 g L⁻¹), mixed for 15 min at room temperature to allow sorption of polyphenols to FN proteins, and centrifuged for 20 min at 4000 rpm. A blank treatment (FN flour mixed with water only) was also prepared. The supernatant was decanted and filtered prior to analysis. The pellet was freeze-dried to afford the polyphenol-rich FTIR spectra, and used for the extraction of the polyphenol-rich fraction.

Characterization of Polyphenol-Rich or Blank FTIR Matrices and FN Flour. The supernatant was evaporated to dryness using a Bürker minispinn MiniSpin (Bürker, MI, USA). Water activity (a₃₃₂) was measured using an Aquapor A-20 device (Decagon Devices, Pullman, WA, USA). To determine protein solubility, 10% dispersions of polyphenol-rich FN matrices or unmodified FN flour in phosphate-buffered saline (PBS, pH 7.5) were prepared. Total nitrogen content of the soluble fractions was determined using a 2400 CHN Elemental Analyzer (PerkinElmer, Norwalk, CT, USA).

FN Protein Distribution and FN-Specific IgE Binding. SDS-PAGE was used to observe protein distribution in polyphenol-rich FN matrices and unmodified FN flour. PBS soluble fractions (from 10% dispersions in 3% w/v) were added 1:1 to Laemmli buffer containing 5% mercaptoethanol. Samples were subsequently subjected to SDS-PAGE with a 12% gel and stained with Coomassie Blue. The protein content of each lane was determined using a Biorad Gel Profiler (Hercules, CA, USA).

Specific Binding Capacity of Cranberry or PAC-Rich Extracts to FN. The binding capacity of FN to PAC-Rich extracts was determined using a Biorad Gel Profiler (Hercules, CA, USA).

Specific Binding Capacity of Cranberry to FN. The binding capacity of FN to cranberry extract was determined using a Biorad Gel Profiler (Hercules, CA, USA).

Reference

PAC compounds were separated on a 5 μm, 250 x 4.6 mm, Luna silica columns (Phenomenex, Torrance, CA, USA). The binary gradient consisted of dichloromethane, MeOH, water, and acetic acid (A, B, V, V/V, v/v, and MeOH). The flow rate was 1 mL min⁻¹ with a gradient as follows: 0–2 min, 90% B; 2–20 min, 90% B; 20–25 min, 90% B; 25–30 min, 90% B; 30–35 min, 90% B; 35–40 min, 90% B; 40–45 min, 90% B; 45–50 min, 90% B; 50–55 min, 90% B; 55–60 min, 90% B; 60–65 min, 90% B; 65–70 min, 90% B; 70–75 min, 90% B; and 75–80 min, 90% B. The peaks were monitored by fluorescence detection with excitation at 276 nm and emission at 316 nm. Quantification of compounds was performed by means of external calibration curves using monomer.
(DP1) through tetramer (DP4) commercial standards. PAC with DP ≥5 including polymers were expressed as DP4 equivalents.

Basophil Degranulation Assay. Because the FN protein polyphenol-
fortified matrices were largely insoluble, an existing basophil activation assay was modified to evaluate solid-phase FN flours. For this assay, 1 mg of cranberry polyphenol-fortified FN matrix (prepared with 1:1 and 1:3 dilutions of cranberry juice concentrate) or unmodified FN flour was added to 200 µL of basophil media (RPMI-10 plus 2 ng/mL human IL-3) and subsequently adjusted to 2% of total well volume with whole blood from FN-allergic subjects who were enrolled in an IRB-approved protocol at UNC-Chapel Hill (5). Antigen and whole blood were mixed by pipetting up and down several times, and then samples were placed in a 37°C humidified CO2 incubator for 30 min. Reactions were stopped by addition of 50 µL of PBS plus 2 mM EDTA. Cells were stained for expression of CD11b, CD133 and CD63 at 4°C in the dark for 30 min and washed twice in staining buffer (PBS plus 0.5% BSA plus 2 mM EDTA). Red blood cells were lysed and white blood cells fixed with 4 µL of FACS lysing solution for 15 min at room temperature. Samples were analyzed on a Canto II flow cytometer (BD, San Jose, CA, USA) with data processing on FlowJo software (TreeStar, Ashland, OR, USA). Basophils were identified as CD11b+ CD133+ lymphocytes, and degranulation was monitored by CD63 expression. Results are expressed as the percentage of basophils that were CD63+.

Simulated Gastric Digestion Assay and Ige Binding Capacity of Digestive Products. To investigate the stability of FN proteins within unmodified FN flour or cranberry polyphenol-fortified FN matrix to peptic digestion, a simulated gastric digestion assay was used. Simulated gastric fluid (SGF) was prepared as reported in the United States Pharmacopeia with minor changes. 0.2% solution of NaCl in water (w/v) was prepared and adjusted to a pH of 2.0 using 6 N or 1 N HCl. A 10 mL dispersion of either unmodified FN flour or cranberry polyphenol-fortified FN matrix was added to SGF (1:19 w/v) and stirred on a stir plate for 5 min. The pH was subsequently adjusted with a few drops of 1 N NaOH (because adding FN flour or cranberry polyphenol-fortified FN matrix to SGF results in a slight pH increase), and the dispersions were completed to equal volume in two separate 15 mL centrifuge tubes using SGF. A 1 mL aliquot from each of the well-distributed dispersions was taken and added to two separate 2 mL centrifuge tubes (controls, no peptic added). Dispersions, in tubes, were subsequently placed into a 37°C water bath and prewarmed for 15 min while a 0.2 mg/mL peptic solution in SGF (w/v) was prepared and also prewarmed (for only 5–10 min to prevent a possible loss of enzyme activity due to autodigestion). One milliliter of peptic solution was subsequently added to each tube containing the dispersions (resulting in a total volume of 10 mL and a 1% dispersion per tube). One milliliter aliquots were taken after 0.5, 1, 3, 9, and 30 min, added to separate 3 mL centrifuge tubes, and immediately subjected to a 90°C water bath for 15 min to inactivate peptic. Each control (a 1 mL aliquot of each dispersion without peptic) underwent the same heat treatment. Throughout the digestion period samples were inverted several times to secure proper mixing of digestion and enzyme. Peptide-inactivated digestive samples and control samples were subsequently lyophilized, and resulting supernatants were used for further analysis. SDS-PAGE and Western blotting were used to evaluate digestive samples and their respective controls for protein digestion and Ige binding capacity. For SDS-PAGE 40 µL of unaltered sample was added to 50 µL of Novex Tris Acetate SDS sample buffer (2X) and 10 µL of NPAGE reducing agent (10X), resulting in a final volume of 100 µL (according to the manufacturer’s instructions). Samples were heated for 2 min at 85°C. Two µL of each sample was loaded onto the gel, subsequently subjected to SDS-PAGE (90 min, 12% V, and stained with SimplyBlue SafeStain. Western blotting was performed as stated earlier in this paper.

Oral Challenges in FN Allergic Mice. Three-week-old C57BL/6 female mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were allowed to acclimatize to their new environment for 2 weeks before being used in protocols. Mice were housed under pathogen-free conditions with free access to water and food while being kept on a FN-free diet for the entire study. All studies were approved by the IACUC at UNC Chapel Hill. To sensitize mice to FN, a FN extract from unmodified FN flour was prepared using PBS with NaCl. Briefly, unmodified FN flour was mixed with PBS (1:6 w/v) and stirred for 1 h while a constant pH of ~8.5 was maintained using 12 M NaOH. The mixture was subsequently centrifuged at 3000g at 4°C for 30 min and the resulting supernatant (FN extract) filtered using 0.8 and 0.2 µm membranes. The protein concentration in the FN extract was determined (Pierce BCA protein assay) and diluted to 10 mg/mL in PBS (w/v). The protein solution was stored frozen until needed. Mice were sensitized to FN proteins by oral gavage using FN extract plus cholera toxin as an adjuvant. On days 1, 8, and 15 mice received 2 mg of FN proteins plus 10 µg of cholera toxin and on day 22 received 5 mg of FN proteins plus 10 µg of cholera toxin. Mice were challenged on day 36 with either unmodified FN flour (50 µg) or polyphenol-fortified FN matrix made with 1:1 dilution of cranberry juice (50 µg) suspended in PBS. Mice were bled 45 min after challenge by subcutaneous bleeding using a 4 mm incision, and sera were subsequently assayed for mouse mast cell protease-1 (MMPC-1) as a marker of intestinal mucosal mast cell degranulation. MMPC-1 was quantified by ELISA according to the manufacturer’s instructions (Bioassays, San Diego, CA, USA). UNC IACUC approval number was 12-176.

Statistical Analysis. All flours and polyphenol-fortified FN matrices were prepared in triplicate, and analysis of variance was performed using JMP 10.0 (SAS, Cary, NC, USA). When significant (p < 0.05), means were separated using Student’s t-test. Basophil data were analyzed on GraphPad (La Jolla, CA, USA), and the data were compared by the Wilcoxon test, and the MMP-1 data were compared by the Mann–Whitney test (one-tailed).

Results and Discussion

Characterization of Polyphenol Sources. Although the polyphenolic sources chosen for this study have some overlapping chemical characteristics, we divided them into three main groups. Cranberry and cinnamon contain high levels of A-type PAC. B-type PAC, on the other hand, can be found in aronia and green tea with the latter being also a rich source of PAC monomers such as catechin, epicatechin, or epigallocatechin gallate. Noble muscadine grape, black currant, and elderberry feature high ANC concentrations and different characteristic ANC profiles. Concentrations of total phenolics (TP), total proanthocyanidins (PAC), total monomeric anthocyanins (ANC), and “Brix values of the diluted juices and extracts before complexing with FN flour are provided in Table 1. TP concentration ranged from 0.9 mg mL−1 (cinnamon powder extract) to 39.0 mg mL−1 (elderberry juice). Green tea leaves extract showed the highest concentration of PAC (59.5 mg mL−1), whereas the PAC content of cinnamon powder extract and muscadine juice was low (0.4 mg mL−1 for each). It should be noted that the DMAC assay has limitations when estimating total PAC levels, as the DMAC reagent reacts only with the terminal monomer of the PAC molecule, regardless of the size of the oligomer or polymer. Therefore, plant sources rich in oligomeric/polymeric proanthocyanidins, such as cinnamon and aronia, are usually underestimated when assayed with the DMAC reagent compared to those rich in monomeric proanthocyanidins such as green tea. The highest amount of ANC was observed in elderberry juice (22.5 mg mL−1), Degrees Brix ranged from 2.5 (cinnamon powder extract) to 39.6 (Noble muscadine grape juice).

