

University of Nebraska - Lincoln

DigitalCommons@University of Nebraska - Lincoln

Dissertations, Theses, & Student Research in
Food Science and Technology

Food Science and Technology Department

5-2013

Risk Assessment of Trace and Undeclared Allergens in Processed Foods

Benjamin C. Remington

University of Nebraska-Lincoln, ben.c.remington@gmail.com

Follow this and additional works at: <https://digitalcommons.unl.edu/foodscidiss>



Part of the [Other Food Science Commons](#)

Remington, Benjamin C., "Risk Assessment of Trace and Undeclared Allergens in Processed Foods" (2013). *Dissertations, Theses, & Student Research in Food Science and Technology*. 32.
<https://digitalcommons.unl.edu/foodscidiss/32>

This Article is brought to you for free and open access by the Food Science and Technology Department at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Dissertations, Theses, & Student Research in Food Science and Technology by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

RISK ASSESSMENT OF TRACE AND UNDECLARED ALLERGENS
IN PROCESSED FOODS

By

Benjamin C. Remington

A DISSERTATION

Presented to the Faculty of
The Graduate College at the University of Nebraska
In Partial Fulfillment of Requirements
For the Degree of Doctor of Philosophy

Major: Food Science and Technology

Under the Supervision of Professors Stephen L. Taylor and Joseph L. Baumert

Lincoln, Nebraska

May, 2013

RISK ASSESSMENT OF TRACE AND UNDECLARED ALLERGENS
IN PROCESSED FOODS

Benjamin C. Remington, Ph.D.

University of Nebraska, 2013

Advisers: Stephen L. Taylor and Joseph L. Baumert

Minimal eliciting doses for objective allergic reactions were found for 13 priority allergens and over 1800 individuals from published clinical literature or unpublished clinical data. Allergic populations did not vary when analyzed by age or geographic region. Results of this study show there are sufficient clinical data from food allergic individuals to use for risk assessment purposes for several allergenic foods.

Of 186 food products bearing advisory statements regarding peanut or 16 products that had peanut listed as a minor ingredient, 8.6% and 37.5% contained detectable levels of peanut (>2.5 ppm whole peanut). An additional market survey of 215 nutrition bars with peanuts as a minor ingredient and/or an advisory statement for peanuts found 24.6% tested positive for peanut compared to 4% of products with no mention of peanuts on the label. Probabilistic risk assessment showed the risk of reaction among peanut allergic consumers from advisory labeled nutrition bars was significant but brand-dependent. The probabilistic approach provides the food industry with a quantitative method to assist with determining when advisory labeling is most appropriate.

Agricultural commodity cross-contamination of soybean was detected in 62.8% of samples representing all forms of wheat flour. Conservative probabilistic risk assessments predict a risk of allergic reaction occurring in the most sensitive soy-allergic individuals. Experimental milling and stream separation with spiked soy in wheat

samples did not produce a soy-free wheat flour stream. Additional cleaning measures will be needed to remove soy before wheat milling begins.

LC-MS/MS identified fourteen known allergens in industry representative soy product samples, including all subunits of Gly m 5 (β -conglycinin) and Gly m 6 (glycinin), the Kunitz trypsin inhibitor, Gly m Bd 28K, and Gly m Bd 30K. Method refinements or different techniques might be necessary to detect low abundance proteins such as Gly m 3 and 4. The relative amount of an allergen in a sample correlated positively to the intensity of IgE binding at the expected molecular weight using sera from soy-allergic individuals.

For my parents, Glen and Jamae.

Thank you for your constant support while I explore the parts of life that make me happy.

ACKNOWLEDGEMENTS

I would like to thank my advisers Drs. Steve Taylor and Joe Baumert for their support and guidance during the completion of my graduate program. Both have been outstanding mentors and their willingness to expose me to multiple aspects of the Food Allergy Research and Resource Program has offered invaluable experience as a graduate student. I will forever be grateful for opportunities and involvements they let me explore and push to be a part of.

I would also like to thank many other members of FARRP. Lynn and Deb, thank you for the endless help and teachings throughout my time in the lab. Julie, thank you for the friendship, lab expertise, and honest conversations over the years. Jamie and Pat, thank you for your patience, support, and ability to make everything run smoothly. To my fellow graduate students, especially Jelena Spiric, Kaye Ivens, Poi-Wah Lee, and Rakhi Panda, thank you for the advice, assistance, and making the office a fun place to work every day. Special thanks goes to Sean, Anna, Ferlie, and all of the analytical lab members that helped with kit techniques and other analytical issues.

I would like to acknowledge Dr. Geert Houben of TNO, Dr. René Crevel of Unilever, and the FARRP Board of Directors for their contributions, comments, and critiques on different aspects of the food industry and food allergen risk assessment within my project.

Finally, I would like to thank my parents, siblings, and other friends and family for their support during my dissertation. I would not be where I am today without them and I am truly grateful for the influences they have had on my life.

TABLE OF CONTENTS

LIST OF TABLES	xi
LIST OF FIGURES	xv
CHAPTER 1: LITERATURE REVIEW	1
I. INTRODUCTION	1
II. FOOD SENSITIVITIES	1
a. Food Intolerances	3
i. Anaphylactoid Reaction	3
ii. Metabolic Food Disorders	3
iii. Idiosyncratic Reactions	4
b. Food Hypersensitivity	5
i. Immune System Overview	5
ii. Cell-Mediated Hypersensitivity	9
iii. IgE-mediated Hypersensitivity and Food Allergy	10
c. Prevalence of IgE-Mediated Food Allergy	13
d. Diagnosis of Food Allergy	16
e. Treatment of Food Allergy	18
f. Legume Allergy	20
g. Soy Proteins and Allergens	25
III. FOOD ALLERGEN LABELING AND THRESHOLDS	38
a. Importance of Thresholds	39
b. Interval-Censoring Survival Analysis	43

IV.	ADVISORY LABELING OF FOOD ALLERGENS IN PROCESSED	
	FOODS	46
	a. History of Use	46
	b. Regulatory Guidance	47
	c. Previous Examples/Studies	49
V.	COMMODITY CONTAMINATION WITHIN FOOD SUPPLY	50
	a. History of Use	50
	b. Regulatory Guidance	50
	c. Previous Examples/Studies	51
VI.	FOOD ALLERGEN RISK ANALYSIS	52
	a. Risk Assessment	55
	i. Hazard Identification	55
	ii. Hazard Characterization.....	56
	iii. Exposure Assessment.....	57
	iv. Risk Characterization.....	58
	1. NOAEL-Based Safety Assessment Method	59
	2. Benchmark Dose method.....	61
	3. Quantitative Risk Assessment.....	63
	b. Risk Management	68
	i. Preliminary Risk Management Activities	69
	ii. Evaluation of Risk Management Options	69
	iii. Implementation of the Risk Management Decision.....	70
	iv. Monitoring and Review	71

c. Risk Communication	72
VII. SUMMARY	73
VIII. REFERENCES	75
CHAPTER 2: PRIORITY FOOD ALLERGEN THRESHOLDS AND METHODS TO IMPROVE CLINICAL FOOD CHALLENGE TRIALS	94
I. ABSTRACT.....	95
II. INTRODUCTION	96
III. MATERIALS AND METHODS.....	99
a. Food Allergen Thresholds.....	99
b. Methods to Improve Clinical Food Challenges	103
i. Ideal Number of Subjects in a Study	103
ii. Food Challenge Dose Scheme Comparisons	103
IV. RESULTS AND DISCUSSION.....	104
V. CONCLUSION	169
VI. REFERENCES	171
CHAPTER 3: QUANTITATIVE RISK ASSESSMENT OF FOODS CONTAINING PEANUT ADVISORY LABELING	180
I. ABSTRACT.....	181
II. INTRODUCTION	182
III. MATERIALS AND METHODS.....	185
a. Peanut Advisory Labeling Studies.....	185
i. 2009 Packaged Food Samples	185
ii. Peanut Analysis.....	185

iii.	2010 Nutrition Bar Market Survey	185
b.	Quantitative Approach to Risk Assessment.....	186
c.	Software and Statistics	190
IV.	RESULTS	190
V.	DISCUSSION	196
VI.	REFERENCES	200
VII.	TABLES AND FIGURES	203
CHAPTER 4: SOY IN WHEAT - CONTAMINATION LEVELS AND RISK		
	ASSESSMENT	209
I.	ABSTRACT.....	210
II.	INTRODUCTION	211
III.	MATERIALS AND METHODS.....	214
a.	Wheat Flour Market Survey.....	214
b.	Quantitative Risk Assessment of Soy in Commercially Available Wheat Flours	214
c.	Stream Analysis During the Milling Process of Clean Wheat Seed Spiked with Soy	217
d.	Software and Statistics	218
IV.	RESULTS	219
V.	DISCUSSION	222
VI.	ACKNOWLEDGEMENTS	228
VII.	REFERENCES	229
VIII.	FIGURES AND TABLES	232

CHAPTER 5: QUANTITATIVE ANALYSIS OF INDIVIDUAL SOY ALLERGENS IN PROCESSED SOY PRODUCTS	241
I. ABSTRACT.....	242
II. INTRODUCTION	243
III. MATERIALS AND METHODS.....	245
a. Soy Products Used in the Study.....	245
b. Relative Quantification of Soy Allergens Using Mass Spectrometry	247
i. Protein Digestion	247
ii. LC-MS/MS Analysis	248
iii. MASCOT Database Analysis	248
c. IgE Binding Studies with Soy Sensitive Subjects.....	249
i. Human Sera.....	249
ii. Extraction of Soy Proteins	252
iii. SDS-PAGE Procedures.....	252
iv. Immunoblotting Procedures.....	253
IV. RESULTS AND DISCUSSION	254
V. CONCLUSSION	276
VI. REFERENCES	278

TABLES

Page

CHAPTER 1

Table 1	Criteria for inclusion in peanut threshold dataset	43
Table 2	Eliciting dose estimations for peanut allergic population.....	45

CHAPTER 2

Table 1	Conversion factors used to normalize the threshold challenge material	101
Table 2	Peanut threshold data gleaned from published DBPCFC clinical records.....	106
Table 3	Milk threshold data gleaned from published DBPCFC clinical records.....	110
Table 4	Egg threshold data gleaned from published DBPCFC clinical records.....	113
Table 5	Hazelnut threshold data gleaned from published DBPCFC clinical records.....	116
Table 6	Soy threshold data gleaned from published DBPCFC clinical records.....	120
Table 7	Wheat threshold data gleaned from published DBPCFC clinical records.....	123
Table 8	Cashew threshold data gleaned from published DBPCFC clinical records.....	126
Table 9	Mustard threshold data gleaned from published DBPCFC clinical records.....	129

Table 10	Lupin threshold data gleaned from published DBPCFC clinical records.....	132
Table 11	Sesame threshold data gleaned from published DBPCFC clinical records.....	135
Table 12	Shrimp threshold data gleaned from published DBPCFC clinical records.....	138
Table 13	Celery threshold data gleaned from published DBPCFC clinical records.....	141
Table 14	Fish threshold data gleaned from published DBPCFC clinical records.....	141
Table 15	Estimated eliciting doses for peanut and hazelnut as affected by age	145
Table 16	Estimated eliciting doses for peanut and milk as affected by the geographic region of the clinic	148
Table 17	Estimated eliciting doses for peanut, milk, and egg as affected by challenge dose material.....	151
Table 18	Estimated eliciting doses for peanut and milk as affected by study population	155
Table 19	Comparison of potential methods for deriving regulatory thresholds for individual allergenic foods	159
Table 20	Probability of a randomly selected subject population being significantly different than the known peanut allergic population (nonparametric methods).....	163
Table 21	Probability of a randomly selected subject population being significantly different than the known peanut allergic population (84% confidence intervals method)	163
Table 22	Dose schemes selected for population simulations.....	165

Table 23	Probability of the dose scheme creating a nonparametric dose distribution from randomly selected subjects that is significantly different than the VITAL egg, peanut, and soy flour populations	167
----------	---	-----

CHAPTER 3

Table 1	Concentration and serving-size doses of peanut in 2009 survey of packaged foods bearing allergy advisory statements for peanut	205
Table 2	Concentration of peanut in packaged nutrition bars in 2010 market survey	206
Table 3	Consumption of nutrition bars per day in the United States based on the 2003-2008 NHANES database	207
Table 4	Inputs and results of the probabilistic modeling approach to risk assessment of nutrition bars with advisory labeling for peanut	208

CHAPTER 4

Table 1	Screen sizes and destinations for higher flour yield with hard wheat.....	234
Table 2	Concentration of soybean in packaged wheat flours	235
Table 3	Consumption of wheat flour in the United States per eating occasion	236
Table 4	Consumption of chocolate chip cookies per day in the United States based on the 2003-2006 NHANES database	237
Table 5	Simulation results.....	238
Table 6	Stream analysis of milled wheat samples	240

CHAPTER 5

Table 1	Soy products acquired for allergen evaluation.....	246
---------	--	-----

Table 2	Sera from 31 soybean-allergic individuals and one control subject without soybean allergy used to investigate IgE binding patterns to a spectrum of soy ingredients and products	250
Table 3	Representative soy-allergic sera used in the IgE binding study	251
Table 4	Allergenic soy proteins of interest identified during LC-MS/MS relative quantification	255