Characterization of Polyphenol-Fortified Matrices. Moisture (%), fat (%), water activity (aw), and soluble nitrogen
moisture content of the prepared PN matrices ($r^2 = 0.82$). This is attributed to the hygroscopicity of residual sugar in the matrices prepared with juices with high sugar content. Additionally, fat content, which ranged from 5.9% (elderberry juice polyphenol-modified PN matrix) to 13.9% (blank flour modified with water only), was negatively correlated with moisture content ($r^2 = 0.71$). The $a_w$ of all polyphenol-modified PN matrices was < 0.4, which is well below the limit for microbial growth. SN concentrations ranged from 0.1 g L$^{-1}$ (both 1:1 and 1:3 dilutions of cranberry polyphenol-modified PN matrices, cinnamon polyphenol-modified PN matrix, and aronia polyphenol-modified PN matrix) to 0.6 g L$^{-1}$ (unmodified PN flour) (Table 2). Protein solubility decreased in all polyphenol-modified PN matrices compared with unmodified flour, suggesting that polyphenols bound to PN proteins, rendering them less soluble. Whereas polyphenols in general have exhibited significant binding affinity for proteins, which can lead to the formation of soluble and insoluble protein–polyphenol complexes, in particular, PAG are known to have a strong affinity for proteins, which likely explains the low SN of cranberry, cinnamon, and aronia-modified matrices.$^{47}$

The concentrations of TP, total PAG, and total monomeric ANC bound to PN flours after complexation with the various diluted juices and extracts are shown in Table 3. The highest amount of total PAG was captured from green tea leaves extract (429.7 mg g$^{-1}$), whereas the lowest concentration of total PAG was observed in aronia polyphenol-modified PN matrix (0.7 mg g$^{-1}$). As noted previously, the DMMAC assay tends to underestimate total PAG for plants such as aronia, which feature oligomeric and polymeric PAC. Elderberry, which has inherently high ANC content (Table 1), resulted in the highest concentration of total ANC when complexed in the PN polyphenol-modified matrices (44.9 mg g$^{-1}$).

### PN Protein Distribution and PN-Specific IgE Binding

To understand the potential for IgE binding modification, polyphenol-modified PN matrices were screened using SDS-PAGE and subsequent Western blots using PN-specific IgE.

### 3. Total Phenolics (TP), Total Proanthocyanidins (PAC), and Total Monomeric Anthocyanins (ANC) Concentrations Sorbed onto Commercial Light Roast 12% Fat Peanut (PN) Flour after Complexation with Different Juices and Extracts

<table>
<thead>
<tr>
<th>Source</th>
<th>TP$^a$ (mg g$^{-1}$)</th>
<th>PAC$^b$ (mg g$^{-1}$)</th>
<th>ANC$^c$ (mg g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cranberry juice 1:3 dilution</td>
<td>14.5</td>
<td>2.3</td>
<td>1.4</td>
</tr>
<tr>
<td>cranberry juice 1:1 dilution</td>
<td>14.6</td>
<td>2.4</td>
<td>1.4</td>
</tr>
<tr>
<td>cinnamon powder extract</td>
<td>5.6</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>green tea leaves extract</td>
<td>13.2</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>aronia juice</td>
<td>23.6</td>
<td>0.7</td>
<td>1.7</td>
</tr>
<tr>
<td>black currant juice</td>
<td>19.8</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>elderberry juice</td>
<td>24.6</td>
<td>1.3</td>
<td>2.0</td>
</tr>
</tbody>
</table>

$^a$Values within each column with different letters are significantly different at p < 0.05. $^b$SN concentrations were determined by preparing 10% (w/v) phosphate-buffered saline (PBS) solutions. PN flour modified with water only.
Ara h 2 and Ara h 6, as observed in cranberry and green tea samples, may significantly reduce allergenicity.

In general, reduced IgE binding to one or more PN proteins in LDS extracts was observed particularly in the PAC-rich samples (cranberry, cinnamon, green tea, and aconia) and also in the ANC-rich black currant sample. In a separate experiment, PN flours were treated with fractions enriched in ANC and PAC (nominally isolated and purified from cranberry juice concentrate) and tested for their polyphenol-class specific contributions to PN-specific IgE binding. Western blotting demonstrated that the PAC-rich fraction was more effective in reducing IgE binding than the ANC-rich fraction in both the soluble and insoluble fractions, but both reduced IgE binding compared to the blank, water-treated PN flour (Figure 3). This supports what we observed in the polyphenol-fortified PN matrices and is likely attributable to the natural affinity of PAC to proteins. Additional experiments are needed to understand the mechanism of reduced IgE binding observed here; however, data suggest that some polyphenol-fortified PN matrices, particularly those prepared with cranberry juice and green tea extract, may potentially be hypoallergenic. Whereas proteins are denatured and bound to a PVDF membrane in this approach (i.e., conformational epitopes are disrupted), SDS-PAGE and Western blotting were shown to be a reproducible screen for IgE binding modifications.

**ATR-FTIR Spectra.** ATR-FTIR spectra of the polyphenol-fortified PN matrices were recorded to evaluate modification to protein secondary structure. This technique allows for evaluation of the modified flour in its natural insoluble powdered state. The most prominent bands were the amide I (1600–1700 cm⁻¹) and amide II (1480–1575 cm⁻¹) bands, which result from vibrations of the protein backbones (Figure 2).
3). Because a mixture of crude proteins was examined as opposed to a single purified PN protein, only qualitative

dependences of conformational changes in PN proteins were gained. Amide I and amide II bands were altered to some extent in all polyphenol-fortified PN matrices compared to unmodified PN flour. Generally, polyphenol-fortified PN matrices that displayed significantly altered IgE binding, including cranberry, aronia, elderberry, and green tea, also displayed altered FTIR profiles in the form of increased or decreased band intensities, band shifting or width changes, and/or appearance of additional peaks compared to the unmodified PN flour. Band shifting can indicate that hydrogen bonds were affected. Because some of the polyphenol-fortified peanut matrices had a relatively high moisture content (up to 21.1% for muscadine modified matrices), it should be taken into account that water absorbs within the amide I region (1645 cm⁻¹, H—O—H bending vibration) and could, besides protein-bound polyphenols, contribute to observed spectrum changes. Width changes are linked to the conformational freedoms of a structure. Molecules that bind to proteins can lead to conformational restriction, thus reducing bandwidth. These findings suggest that the decreased IgE binding observed in polyphenol-fortified PN matrices may be partly due to changes in protein secondary structure. However, because modified matrices were screened on a qualitative level, a water reference spectrum as well as further IR spectral data analysis methods would be needed to evaluate water interference and to break down and analyze overlapping bands in amide regions in a more qualitative and quantitative manner, respectively.

Characterization of Cranberry PAC Bound to PN Matrices. On the basis of decreased IgE binding specifically to Ara h 2 and Ara h 6 (Figure 1) and significantly altered FTIR spectra (Figure 3), the cranberry polyphenol-fortified PN matrix (rich in A-type PAC) was selected for further experiments. PAC isolated from cranberry juice (1:1 dilution) and from supernatants (spent juice) obtained after complexation with PN flour were analyzed to evaluate the capacity of individual PAC to bind to PN flour. HPLC chromatograms of PAC in the cranberry juice before (A) and after (B) treatment with PN flour are shown in Figure 4. PAC that were more highly polymerised showed a higher level of binding to PN flour (Figure 4B). Table 4 shows the amount of individual PAC bound to PN flour (mg g⁻¹). Polymerised PAC have a stronger binding capacity to proteins than PAC monomers or oligomers. Because noncovalent interactions between proteins and monomeric phenolic compounds such as catechins require relatively high molecular ratios of phenolic compounds to proteins (>100) to affect the functional properties of proteins, it is more likely that PAC and particularly polymerised PAC interacted with PN proteins. Further experiments must investigate the binding nature of polyphenols to PN proteins.

Basophil Degranulation Assay. A modified basophil assay provided an ex vivo assessment of the ability of antigens to trigger degranulation of human basophils found in peripheral blood and allowed for testing the nontoxic matrices in a whole blood basophil assay. The basophil activation test augments the IgE binding and FTIR data described above, as it allows for a functional assessment of the polyphenol-fortified PN matrices' capacity to cross-link IgE on basophils, leading to degranulation. PN protein—polyphenol matrices created with cranberry juice triggered significantly less basophil degranulation (median = 37.2% and range = 5.2–70.5% for the 1:1 dilution; median = 45.5% and range = 8.0–78.4% for the 1:3 dilution) than the unmodified PN flour (median = 66.1%, range = 15.5–94.0%) in the seven PN-allergic subjects studied with the basophil assay (Figure 5). Statistically significant reduction in activation level was found by comparing the cranberry polyphenol-fortified PN matrices to the unmodified PN flour (p = 0.015). Although basophil activation was not completely abrogated by cranberry modification of PN flour, the significant decrease in IgE cross-linking suggested enhanced hypoallergenicity. The basophil assay results indicate that PN proteins complexed with cranberry phytochemicals have a decreased capacity to cross-link IgE, although there are important limitations to this finding. For example, the assay is conducted using edible PN matrices that are not exposed to acidic pH or digestive enzymes, which would be encountered following oral ingestion. Exposure to a low pH, such as that found in the stomach, as well as exposure to various digestive enzymes such as pepsin (stomach) may disrupt the PN protein—polyphenol
interactions and lead to the release of allergens that can cause reactions.

**Simulated Gastric Digestion Assay.** An in vitro digestion method was used to mimic the gastric digestion of unmodified PN flour or cranberry polyphenol-fortified PN matrix by pepsin in an acidic environment at a physiologic temperature. This assay was used to evaluate and mimic protein stability and digestibility under harsh conditions such as those present in the stomach. Pepsin was obtained with the highest specific enzyme activity commercially available (3802 U mg\(^{-1}\), protein), whereas enzyme concentration (0.2 mg mL\(^{-1}\)) was optimized for a more gradual digestion of proteins. The digestibility of proteins can be evaluated by keeping track of the disappearance of intact protein bands on SDS-PAGE. PN proteins in unmodified PN flour as well as in cranberry polyphenol-fortified PN matrix were rapidly digested into smaller molecular weight digestive fragments (Figure 6A). In particular, Ara h 2 and Ara h 6 (acidic subunits) as indicated in the figure were significantly digested by pepsin in both unmodified PN flour and cranberry polyphenol-fortified PN matrix after 0.5 min. However, as expected, Ara h 2 and Ara h 6 in both unmodified PN flour and cranberry polyphenol-fortified PN matrix were highly resistant to enzymatic proteolysis. In addition, digestive fragments at ~10 and ~4 kDa were observed that likely correspond to pepsin-resistant fragments of Ara h 2 and Ara h 6 as previously observed in studies working with purified individual peanut allergens.\(^{59,60}\) In fact, similar findings for Ara h 2 and Ara h 6 are not surprising, because both, although independent allergens, belong to the 2S albumin family and are 59% homologous with respect to their amino acid sequence.\(^{59}\) Even
Table 4. Amount of Individual Proanthocyanidins Sorbed onto Commercial Light Roast 12% Fat Peanut Flour after Complexation with Cranberry Juice (1:1 Dilution)

<table>
<thead>
<tr>
<th>Proanthocyanidin*</th>
<th>Concentration (mg g⁻¹)</th>
<th>N⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP1</td>
<td>0.2</td>
<td>117</td>
</tr>
<tr>
<td>DP2 A</td>
<td>5.2</td>
<td>40.6</td>
</tr>
<tr>
<td>DP2 B</td>
<td>1.4</td>
<td>29.7</td>
</tr>
<tr>
<td>DP3</td>
<td>6.8</td>
<td>52.4</td>
</tr>
<tr>
<td>DP4</td>
<td>5.3</td>
<td>63.0</td>
</tr>
<tr>
<td>DP&gt;4</td>
<td>8.0</td>
<td>80.1</td>
</tr>
<tr>
<td>Polymers</td>
<td>5.3</td>
<td>79.1</td>
</tr>
</tbody>
</table>

*DP, degree of polymerization; DP1, monomers; DP2 A, dimer type A; DP2 B, dimer type B; DP3, trimers; DP4, tetramers; DP>4, oligomers. N⁺ was estimated by external standard curves as mg proanthocyanidin sorbed to 1 g of matrix. Calculated as percent proanthocyanidin of untreated juice complexed with peanut flour.

Figure 5. Basophil activation assay of unmodified light roast 12% fat peanut (PN) flour and polyphenol-fortified peanut matrices modified with 1:1 and 1:5 dilutions of cranberry juice (CB). Data shown are percentage of basophils that are degranulated on the basis of CD63⁺ expression from seven peanut allergic subjects. Individual data are shown with significant differences indicated.

through our findings were observed in both unmodified PN flour and cranberry modified PN matrix, more conclusive interpretations of resulting digestive fragments cannot be made on the basis of a mixture of various allergic and nonallergic peanut proteins present in our samples. Whereas digestion of PN proteins resulted in digestive fragments with various molecular weights, not all showed IgE binding on the respective Western blots (Figure 6B), Pepin-resistant allergens Ara h 2 and Ara h 6 (see Figure 6A) both retained their IgE-binding capacity over the digestion period when unmodified PN flour was used, however, Ara h 6 to a much lesser extent. Ara h 2 remained immunoreactive throughout the digestion of cranberry polyphenol-fortified PN matrix, whereas IgE binding to Ara h 6 could barely be observed. Other studies also have shown that extensive digestion of peanut allergens by gastrointestinal enzymes can result in the survival or formation of peptide fragments that remain immunoreactive (intact and accessible IgE binding epitopes). SDS-PAGE provided us with general information about how or to what extent PN proteins present in unmodified PN flour or cranberry polyphenol-fortified peanut matrix were broken down by pepsin, but only limited data could be obtained as to which digestive fragment (band) belongs to which intact allergic or nonallergic PN protein. Additionally, results gained from Western blots do not determine if digestive fragments remain or do not remain immunoreactive in vivo. Pepin, trypsin, and chymotrypsin are all important enzymes that could influence the allergenicity of these cranberry-PN complexes in vivo. Thus, we next investigated the cranberry polyphenol-fortified PN matrix's allergenicity in an oral challenge in PN-allergic mice.

Oral Challenges in PN-Allergic Mice. To further evaluate the allergenic potential of the cranberry-fortified PN matrix in vivo, C3H/HeJ mice (n = 4 per group) were made allergic to PN proteins and challenged orally with either unmodified PN flour (control) or polyphenol-fortified PN matrix modified with 1:1 dilution of cranberry juice. No difference was observed in groups of mice for PN-specific IgE levels prior to oral challenge (data not shown). Levels of MMCP-1 were measured in sera of mice to gauge the ability of the cranberry polyphenol-fortified PN matrix to attenuate intestinal mucosal mast cell degranulation. Results showed that mice challenged with cranberry polyphenol-fortified PN matrix had significantly lower levels (using the Mann-Whitney test (one-tailed), we find a p value = 0.041) of MMCP-1 in their sera than mice challenged with unmodified PN flour (Figure 7). These results demonstrate that the cranberry-PN complexes are hypoallergenic in vivo because mast cells are degranulated to a lesser extent.
extent than in mice challenged with the unmodified PN flour. Together, our data showing decreased IgE binding to the major PN allergens, decreased capacity to degranulate human basophils, and decreased allergic response in vivo and in a mouse model led us to conclude that the cranberry polyphenol- fortified PN matrix is hypoallergenic.

To our knowledge, no other modified PN materials have been assessed for allergenicity in an oral challenge mouse model. However, similar assays were utilized to study egg allergens and the effects of heat-induced modifications. The authors found that heating egg allergens caused reduced IgE binding and reduced responses in basophil assays, as well as reduced allergic symptoms following an oral challenge. The research suggests that structural modifications following heating may eliminate conformational IgE-binding epitopes. It is interesting that in our work, we also observed structural changes in proteins upon complexation of PN flour with cranberry polyphenols, without heating, as evidenced by ATR-FTIR.

Collectively, these results suggest that the cranberry polyphenol-fortified PN matrix is hypoallergenic and may have potential to serve as a safer second-generation ingredient for oral immunotherapy trials. Future trials with the other promising polyphenol sources are warranted to validate their efficacy as well. Although other technologies for producing hypoallergenic PN proteins have been described, including genetic engineering, high-pressure processing, enzymatic hydrolysis, and controlled Maillard type modifications, among others, there are challenging regulatory barriers, costs, and negative public perceptions associated with many of these approaches. In contrast, the technology we describe here is of food grade, is economical, and could be readily adapted to produce sufficient quantities of polyphenol-fortified PN matrices necessary for clinical applications. Importantly, this technology has also been demonstrated for a range of food grade plant and animal proteins, suggesting it could be readily adapted to treat other food allergies.

Figure 7. MMCP-3 levels in serum 45 min following oral challenge with unmodified light roast 12% fat peanut (PN) flour or polyphenol-fortified peanut matrix modified with 1:1 dilution of cranberry juice (CB) in peanut-allergic mice (n = 4 per group). Data shown are means plus standard deviation and showed significant difference when using the Mann–Whitney test (one-tailed) at p = 0.041.

REFERENCES


CHAPTER 3

Protein-Polyphenol Interactions in Hypoallergenic Edible Peanut Matrices and their Stability during Simulated Pepsin Digestion

The content of this paper is in preparation for submission to a scientific journal
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Abstract

Allergenic peanut proteins are relatively resistant to digestion (compared to non-allergenic proteins), and if digested, metabolized peptide fragments remain relatively large and immunoreactive, triggering IgE-mediated allergic reactions in peanut-sensitive individuals. In this study, hypoallergenic peanut protein-polyphenol matrices were evaluated to determine the nature of their protein-polyphenol interactions and their stability prior to and during simulated gastric digestion (*in vitro*). Urea (1-10 M) had little effect on protein solubility of protein-polyphenol complexes, suggesting that interactions between peanut proteins and polyphenols are partially hydrophobic in nature. Polyphenol-fortified protein complexes created with cranberry and green tea polyphenols were significantly more stable (reduced protein solubility) in simulated gastric fluid at pH 2 than proteins in uncomplexed peanut flour. When subjected to pepsin, the Ara h 3 basic subunit was more rapidly digested in cranberry and green tea complexed peanut matrices compared to uncomplexed peanut flour. In addition, Ara h 2 was hydrolyzed more quickly in the cranberry complexed peanut matrix than in uncomplexed peanut flour. However, digestive fragments from cranberry and green tea complexed peanut matrices appeared to be substantially less immunoreactive (based on the capacity to bind to peanut-specific IgE from patient plasma) compared to the digestive fragments from cinnamon-peanut complexes or uncomplexed peanut flour samples.

Keywords: Peanut allergens, plant polyphenols, protein-polyphenol interactions, simulated gastric pepsin digestion, OIT
Introduction

Peanuts are widely used in a variety of foods in the United States and are a good source of protein. However, they also are one of the “big eight” allergenic foods and their ingestion by allergic individuals can result in severe consequences such as anaphylactic shock or death.¹ The prevalence of peanut allergy in North America is increasing and is currently estimated to occur in about 1% of infants or children and about 0.6% of adults.² The peanut components that trigger the allergic reaction are primarily storage proteins found in the edible seed. Peanut allergy is considered a type I hypersensitivity and is mediated by immunoglobulin E (IgE). Upon peanut consumption by a peanut allergic individual, certain parts of the allergenic proteins, known as epitopes, bind and cross-link peanut-specific IgE antibodies located on mast cell and basophil surfaces. This results in a cascade of reactions which trigger mast cells and basophils to degranulate and to release immunological mediators (such as histamine) responsible for local and/or systemic allergic symptoms.³

To date, 12 peanut allergens have been recognized by the Allergen Nomenclature Sub-Committee of the International Union of Immunological Societies.⁴ Of those, Ara h 1, Ara h 2, Ara h 3, and Ara h 6 are the major allergens of concern. Ara h 1 affects 35-95% of peanut-allergic individuals in different populations.⁵ It initially was considered to be the major allergen of concern⁶, however, several other studies have shown that Ara h 2 and Ara h 6 have a higher ability to release histamine from human basophils containing peanut-specific IgE and have a greater reactivity in skin tests in peanut-sensitive individuals compared to Ara h 1.⁷-¹¹ In addition, more than 95% of peanut-sensitive patients living in the United States have specific IgE to Ara h 2.¹⁰ Ara h 3, on the other hand, is recognized in more than 50% of
sera from peanut-allergic individuals.\textsuperscript{12,13} Ara h 3, similar to Ara h1, releases less histamine than Ara h 2 and Ara h 6 \textit{in vitro} and \textit{in vivo}.