FIGURES

	Page
CHAPTER 1	
Figure 1	Classifications of different types of food sensitivities2
Figure 2	Classes of hypersensitivity reactions8
Figure 3	Mechanism of IgE-mediated food allergy12
Figure 4	Individually weighed particulate peanut samples42
Figure 5	Diagram of Interval Censoring Survival Analysis.....44
Figure 6	Dose distribution of the peanut population threshold curve45
Figure 7	The interactive processes involved in risk analysis53
Figure 8	Probabilistic risk assessment model for food allergens64
Figure 9	Quantitative risk assessment step-by-step decision tree68
CHAPTER 2	
Figure 1	Peanut log-logistic, log-normal, and Weibull (A) probability distribution models and (B) ED estimates with 95% confidence intervals107
Figure 2	Log-normal probability distribution models of peanut from 3 dataset expansions.....108
Figure 3	Milk log-logistic, log-normal, and Weibull (A) probability distribution models and (B) ED estimates with 95% confidence intervals111
Figure 4	Egg log-logistic, log-normal, and Weibull (A) probability distribution models and (B) ED estimates with 95% confidence intervals114
Figure 5	Hazelnut log-logistic, log-normal, and Weibull (A) probability distribution models and (B) ED estimates with 95% confidence intervals117

Figure 6	Soy flour log-logistic, log-normal, and Weibull (A) probability distribution models and (B) ED estimates with 95% confidence intervals.....	121
Figure 7	Wheat log-logistic, log-normal, and Weibull (A) probability distribution models and (B) ED estimates with 95% confidence intervals.....	124
Figure 8	Cashew log-logistic, log-normal, and Weibull (A) probability distribution models and (B) ED estimates with 95% confidence intervals.....	127
Figure 9	Mustard log-logistic, log-normal, and Weibull (A) probability distribution models and (B) ED estimates with 95% confidence intervals.....	130
Figure 10	Lupin log-logistic, log-normal, and Weibull (A) probability distribution models and (B) ED estimates with 95% confidence intervals.....	133
Figure 11	Sesame log-logistic, log-normal, and Weibull (A) probability distribution models and (B) ED estimates with 95% confidence intervals.....	136
Figure 12	Shrimp log-logistic, log-normal, and Weibull (A) probability distribution models and (B) ED estimates with 95% confidence intervals.....	139
Figure 13	All 11 allergen log-logistic, log-normal, or Weibull (A) probability distribution models and (B) ED10 estimates with 95% confidence intervals.....	143
Figure 14	Probability distribution models based on the age of the allergic individual at challenge (a) Peanut (b) Hazelnut.....	146
Figure 15	Probability distribution models based on the geographic region during challenge (a) Peanut (b) Milk	149
Figure 16	Probability distribution models based on the dose material given at challenge (a) Peanut (b) Milk (c) Egg.....	152
Figure 17	Probability distribution models based on the study population (a) Peanut (b) Milk	156

Figure 18	Predicted log-normal ED10 after random selection of 10, 20, 30, 50, 100, or 200 subjects from the known peanut dataset	165
-----------	--	-----

Figure 19	Visual depiction of selected scheme fits	168
-----------	--	-----

CHAPTER 3

Figure 1	Probabilistic risk assessment model for food allergens	203
----------	--	-----

Figure 2	Quantitative risk assessment step-by-step decision tree	204
----------	---	-----

CHAPTER 4

Figure 1	Probabilistic risk assessment model for food allergens	232
----------	--	-----

Figure 2	Diagram of the Bühler Experimental Mill (Model MLU-202)	233
----------	---	-----

Figure 3	Thresholds of predicted reactions during probabilistic risk assessment	239
----------	--	-----

CHAPTER 5

Figure 1	Relative quantitation of soy allergens grouped by allergen	256
----------	--	-----

Figure 2	Relative quantitation of soy allergens grouped by individual products	258
----------	---	-----

Figure 3	SDS-PAGE gels of soy products under non-reducing (A) and reducing (B) conditions	260
----------	--	-----

Figure 4	Serum 668 IgE immunoblot of soy products under non-reducing (A) and reducing (B) conditions	261
----------	---	-----

Figure 5	Serum 422 IgE immunoblot of soy products under non-reducing conditions	263
----------	--	-----

Figure 6	Serum 681 IgE immunoblot of soy products under non-reducing conditions	264
----------	--	-----

Figure 7	Serum SP902 IgE immunoblot of soy products under non-reducing (A) and reducing (B) conditions.....	265
Figure 8	Serum SP909 IgE immunoblot of soy products under non-reducing conditions.....	266
Figure 9	Serum SP903 IgE immunoblot of soy products under non-reducing conditions.....	267
Figure 10	Serum ACG IgE immunoblot of soy products under non-reducing conditions.....	268
Figure 11	Serum 893 IgE immunoblot of soy products under non-reducing conditions.....	269
Figure 12	Control Serum 1 IgE immunoblot of soy products under non-reducing conditions.....	270

CHAPTER 1: LITERATURE REVIEW

Introduction

Recognized by the “Father of Medicine” Hippocrates, food allergies and intolerances have been documented for millennia (Adams, 1886). However, the incidence of food allergies and related diseases did not take prominence in medical literature until the 20th century. Currently, the only treatment available for food allergic individuals is a strict avoidance diet which can cause anxiety issues for all involved (de Blok et al., 2007; Taylor et al., 1986a). The prevalence of food allergies is globally on the rise and a significant amount of research is dedicated to find the cause, treatment, and cure for food allergies. This review will focus on the types of adverse reactions to food, with special attention paid to food allergy: its mechanisms, prevalence, diagnosis, and treatments. Additionally, thresholds of reactivity for allergic individuals and populations and risk assessment methods for food allergens based upon the threshold distribution will be presented within the framework of current regulations and relevant food industry situations.

Food sensitivities

As stated by Taylor (1987), “food sensitivity has become the accepted term to describe the broad range of individualistic adverse reactions to foods.” Food sensitivities are separated into primary and secondary subclassifications. Primary sensitivities are more common than secondary sensitivities and include true immunologically mediated food allergies and other non-immunological food intolerances (Figure 1). Food hypersensitivities/allergies are separated into immunoglobulin E (IgE)-mediated immediate reactions and non-IgE cell-mediated delayed reactions. Non-immunological

food intolerances are further classified as anaphylactoid reactions, metabolic food disorders, and idiosyncratic reactions. Most food intolerances have mild symptoms and individuals can tolerate minor exposures as part of a restrictive diet without eliciting symptoms. In comparison, allergic individuals must follow a more restrictive avoidance diet due to the potential severity of their disease and lower thresholds for some individuals (Taylor and Hefle, 2006b). Secondary sensitivities can result in the same ailments as primary sensitivities due to the effects from numerous gastrointestinal conditions or drug treatments. Secondary conditions are usually temporary but can enhance the chances of developing permanent food allergies, lactose intolerance, or celiac disease (Taylor, 1987).

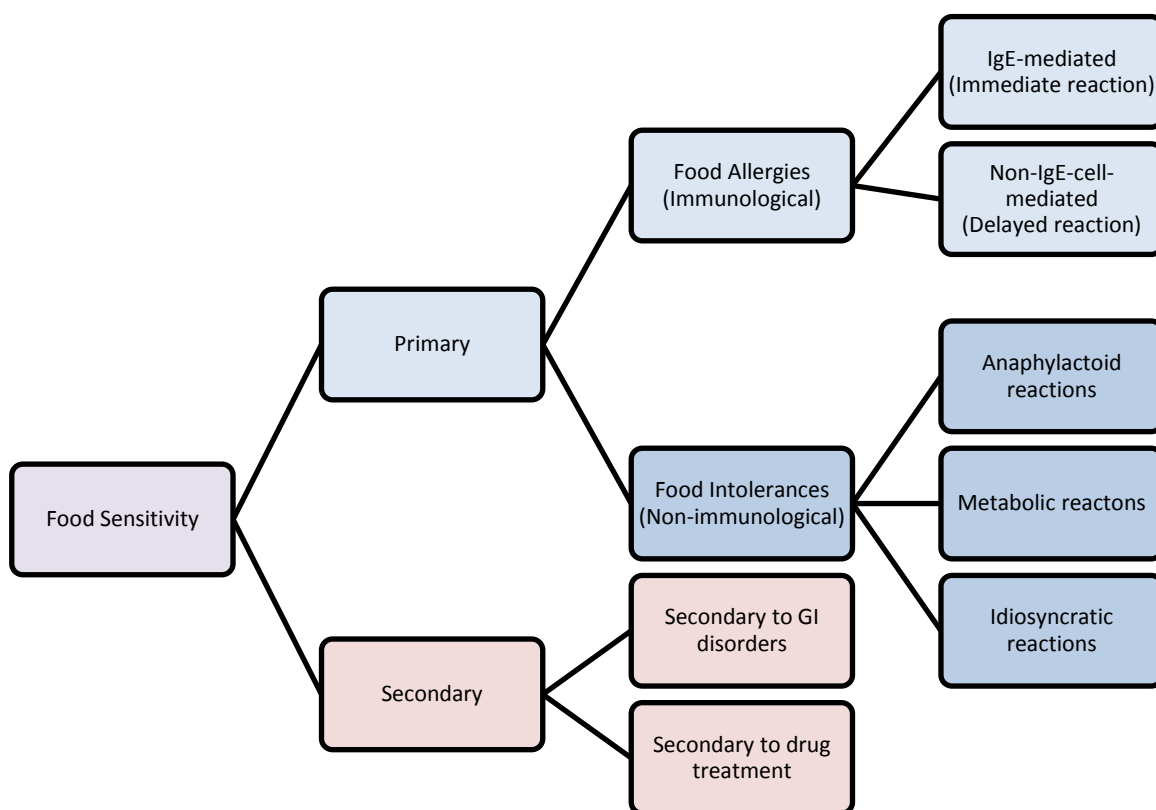


Figure 1 – Classifications of different types of food sensitivities (Modified from Taylor and Hefle (2001)).

Food intolerance

Food intolerances include all individualistic adverse reactions to foods where an abnormal immune system response is not the cause of the symptoms (Taylor and Hefle, 2006b). As previously stated, the major categories of food intolerances include anaphylactoid reactions, metabolic food disorders, and idiosyncratic reactions.

Anaphylactoid reactions

Anaphylactoid reactions are characterized by the release of histamine and other mediators from mast cells and basophils without the interaction of IgE or other immunoglobulins (Taylor et al., 1989). The occurrence of anaphylactoid reactions to foods is debatable as the release of mediators occurs through an as of yet unknown mechanism and there have been no foodborne substances identified that trigger the spontaneous release of histamine (Taylor and Hefle, 2006b; Taylor et al., 1989). The most evidence available for the existence of anaphylactoid reactions is the lack of immunological evidence in certain possible cases of food allergy, such as strawberries, shellfish, and chocolate (Hefle, 1996; Taylor et al., 1989). As the mechanism behind these reactions is unknown, an avoidance diet is required to avoid any adverse reactions.

Metabolic food disorders

Metabolic food disorders are the result of inherited genetic deficiencies and the loss of ability to metabolize a food component or an enhancement of the individual's sensitivity to a foodborne chemical due to the loss of normal cellular or enzymatic function (Taylor et al., 1989). The two best known metabolic food disorders are lactose intolerance and favism, with favism affecting over 100 million individuals worldwide (Taylor et al., 1989).

Lactose intolerance is characterized through the deficiency of the lactase (β -galactosidase) enzyme in the intestinal mucosa (Lomer et al., 2008; Taylor and Hefle, 2006b). Lactase will hydrolyze lactose into glucose and galactose to be absorbed in the small intestine. Unhydrolyzed lactose will reach the colon, where it is fermented by the natural gut microbiota into carbon dioxide, hydrogen, and water which causes abdominal pain, diarrhea, and flatulence (Lomer et al., 2008). Some level of lactose can be tolerated by many individuals with lactose intolerance. Avoidance of milk products with large doses of lactose is necessary to treat lactose intolerance. Additionally, ingesting another source of β -galactosidase, such as a purified lactase enzyme in pill form or the bacterial form of the enzyme found in yogurt, can also lessen symptoms. Lactose is then hydrolyzed by the ingested enzyme before it reaches the gut bacteria (Lomer et al., 2008).

Favism affects individuals with a deficiency of the glucose-6-phosphate dehydrogenase enzyme (G6PDH) in their red blood cells (Taylor et al., 1989). These individuals' red blood cells are then susceptible to oxidative damage. Fava beans produce natural oxidants and favism symptoms are consistent with hemolytic disease, including pallor, fatigue, and dyspnea. Favism is self-limiting and usually not serious but renal failure can occur in severe cases (Taylor et al., 1989). Avoidance of fava beans is necessary for G6PDH-deficient individuals and is not particularly difficult for most on a Western diet.

Idiosyncratic reactions

Idiosyncratic reactions are adverse reactions to food that occur through unknown mechanisms (Taylor and Hefle, 2006b). Symptoms can range from mild and self-limiting to severe, life-threatening reactions. Just as the symptoms vary, a wide range of

underlying mechanisms could be involved with these reactions (Taylor et al., 1989).

While many cause-effect relationships have not been clearly established for idiosyncratic reactions, sulfite-induced asthma and aspartame-induced urticaria are two of the proven relationships. Double-blind placebo-controlled food challenges (DBPCFC) are the method of choice to prove or disprove the cause-effect relationships in idiosyncratic reactions and have been successful in proving sulfite-induced asthma (Taylor et al., 1986b; Taylor et al., 1989). Sulfites are used by the food industry to prevent enzymatic and nonenzymatic browning, as broad spectrum antimicrobial agents, as dough conditioning agents, to provide antioxidant protection, and as bleaching agents and must be labeled if used in a fashion that leads to residual levels above 10 parts per million (ppm) or mg/kg in the product (Taylor et al., 1986b).

Food hypersensitivity

Immune system overview

The immune system is a collection of effector cells and molecules that protect the host from infectious agents and other harmful substances. For the host to be properly protected, the immune system must accomplish four main tasks. The first task, immunological recognition, is the detection of an infection by white blood cells and/or lymphocytes. The second task, if possible, is to contain and eliminate the infection. The third task is immune regulation. Failure of proper immune regulation can be physically destructive to the hosts and in some cases lead to allergic responses or autoimmune diseases. The fourth task is to generate immunological memory and protect the host against recurring disease from a single pathogen. Both the innate and adaptive immune

systems are involved in these tasks and will be discussed further below (Murphy et al., 2008).