A characteristic which peanut allergens (or food allergens) have generally in common (and which is recognized as a means to distinguish between potentially allergenic and non-allergenic proteins) is their relative resistance to digestion.\textsuperscript{14} Allergenic proteins can withstand acidic conditions found in the gastric system (stomach) and are relatively resistant to proteolytic hydrolysis (digestion) by various enzymes in the gastrointestinal tract (compared to non-allergenic proteins).\textsuperscript{15} While pepsin has been shown to be the active enzyme involved in the proteolysis of peanut allergens and substantial degradation was observed during the digestion of a protein extract from roasted peanuts, IgE binding properties of digestive peptide fragments were not affected.\textsuperscript{16,17} Allergenic proteins can be rendered less digestible by processing, and/or the structure of some peanut allergens may naturally resist digestion, allowing them to remain intact for a longer period than non-allergenic proteins. If subjected to prolonged and extensive (i.e. high pepsin concentrations) digestion conditions, these allergenic proteins may decompose into (relatively large) peptide fragments that remain immunoreactive (intact and accessible IgE binding epitopes), triggering allergic reactions in peanut-sensitive individuals.\textsuperscript{18,19}

Polyphenols found in fruits, vegetables and other edible plant sources have been shown to provide health-promoting and disease preventative benefits mainly attributable to their antioxidant and anti-inflammatory properties.\textsuperscript{20} These compounds hold promise as natural allergy-alleviating agents since they may have modulating effects on different biological pathways, and immune cell functions in an allergic immune response.\textsuperscript{21} Dietary
polyphenols have significant binding capacity to proteins which can result in the creation of soluble and insoluble protein-polyphenol complexes. Complex formation can result either from reversible non-covalent forces such as hydrogen bonding, hydrophobic interactions, or cross-linking of polyphenols between proteins\textsuperscript{22,23}, or from irreversible covalent bonds formed between polyphenols and proteins.\textsuperscript{24} These interactions can change structural, functional and nutritional properties of both proteins and polyphenols. Changes in secondary and tertiary protein structure, decreased protein solubility, reduced protein enzymatic digestibility and a loss of some amino acids may occur due to protein-polyphenol interactions.\textsuperscript{25}

Oral immunotherapy (OIT) is a strategy for treating peanut allergy that involves the administration of small doses of peanut flour, in a clinical setting, and has been reported to result in induction of clinical tolerance to different food proteins, including peanut, in some allergic patients.\textsuperscript{26} While OIT can be a promising therapeutic strategy for desensitizing peanut-sensitive individuals (preventing potentially life-threatening reactions due to an accidental ingestion of peanut), there is a risk for side effects (such as gastrointestinal problems or even anaphylactic shock). These risks currently limit wider application of OIT beyond highly controlled clinical settings.

In a recent study, we described an approach to bind selected polyphenolic plant bioactive compounds to light roasted 12% fat peanut flour, the current ingredient used for peanut-specific OIT, to create dry edible peanut protein-polyphenol matrices with reduced allergenicity (modulated IgE binding epitopes).\textsuperscript{27} We demonstrated that certain polyphenol-fortified peanut complexes showed reduced peanut-specific IgE binding in Western blots.
(particularly cranberry, cinnamon and green tea polyphenol-peanut protein matrices) and reduced basophil degranulation (cranberry polyphenol-peanut protein matrix). Cranberry polyphenol-peanut protein complexes triggered less mast cell degranulation, a marker for allergic reaction, compared to peanut flour when used to orally challenge peanut-allergic mice in vivo.\textsuperscript{27}

In the present work, select polyphenol-fortified peanut flour matrices previously identified as promising OIT candidates\textsuperscript{27} were evaluated to determine the nature of their protein-polyphenol interactions, and stability prior to and during simulated gastric digestion (\textit{in vitro}), in an effort to elucidate possible \textit{in vivo} mechanisms for reduced allergenicity. We hypothesized that certain polyphenol-fortified peanut protein complexes would promote gastric digestion of peanut-allergenic proteins (compared to unmodified peanut allergens) rendering digestive fragments less allergenic when screened for peanut-specific IgE binding capacity.

**Materials and Methods**

**Materials and Reagents**

Procyanidin A2 was obtained from Chromadex (Irvine, CA, USA). Procyanidin trimer [epi-(4β → 6, 2β → O→7)-epi- (4β → 8, 2β → O→7)-cat] and tetramer [epi-(4β → 8, 2β → O→7)-epi-(4α → 6)-epi-(4β → 8, 2β → O→7)-cat] were obtained from Planta Analytica (Danbury, CT, USA). Catechin, pepsin from porcine gastric mucosa (3802 U mg
protein\(^1\), 92% purity), urea and sugar standards myo-inositol, glucose, and sucrose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sugar standard fructose was purchased from Fisher Scientific (Pittsburgh, PA, USA). Internal sugar standards lactose and cellobiose were obtained from Eastman Kodak (Rochester, NY, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Criterion Precast Tris-HCl gels (10-20% polyacrylamide), Laemmli sample buffer and Precision Plus protein unstained standard were obtained from Bio-Rad (Hercules, CA, USA). Biotinylated goat IgG-anti-human-IgE was obtained from Kirkegaard & Perry Laboratory, Inc (Gaithersburg, MD, USA). NeutrAvidin HRP (horseradish peroxidase) and Super Signal West Pico Chemiluminescent Substrate were purchased from Thermo Scientific (Rockford, IL, USA). Bovine serum albumin (BSA), Novex 16% Tricine gels, Tricine SDS running buffer (10\(\times\)), NuPAGE reducing agent (10\(\times\)) and Novex Tricine SDS sample buffer (2\(\times\)) were purchased from Life Technologies (Grand Island, NY, USA). Tris/glycine/SDS running buffer was purchased from Invitrogen (Carlsbad, CA, USA).

**Polyphenol-Fortified Peanut Matrices Tested**

Cranberry (*Vaccinium macrocarpon* Ait.) juice concentrate (50 °Brix) was provided by Ocean Spray (Lakeville-Middleboro, MA, USA), cinnamon powder (*Cinnamomum burmannii* Blume, A grade, 3% oil) was purchased from Frontier Co-op (Norway, IA, USA) and organic green tea leaves (*Camellia sinensis* (L.) Kuntze) from Sri Lanka were provided by QTrade Teas & Herbs (Cerritos, CA, USA). Light roasted 12% fat peanut (PN) flour (*Arachis hypogaea* L.) containing 50% ± 2% protein was obtained from Golden Peanut Co.
LLC (Alpharetta, GA, USA). Cinnamon powder and green tea leaves extracts as well as cranberry juice, cinnamon powder extract or green tea leaves extract polyphenol-fortified PN matrices used in this study were prepared as described in our previous study. Briefly, PN flour was combined with (1:1) diluted cranberry juice concentrate or extracts (30 g L\(^{-1}\)), mixed for 15 min at room temperature to allow sorption of polyphenols to PN proteins, and centrifuged for 20 min at 3,434 g. The supernatant was decanted and the pellet was freeze-dried to yield the polyphenol-fortified PN matrices. A blank PN matrix was also prepared by complexing PN flour with water only.

**Protein and Sugar Quantification in Polyphenol-Fortified PN Matrices**

Percent total nitrogen in polyphenol-fortified PN matrices, blank PN matrix and PN flour was determined using a 2400 CHN Elemental Analyzer (Perkin Elmer, Norwalk, CT, USA) and converted to %protein (n=2, conversion factor 5.46 for peanut). Ion chromatography (IC) was used for the identification and quantification of mono- and disaccharides in polyphenol-fortified PN matrices, blank PN matrix and PN flour according to the method by Pattee et al. with minor changes. Briefly, residual oil in PN matrices or PN flour was extracted with hexane (n=3), and the oil-free wet pellets were subsequently dried under nitrogen and 15 mL of sugar extraction solvent (methanol:chloroform:H\(_2\)O; 60:25:15; v/v/v) was added. Samples were briefly vortexed, sonicated for 15 min, centrifuged for 10 min at 165 g and the supernatants decanted. Supernatants were evaporated overnight and residuals subsequently re-suspended in an internal sugar standard solution (containing lactose and cellobiose). Samples were filtered using Dionex OnGuard II H syringe filters.
(Dionex, Sunnyvale, CA, USA) before loading onto the column to eliminate insoluble particles and sugar interfering compounds such as free amino acids. A Dionex BioLC chromatographic system with a Dionex Pulsed Amperometric Detector (PAD) equipped with an Au electrode, a Dionex CarboPac PA1 column (250 mm x 4.6 mm) was used (Sunnyvale, CA, USA). The column oven temperature was set to 30 °C and the injection volume was 10 µL. The mobile phase consisted of NaOH (200 mM) at a constant flow rate of 1.0 ml min⁻¹. Myo-inositol, glucose, fructose and sucrose were used as standard references and internal standards used were lactose and cellobiose.

**PN Protein Precipitation Capacity**

The capacity of juice or extracts to precipitate PN proteins was investigated by complexing PN flour with juice or extracts and measuring the protein content in the soluble portion. For this, PN flour (30 g L⁻¹) was added to cranberry juice, cinnamon powder extract or green tea leaves extract, mixed, and the dispersion centrifuged (20 min at 6064 g) as previously described.²⁷ A blank PN matrix (PN flour complexed with water only) was also prepared and evaluated. Soluble protein in the supernatants after centrifugation was determined using the EZQ protein quantitation kit and ovalbumin as a reference standard (Life Technologies, Grand Island, NY, USA).

**PN Protein-Polyphenol Interactions**

To investigate non-covalent PN protein-polyphenol interactions in the polyphenol-fortified PN matrices, PN matrices or PN flour were dispersed in urea solutions and assayed
for soluble protein. Two separate experiments were conducted. Either equivalent amounts (normalized by weight) of PN matrix or PN flour (1%, w/v), or the amount of PN matrix or PN flour to provide equivalent protein contents (50 mg), was used. Samples were dispersed in 1, 2, 4, 6, 8 and 10 M aqueous urea solutions. Control dispersions in water were also prepared. Dispersions (1 mL) were vortexed for 5 min and sonicated for 10 min and subsequently centrifuged for 10 min at 13,793 g. Soluble protein, in the supernatants, was determined using the EZQ assay. Supernatants were also evaluated for simple or higher polymerized procyanidins using HPLC. Samples were filtered through 0.2 µm syringe filters and dissolved 1:1 (v/v) in deionized water prior to HPLC analysis.

Proanthocyanidins (PAC) were analyzed with a Dionex Summit HPLC system (Sunnyvale, CA, USA) consisting of a model P680 quaternary pump, an ASI-100 autosampler, a TCC-100 column oven, and an RF2000 fluorescence detector according to the method of Hammerstone et al.\textsuperscript{29} with modifications described by Constanza et al.\textsuperscript{30} Compounds were separated on a 5 µm, 250 mm × 4.6 mm, Luna silica column (Phenomenex, Torrence, CA, USA). The binary gradient consisted of dichloromethane, MeOH, water, and acetic acid (A, 82:14:2:2; v/v/v/v) and MeOH, water, and acetic acid (B, 96:2:2; v/v/v). The flow rate was set at 1 mL min\textsuperscript{−1} with a gradient as follows: 0–20 min, 0–11.7% B; 20–50 min, 11.7–25.6% B; 50–55 min, 25.6–87.7% B; 55–65 min, 87.7% B, isocratic; 65–70 min, 87.7–0% B. The peaks were monitored by fluorescence detection with excitation at 276 nm and emission at 316 nm. Quantification of compounds was performed by means of external calibration curves using monomer (DP1) through tetramer (DP4) commercial standards. PAC with DP ≥4 including polymers were quantified as DP4 equivalents.
PN Protein-Polyphenol Complex Stability

The stability of PN protein-polyphenol complexes was compared to uncomplexed PN flour under acidic conditions using simulated gastric fluid (SGF). SGF was prepared according to the United States Pharmacopeia with minor changes. A solution of 0.2% NaCl (w/v) in deionized water was prepared and adjusted to a pH of 2.0 using 6 N or 1 N HCl. In one experiment, the amount of PN matrix or PN flour to provide equivalent protein content (5, 10, 25, 50 and 100 mg) was used to evaluate possible pH changes (such as could occur in the gastric tract) and their effect on protein stability in solution. Subsequently, 1 mL dispersions of PN matrices or PN flour in SGF (w/v) were prepared without pH adjustment. The dispersions were stirred for about 10 min at room temperature, centrifuged (15 min at 13,793 g), and protein content in soluble fractions was quantified using the EZQ assay. SDS-PAGE was used to observe protein distribution in the soluble fractions following the method by Plundrich et al. with minor changes. Samples were added 1:1 to Laemmli buffer containing 5% mercaptoethanol (v/v) and subsequently heated for 5 min at 95 °C. Equivalent volume (25 µL) was loaded per lane. Loaded samples were subjected to SDS-PAGE (BioRad Criterion Precast Tris-HCl gel, 55 min, 200 V) and subsequently stained with Coomassie Blue.

In another experiment pH was adjusted to 2 after adding PN matrices or PN flour to SGF. Either equivalent amounts of PN matrix or PN flour (1, 5, 10, 15, 20 and 50 mg) was used or the amount of PN matrix or PN flour to provide equivalent protein content (5, 10, 25, 50 and 100 mg) was used. Subsequently, 10 mL dispersions of PN matrices or PN flour in
SGF (w/v) were prepared. The dispersions were stirred, centrifuged (20 min at 3434 g) and analyzed for soluble protein using the EZQ assay.

**Digestibility and IgE Binding Capacity of PN Protein-Polyphenol Complexes during Simulated Gastric Digestion**

To investigate the digestibility of PN proteins in PN protein-polyphenol complexes by pepsin compared to native proteins in uncomplexed PN flour, a simulated gastric digestion assay was used according to Plundrich *et al.* with minor changes. A dispersion of PN matrix or PN flour was prepared in SGF resulting in 55.5 mg total protein each in a total volume of 40 mL. Dispersions were adjusted to pH 2, stirred on a stir plate for 5 min, and then a 4 mL aliquot from each of the dispersions was taken and added to separate 15 mL centrifuge tubes (controls, no pepsin added). Remaining dispersions (36 mL, 50 mg protein) were placed into a 37 °C water bath and pre-warmed for 15 min while a 0.2 mg mL$^{-1}$ pepsin solution (14 U mg protein$^{-1}$) in SGF (w/v) was prepared and also pre-warmed (for only 5–10 min to prevent a possible loss of enzyme activity due to autodigestion). Four milliliters of pepsin solution were added to each tube containing the dispersions. Four milliliter digestive aliquots were taken after 0.5, 1, 2, 4, 8, 16, 30 and 60 min, added to separate 15 mL centrifuge tubes, and immediately subjected to a 90 °C water bath for 15 min to inactivate pepsin. Each control (a 4 mL aliquot of each dispersion without pepsin) underwent the same heat treatment. Throughout the digestion period samples were inverted several times to ensure proper mixing of dispersion and enzyme. Pepsin-inactivated digested samples and controls were centrifuged for 20 min at 7,921 g, transferred to 2 mL centrifuge tubes and
centrifuged a second time for 5 min at 11,750 g to remove any remaining insoluble particles. The resulting supernatants were used for further analysis. SDS-PAGE and Western blotting were used to evaluate digested samples and controls for protein distribution and IgE binding capacity. For SDS-PAGE 40 μL of undiluted sample was added to 50 μL of Novex Tricine SDS sample buffer (2×) and 10 μL of NuPAGE reducing agent (10×), resulting in a final volume of 100 μL (according to the manufacturer’s instructions). Samples were heated for 2 min at 85 °C. Ten microliters of each sample was loaded onto the gel, subjected to SDS-PAGE (Novex 16% Tricine gel, 90 min, 125 V), and stained with SimplyBlue SafeStain.

For the detection of peanut-specific IgE binding by PN proteins, Western blotting was performed with pooled plasma from two peanut-allergic individuals obtained from PlasmaLab (Everett, WA, USA). Both individuals had peanut-specific IgE > 100 kU L\(^{-1}\) as determined via ImmunoCAP (Phadia, Uppsula, Sweden). SDS-PAGE-resolved proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (iBlot, Invitrogen, Carlsbad, CA, USA). The membrane was allowed to dry (30 min), rehydrated in MeOH (10-15 sec) and washed with phosphate buffered saline (PBS) containing 0.5% Tween20 (PBST, 1x) for 5 min. The membrane was then blocked in PBST containing 2% bovine serum albumin (BSA) for 1-2 h at room temperature. The membrane was incubated overnight with a 1:40 dilution (v/v) of pooled human plasma in PBST containing 2% BSA (primary antibody), then washed in PBST four times for 5-10 min and subsequently incubated with biotinylated goat IgG-anti-human IgE (secondary antibody, 1:4,000; v/v in PBST with 2% BSA) for 1 h. The membrane was again washed four times for 5-10 min and incubated with NeutrAvidin-horseradish
peroxidase (HRP) conjugate (NA-HRP, 1:10,000; v/v in PBST with 2% BSA) for 30 min. Finally the membrane was washed for 5-10 min, incubated with Super Signal West Pico Chemiluminescent Substrate for 8 min and developed on a ChemiDoc MP system (Bio-Rad, Hercules, CA, USA).

In addition, digested samples were screened for simple or higher polymerized procyanidins using HPLC as described above. Samples were prepared in triplicate for analysis to evaluate consistency of HPLC chromatograms.

**Statistical Analysis**

All samples were prepared in triplicate, and analysis of variance (ANOVA) was performed using JMP 11.0 (SAS, Cary, NC, USA). When significant (p < 0.05), means were separated using Student’s t test.
Results and Discussion

Protein and Sugar Quantification in PN Matrices

Concentrations of total protein (%), total sugars (%), and individual sugars (mg g⁻¹) in flours or matrices are provided in Table 1. The protein content in untreated PN flour was 51.3%. Protein concentrations remained comparable in cinnamon (56.3%) and green tea (47.7%) matrices, but were significantly reduced in cranberry matrices (32.0%). Total sugars were significantly higher in cranberry and green tea matrices (9.59 mg g⁻¹ and 13.63 mg g⁻¹, respectively) as compared to the untreated peanut flour (1.61 mg g⁻¹) or the blank peanut matrix (0.41 mg g⁻¹). High levels of glucose were sorbed from cranberry juice and green tea leaves extract to PN flour, 67.9 mg g⁻¹ and 116 mg g⁻¹, respectively. It should be noted that the glucose level and total monomeric/dimeric sugars were likely overestimated in green tea matrix since one unidentified (possibly a non-acid hydrolyzable conjugated sugar) compound co-eluted with glucose and fructose and was calculated as glucose. PN matrices were also enriched with fructose from cranberry juice and cinnamon powder extract while myo-inositol and sucrose were washed out from PN flour during preparation of the fortified matrices.