When an infectious agent overcomes an individual's physical and chemical barriers to enter the body, the first defenses they encounter are the phagocytic white blood cells (the neutrophils, monocytes, and macrophages) of the innate immune system. The phagocytic cells ingest and kill microbes through the production of toxic chemicals and degradative enzymes. Natural killer cells (NK cells) lack antigen-specific receptors found in the adaptive immune system but are able to recognize general receptor patterns and kill an abnormal cell when present. Additionally, the innate immune system provides the ability to discriminate between self and non-self through the help of pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs) that recognize specific sections of microbes' cell walls and unmethylated DNA common in many pathogens but not associated with the human body. Activation of the PRRs will in turn initiate the adaptive immune system. Dendritic cells and macrophages are additional innate cells that will activate the adaptive immune system through the uptake and presentation of antigens to other cells. The innate immune system only takes hours to respond, but is unable to form memory and relies on the adaptive immune system to develop specific defenses towards individual pathogens. There are two main types of adaptive immune cells, B lymphocytes (B cells) and T lymphocytes (T cells). The B cells bind to antigen presenting cells and differentiate into antibody producing plasma cells. Antibodies are Y-shaped proteins specific to a particular antigen and help facilitate a pathogen's destruction and removal. Antibodies can bind and neutralize a pathogen, enhance phagocyte uptake, or activate the complement system to remove an infectious

agent. Separate T cells provide regulation to the immune system, kill infected cells, or help and instruct the B cells as to what immunoglobulin type to produce and when to secrete antibodies. The adaptive immune system also facilitates the differentiation of some cells into memory cells that provide the long-lasting immunity against a specific pathogen (Murphy et al., 2008).

Proper immune system function is required for a healthy and stable host to be protected from pathogens, but a hypersensitivity reaction can be elicited by an adaptive immune system response to inherently harmless antigens such as pollen, food, and drugs. Coombs and Gell (1975) separated hypersensitivity reactions into four classes displayed in Figure 2. Type I (IgE, soluble antigen), II (IgG, cell-surface antigen), and III (IgG, soluble antigen) hypersensitivity reactions are humoral immune responses and antibody-mediated with the distinctions coming from the type of antigen recognized and the class of antibody interacting. Type IV hypersensitivities are delayed, T cell mediated reactions and can further be divided by the type of antigen and T cell involved.

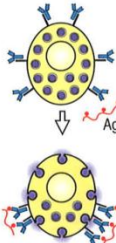
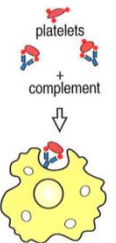
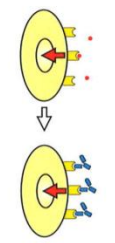
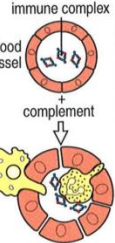
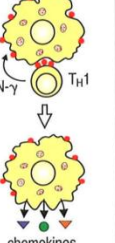
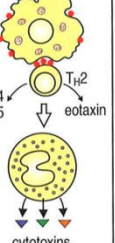
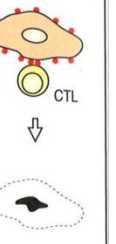
	Type I	Type II		Type III	Type IV		
Immune reactant	IgE	IgG		IgG	T _H 1 cells	T _H 2 cells	CTL
Antigen	Soluble antigen	Cell- or matrix-associated antigen	Cell-surface receptor	Soluble antigen	Soluble antigen	Soluble antigen	Cell-associated antigen
Effector mechanism	Mast-cell activation	Complement, FcR ⁺ cells (phagocytes, NK cells)	Antibody alters signaling	Complement, phagocytes	Macrophage activation	IgE production, eosinophil activation, mastocytosis	Cytotoxicity
							
Example of hypersensitivity reaction	Allergic rhinitis, asthma, systemic anaphylaxis	Some drug allergies (e.g. penicillin)	Chronic urticaria (antibody against FcεR1α)	Serum sickness, Arthus reaction	Contact dermatitis, tuberculin reaction	Chronic asthma, chronic allergic rhinitis	Graft rejection

Figure 2 – Four classes of hypersensitivity reactions. From Murphy et al. (2008).

Food hypersensitivity, also known as true food allergies, are a worldwide public health concern in westernized nations and defined as abnormal immunologic responses to a particular food or food component (Taylor and Hefle, 2006b). Food allergies are typically reactions against naturally occurring proteins in food and can be immediate hypersensitivity, IgE-mediated reactions (Type I) or cell-mediated, delayed hypersensitivities (Type IV). Immediate, IgE-mediated reactions involve the formation of IgE antibodies and recognition of specific allergenic proteins in food. IgE-mediated reactions are the most important of food sensitivities due to the rapid onset time of possible life-threatening symptoms (Taylor and Hefle, 2006b). Cell-mediated reactions are usually confined to the gastrointestinal tract with localized, non life-threatening symptoms occurring hours after ingestion of the antigen due to the interaction of a sensitized T cell lymphocyte and a specific antigen (Taylor and Hefle, 2006b).

Cell-mediated hypersensitivity

Non-IgE-mediated food allergies are Type IV food hypersensitivity reactions. Reactions are T cell-mediated with symptoms occurring 6-24 hours after the ingestion of the antigen. Symptoms often peak after 48 hours and the associated inflammatory response slowly subsides. Currently, an avoidance diet is the only treatment for complete symptom prevention associated with cell-mediated food allergies. Examples of cell-mediated hypersensitivity reactions include celiac disease, food protein-induced enterocolitis, food protein-induced enteropathy, food protein-induced proctitis, allergic eosinophilic gastroenteritis, and allergic eosinophilic esophagitis. Cell-mediated reactions and/or IgE-antigen complexes could play a combined role in some of these diseases. (Sampson, 2004). Delayed hypersensitivities are not as well studied as IgE-mediated food allergies. Most mechanisms are unknown, although the cause-and-effect relationship is established. Celiac disease, also known as celiac sprue or gluten-sensitive enteropathy, is a malabsorption syndrome associated with ingestion of gluten from a number of grains but mostly wheat, barley, and rye. Celiac disease affects 0.7% of individuals in the United States and is the most studied of the delayed hypersensitivities (Rubio-Tapia et al., 2012; Taylor and Hefle, 2006b).

Celiac disease is clinically presented as a local inflammatory response in the intestinal tract. The inflammation damages and reduces the number of epithelial cells and levels of mucosal enzymes critical to digestion and absorption (Taylor and Hefle, 2006b). Individuals genetically susceptible to celiac disease express the human leukocyte antigen (HLA)-DQ2 or HLA-DQ8. Up to 40% of individuals carry these HLA haplotypes suggesting environmental factors play a large role in the development of celiac disease.

These HLA types are necessary for the development of celiac disease, but their absence virtually excludes the diagnosis (Niewinski, 2008). Diagnosis of celiac disease is confirmed by observation of and subsequent reversal of villous atrophy, crypt hyperplasia, and cellular infiltrate after the removal of gluten from the diet (Spergel, 2006). Gluten peptides elicit both an innate and adaptive immune response in celiac disease, with CD4⁺ T cells in the lamina propria recognizing gluten peptides processed and presented by antigen-presenting cells (Niewinski, 2008). Symptoms generally include diarrhea, abdominal distension, and failure to thrive in children with diarrhea, constipation, weight loss, weakness, short stature, flatus, abdominal pain, and vomiting presenting in adults (Niewinski, 2008). The only proven treatment for celiac disease is a strict, life-long avoidance diet of gluten. However, future therapies could degrade gluten into peptides safe for consumption, inhibit activation of gluten-reactive T cells, or block gluten from binding to the HLA-DQ2 or HLA-DQ8 molecules (Sollid and Khosla, 2005).

IgE-mediated hypersensitivity and food allergy

The protective immune response involves a myriad of cells, proteins, signals, and antibodies. The biological role of IgE involves protective immunity, especially in response to parasitic worms. However, certain individuals will produce IgE antibodies to innocuous, non-parasitic antigens that trigger inappropriate IgE responses known as Type I hypersensitivity reactions (Murphy et al., 2008). A true food allergy is an immediate Type I, IgE antibody mediated hypersensitivity reaction to a naturally occurring food component, most often a protein. Type I reactions are distinguished by IgE recognition of specific epitopes (linear or conformational) within a soluble antigen to trigger mast cell activation (Murphy et al., 2008; Pomes, 2010; Untersmayr and Jensen-Jarolim, 2006). In

addition to foods, certain drugs and environmental substances such as pollens, molds, bee venoms, dust mites, and animal danders have been shown to elicit IgE formation and cause allergic reactions (Taylor and Hefle, 2006b). In food allergy, symptoms typically develop in less than 10 minutes up to 2 hours after consumption of the offending food. Symptoms of a food allergy can be mild to severe and include hives, itching, skin rash, swelling of the lips, face, tongue, throat, and other body parts, wheezing, nasal congestion, abdominal pain, diarrhea, nausea, vomiting, dizziness, lightheadedness, or fainting. Anaphylaxis is a reaction that involves multiple organ systems, including any combination of the respiratory, cardiovascular, gastrointestinal, and cutaneous systems (FDA, 2009; Taylor and Hefle, 2006b). An anaphylactic reaction and its associated symptoms do not have to be life-threatening. However, most life-threatening or fatal food allergic reactions are anaphylactic in nature and disrupt respiratory and/or cardiovascular function (Taylor and Hefle, 2006b).

The mechanism involved in IgE-mediated food allergic reactions is shown in Figure 3. There are two stages of developing an IgE-mediated food allergy, the sensitization phase and the elicitation phase (Taylor and Hefle, 2006b). Sensitization can occur at any age in life and does not always occur upon the first exposure to an allergen. Sensitization is symptomless and consists of allergen absorption, processing and presentation, T cell and B cell activation, development of oral tolerance or allergic sensitivity, and synthesis of antigen-specific IgE antibodies by plasma cells (Fraser et al., 2001; Taylor and Hefle, 2006b). The allergen-specific IgE attaches to the surface of mast cells in various connective tissues (gastrointestinal system, respiratory tract, skin) and basophils in the blood. Cross-linking of allergens to IgE on the surface of the mast cell or

basophil membrane triggers the release of histamine and other chemotactic mediators responsible for clinical allergic symptoms (Fraser et al., 2001; Taylor and Hefle, 2006b). Histamine, prostaglandins, and leukotrienes can elicit contraction of the smooth muscles in the blood vessels, gastrointestinal tract, and respiratory tract, as well as increase vascular permeability and vasodilation, increase mucus production, and increase chemotaxis of eosinophils, neutrophils and mononuclear cells. The mediators are released into the bloodstream and can trigger systemic reactions involving multiple tissues and organs (Fraser et al., 2001; Taylor and Hefle, 2006b).

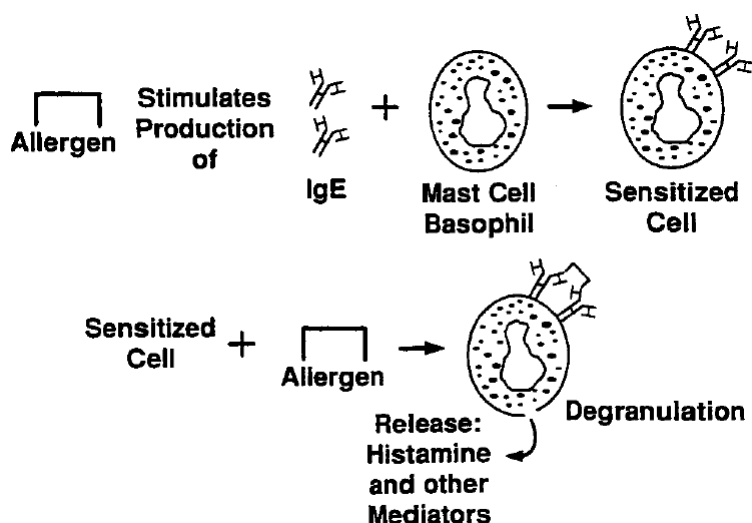


Figure 3 – Mechanism of IgE-mediated food allergy. Adapted from Taylor and Hefle (2006b).

Nearly all foods with naturally occurring protein could potentially elicit allergic reactions in specific individuals. However, exposure to food proteins does not always result in the formation of protein-specific IgE antibodies and only a small percentage of food proteins have been identified as allergens (Hefle et al., 1996). Most food allergens are water- or salt-soluble glycoproteins with acidic isoelectric endpoints that are comparatively stable to processing, cooking, proteolysis, and the digestive processes (Taylor and Lehrer, 1996). However, classes of allergens do exist that are heat labile,

most notably allergens in fresh fruits, raw vegetables, and soy that are cross-reactive with the major birch pollen allergens (Geroldinger-Simic et al., 2011; Mittag et al., 2004; Vieths et al., 1996). While a wide variety of foods are consumed across the globe, relatively few are frequent causes of allergies (Hefle et al., 1996). The most common allergenic foods or food groups, referred to as the “Big 8” food allergens consists of milk, eggs, wheat, peanuts, tree nuts, soybeans, fish, and crustacean shellfish. The major allergenic foods are responsible for 90% of IgE-mediated food allergies (FAO, 1995). In addition to the “Big 8” there have been over 150 other allergenic foods reported (Hefle et al., 1996).

Prevalence of IgE-mediated food allergy

Food allergies are a worldwide health concern as an estimated 5 – 10% of children and 3 – 4% of adults in westernized countries are affected (Osborne et al., 2011; Rona et al., 2007; Sicherer and Sampson, 2010). Awareness of food allergy is increasing and up to 35% of individuals self-diagnose a food allergy. While specific causes have not been identified, the prevalence of food allergies is increasing across the globe (Altman and Chiaramonte, 1996; Lack, 2008; Rona et al., 2007; Sicherer and Sampson, 2010; Sloan and Powers, 1986).

Multiple theories exist regarding the increasing prevalence of food allergy: genetic factors, Caesarean section births, the hygiene hypothesis, time and route of first exposure to food allergens, changes in dietary habits, food processing, and levels of vitamin D exposure (Lack, 2008; Sicherer and Sampson, 2010). No large scale change in population genetics can account for the rise in food allergy. Epigenetics is the study of heritable and non-heritable changes of gene function that occur without a change in the

nucleotide sequence of DNA. Epigenetic changes due to shifts in diet and environmental exposures have been linked to the development of asthma and allergic rhinitis but not food allergy (Kouzarides, 2007; North and Ellis, 2011). The hygiene hypothesis proposed that increased rates of infection early in life had a protective effect on the development of allergies, asthma, and other atopic diseases (Strachan, 2000). Germ free mice had abolished TH1 immune responses and were unable to achieve oral tolerance. However, exposure to intestinal bacteria during the neonate stage restored proper regulation of the TH2 immune response and development of oral tolerance (Pistiner et al., 2008; Renz-Polster et al., 2005; Sudo et al., 1997). The dual-allergen-exposure hypothesis states that tolerance occurs due to oral exposure to food and allergic sensitization occurs due to cutaneous exposure (Lack, 2008). Low dose cutaneous exposure is taken up through Langerhans cells and leads to a TH2 response and IgE production by B-cells. Oral exposure leads to TH1 and regulatory T-cell response in the gut to induce tolerance. Cutaneous exposure to peanut and peanut oil on abraded skin increased peanut sensitization and the risk of food allergies to peanut in mice and humans (Lack, 2008; Strid et al., 2004). Inflammation due to eczema reduces the effectiveness of the epidermal barrier protein and opens an opportunity for allergen protein exposure and creation of food-allergen specific T-cells in the open skin (Howell et al., 2007; van Reijssen et al., 1998). Low levels of peanut are accessible in the household environment to infants after cleaning, providing a cutaneous exposure to those at risk (Perry et al., 2004). Time of peanut introduction into the diet had a significant effect on the prevalence of peanut allergy among Jewish school children (du Toit et al., 2008). Israeli Jewish children consumed more peanut in their first year of life than their UK counterparts and the

prevalence of peanut allergy was 0.17% in Israel versus 1.85% in the UK; variations in atopy, social class, or genetic background did not have a significant effect (du Toit et al., 2008). Additionally, the form of peanut consumed may determine if an allergic response is formed. The stability and allergenicity of allergenic proteins may be altered through food processing. For example, the roasting of peanuts modifies the stability of peanut allergens through the Maillard reaction and the modified peanut allergens have increased IgE binding capacity (Maleki et al., 2000). However, convincing evidence does not exist to link changes in dietary habits or food processing and a rise in the prevalence of food allergy (Lack, 2008).

In the U.S., milk, eggs, and peanuts are the most frequent allergenic foods in children, while adults are more likely to have allergies to crustacean shellfish, peanuts, and tree nuts (Sicherer and Sampson, 2010). Many children will outgrow their food allergies and become tolerant to milk, eggs, soy, and wheat. Allergies to peanut, tree nuts, and crustacean shellfish are rarely outgrown (Sicherer and Sampson, 2010; Skolnick et al., 2001; Wood, 2003). Milk and egg allergies are common across the globe but other major food allergens will vary by region based on cultural and dietary habits (Lack, 2008). Food allergies are potentially life-threatening. In the past 25 years, three studies found an average of 6 identified fatal anaphylactic reactions per year to foods in the U.S. (Bock et al., 2001; Sampson et al., 1992; Yunginger et al., 1988); additional fatalities may occur as the system for recording such events is faulty. Restaurants and educational settings were, and still are, the most common locations of fatal allergic reactions, and peanut is responsible for over 50% of food allergy related fatalities in the U.S. (Keet and Wood, 2007). Food companies are required to declare when an ingredient is derived from

the major allergenic foods of milk, eggs, fish, crustacean shellfish, peanuts, soybeans, tree nuts, and wheat, plus or minus a few others depending on the country (Gendel, 2012). However, these labeling laws apply only to packaged foods and ingredients and do not require allergen labeling within a restaurant or cafeteria setting.

Diagnosis of Food Allergy

The diagnosis of food allergy is a challenging task, but the effects of a correct medical diagnosis of food allergy, positive or negative, have been shown to positively impact an individual's quality of life (Hourihane et al., 2011). Diagnosing a food allergy begins with a detailed review of the patient's medical history and a physical examination to determine causal foods and distinguish allergy from other diseases and conditions. *In vitro* and *in vivo* laboratory tests are used to confirm the diagnosis of a specific causal food. However, *in vitro* and *in vivo* tests only detect a patient's sensitization (presence of specific IgE antibodies to a food) and absolute clinical reactivity cannot be predicted (Sicherer and Sampson, 2010). A physician-supervised food challenge provides the strongest evidence of a clinical food allergy.

For IgE-mediated disorders, skin prick tests (SPTs) provide a rapid *in vivo* method to detect sensitization. Negative SPT responses accurately predicted the absence of IgE-mediated allergic reactivity (>90%), but a positive test response does not prove food allergy (specificity and positive predictive accuracy, <100%) (Pucar et al., 2001; Sicherer and Sampson, 2010). A negative SPT or a negative physician-supervised food challenge (or both) confirm the absence of clinical allergy (Sicherer and Sampson, 2010). *In vitro* diagnostic tests for allergen-specific IgE are potentially more expensive and less sensitive than SPT methods but may be preferred for safety reasons in patients with

extreme sensitivity (Sicherer and Sampson, 2010; Taylor and Hefle, 2006b). *In vitro* methods include the radioallergosorbent test (RAST), the ImmunoCAP® test (Thermo Fisher Scientific), and the enzyme-linked immunosorbent assay (ELISA). The most common test used is the ImmunoCAP®, in which the patient's blood serum reacts with the allergen of interest, covalently coupled to the solid phase matrix. Fluorescent enzyme-labeled anti-IgE is used to detect the degree of binding by allergen-specific IgE (Thermo-Fisher-Scientific, 2011).

Oral food challenges are the best method to establish or rule out an adverse reaction to foods (Bindslev-Jensen et al., 2004). Challenges can be conducted in an open, single-blind, or double-blind procedures. Double-blind, placebo-controlled food challenges (DBPCFCs) are considered the gold standard for diagnosing IgE mediated food allergy. A DBPCFC is reliable, consistent, and indisputably associates the ingestion of a specific food to a set of food allergic symptoms (Taylor and Hefle, 2006b). In case of a severe reaction, DBPCFCs should be performed in a clinical setting that has access to emergency care. However, severe reactions are rare in low-dose protocols (<1 mg protein) that have been found to provoke only mild objective symptoms in sensitive subjects, regardless of prior reaction history. Objective symptoms are discernible to clinical observers e.g. vomiting, urticaria, rash, angioedema, etc. Dosing protocols should stop at the observation of objective symptoms during challenge and follow a doubling or semi-logarithmic scheme to mitigate the chance of a severe reaction. In addition to allergy diagnosis, oral food challenges with objective endpoints can provide risk assessors and regulators with valuable data regarding the thresholds and minimal eliciting

doses of food allergic individuals and populations (Bindsvlev-Jensen et al., 2004; Taylor et al., 2004; Taylor et al., 2010).

Treatment of Food Allergy

The most basic and successful treatment of a food allergy is avoiding the offending food(s) (Taylor et al., 1986a). Avoidance diets are successful but significant responsibility is placed on the patient and complete avoidance is not always possible due to cross-contamination of allergens in cafeterias, restaurants, packaged foods, and other settings (Sicherer and Sampson, 2010; Taylor et al., 1986a; Taylor and Hefle, 2006b).

The onus for the implementation of a safe and effective avoidance diet falls upon the consumer causing stress and a diminution of their quality of life (Dunn-Galvin et al., 2008). The patient should consult with an expert allergist and dietician when developing a proper avoidance diet as proper diagnosis and a limited the number of avoided foods helps ensure compliance and a nutritionally complete diet (Taylor et al., 1986a).

Education of others is key to a successful avoidance diet. An estimated 50% of reactions in infants followed through preschool were to foods provided by individuals other than the parent, including relatives and teachers (Fleischer et al., 2012). In addition to the avoidance diet, pharmacologic treatments exist to help manage food allergies in case of accidental exposures. Pharmacologic methods such as antihistamines can block the histamine receptors in tissues and relieve symptoms of itching and inflammation from oral allergy syndrome and IgE-mediated skin reactions (Sicherer and Sampson, 2010; Taylor and Hefle, 2006b). Antihistamines only block one inflammatory mediator associated with allergic reactions and are not fully effective in treating more severe allergic reactions. Epinephrine (adrenaline) is a powerful drug that can resolve many

severe anaphylactic reactions to foods. Individuals with a food allergy are advised to carry an EpiPen® (epinephrine auto-injector) with them at all times as past reaction symptoms are not an indicator for the severity of future reactions (Taylor and Hefle, 2006b).

Hypoallergenic foods are available for use by food allergic infants (Kleinman et al., 1991). But, a few other categories of food including highly refined peanut oil and fish gelatin also are known to contain insufficient amounts of allergenic protein to provoke reactions (Hansen et al., 2004; Hourihane et al., 1997). This is especially important for infant formulas as milk-allergic infants cannot substitute a wide variety of foods in their diet and maintain adequate nutrition. To be labeled hypoallergenic, these formulas must not provoke reactions in 90% of infants or children with confirmed cow's milk allergy with 95% confidence (American Academy of Pediatrics et al., 2000; Kleinman et al., 1991). Milk-allergic infants have multiple hypoallergenic formulas available, including soybean, rice, and protein hydrolysate-based products. Elemental amino acid-based formulas are an option if all other hypoallergenic formulas are rejected. Practical experience has shown that a small number of milk-allergic infants do react to ingestion of hypoallergenic formula based on extensively hydrolyzed casein (Ellis et al., 1991; Saylor and Bahna, 1991). This observation is not surprising since testing assures only that more than 90% of infants will tolerate the formula.

At present, a clinically proven treatment or cure of food allergies does not exist. However, multiple approaches are being studied as a means to treat food allergy including anti-IgE injections, Chinese herbal remedies, probiotics, modified protein vaccines, and immunotherapy via the oral, sublingual, and epicutaneous routes. Oral

immunotherapy has undergone the most clinical research and involves the daily feeding and gradual increase of doses of specific allergenic foods to an allergic-individual over the period of months to years. Once a maintenance dose is reached, the individual is protected from accidental consumptions of the offending food (Sicherer and Sampson, 2010). One key question that remains about oral immunotherapy is the development of true long-term oral tolerance versus short-term desensitization (Jones et al., 2010; Sicherer and Sampson, 2010; Varshney et al., 2009). If tolerance is not achieved, it is unclear how long desensitization will continue if the maintenance dose is discontinued. Desensitization is temporary and would require a constant, possibly daily, maintenance dose. Tolerance is a complete lack of reactivity and could take years to achieve. However, desensitized individuals are provided with an increased level of safety that allows them to remove a large stress from their daily life. Preliminary studies in the U.S. with peanut, milk, or egg and a multitude of studies in Europe indicate that oral immunotherapy could be beneficial for the majority of food allergic individuals (Blumchen et al., 2010; Caminiti et al., 2009; Clark et al., 2009; Hofmann et al., 2009; Jones et al., 2010; Morisset et al., 2007; Skripak et al., 2008; Varshney et al., 2009).

Legume Allergy

The term legume refers to a leguminous plant, with seeds in pods, or directly to the seed, pod, or other edible part a leguminous plant. Common legumes include peanuts, soybeans, peas, lentils, lupin, chickpeas, and dry edible beans. Legumes are a staple food category in the human diet and have been cultivated for over 10,000 years (Graham and Vance, 2003). They are grown on 15% of the Earth's arable surface, account for 27% of the world's primary crop production, and contribute 33% or more of the dietary protein

nitrogen (N) needed by humans. Soybean and peanut account for more than 33% of the world's processed vegetable oil (Graham and Vance, 2003). Legumes are extremely versatile and can be popped like popcorn, candied, or utilized in a number of products as a flour (bread, tortillas, chips, spreads, and extruded snacks), in liquid form (milks, yogurt, and infant formula), and as a modified protein concentrate or isolate to add protein and functional properties to a product (Egbert, 2004; Genta et al., 2002; Graham and Vance, 2003; Keshun, 1997; Popenoe et al., 1989). Industrial uses are just as diverse with legumes in biodegradable plastics, gums, dyes, pharmaceuticals, pesticides, phytochemicals, and inks (Morris, 1997). Soybean oil, and products from other legumes, are utilized as biodiesels and alternative fuels, but more research is needed in this area to commercially produce a fuel with an overall positive energy output compared to required inputs (Graham and Vance, 2003; Pimentel and Patzek, 2005; Scott et al., 2008). Legumes are also a primary source of cheap dietary protein and nutrients for industrial farm animal production (Barac et al., 2004; Graham and Vance, 2003).

Nitrogen is required to biosynthesize basic building blocks of life and is the primary nutrient limiting plant production in most natural ecosystems (Emsley, 2011; Lawlor et al., 2001). Nitrogen fixation, or the conversion of nitrogen from its stable gas form (N_2) to a usable form such as ammonia (NH_3), is essential for agriculture (Lawlor et al., 2001). Legumes achieve nitrogen fixation through a symbiotic relationship with rhizobia, and other diazotrophs, and play an important role in colonizing disturbed ecosystems (Graham and Vance, 2003). The use of legumes in pastures and for soil improvement dates back to the Romans, who noted their benefit to future crops (Graham and Vance, 2003). Proper crop rotation and till conditions can increase the levels of

nitrogen in the soil, increase crop yields, and/or reduce the amount of fertilizer required to optimize crop yields (Havlin et al., 1990; López-Bellido et al., 1996; Smil, 1999). Annually, up to 60 million metric tons N_2 are fixed by legumes and modest crop rotation could save U.S. farmers at least \$300 million in fertilizer costs (Peterson and Russelle, 1991; Smil, 1999).