PN Protein Precipitation Capacity

The potential of cranberry juice, cinnamon powder extract and green tea leaves extract to precipitate or solubilize PN proteins during the complexation process (compared to water alone, for the blank PN matrix) was investigated to determine if a loss of soluble PN
proteins contributed to the decreased protein contents for some of the polyphenol-fortified PN matrices described above. Table 2 shows amounts of PN proteins present in supernatants (which corresponds to the proteins that remained dispersed after the complexation process). The highest concentration of soluble protein (0.85 mg mL\(^{-1}\)) was found in the supernatant of the blank PN matrix (which was formed after mixing PN flour with water only). Significantly less PN protein was solubilized and lost to the supernatant when the PN flour was treated with juice or extracts, rather than plain water. While some PN proteins were extracted (solubilized) from PN flour during complexation with aqueous juice or extracts, there was much less protein solubilization and more protein precipitation than when water alone was used. These results are likely due to a) various fruit or plant compounds already present in juice or extracts, likely making it more difficult for proteins to go into solution without oversaturation and b) known protein precipitating properties of plant polyphenols.\(^{32}\)

Since the complexation process did not lead to a significant loss of proteins into solution, it is likely that observed reduced protein content in some polyphenol-fortified PN matrices (i.e. cranberry PN matrix) was a result of sorbed plant compounds (such as polyphenols or sugars) which had a diluting effect on proteins present. For example, an increase of sorbed total sugars (%) generally led to a decrease of total protein (%) compared to PN flour (Table 1). A study by Grace et al. reported similar findings working with cranberry-fortified pea or soy protein isolate matrices.\(^{33}\) On the other hand, proteins originally present in cinnamon powder extract are likely responsible for the increased protein content of cinnamon matrix compared to PN flour.\(^{34}\) Hence, based on these findings, further
experiments with these matrices were normalized to equivalent protein content and compared to findings when equal dispersions were used.

**Evaluation of PN Protein-Polyphenol Interactions**

Polyphenols can interact with proteins both irreversibly and reversibly.\(^2^4\) At alkaline pH, polyphenols are known to react with side chain amino groups of peptides (under oxidizing conditions), resulting in the formation of covalent (irreversible) protein cross-links.\(^3^5,3^6\) At pH \(\leq 7\), formation of non-covalent (reversible) bonds is favored.\(^3^7\) Since complexation of PN proteins with polyphenols from juice or extracts was performed at an acidic pH, non-covalent forces, including hydrogen bonding, Van der Waals forces, or hydrophobic effects were thought to be prevalent.\(^2^2,2^3\) Accordingly, urea was used to attempt to disrupt protein-polyphenol interactions, and soluble protein and soluble free polyphenols released were subsequently measured. Urea is an organic agent with both polar and apolar properties. It has been suggested that hydrophobic interactions with apolar protein residues as well as hydrogen bonds formed to the protein backbone (and to water molecules in the water hydrogen bond network) result in a weakened hydrophobic effect. Hence, protein stability is decreased ultimately resulting in protein denaturation (unfolding) and increased solubility in solution.\(^3^8\)

Generally untreated PN flour produced the highest yield of solubilized PN protein across all urea concentrations tested (Figure 1 A and B). Proteins from PN flour were 100% solubilized (50 mg mL\(^{-1}\)) when 8 M and 10 M urea solutions were used (Figure 1 B) indicating that all proteins were denatured into small enough particles that remained
dispersed after centrifugation. Proteins in polyphenol-fortified PN matrices were less affected by urea. In fact, even high urea concentrations did not lead to a disruption of all protein-polyphenol interactions (and protein denaturation) since significantly less proteins remained dispersed after centrifugation indicating that some particles (more or less intact protein-polyphenol complexes) remained big enough to separate from the dispersion by centrifugation (Figure 1 B). At this point, it is not clear why soluble protein from the cranberry PN matrix (Figure 1 A and B) appeared to initially increase with increased urea concentration and then decreased. This observation warrants further investigation.

Interestingly, the protein content in the cinnamon matrix was very similar to that of PN flour (56.3% and 51.3% respectively), yet proteins in the cinnamon matrix were significantly less affected by low to relatively high urea concentrations than proteins in the control sample (Figure 1 A). Since 50 mg protein corresponded to 10.0%, 18.3%, 9.43% and 11.1% dispersions (w/v) of PN flour or cranberry, cinnamon and green tea matrices, respectively, it is unlikely that a decrease in protein solubility in matrices was due to oversaturation in urea solutions used. Instead, it may be likely that the plant polyphenols formed complexes with PN proteins, causing them to have increased hydrophobicity, and making it harder for urea molecules to affect protein structure. In fact, a study by Oh et al. and later by Bennion and Daggett suggested that the mechanisms by which protein is denatured by urea are yet not fully understood. Early studies suggested that urea, based on its structure, may act as a proton donor or a proton acceptor, with the ability to form strong hydrogen bonds while they are not intrinsically hydrophobic. Bennion and Daggett further suggested that urea may act directly by protein binding or indirectly by disrupting hydrogen bond networks around the
protein, and therefore weaken hydrophobic effects as mentioned earlier. Urea was able to bind to the test protein, competing with native interactions which promoted the protein unfolding process. Both mechanisms could potentially depend on urea concentration present in solution. However, when Oh et al. investigated interactions in protein-tannin complexes they found that 6 M urea had no effect on protein-tannin complex dissociation. These investigators suggested that steric factors could have prevented urea molecules from having access to hydrogen bonds between proteins and tannins and proposed that, for proteins and tannins tested, interactions were hydrophobic in nature.

Another study by Shamanthaka Sastry et al. showed that the addition of urea (8 M) and dioxane (a hydrogen bond breaker) completely abolished the binding of chlorogenic acid to the 11S protein of sunflower seed. Results indicated that sunflower seed protein-chlorogenic acid interactions were based on hydrogen bonding. To date, mechanisms of urea-induced protein denaturation are highly discussed. Stumpe and Grubmüller as mentioned above more recently described a combination of currently viewed mechanisms (direct and indirect action of urea).

In addition, Plumb et al. evaluated the hydrophobicity of some catechins and oligomeric procyanidins by means of partition coefficient measurements between n-butanol and water and found that hydrophobicity increased with degree of procyanidin polymerization and galloylation. It is possible that hydrophobic polyphenols are associated with hydrophobic sites on PN proteins.

Our results suggest that PN protein-polyphenol interactions are partially hydrophobic in nature, however further experiments are needed to verify this and other possible non-
covalent mechanisms (such as hydrogen bonding). It should also be noted that procyanidins dissociated from protein-polyphenol complexes in all PN matrices treated with urea (Table 3). Generally, soluble procyanidin concentrations increased with an increase in urea concentration. It is possible that urea was able to dissociate protein-polyphenol complexes while protein structure or protein solubility was relatively unaffected.\textsuperscript{44} Since the urea molecule has technically five sites where hydrogen bonds may form (water has only three), it is possible that urea molecules were able to bind to protein-bound polyphenols (such as potential free hydroxyl groups) resulting in the solubilization of polyphenols but not (or to a lower extent) in protein denaturation. At this point it is unclear why in many cases the amount of solubilized procyanidins from matrices exceeded the theoretically possible highest amount soluble (PN flour-sorbed plant procyanidins plus naturally present procyanidins in PN flour). One explanation could be that procyanidins in samples used to determine the amount of procyanidins sorbed to PN matrix degraded after prolonged storage at -20 °C. This could have led to an underestimation of the amount of procyanidins present in the PN matrix. While urea was present in samples tested, HPLC analysis of urea solutions only showed no absorbance of urea at excitation and emission wavelengths used. It may also be possible that urea underwent chemical transformations in sample preparations (i.e. formation of cyanates) which may have interfered with accurate HPLC analysis of procyanidins.

**PN Protein-Polyphenol Complex Stability in Simulated Gastric Fluid**

Since allergenic proteins are known to be resistant to harsh conditions such as the highly acidic environment present in the stomach, the stability of PN protein-polyphenol
complexes compared to PN flour (= solubility capacity of PN proteins) in SGF (pH 2) was investigated using two separate approaches. In one experiment, matrices or PN flour were added to SGF in order to evaluate their capacity to possibly alter the pH of the latter and to investigate protein-polyphenol complex stability in those dispersions (Table 4). Results showed that PN flour as well as cinnamon and green tea matrices increased pH even at relatively low levels with higher levels resulting in pH values well over 5. PN flour complexed with cranberry juice, however, only resulted in a moderate pH increase, with low matrix to SGF ratios barely affecting pH. This is likely due to the acidic nature of cranberry juice (pH 2.38) compared to green tea leaves or cinnamon powder extracts (pH 5.25 and pH 4.91, respectively) used for complexation with PN flour. Soluble protein concentrations (mg mL\(^{-1}\)) are provided in Table 4. Generally, an increase in pH resulted in a decrease in protein solubility. A decrease in protein solubility in SGF generally resulted in an increase of the amount of proteins found in the insoluble portion. The isoelectric points (pI), of Ara h 1, Ara h 2, Ara h 3 and Ara h 6 are between pH 4.55 and pH 5.50\(^{15}\) hence, they will precipitate at these pH levels. In fact, Ara h 1 is thought to precipitate first (pI 4.55), followed by Ara h 6 (pI 5.00), Ara h 2 (pI 5.20) and Ara h 3 (pI 5.50). Solubility and precipitation (insolubility) of PN proteins was tracked by SDS-PAGE (Figure 2). The distribution pattern of proteins on the gel confirmed data obtained and presented in Table 4. Major PN allergens precipitated in cinnamon and green tea PN matrix samples at elevated amounts of protein tested (25 – 100 mg PN protein within matrix, reflecting high pH increase) and did not show up on the gel. Cranberry PN matrix samples (with only a moderate pH increase), on the other hand, showed a steady increase in the amount of soluble protein on the gel as amounts of protein increased.
PN proteins in PN flour were the least soluble at 25 mg protein (corresponding to a 5% dispersion; w/v), yet, increased in the 50 mg and 100 mg protein samples (corresponding to 10% and 20% dispersions). Likely, in the latter samples pH moved above the pI of some proteins, rendering some proteins more susceptible to solubilization.