For centuries, legumes have been used in folk medicine (Kindscher, 1992). Recently, legumes have been shown to impart a number of health benefits on consumers. Elevated intake levels of soy protein have been shown to lower total cholesterol levels and low-density lipoprotein (LDL) cholesterol levels in serum (Weggemans and Trautwein, 2003). In 1999, the FDA concluded that soy protein included in a diet low in saturated fat and cholesterol may reduce the risk of coronary heart disease (CHD) by lowering blood cholesterol levels (FDA, 1999). Legumes, in part with a low-Glycemic Index diet, improved glycemic control and reduced the risk of CHD in individuals with type 2 diabetes (Jenkins Da and et al., 2012). Legume consumption, including lentils, peas, chickpeas, and beans, is inversely associated with serum concentrations of adhesion molecules and biomarkers of systemic inflammation (Esmailzadeh and Azadbakht, 2012). Consumption of dry beans, peas, and peanuts significantly reduced the risk of CHD and cardiovascular disease (CVD) (Bazzano et al., 2001). Increased consumption of peanut butter is correlated with a reduction in risk of death from CVD and CHD (Blomhoff et al., 2006). Soy isoflavones, genistein in particular, have been reported to prevent the growth of prostate cancer cells in vitro and in rodents (Prezioso et al., 2007). Japanese men (high consumers of soy foods) rarely die from prostate cancer while frequently being diagnosed with small tumors in the prostate (Pisani et al., 1999). Due to

the early results in human studies, the American Cancer Society includes eating soybeans as one of the seven steps to reduce the risk of developing prostate cancer (Liu, 2004a).

Soy saponins are important regulators of the promotional stages of cancer formation (Liu, 2004a). Lunasin, a 43-amino acid peptide found in soy, barley, wheat, and other plants, has been shown to prevent skin cancer in a mouse model and shown to be a strong tumor suppressor in vitro (Galvez et al., 2001). While legumes have a high nutritional value, they contain relatively low quantities of the essential amino acid methionine, and supplements or protein sources other than legumes must be consumed to obtain adequate methionine intake (Hove et al., 1978).

Legumes, especially peanut, are some of the most prevalent and potent allergenic foods. Allergenic responses to legume proteins can provoke a spectrum of symptoms, from mild to life-threatening (Sicherer and Sampson, 2010). Proteins associated with plant food allergens, including legumes, can be mostly classified into four protein families and superfamilies: prolamins, cupins, profilins, and the Bet v 1 superfamily. The majority of allergenic proteins come from the seed storage proteins, albumins and globulins, within the prolamin and cupin superfamilies (Breiteneder and Radauer, 2004; Radauer and Breiteneder, 2007). Legume seed storage proteins are often found in high abundance and are resistant to thermal and proteolytic denaturation (Burks et al., 1992; Lehmann et al., 2006; Sen et al., 2002). Additionally, the four superfamilies include allergens that are defense proteins such as pathogenesis-related proteins, proteases, and protease inhibitors (Breiteneder and Radauer, 2004; Radauer and Breiteneder, 2007). Cross-reactive allergenic proteins, between legumes as well as other food groups, are found in all four superfamilies.

IgE from legume-allergic individuals have shown high cross-reactivity between the proteins of peanut, soybean, lima bean, pea, garbanzo bean, and green beans, but only 5% of the study population was clinically reactive to more than one legume (Bernhisel-Broadbent et al., 1989). Individuals with persistent peanut allergy have higher risk of cross-reactivity to other legumes than most legume-allergic subjects. In the U.S., the prevalence of peanut allergy is estimated at 0.6 – 1.0%, with up to 6.4% of peanut-allergic individuals having clinical reactivity to soy, 2.4% to pea, and less than 1% to lentil, chickpea, and green bean. Of peanut-allergic individuals with clinical cross-reactivity, 8% were cross-reactive to multiple legumes (Neuman-Sunshine et al., 2012; Sicherer and Sampson, 2010). European peanut-allergic individuals are more inclined to have cross-reactivity to lupin (up to 44%) (Fiocchi et al., 2009; Moneret-Vautrin et al., 1999; Shaw et al., 2008). Lupin proteins, β -conglutin and the pathogenesis-related protein PR-10, have homologs in peanut, Ara h 1 and Ara h 8, respectively. Soybean β -conglycinin is also homologous with lupin β -conglutin, but less clinical cross-reactivity between soy and lupin has been reported (Goggin et al., 2008; Guarneri et al., 2005). Legume cross-allergic symptoms in mice were milder than the primary allergic responses, but human fatalities have been reported in severe peanut-allergic individuals when exposed to soy (Sicherer et al., 2001; Vinje et al., 2012). Spanish populations demonstrate a significantly higher prevalence of clinical reactivity to multiple legumes (82%) including lentil, chickpea, pea and peanut with, with 69% allergic to lentil and chickpea (Ibáñez et al., 2003; Martinez San Ireneo et al., 2008). The prevalence of reactivity to multiple legumes is estimated to be higher than the reported 12% cross-reactivity in tree nuts (Fleischer et al., 2005).

Soy proteins and allergens

Soybeans are a staple food in many cultures and play a large role in the diet of food-producing animals (Barač et al., 2004; Friedman and Brandon, 2001). In 2009, farmers planted a record breaking 77.5 million acres of soybean varieties and the U.S. led the world in soybean exports (USDA, 2010). Soybean meal is added to animal feeds and processed foods as a cheap source of protein (Barač et al., 2004). Soy has high nutritional value, but is deficient in the essential amino acid, methionine (Friedman and Brandon, 2001). A typical dry soybean is composed of 40% protein, 20% oil, 35% carbohydrates, and 5% ash (Liu, 2004b). Different varieties of soy can be grown to contain up to 48% protein, but the easiest way to obtain higher protein levels is to process the bean into defatted soy flour, soy protein concentrate (SPC), or soy protein isolate (SPI). Most soy flours are made by grinding dehulled and defatted soy flakes, containing at least 50% protein (dry basis), and are traditionally used as an ingredient in the baking industry. Soy protein concentrates are made by removing the soluble sugars from the defatted flake with an aqueous alcohol extraction. They contain at least 65% protein, and are used to bind water while adding protein, texture, and body to many different products. Soy protein isolates have the soluble and insoluble carbohydrates removed from the defatted flake, contain at least 90% protein, and are used for gelation, emulsification, water binding, viscosity, foaming, and whipping (Egbert, 2004).

Soy flours, SPC, and SPI are used in everything from protein shakes to soups, baked products, meats, and cheeses. Soy products can be used for protein fortification, but other applications include improving texture, gelation, emulsification, water binding, viscosity, foaming, and whipping. Soy sauce and hydrolyzed vegetable protein are used

for flavor enhancement. The traditional methods to modify the functional properties of soy products include adjusting the protein content, heat denaturation, full or partial protein hydrolysis, and pH adjustment. Newer methods include jet-cooking/flash cooling, high pressure treatment, and different solvent extractions (Egbert, 2004). Due to the widespread use of soy, it is necessary to understand the effects that these treatments have on the different proteins found in soybeans, including the allergens.

Mature soybeans contain a mixture of seed storage and bioactive proteins. Storage proteins include α -, β -, and γ -conglycinins, glycinin, and other globular proteins that range in molecular weight from 140 to 300 kDa. Bioactive proteins include β -amylase, cytochrome c, lectin, lipoxygenase, lunasin, urease, Kunitz trypsin inhibitor (SKTI), and Bowman-Birk chymotrypsin/trypsin inhibitor (BBI). The major seed storage proteins of glycinin and β -conglycinin affect the properties of any soy flour, SPC, or SPI. The food industry is able to achieve a wide range of desired functional properties for soy products by varying the composition, structure, and modification of major seed storage proteins such as glycinin and β -conglycinin. Health promoting, bioactive compounds found in soy include, but are not limited to lecithin, isoflavones, saponins, phytosterols, phytate, and lunasin. Antinutritional factors of SKTI, BBI, and lectins are detrimental at high levels but have been shown to provide anti-cancer benefits at low levels (Barać et al., 2004; Friedman and Brandon, 2001; Liu, 2004b; Mikić et al., 2009). Due to the commercial nature of soybeans, many varieties of the plant have been developed that express custom protein profiles. Certain proteins can be expressed at high levels and others can be removed all together (Mikić et al., 2009). Soybean has become one of the most versatile foods in the industry and can be added to products for a number of different purposes.

Asthma and food allergy to soy have been reported since 1934 (Duke, 1934). Soy is listed among the most common allergenic foods on a worldwide basis and is on the priority allergenic foods list in the USA, the EU, and Australia among others (Gendel, 2012). However, soy is not listed as a priority allergen in Japan where soy is commonly consumed and the 7th most common cause of food allergen anaphylaxis (Gendel, 2012; Imamura et al., 2008). While considered a major allergenic food, the data for soy used by the Codex Alimentarius Commission in 1999 were fragmentary and mostly focused on comparative prevalence in populations of food-allergic infants (Sampson and McCaskill, 1985). Subsequently, the prevalence of soy allergy appears to be lower than that of most other commonly allergenic foods. In unpublished data from the large EuroPrevall study just completed in the EU, the prevalence of soy allergy was very low and below that of several foods not currently on allergen priority lists. However, an emergence of soy allergies to certain brands of soy milks and soy nutritional drinks has occurred in several EU countries (Mittag et al., 2004). Recent unpublished clinical observations from a Dutch clinic indicate that many of these patients can tolerate soy flour and confirm the previous observations that reactivity is associated with certain specific types of soy products. Clearly, this aspect deserves further evaluation.

The clinical manifestations of soy allergy are broad, and include atopic dermatitis and eczema, asthma, severe enterocolitis of infancy, and immediate IgE-mediated reactions. Unlike peanut, soy is not believed to be common cause of severe or fatal allergic reactions (Sicherer et al., 2001). However, anaphylaxis and exercise-induced anaphylaxis to soy have been reported (Adachi et al., 2009; Pumphrey and Stanworth, 1996; Sicherer et al., 2001). Additionally, fatalities to soy have been reported in

individuals with asthma and severe peanut allergy (Foucard and Yman, 1999; Yunginger et al., 1991). Serum IgE binding to at least 16 soy proteins has been shown in soy-sensitive individuals with atopic dermatitis (Ogawa et al., 1991). There are six proteins in soy recognized as allergens by the International Union of Immunological Societies (IUIS) including two soy hull proteins (Gly m 1 and Gly m 2), profilin (Gly m 3), a stress induced, pathogenesis-related starvation associated message protein (Gly m 4), β -conglycinin (Gly m 5), and glycinin (Gly m 6). In addition to the official IUIS allergens, there are multiple other proteins not recognized by the IUIS that have been shown to cause reactions or bind IgE from soy allergic individuals. These proteins include the vacuolar protein P34 (Gly m Bd 30 K), Gly m Bd 28 K, the Kunitz trypsin inhibitor, and lectin.

The soy hull proteins, Gly m 1 and Gly m 2, are inhalation allergens associated with environmental or occupational exposure to dust from soy hulls. Gly m 1 has 2 isoforms, both hydrophobic proteins from soybean hulls (Accession No. AAB34755 and AAB34756) (Gonzalez et al., 1995). Gly m 1A is 42 amino acids (AA) long and 7.5 kDa while Gly m 1B is missing the amino-terminal ALI tripeptide sequence, leaving it 39 AA in length and 7.0 kDa (Gonzalez et al., 1995). Gly m 2, or soybean defensin, is an 8 kDa hull protein (Accession No. A57106) (Codina et al., 1997). Collectively these 3 proteins were responsible for several asthma outbreaks during the 1980s in the Spanish cities of Barcelona and Cartagena after soy dust spread through the cities during unloading and transport of soybeans from the seaports (Codina et al., 1997; Gonzalez et al., 1995). Additionally, bakers working with soy flour have had occupational asthma to Gly m 1 and 2, but the bakers with asthma and IgE mediated food allergy to soy were also

sensitized to higher molecular weight proteins in soybean (Quirce et al., 2000). Thus, Gly m 1 and 2 are determined to be respiratory allergens and not significant food allergens in IgE mediated soy allergy.

Gly m 3, or soybean profilin, is a 14 kDa hydrophilic, heat-labile protein. Gly m 3 is present in the seed at levels of 0.6 – 0.8% of total soluble protein (Accession No. CAA11755) (Amnuaycheewa and Gonzalez de Mejia, 2010). A recombinant form of this protein (rGly m 3) was shown to bind IgE from sera of 9 of 13 (69%) food-allergic subjects who had positive IgE binding to soy proteins by ImmunoCAP® tests (Rihs et al., 1999). IgE binding to rGly m 3 depends on the availability of the full length protein in its original conformational structure as no IgE binding was observed to profilin fragments (Rihs et al., 1999). The rGly m 3 was cross-reactive with Bet v 2, birch pollen profilin, and Bet v 2 binding was inhibited by rGly m 3 (Mittag et al., 2004; Rihs et al., 1999). Multiple soy milk reactive individuals display sensitization to rGly m 3 but not rGly m 4, the major birch pollen cross-reactive protein in soy, suggesting Gly m 3 is also involved in cross-reactions between birch pollen and soy (Mittag et al., 2004). Soy milk is a potentially dangerous product to Gly m 3 sensitive individuals as the food matrix of soy milk affects profilin's thermal stability. Pasteurization does not alter the conformational structure of Gly m 3 and boiling is necessary to reduce conformational epitopes (Amnuaycheewa and Gonzalez de Mejia, 2010). While a concern in less processed foods, Gly m 3 is not considered a major allergen in boiled or highly processed soy products.

Gly m 4 is a 17 kDa stress induced, pathogenesis-related starvation associated message protein (SAM22) (Accession No. P26987). Gly m 4 is found in the roots and leaves of maturing plants and can be induced by wounding or stressing young leaves

(Crowell et al., 1992). Published and unpublished data estimate Gly m 4 at 0.01 – 0.1% of total soy protein (Mittag et al., 2004). While most soy allergic individuals must avoid all forms of soy, Kleine-Tebbe et al. (2002) reported certain consumers characterized by an existing allergy to birch pollen who experienced severe reactions to a specific SPI in a specific brand of soy-based nutritional drink. These reactions occurred on the first time that these consumers ingested this particular SPI. The soy allergen Gly m 4 was identified as a cross-reactive protein with Bet v 1, a major birch pollen allergen (Kleine-Tebbe et al., 2002). After the initial study, soy allergy to Gly m 4 and this particular SPI was confirmed in 16 adults with birch pollen allergy by DBPCFC (Mittag et al., 2004). Allergic reactions in children to soy milk made from filtered whole soybeans have been attributed to Gly m 4 sensitization (Kosma et al., 2011). In this case, the reactions occurred to the ingestion of soy during pollen season. More recent, unpublished evidence suggests that the number of consumers with allergic reactions to certain types of soy milk is increasing in some European countries and is possibly surpassing the prevalence of more typical soy allergy.

The prevalence of birch pollen allergy can be estimated at 2 – 20% in North, Central, and Eastern Europe (D'Amato et al., 2007). It is reported that 10% of highly sensitized birch pollen patients have soy allergy and cross-reactivity correlated with IgE reactivity to one of the major birch pollen allergens (Geroldinger-Simic et al., 2011; Mittag et al., 2004). It is believed that individuals are first sensitized to Bet v 1 in its native form through inhalation of birch pollen and then experience reactions on repeated exposure to birch pollen and Bet v 1 homologs in food (Jenkins et al., 2005). Allergic reactions against pollen lead to clinical syndromes like hay fever, asthma, and dermatitis

while cross-reactive foods can produce symptoms including oral allergy syndrome, itching and swelling of the lips, tongue, and throat, hives, and anaphylaxis (Kleine-Tebbe et al., 2002; Neudecker et al., 2003).

Gly m 4 and Bet v 1 share 47% sequence homology and demonstrate a conserved conformational structure including multiple IgE binding epitopes (Jenkins et al., 2005). Gly m 4 content is increased during the ripening and storage of soybeans, but its levels can be reduced by cooking or other processing methods. Highly fermented foods or roasted soybeans had no detectable Gly m 4, tofu and soy flakes had levels near 10 ppm, and the SPI implicated by Kleine-Tebbe et al. (2002) had 140 ppm Gly m 4 (Mittag et al., 2004). Strong heating reduced antibody binding to Gly m 4, as detection was reduced after 30 minutes of cooking and eliminated after 4 hours of cooking (Mittag et al., 2004). The level of Gly m 4 in the soy milk made from filtered whole soybeans was not determined (Kosma et al., 2011). Due to the versatility of soy protein, a wide variety of production processes are used to obtain soy protein concentrates and isolates with different functional properties. The SPI product involved in these cases of European soy nutritional drink allergy is minimally processed compared to traditional SPIs (Egbert, 2004; Kleine-Tebbe et al., 2002). Additionally, these soy allergic patients have reported reactions to raw soybean sprouts, tofu, soy milk, and a soy pudding but many could tolerate cooked or highly processed soy products (Mittag et al., 2004). Additionally, recent data from Japan identifies birch pollen sensitivity and Gly m 4 sensitivity in an area lacking atmospheric birch pollen (Fukutomi et al., 2012; Yamagiwa et al., 2002). Alder pollen (Aln g 1) was been identified as the sensitizing agent and as another Gly m 4 cross-reactive protein (Fukutomi et al., 2012). Twenty-one Japanese soy-allergic adults,

some with anaphylactic reactions, were more likely to be sensitized to Gly m 4, alder, and birch pollen (100%) than to Gly m 5 or 6 (5%), providing more evidence that Gly m 4 is of clinical significance (Fukutomi et al., 2012).

Gly m 5, or β -conglycinin, makes up 30% of the total seed proteins and is densely packed into trimers composed of three glycosylated subunits, α (67 kDa), α' (71 kDa), and β (50 kDa) (Accession No. CAA35691, AAB01374, and AAB23463) (Holzhauser et al., 2009; Maruyama et al., 1998). European and Japanese IgE binding studies respectively found 43% and 100% recognition of Gly m 5 by soy allergic sera (Holzhauser et al., 2009; Ito et al., 2011). Early studies found IgE reactivity to the α but not the α' or β subunits of Gly m 5 (Ogawa et al., 1995). Recent studies have shown sensitivity and IgE reactivity to all 3 subunits of Gly m 5 through immunoblotting (Holzhauser et al., 2009; Krishnan et al., 2009; Zheng et al., 2012), ImmunoCAP® (Ito et al., 2011), and basophil histamine release (Zheng et al., 2012). All purified subunits induced dose-dependent histamine release in basophils from soy-allergic patients (Zheng et al., 2012). Purified or recombinant α , α' , and β subunits of β -conglycinin retained allergenic activity and could be used for in vitro and in vivo component resolved diagnosis of soy allergy (Holzhauser et al., 2009; Krishnan et al., 2009; Zheng et al., 2012). Deglycosylation of the β -subunit, by glycosidases or recombinant protein expression in *E. coli*, did not abolish IgE reactivity to the β -subunit (Krishnan et al., 2009). Gly m 5 has been implicated in a case of exercise-induced anaphylaxis after consumption of tofu. Alterations in the resistance of Gly m 5 to pepsin occurred after tofu processing. Gly m 5 in soy milk was digested and IgE reactivity abolished after 20 minutes of exposure to pepsin. Gly m 5 from tofu was intact after 120 minutes or more of

pepsin digestion and IgE reactivity remained up to 240 minutes into digestion (Adachi et al., 2009).

Gly m 6, or glycinin, makes up 40% of the total seed protein, is a hexameric protein, and each subunit (G1, G2, G3, G4, and G5) has at least one basic and acidic subunit linked by a disulfide bond (Holzhauser et al., 2009; Prak et al., 2005). In a recent study, Gly m 6 was recognized by 36% of soy-allergic individuals subjects (Holzhauser et al., 2009). Five major subunits have been identified from soybean: G1 (A1aB1b, 53.6 kDa), G2 (A2B1a, 52.4 kDa), G3 (A1bB2, 52.2 kDa), G4 (A3B4, 61.2 kDa), and G5 (A5A4B3, 55.4 kDa) (Accession No. CAA26723, CAA26575, CAA33217, CAA37044, and AAA33964) (Adachi et al., 2003). Glycinin is not glycosylated and each subunit is composed of acidic (A1a, A1b, A2, A3, A4, A5) and basic (B1a, B1b, B2, B3, B4) chains linked by a disulfide bond (Maruyama et al., 2003). IgE binding studies respectively found 36% and 100% recognition of Gly m 6 by soy allergic sera (Holzhauser et al., 2009; Ito et al., 2011). All five subunits are known to react with IgE (Holzhauser et al., 2009). One study found IgE reactivity in all acidic subunits, but not in the basic subunits (Pedersen and Djurtoft, 1989). Conversely, another study found IgE reactivity in all five basic chains, but none of the acidic chains (Helm et al., 2000a). The acidic chain of G1 (A1a) was found to have a single IgE-binding fragment of approximately 15 kDa corresponding to AA residues 192 to 306. Binding to A1a was stronger than to A2, the acidic chain of G2 (Zeece et al., 1999). Eleven linear IgE binding epitopes (4 immuno-dominant), were found distributed asymmetrically on the surface of G2 trimers (Helm et al., 2000b). These epitopes were predicted to be distributed asymmetrically on the surface of G2 trimers. Subunits of Gly m 6 have high sequence similarity with peanut Ara h 3.

The Gly m 6 acidic chains of A1a and A2 shares IgE binding epitope regions with Ara h 3 (Beardslee et al., 2000; Rabjohn et al., 1999; Xiang et al., 2002). These shared epitopes could in part explain the cross-reactivity found between peanut and soy in some allergic individuals.

Both Gly m 5 and 6 can bind IgE through linear and conformational epitopes (Helm et al., 2000b; Ogawa et al., 1995; Zeece et al., 1999). Gly m 5 and 6 are stable proteins and potent allergens due to the combination of linear and conformational epitopes and their structural resistance to denaturation from tight packing and disulfide bonds. Significantly higher levels of soybean specific IgE was found in individuals with severe reactions when compared to mild reactors. Severe reactors had higher levels of IgE binding to Gly m 5 and Gly m 6 when compared to individuals sensitized to soybean without clinical allergy symptoms (Ito et al., 2011). Similarly, Holzhauser et al. (2009) found that 86% of subjects with anaphylaxis to soy and 55% with moderate reactions had IgE to Gly m 5 or 6. However, only 33% of mild reactors had IgE to Gly m 5 or 6 and 92% of mild reactors had IgE specific to Gly m 4. Their sample size was extremely small so drawing any concrete conclusions is impossible, but allergy to Gly m 5 and 6 should translate worldwide and not be heavily influenced by regional differences of birch or alder pollen levels.

Soy Kunitz trypsin inhibitor (SKTI), consists of 181 amino acids (AA), has a molecular weight of 20 kDa, an isoelectric point of 4.5, and represents 4-7% of the total extractable protein in soy. The protein is tightly packed, not glycosylated, and trypsin inhibition is achieved through reversible binding of SKTI to the trypsin enzyme (Barač et al., 2004; Friedman and Brandon, 2001; Kunitz, 1947; Mikić et al., 2009). SKTI is an

inhalation allergen associated with occupational exposure to flour dust in bakers (Baur et al., 1996; Quirce et al., 2006). There are three major isoforms of SKTI, A,B, and C, with only one AA difference between A and C and eight AA differences between A and B (Accession No. P01070, P01071, TISYC) (Kim et al., 1985). There are two disulfide bonds in SKTI between Cys39-Cys86 and Cys138-Cys145, both of which are critical for trypsin inhibition and resistance to denaturation (Sessa and Ghantous, 1987). While dry heat does not have an effect on trypsin inhibition, wet heat has been shown to reduce trypsin inhibition. Cooking parameters of 30 minutes at 100°C or 143°C for 62 seconds have been shown to achieve a 90% reduction in inhibitor activity (Keshun, 1997; Kwok et al., 2002). Proper rapid cooling methods must be followed to insure deactivation as slow cooling rates after heating allow nearly all of the SKTI to refold and remain intact (Roychaudhuri et al., 2004). High pressure processing has been tested for use in a soy milk system where extensive heating would ruin the sensory qualities of the milk. van der Ven et al. (2005) found 600 MPa at 60°C for one minute reduced inhibitor activity 40%. Although higher temperature, pressure, and slightly longer times would be required to reach 90% inactivation, it is predicted there would not be an effect on sensory qualities. As an allergen, SKTI primarily affects bakers exposed to large amounts of inhaled soy flour. The manifestation of baker's asthma has led to individuals with IgE binding patterns specific for SKTI, positive SPT for SKTI, and reactions during a specific inhalation challenge with purified SKTI (Baur et al., 1996; Quirce et al., 2006). The incidence of inhaled SKTI related allergic reactions is very low. Ingestion of SKTI has only been confirmed to cause an allergic reaction in one individual, although symptoms were severe in their case (Moroz and Yang, 1980).

Gly m Bd 30K, also known as soybean vacuolar protein P34, is an oil body protein with IgE binding characteristics (Accession No. ABC56139) (Helm et al., 1998a; Ogawa et al., 1993). Gly m Bd 30K is a monomeric glycoprotein with thiol protease activity in the papain family. It has been shown to react with 65% of soy-sensitive patients with atopic dermatitis (Helm et al., 1998a; Ogawa et al., 1993; Wilson et al., 2005). Gly m Bd 30K is accumulated during seed maturation and is present at 5% of total protein levels in seed cotyledons, which become the leaves of the plant embryo. Initially the protein is present as a molecular mass 34 kDa polypeptide and is processed to a molecular mass of 32 kDa with the onset of oil mobilization, the fourth through sixth days of seedling growth (Herman et al., 1990; Ogawa et al., 1993). B cell epitope mapping with overlapping 15-mer peptides found 10 regions with IgE binding, 10-mer peptides revealed 16 distinct linear epitopes. Individual patient serum identified 5 immunodominant epitopes (Helm et al., 1998b). Due to the complex structure and the number of epitopes in Gly m Bd 30K, heat does not significantly denature the protein or reduce IgE binding. Additionally, soybeans exposed to superheated steam during the autoclave process demonstrated increased binding to Gly m Bd 30K (Yamanishi et al., 1995). While resistant to a number of denaturation treatments, Gly m Bd 30k may be coded by a single gene and represents 2% to 3% of the total protein content (Wilson et al., 2005). Transgene-induced gene silencing had been successfully used to prevent the accumulation of Gly m Bd 30 K protein in soybean seeds. The Gly m Bd 30 K-silenced plants were equivalent to control plants with no compositional, developmental, structural, or ultrastructural phenotypic differences during comparison (Herman et al., 2003).

Although other allergenic proteins would still be present, these results demonstrate an opportunity for cultivators to grow soybeans without one of the major allergens.