Solubility of proteins depends on factors including protein type, solvent, protein to solvent ratio, presence of other compounds, or pH\textsuperscript{46-48}, and it should be noted that protein-polyphenol interactions are also affected by many of the same factors.\textsuperscript{49} Our findings show that polyphenol-fortified PN matrices and PN flour can alter the pH of SGF in a dose-dependent manner and that solubility of proteins in both PN matrices and PN flour depends on pH. Based on our findings, pH not only affects PN protein solubility but also activity and stability of the active gastric digestive enzyme, pepsin. The optimum pH for maximum activity of pepsin derived from porcine gastric mucosa used in our study, based on supplier specifications, is between pH 2 and pH 4. A study by Piper and Fenton reported a maximum peptic activity of pepsin from human gastric fundic mucosa in the range of pH 1.5 to pH 2.5, yet, noted that values may slightly vary based on method used.\textsuperscript{50} They also showed that pepsin lost about 30% of its maximum activity at a pH between 2.5 and 5, while at pH 5 to pH 7.5 no peptic activity was observed. (It should be noted that the gastric pH changes over time between the fasted and the fed state \textit{in vivo}).\textsuperscript{51}

Since the \textit{in vitro} simulated gastric digestion assay is a commonly accepted tool to evaluate digestibility of proteins\textsuperscript{14}, further experiments using SGF were performed at pH 2. Stability of proteins in polyphenol-fortified PN matrices or PN flour in SGF was evaluated on the basis of either 1) equivalent amounts of PN matrix or PN flour (Figure 3 A) or 2) a
normalized protein content in a sample of PN matrix or PN flour (Figure 3 B). Proteins from PN flour and cinnamon PN matrix were highly soluble under both conditions across all treatments in a dose-dependent manner (Figure 3 A and B). Proteins in green tea and cranberry PN matrices generally were significantly less soluble in SGF. In addition, proteins in green tea PN matrix appeared to reach their maximum solubility at 10 mg ml⁻¹ (equal to 1% dispersion, w/v); protein solubility decreased at concentrations used >10 mg ml⁻¹. PN flour complexed with cranberry juice rendered PN proteins least soluble in both conditions investigated. In fact, when equivalent amounts of protein were tested, the amounts of soluble protein from cranberry PN matrices were too close to the detection limit of the assay (close to zero), thus values were not displayed in Figure 3 B. No protein appeared to be soluble or below detection limits at 5 mg protein across all samples tested, and while for all, PN matrices and PN flour, up to 100 mg protein was used, proteins were only partially soluble across all samples. The maximum yield was observed in PN flour (100 mg) with 6.67 mg mL⁻¹ soluble protein (Figure 3 B). The reduced solubility capacity of proteins from roasted PN flour was not surprising, since it has been shown that progressive roasting can lead to decreased protein solubility (compared to raw peanuts).⁵²,⁵³ Proteins in cranberry and green tea PN matrices were significantly less soluble than proteins in PN flour or cinnamon PN matrix. However these experimental designs were possibly limited by the volume of SGF used (10 mL). Solvent volume was chosen to create dispersions without under -or oversaturation. Effects of various solvent volumes (resembling volume of gastric fluid in stomach) needs to be further addressed.
Digestibility and IgE Binding Capacity of PN Protein-Polyphenol Complexes during Simulated Gastric Digestion

Since digestion plays a crucial role in determining allergenicity of proteins, it is important to understand how polyphenols interact with PN proteins and how this interaction affects digestibility of particularly allergenic PN proteins. An in vitro simulated gastric pepsin digestion assay was used to mimic in vivo gastric pepsin digestion under acidic conditions and at a physiological temperature, and to investigate IgE binding capacity of digestive products. In contrast to previous studies, which have typically used isolated PN allergens for in vitro digestion, we used whole polyphenol-fortified PN matrices or PN flour in this work, which more closely mimics human consumption. The digestibility of proteins was evaluated by monitoring the disappearance of intact protein bands on SDS-PAGE while a potential release of complexed polyphenols was tracked by measuring their concentration in digestive samples.

Proteins from PN matrices and PN flour were rapidly digested into smaller molecular weight fragments (Figure 4 A, C, E, G). However, the degradation of some peanut allergens into peptide fragments generally occurred more rapidly in PN matrices compared to allergenic proteins in PN flour. As expected, Ara h 1, which appears as a 64 kDa band under reducing conditions\(^{19}\), was quickly digested in all samples tested after 0.5 min. This also applied to Ara h 3 acidic subunits (at 42 kDa and 45 kDa\(^{54}\)) however, the Ara h 3 basic subunit (at 25 kDa\(^{54}\)) was only rapidly digested in cranberry and green tea PN matrices. Ara h 2 and Ara h 6 in both unmodified PN flour and polyphenol-fortified PN matrices were highly resistant to enzymatic proteolysis. Ara h 2, consisting of two isomers with 16.7 kDa
and 18 kDa, remained intact after 2 min in PN flour, while after 4 min its higher molecular weight isomer disappeared. Only after 16 min was Ara h 2 entirely digested into smaller fragments. In cranberry and cinnamon PN matrices, Ara h 2 disappeared after 8 min and appeared blurry in the green tea PN matrix sample after only 1 min, making it impossible to distinguish between the two allergen isomers.

It has been suggested that protein modifications such as glycosylation or complexation with polyphenols may result in diffuse appearance of protein bands. Alvarez observed that honey protein bands appeared diffuse on SDS-PAGE, however, after polyphenol removal, clear bands appeared, indicating that honey proteins naturally associate with polyphenols. Ara h 6 was not affected by pepsin digestion until after 60 min in the cranberry PN matrix while the allergen in PN flour and cinnamon and green tea PN matrices was decomposed into smaller peptides after 16 min. In addition, digestive peptides at ~10 and ~4 kDa were seen that likely corresponded to pepsin-resistant fragments of Ara h 2 and Ara h 6. Similar observations for Ara h 2 and Ara h 6 were not surprising since they belong to the 2S albumin family and are 59% homologous with respect to their amino acid sequence. More conclusive interpretations of resulting digestive fragments cannot be made on the basis of a mixture of various allergenic and non-allergenic PN proteins present in our samples.

Whereas digestion of PN proteins resulted in generally smaller digestive fragments with different molecular weights, not all showed IgE binding on the respective Western blots (Figure 4 B, D, F, H). Studies have shown that even extensive digestion of PN allergens by gastrointestinal enzymes can result in the survival or formation of peptide fragments that
remain immunoreactive (intact and accessible IgE binding epitopes).\textsuperscript{60,61} In the present study, Ara h 2 retained its IgE binding capacity across all samples, however, compared to PN flour, it was far more pronounced in the digestive samples from the cinnamon PN matrix (even at 16 min), while no IgE binding was observed in digestive samples of cranberry PN matrix after 4 min (Figure 4 D) and after 0.5 min in digestive aliquots from green tea PN matrix (Figure 4 H). Ara h 3 appeared to remain immunoreactive in both PN flour and the cinnamon PN matrix (Figure 4 B and F). In fact, distinct protein smearing on the cinnamon SDS-PAGE was observed, which may be attributed to protein modification by polyphenols\textsuperscript{62}, and this smearing was partially recognized by IgE on the respective Western blot.

In summary, digestive samples from cranberry and green tea PN matrices showed less IgE binding compared with PN flour, whereas cinnamon PN matrix samples showed increased IgE binding capacity compared with the control. It is possible that these findings are linked to the increased stability or protein-polyphenol interactions in cranberry and green tea PN matrices as described earlier. Interestingly, although polyphenols, particularly procyanidins, were sorbed to PN flour, during the course of digestion, it appeared that fewer total procyanidins were released from PN matrices compared to PN flour by the proteolytic activity of pepsin (Table 5). This is not surprising since diffused protein bands and band smearing indicated protein modification by complexed polyphenols. Accelerated and altered degradation of certain allergens in some PN matrices compared to PN flour could possibly be due to enhanced pepsin activity by certain polyphenols present in digestive preparations. For example, a study by Tagliazucchi \textit{et al.} showed that phenolic compounds such as catechin, quercetin, epigallocatechin-3-gallate but also phenolic-rich beverages such as red wine and
green tea were able, in a dose-dependent manner, to affect $V_{\text{max}}$ of pepsin by increasing its initial velocity when denatured hemoglobin was degraded.\textsuperscript{63} Another study, working with PN extract from raw peanuts revealed that a catechin-enriched polyphenol green tea extract promoted pepsin activity in a concentration-dependent manner and resulted in a more rapid digestion of major PN allergens Ara h 1 and Ara h 2.\textsuperscript{64} Interestingly, phenol oxidase products of catechin-enriched polyphenol green tea extract (condensed polyphenols) and laccase/green tea cross-linked proteins had interfering effects on proteolysis of $\alpha$-lactalbumin and $\beta$-lactoglobulin but not on proteins in the PN extract. In general, polyphenols are able to bind to enzymes (proteins), potentially changing their conformation and activity and rendering them either more active or impaired.\textsuperscript{63,65}

SDS-PAGE revealed general information about how or to what extent PN proteins present in PN matrices or PN flour were broken down by pepsin, but only limited data could be obtained as to which digestive fragments belong to which intact allergenic or non-allergenic PN proteins. It should also be recognized that results gained from Western blots do not determine if digestive fragments remain or do not remain immunoreactive \textit{in vivo}. While these blots allow for evaluation of IgE binding capacity of different samples (to select potential hypoallergenic candidates) it does not imply that a PN-allergic reaction would or would not occur since the critical step \textit{in vivo} is the cross-linking capacity of peanut-specific IgE with receptors on mast cells or basophils, triggering them to release inflammatory mediators such as histamine responsible for allergic symptoms.

When food is ingested and has orally been pre-digested and partially been broken down, it is subjected initially to gastric processing for variable times. The pH in the stomach
during digestion can vary based on factors including food volume, meal composition, presence of pH-influencing drugs such as antacids, or even on the individual.\textsuperscript{66,67} Since PN-allergic patients undergoing OIT are advised to ingest PN flour (mixed in a food vehicle such as yoghurt or apple sauce) on a full stomach\textsuperscript{26}, these factors should be taken into account for future digestion studies on PN matrices. In addition, trypsin, and chymotrypsin were not evaluated in this study, but are, together with pepsin, all important enzymes that could influence the digestibility and allergenicity of tested PN protein-polyphenol complexes \textit{in vivo}. Furthermore, since protein-bound polyphenols altered digestibility of PN proteins, follow-up experiments need to investigate if protein-polyphenol interactions possibly create new allergens towards which a sensitive individual could develop a reaction to.