Gly m Bd 28 K is a 26 kDa Asn-linked glycoprotein that has been shown to bind IgE from 25% of soy allergic subjects (Accession No. BAB21619) (Hiemori et al., 2000; Ogawa et al., 2000; Tsuji et al., 1997). Purification of Gly m Bd 28K led to a number of proteins with different isoelectric points but identical N-terminal AA sequences, suggesting that the protein is unstable (Tsuji et al., 1997). High levels of Gly m Bd 28K were detected in tofu, yuba (tofu skin), abura-age (fried tofu), SPI and soy milk. However, Gly m Bd 28K seems to be digested during fermentation as detection was not found in natto and soy sauce (Tsuji et al., 1997). Both Gly m Bd 30K and Gly m Bd 28K are N-linked glycoproteins with respective sugar chain binding to the Asn170 and Asn20 residues (Bando et al., 1996; Hiemori et al., 2000). The sugar moiety of Gly m Bd 30K was shown to consist of mannose, N-acetylglucosamine, fucose, and xylose at a molar ratio of 3:2:1:1 (Bando et al., 1996). The glycan moiety on Gly m Bd 28K is composed of the same sugar ratios as the chain on Gly m Bd 30K (Ogawa et al., 2000; Tsuji et al., 1997). The glycopeptide of Gly m Bd 28K reacted with the sera of soybean-sensitive patients, but did not show IgE reactivity in its deglycosylated form. Additionally, a 23 kDa C terminal fragment of Gly m Bd 28K has been shown to have the same IgE reactivity as the 26 kDa form (Hiemori et al., 2000; Hiemori et al., 2004). IgE antibodies recognized epitopes on the protein peptide sequences of Gly m Bd 30K at a ratio of 1:4 to those recognizing the glycan moiety, meaning that 80% of Gly m Bd 30K IgE antibodies are carbohydrate-determinant specific. Similar glycan moieties could react with soy-

sensitive IgE as a CCD but the clinical relevance of the CCD-directed IgE in soy-sensitive individuals is not fully known (Ogawa, 2006).

Food allergen labeling and thresholds

Food allergen risk assessment and proper labeling is an important process for members of the food industry, regulatory agencies, and food allergic consumers. Allergic consumers have no choice but to adhere to a strict avoidance diet and carefully read the ingredient labels of the food they eat (Hefle et al., 2007; Pieretti et al., 2009; Taylor et al., 1986a; Yu et al., 2006). The presence of allergens in mislabeled or unlabeled packaged products has led to allergic reactions in consumers relying on clear and accurate ingredient statements (Kemp and Lockey, 1996; Yu et al., 2006).

The Food Allergen Labeling and Consumer Protection Act (FALCPA) was passed in 2004 by the U.S. Congress to protect allergic individuals from unclear or unlabeled products and became effective January 1, 2006 (FDA, 2006). For public health authorities, the primary strategies have been to develop lists of priority allergenic foods and enact regulations to assure that any ingredients derived from these foods are declared on the labels of packaged foods (Gendel, 2012). Allergen labeling laws (i.e. FALCPA in the U.S., EU directive 2003/89/EC, Australian Food Standards 1.2.3 and 1.2.4) were implemented to address the issue of hidden allergens in food (EU, 2003a; FDA, 2006; FSANZ, 2002). A company is required to declare when an ingredient is derived from the Big 8 food allergens plus a few others depending on the country (Gendel, 2012). Industry compliance helps consumers identify allergens hidden in hard to identify mixtures, such as spices and minor flavorings in processed food but these labeling laws essentially create a zero tolerance for unlabeled food allergens. With a de facto zero tolerance, the

legislation requires labeling of allergens even if present at levels that will likely not pose any allergic risk to the allergic consumer. Safe consumption of these foods declaring the presence of an allergen could mislead allergic consumers to believe their allergy has resolved, relax their avoidance diet, and increase their risk of an allergic reaction (Noimark et al., 2009; Taylor and Hefle, 2006a).

Importance of Thresholds

Threshold-based risk approaches have long been used for the management of chemical and microbial hazards in food (EU, 2003b; Kroes et al., 2000; Lammerding and Fazil, 2000; Larsen, 2006; Notermans et al., 1995). More recently, the importance of food allergy as a public health and food safety issue has placed pressure on the food industry and regulatory agencies to implement threshold-based strategies to protect the food allergic consumer. Food allergen thresholds can have different meanings to different stakeholders. To the food allergic consumer, their personal threshold or Minimal Eliciting Dose (MED) is the amount of food required to cause an allergic reaction. The population threshold could be the amount of food required to cause a reaction in the most sensitive individual or in a determined percentage of the food allergic population. To the food industry and regulatory bodies, the term threshold could determine how much allergen would trigger a product recall if unlabeled or when to place an advisory statement on the label if allergens are possibly present due to cross-contamination. Two countries have attempted to establish action levels for undeclared allergens. Switzerland has defined an action limit of 1,000 ppm for allergens. This limit states that if unavoidable, contamination above 1,000 ppm (0.1%) must be declared as an ingredient, but contamination below 1,000 ppm may be declared if desired (Kerbach et al., 2009). Levels

of 1000 ppm may provide enough protein (low mg doses) to cause reactions at moderate consumption levels in multiple foods (Taylor et al., 2004). Japan has taken a stricter approach and limited undeclared allergens to 10 ppm (0.001%) in foods (Kerbach et al., 2009). At the present time, the U.S. has not adopted legislation or regulations regarding regulatory thresholds for food allergens and there is no regulatory guidance for trace levels of allergens due to cross-contact. In the absence of guidance from public health authorities regarding thresholds, the food industry has implemented the widespread use of various forms of voluntary advisory or precautionary “may contain” labeling in an attempt to manage the risk and protect food-allergic consumers. As a result, the quality-of-life of food-allergic consumers has decreased and some are ignoring these advisory statements (Hefle et al., 2007; Hourihane et al., 2011). Additionally, the widespread use of advisory labeling has led to varying advice within the medical community on whether patients should avoid all foods with advisory labeling (Koplin et al., 2010; Vierk et al., 2007). Regulatory establishment of thresholds could benefit allergic consumers but they should never be advised to ignore advisory statements on package labels (Taylor and Hefle, 2006a). All stakeholders (regulators, food industry, clinical researchers and patients) agreed it is essential to address the current lack of action levels and thresholds for food allergen labeling, but it is difficult to define and quantify a level of tolerable risk (Madsen et al., 2012). There is an obvious need for research and scientific advancement in the area of food allergen thresholds.

The FDA Threshold Working Group examined four approaches to allergen risk assessment and the establishment of thresholds (analytical methods-based, statutorily derived, safety assessment-based, and risk assessment-based) but came to the conclusion

that a quantitative risk assessment-based approach provides the strongest, most transparent scientific analyses to establish thresholds for the major food allergens (Gendel et al., 2008). Others have agreed that the use of allergic population threshold distributions and probabilistic risk assessment is the best approach to establish thresholds and determine the risk from food allergens (Madsen et al., 2009; Spanjersberg et al., 2007). However, the probabilistic approach has only recently been applied to food allergens (Kruizinga et al., 2008; Rimbaud et al., 2010; Spanjersberg et al., 2010; Spanjersberg et al., 2007). The FDA Threshold Working Group stated that data available in 2006 were not sufficient to meet the requirements of the quantitative approach and that a research program should be initiated to develop applicable risk assessment tools and to acquire and evaluate the clinical and epidemiological data needed to support the quantitative risk assessment-based approach (Gendel et al., 2008).

After the declarations of the FDA Threshold Group, the Food Allergy Research and Resource Program (FARRP) at the University of Nebraska began gathering data on published low dose DBPCFCs for peanut (Taylor et al., 2009). The DBPCFC can be used to deduce an individual's MED for a specific food. FARRP gathered additional data from an allergy clinic in Nancy, France and accumulated a total of 450 individual allergic thresholds from DBPCFCs (Taylor et al., 2010). It was reported that the MED for peanut-allergic individuals in clinical trials spans 5 orders of magnitude – 0.4 mg up to 30,000 mg of whole peanut (Taylor et al., 2009; Taylor et al., 2010). Additionally, a single individual's allergic threshold can range over time but usually not in a significant fashion (Crevel et al., 2010). Small particulates of less than 1.0 mg peanut can cause a reaction and are displayed in Figure 4.

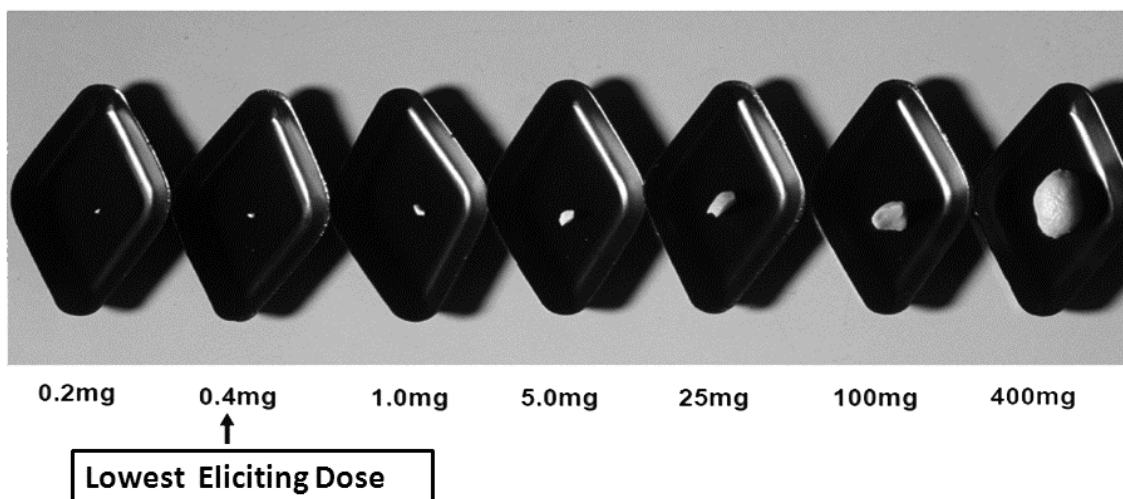


Figure 4 – Individually weighed particulate peanut samples up to ½ of a peanut. The lowest MED found in DBPCFC trials is labeled. (Photo credit: Barbara Ballmer-Weber)

Clinicians have confirmed the existence of safe doses, or No Observed Adverse Effect Level (NOAEL), during low dose DBPCFC for all foods (Bindeslev-Jensen et al., 2004; Taylor et al., 2004; Taylor et al., 2010). The DBPCFC trials can be used to derive the NOAEL and Lowest Observed Adverse Effect Level (LOAEL) for each allergic individual. In clinical challenges, there is no significant correlation between the severity of a reaction and individual LOAELs (Taylor et al., 2010). As stated before, physicians recommend a complete avoidance of peanut but every allergic individual is able to tolerate a dose of peanut below their personal MED. The population threshold dose for peanut has been determined based on individual DBPCFC of 450 peanut-allergic individuals based on elicitation of objective symptoms (Taylor et al., 2009; Taylor et al., 2010). Criteria for inclusion in the dataset are described in Table 1. Clinical literature on provoking doses from DBPCFC now exists for other major food allergens. Similar methods were used for 12 other allergenic foods (milk, egg, hazelnut, soybean, wheat,

cashew, mustard, lupin, sesame, shrimp, celery, and fish) to characterize the risk of each food (Australian Allergen Bureau, 2011; Taylor et al., 2013 (In prep)).

Table 1 - Criteria for inclusion in peanut threshold dataset

Published study
Supplemented with unpublished results
Peanut allergic by history or other factors
Double-blind, placebo-controlled food challenges (DBPCFC) for peanut
Open challenge allowed if patient is under 3 years old
Description of NOAEL and/or LOAEL (if dosing regimen provided, then can determine NOAEL from LOAEL)
Data on individual patients
Objective symptoms @ doses

Interval-Censoring Survival Analysis

The NOAELs and LOAELs from each individual were used as part of an Interval-Censoring Survival Analysis (ICSA) approach to generate a population threshold for peanut (Collett, 1993). The ICSA method is appropriate as the exact dose that provokes a reaction in an individual is not known but it is known to fall into a particular interval dependent on the dosing scheme used in the challenge (Taylor et al., 2009). As shown in Figure 5, left -censoring occurs when an individual experiences an objective reaction at the first dose in a challenge trial. If left-censored, the individual NOAEL is set to zero (left blank in the ICSA program) with the LOAEL set as that first dose. An individual is interval-censored when they experience a reaction to one of the doses in the middle of a dosing scheme and that individual threshold dose is bounded by the NOAEL and LOAEL. Right-censoring occurs if an individual does not experience an objective reaction after the largest challenge dose. In such cases, the NOAEL is set to that largest

challenge dose. An individual is considered as right-censored if they experience a subjective reaction to the largest dose or if a challenge was stopped early due to persistent subjective symptoms. The individual LOAEL is then set to infinity (left blank in the ICSA program) for right-censored subjects. The LIFEREG procedure (SAS v9.2) was used to fit cumulative probability function models to the interval-censored data. Multiple distributions are evaluated and the Log-Normal and Log-Logistic models were determined to best fit the peanut dataset at the lower end of the dose distribution. The models were used as shown in Figure 6 to estimate the ED_{10} , the dose predicted to provoke reactions in 10% of the peanut-allergic population (Taylor et al., 2009; Taylor et al., 2010). Similar methods can be applied to other food allergens where sufficient DBPCFC data exist.