### Conclusions

The aim of the present study was to obtain better insights into mechanisms responsible for the hypoallergenic potential of certain polyphenol-fortified PN matrices. The ultimate goal of our work is to create hypoallergenic chimeric PN protein-polyphenol complexes, which potentially could find use as safer OIT treatments with reduced side effects or complications. To our knowledge, no \textit{in vitro} stability/digestive studies have previously been performed on PN protein-polyphenol complexes using whole PN flour (as opposed to PN extract or isolated PN allergens). Thus, we investigated the nature of protein-polyphenol interactions as well as the stability of protein-polyphenol complexes prior to and during gastric digestion in a well-established simulated gastric digestion assay. Collectively, our results suggest that PN protein-polyphenol complexes may be less immunoreactive passing
through the digestive tract in vivo possibly resulting in decreased IgE binding and cross-linking capacities and ultimately less or alleviated allergic reactions. Extended, similar studies in vivo need to be done to further evaluate the hypoallergenic potential and mechanism of the most promising polyphenol-fortified PN matrices, which appear to be cranberry and green tea PN matrices. Furthermore, this complexing technology has also been demonstrated for a range of other food grade plant and animal proteins proposing a potential means to treat other food allergies.

Acknowledgments

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### Table 1. Total Protein, Total Sugars and Individual Sugars Present in Peanut Flour and Polyphenol-Fortified Peanut Matrices.\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>total protein(^b) (%)</th>
<th>total sugars(^c) (%)</th>
<th>individual sugars(^d) (mg g(^{-1}))</th>
<th>others(^e) (%)</th>
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<tbody>
<tr>
<td>PN flour</td>
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<td>1.61 c</td>
<td>3.40 a</td>
<td>0.23 c</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0.12 c</td>
<td>12.07 a</td>
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<td>blank(^f)</td>
<td>57.77 a</td>
<td>0.41 c</td>
<td>0.05 c</td>
<td>0.00 c</td>
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<td></td>
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<td>cranberry PN matrix</td>
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<td>9.59 b</td>
<td>0.65 b</td>
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<td>0.80 b</td>
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<td>2.49 b</td>
<td>0.96 c</td>
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<tr>
<td>green tea PN matrix</td>
<td>47.74 d</td>
<td>13.63 a</td>
<td>3.36 a</td>
<td>115.99(^e) a</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>ND(^h)</td>
<td>1.63 c</td>
</tr>
</tbody>
</table>

\(^a\) Values, on dry-weight basis, within each column with different letters are significantly different at p < 0.05.

\(^b\) Determined by %total nitrogen.

\(^c\) Total and individual sugars were determined by ion chromatography using internal standard references.

\(^d\) Estimated by comparison with internal standards as mg sugar type present in one gram of matrix or flour.

\(^e\) Calculated by subtracting %total protein and %total sugars from 100%, dry-weight basis.

\(^f\) Peanut flour complexed with water only.

\(^g\) Peak co-eluted with glucose indicated conjugated compound/s and was combined and expressed as glucose.

\(^h\) ND, not detectable (peak was overlapped by undefined conjugated compound/s).
Table 2. Protein Precipitation Capacity of Juice or Extracts during the Preparation of Protein-Polyphenol Complexes Evaluated as Protein Remaining Dispersed after the Complexation Process.$^a$

<table>
<thead>
<tr>
<th></th>
<th>soluble protein$^b$ (mg mL$^{-1}$)</th>
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</thead>
<tbody>
<tr>
<td>blank$^c$</td>
<td>0.85 a</td>
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<tr>
<td>cranberry PN matrix</td>
<td>0.19 b</td>
</tr>
<tr>
<td>cinnamon PN matrix</td>
<td>0.15 b</td>
</tr>
<tr>
<td>green tea PN matrix</td>
<td>0.01 c</td>
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</tbody>
</table>

a) Values within each row with different letters are significantly different at $p < 0.05$.
b) Measured via fluorescence with excitation and emission wavelengths 485 and 590 nm.
c) Peanut flour complexed with water only.
### Table 3. Individual Procyanidins from Juice or Extracts Sorbed to Peanut (PN) Matrices and Stability of Protein-Polyphenol Complexes in Urea Evaluated by Means of Free Procyanidins (µg mL⁻¹). a

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<th>2 M</th>
<th>4 M</th>
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<th>8 M</th>
<th>10 M</th>
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</tr>
</tbody>
</table>

a) Values within each row with different letters are significantly different at p < 0.05.

b) Free polyphenols in samples pre and post urea treatment were measured by HPLC using external standard curves and expressed as µg procyanidin per mL solvent.

c) DP, degree of polymerization; DP1, monomers; DP2, dimers; DP3, trimers; DP≥4, tetramers and oligomers.

d) Concentration of procyanidins in supernatant (left after complexation with peanut flour) subtracted from procyanidin concentrations in original juice or extracts (before complexation) in µg mL⁻¹.

e) Control, peanut matrix in deionized water only.

f) NA, not applicable.

g) ND, not detectable.
Table 4. Stability of Protein-Polyphenol Complexes in pH 2 Simulated Gastric Fluid without pH Adjustment.\(^a\)

<table>
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<tr>
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<th>% dispersion (w/v)</th>
<th>pH(^c)</th>
<th>protein in dispersion (mg)</th>
<th>protein fraction(^b) (mg mL(^{-1}))</th>
<th>protein fraction(^b) (mg mL(^{-1}))</th>
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<td>0.27 b</td>
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</table>

\(^a\) Values within each column (with equivalent amount of protein in matrix) with different letters are significantly different at \(p < 0.05\).

\(^b\) Stability was measured by means of protein solubility in both, soluble (supernatant, in pH 2 simulated gastric fluid) and insoluble (pellet, solubilized in LDS) fractions.

\(^c\) Approximate pH value ranges are given based on repeated experiments.

\(^d\) Sample exceeded effective range of the assay used.

\(^e\) NE, not evaluated (dispersions too thick in LDS).
Table 5. Individual Procyanidins from Juice or Extracts Sorbed to Peanut (PN) Matrices and Stability of Protein-Polyphenol Complexes during Simulated Gastric Pepsin Digestion Evaluated by Free Procyanidins (µg mL\(^{-1}\)) in Digestive Aliquots Taken at Different Time Points.\(^{a}\)

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<th>1 min</th>
<th>2 min</th>
<th>4 min</th>
<th>8 min</th>
<th>16 min</th>
<th>30 min</th>
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<td>55 ab</td>
<td>52 c</td>
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a) Values within each row with different letters are significantly different at p < 0.05.
b) Free polyphenols in samples pre digestion and post digestion were measured by HPLC using external standard curves and expressed as µg procyanidin per mL solvent.
c) DP, degree of polymerization; DP1, monomers; DP2, dimers; DP3, trimers; DP≥4, tetramers and oligomers.
d) Concentration of procyanidins in supernatant (left after complexation with peanut flour) subtracted from procyanidin concentrations in original juice or extracts (before complexation) in µg mL<sup>-1</sup>.
e) Control, peanut matrix in simulated gastric fluid (pH 2) only (time point 0 min, before pepsin was added).
f) NA, not applicable
g) ND, not detectable
Figure 1. Protein concentration of dispersions of polyphenol-fortified peanut (PN) matrices and PN flour in urea. (A) normalized to weight (1% wet weight; w/v) or (B) normalized to protein (50 mg). Data shown are means plus standard deviation. Values within each treatment with different letters are significantly different at p < 0.05.
Figure 2. SDS-PAGE showing protein stability of proteins from polyphenol-fortified peanut (PN) matrices and PN flour in pH 2 simulated gastric fluid without pH adjustment. Top row: in pH 2 simulated gastric fluid; Bottom row: insoluble fractions, solubilized in LDS. Approximate locations for peanut allergens are shown.
Figure 3. Protein concentration of dispersions of polyphenol-fortified peanut (PN) matrices and PN flour in simulated gastric fluid at pH 2 with pH adjustment. (A) normalized to weight (1, 5, 10, 15, 20 and 50 mg) or (B) normalized to protein (50 mg). Data shown are means plus standard deviation. When significantly different at p < 0.05 values within each treatment were letter separated.
Figure 4. Pepsin simulated gastric digestion of polyphenol-fortified peanut (PN) matrices and peanut PN flour and their respective controls (CTL; PN matrices or PN flour in simulated gastric fluid at pH 2 and no pepsin added) at time points 0.5, 1, 2, 4, 8, 16, 30 and 60 min: (A, C, E, G) SDS-PAGE under reducing conditions; (B, D, F, H) associated Western blot. Approximate locations for peanut allergens are shown.
REFERENCES


57. Alvarez, L.M. Honey proteins and their interaction with polyphenols. *Brock University, Ontario, Canada 2010*,


CHAPTER 4

Conclusions and Future Perspectives
With this work we successfully demonstrated the feasibility for creation of hypoallergenic plant polyphenol-fortified peanut matrices using light roasted 12% fat peanut flour, the current active ingredient in peanut oral immunotherapy, and plant-derived polyphenols. Future studies need to be performed to more extensively evaluate the hypoallergenic potential of the most promising matrices tested. *In vivo* work using murine models (such as peanut allergic mice), in particular, will be required to evaluate if reduced IgE binding capacity observed *in vitro* exclusively results in decreased cross-linking with mast or basophil cells, their degranulation and ultimately reduced allergic reactions. Additional *in vitro* experiments will be required to better understand mechanisms by which plant polyphenol-modified peanut allergenic proteins are rendered less immunoreactive in peanut sensitive animals. For example, circular dichroism (CD) in the UV wavelength range can be used to further investigate changes in secondary or tertiary protein structure. This method takes advantage of optical active (chiral) molecules, such as amino acids, and their associated differential absorption of left and right circularly polarized light. It could also be used to explore further how denaturing agents such as urea affect protein structure of native proteins (in peanut flour) versus altered proteins (in polyphenol-fortified peanut matrices).

IgE epitope mapping can potentially be used to address possible IgE binding epitope modifications resulting from protein-polyphenol complexation (and which at least partially could be responsible for the hypoallergenic nature of polyphenol-fortified peanut matrices). Other digestive models, including additional physically relevant proteolytic enzymes such as trypsin or chymotrypsin, can be a means to mimic digestion of modified proteins and to get more insight into their degradation behavior.
Finally, clinical OIT trials will be required to definitively assess whether polyphenol-fortified peanut matrices and their respective modified peanut allergens result in reduced or alleviated allergic reactions and promote allergen desensitization in sensitive individuals.

Furthermore, other food allergies including milk, wheat or egg allergy are common and are also triggered by specific allergenic proteins. Thus, it can be hypothesized that the presented novel technology could potentially be used to create other polyphenol-protein matrices that could find applications in allergen-specific oral immunotherapy.