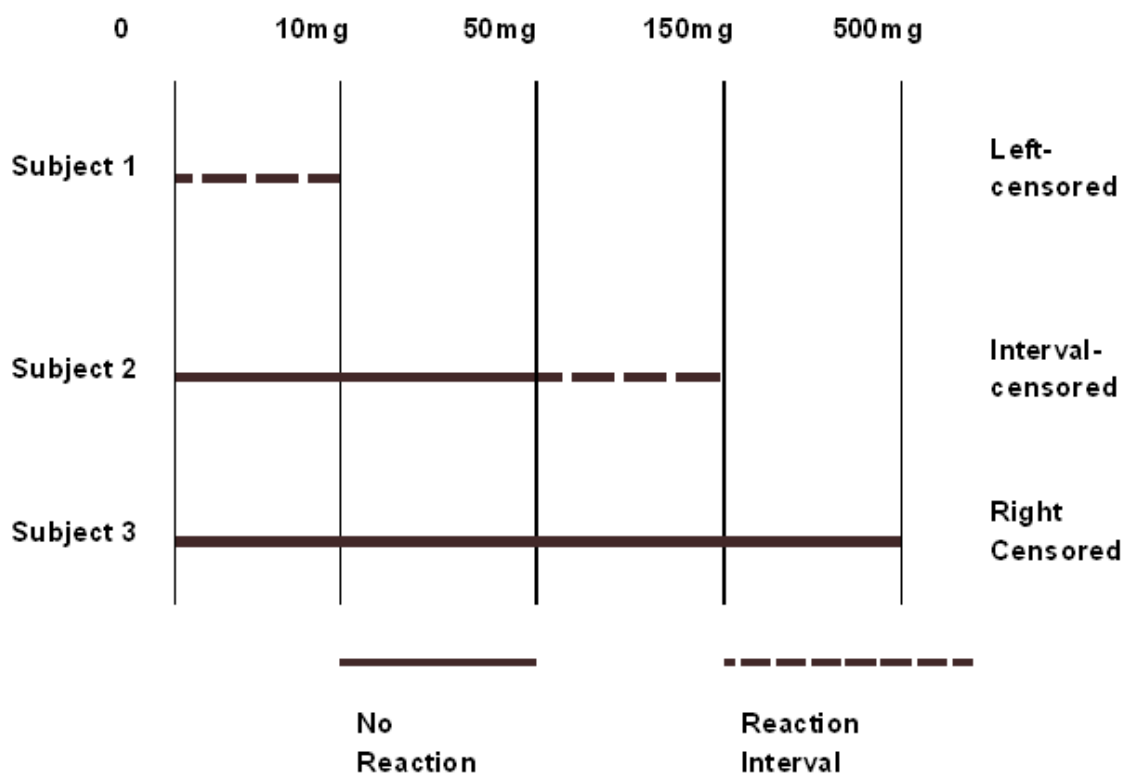


Figure 5 – Diagram of the Interval Censoring Survival Analysis and how it assigns censoring values. Sample dosing scheme progresses from 10 mg – 50 mg – 150 mg – 500 mg.

The Log-Normal ED₁₀ and ED₀₅ were 12.3 mg and 5.2 mg whole peanut, respectively (Table 2) (Taylor et al., 2010), while the Log-Logistic ED₁₀ and ED₀₅ were 12.6 mg and 4.5 mg whole peanut, respectively. The mathematical model chosen begins to heavily influence the predicted ED₀₁ values when less experimental data are available, especially from subjects with low individual thresholds. The established Log-Normal or Log-Logistic threshold curve could be used in quantitative risk assessment models to set regulatory and food industry action levels for peanut.

[illegible]

Advisory labeling of food allergens in processed foods

History of Use

As previously stated, physicians recommend complete avoidance of the allergenic food(s) and avoidance of the allergenic food is the only treatment option (Taylor et al., 1986a). Advisory “may contain” labeling is placed on products by food companies that cannot guarantee the absence of allergens due to incidental cross-contact during processing (Taylor and Baumert, 2010). With no guidance on food allergen thresholds from public health authorities, the food industry has placed voluntary advisory labeling on an ever-increasing number of packaged food products in efforts to alert food-allergic consumers to the possible presence of residues of the allergenic food with no consideration of the magnitude of any risk. While this situation assures to the maximum extent possible the safety and well-being of the food-allergic consumer, it serves to seriously limit food choices. Consequently evidence exists that some food-allergic consumers are ignoring precautionary allergen statements on labels, the exact opposite of the intent (Hefle et al., 2007; Sheth et al., 2012). Food-allergic individuals must try to interpret a variety of advisory labels causing confusion. Because of the proliferation of different forms of the wording of these voluntary advisory statements, some food-allergic consumers have the false impression that some foods with specific advisory statements (e.g. manufactured in a shared facility) are safer than foods with other statements (e.g. may contain) (Hefle et al., 2007). Despite the variety of statements that are used, all such statements are meant by the food industry to alert food-allergic consumers to foods possibly containing allergen residues so that they may avoid those foods. However, analytical surveys have documented, for products with advisory labels for peanut, that

only a small percentage contain detectable peanut residues and that some products without advisory labels possess similar allergen residue levels (Hefle et al., 2007; Pele et al., 2007; Pieretti et al., 2009).

Regulatory Guidance

Advisory labeling for allergens is voluntarily used by the food industry and not directly regulated or addressed by FALCPA (Gendel, 2012). With the exception of Japan, international allergen labeling regulations do not address advisory labeling (Gendel, 2012; Japan Consumer Affairs Agency, 2011). Japan states that “Possibility Labeling” is not allowed as it would allow manufacturers an easy way to escape the Product Liability Act and narrows options for allergic patients (Japan Consumer Affairs Agency, 2011). The U.S. Food, Drug, and Cosmetics Act requires that label statements be “truthful and not misleading” (Chapter II, Sec. 201) but the broad statement allows for a wide variety of advisory statements to appear on labels. Food allergic individuals are left to interpret advisory labels which can cause confusion and lead to weighted opinions of differing label styles. In a report by the U.S. Food and Drug Administration (FDA), both allergic and non-allergic consumers indicate that shorter “may contain” advisory labels were more likely to contain peanuts or other listed allergens. Additionally, both allergic and non-allergic consumers were more likely to serve a product with a longer statement such as “shared facility” or “shared equipment” to an allergic individual than a product with a shorter “may contain” statement (FDA, 2006). Additional studies show that allergic consumers avoid products that state they “May contain” or were “Manufactured on the same/shared equipment” more so than products that were “Manufactured in a facility that also processes/uses”. Health Canada and the UK Food Standards Agency have provided

guidance on the wording of advisory labels to address this issue but no further direction is available (Gendel, 2012).

The Voluntary Incidental Trace Allergen Labeling (VITAL) program in Australia was the first food allergen management tool developed to assist the food industry with the use of advisory labeling and declaration of the possible presence of allergens in products (Australian Allergen Bureau, 2009). VITAL's goal is to limit the use of advisory labeling through rigorous investigation of cross contact allergen presence in products before their release to the public. Initial risk management action levels were established for VITAL through the use of lowest reported individual threshold doses of protein from allergenic foods for subjective and objective allergic responses as cited by Gendel et al. (2008) in the 2006 U.S. Food & Drug Administration (FDA) Threshold Working Group (Australian Allergen Bureau, 2009). In 2011, a scientific panel was established to review the recommended reference doses for advisory labeling in VITAL as new data had become available. Between the panel and allergic consumer groups, it was decided that a protection level of 99% (the ED₀₁) is ideal. FARRP and TNO, in the Netherlands, reviewed allergic individuals with objective symptoms reported in blinded oral challenges from published literature and unpublished clinical data for the VITAL database. The collection of data revealed that the ED₀₁, a protection level of 99%, could be applied to several allergens. When the ED₀₁ was not a viable option due to limited data, the lower 95% confidence interval of the ED₀₅ was used to likely protect 97 – 99% of the allergic population. These general guidelines are available for companies to use when evaluating existing control measures and making decisions about the necessity of advisory labeling on their individual products. In using the VITAL reference dose, a

company must still assess their own internal control measures and their ability to consistently have allergen levels under the reference dose (Taylor et al., 2013 (In prep)).

Previous Examples/Studies

Products tested that contain advisory labels have been shown to have allergens present at low and high levels for all three major advisory labeling forms (“May contain,” “Shared Equipment,” and “Shared Facility”) (Ford et al., 2010; Hefle et al., 2007; Pele et al., 2007; Pieretti et al., 2009). A U.S. supermarket survey found 17% of products contain an advisory label statement for food allergens. The wording of such statements was split evenly among products as 38% had “May contain”, 33% had “Same/shared equipment”, and 29% had “Shared facility” labels. Certain categories, such as chocolate candy, cookies, and baking mixes, had the highest prevalence of advisory statement usage with 40-54% of the products having an advisory label (Pieretti et al., 2009). While the use of advisory labeling is high, Hefle et al. (2007) found in a 2005 survey that only 7.3% of products with peanut advisory statements tested had detectable levels of peanut. Conversely, a similar study with products containing milk advisory statements found detectable levels of milk in 42% of products (Crotty and Taylor, 2010). Detectable levels of milk were found in 78% of dark chocolates with advisory labeling for milk (Crotty and Taylor, 2010). Ford et al. (2010) found detectable levels of allergen in 1.8% of products with egg advisory statements, 10.2% of products with milk advisory statements, and 4.5% of products with peanut advisory statements. Products with milk advisory labels were more likely to contain detectable levels of milk if they were made by small companies in contrast to large food companies (Crotty and Taylor, 2010; Ford et al., 2010). A higher prevalence of detectable allergen was found in European products with

advisory labeling for peanut (25% of cookies, 43% of chocolates) and hazelnut (36% of cookies, 79% of chocolates) (Pele et al., 2007). The absence of advisory labeling does not protect allergic consumers as a survey found detectable levels of peanut (11% of cookies, 25% of chocolates) and hazelnut (25% of cookies, 53% of chocolates) in foods with no reference to the two allergens (Pele et al., 2007). Among U.S. products with no allergen declarations, only 1.9% contained detectable levels of peanut, milk, and egg products (Ford et al., 2010). Consumer avoidance of advisory labeled products has decreased and the prevalence of detectable allergen is low, but a risk of an allergic reaction still exists when consuming advisory labeled products.

Commodity contamination within the food supply

History of Use

Due to the nature of agricultural production supply chains, raw agricultural commodities can become contaminated with other agricultural commodities during harvest, transport, and storage with shared equipment and facilities. Although direct ingredients derived from commonly allergenic sources must be labeled in clear terms when added to food, raw agricultural commodities are exempt from FALCPA (FDA, 2006).

Regulatory Guidance

The U.S. Department of Agriculture (USDA) Grain Inspection Handbook allows up to 10% of other grains with established standards to be present in wheat (USDA, 2004). The 10% level equates to 100,000 parts per million (ppm) or 100,000 mg/kg ($\mu\text{g/g}$) of other grains and would cause visual contamination within containers of wheat.

Other grains with established standards include barley, canola, corn, flaxseed, oats, rye, sorghum, soybeans, sunflower seed, and triticale (USDA, 2004).

Before the removal of dockage (all matter other than the desired grain that can be removed), up to 25% of other grains are allowed in oats and barley, 20% in flaxseed, and 10% in corn, canola, soy, sorghum, rye, sunflower seed, and triticale (USDA Grain Inspection Handbook). CODEX international grain standards allow 1.5% or 15,000 ppm (mg/kg) other grains in wheat and corn (CODEX Standard 199-1995; CODEX Standard 153-1985). CODEX standards serve as guidance for all countries around the world.

While Japan and Switzerland have established regulatory threshold levels, neither country is known to have applied these levels to commodity grains. Commodity grain shipments may exceed the Japanese limit. The economics of buying and selling commodity grains have kept comingling below these allowed limits as food processors demand a cleaner, higher grade of wheat and other grains. However, the extent of the risk from commodity comingling to allergic consumers has not been extensively investigated.

Previous Examples/Studies

The issue of soy in other grains has become an issue for food safety inspection as highlighted by numerous food alerts within the European Union Rapid Alert System for Food and Feed (RASFF) and the Canadian Food Inspection Agency (CFIA). The Food Safety Authority of Ireland has issued multiple food alerts for the presence of soy in wheat and corn based products (white bread flour, flour and corn tortillas, corn chips, and a batter mix) (RASFF Reference 2011.0015; 2011.0019; 2011.0022; 2011.0023; 2011.0215). The CFIA also issued a food recall of a wheat-based cereal due to undeclared soy (Reference Number: 6848). Despite the lack of consumer complaints

associated with this cereal, products were recalled. In all likelihood, this cereal product has been produced for years with similar levels of soy. While levels of soy and other grains are known to occur in grain-based products, few studies have reported the levels of allergenic contaminants in agricultural commodities or finished products manufactured from these commodities. One exception is gluten, in large part due to “gluten-free” labeling, with North American levels of gluten contamination in other grains reported by multiple studies. Thompson et al. (2010) reported gluten contamination in 9 of 22 (41%) inherently gluten-free grain samples with levels up to 2925 ppm gluten. In a study by Health Canada, Koerner et al. (2011) reported 117 of 133 (88%) retail oat products contained levels up to 3784 ppm gluten. However, only one oat variety with a “gluten-free” label was tested and it was consistently below the limit of quantitation for gluten. Recent IgE-mediated allergic reactions due to commodity contamination of wheat in infant foods have led the CFIA to encourage manufacturers and importers of grain-based products to inform consumers and transition towards the inclusion of precautionary labeling (a 'may contain wheat' statement) on their products containing cereal grains, such as oats or barley, to indicate the potential presence of wheat at low levels (CFIA, 2011). While the actual levels of co-mingling are much lower than the 10% and 1.5% allowed by the USDA Handbook and CODEX, allergic individuals could still be at risk by consuming these products.

Food Allergen Risk Analysis

Risk analysis is a three part, interactive process that consists of a scientific risk assessment, a risk management strategy, and an exchange of information through risk communication (Figure 7)(FAO/WHO, 2008). All manner of risks are evaluated using

this same process. Risk analysis for food allergens does not, in concept, differ from other risks associated with foods.

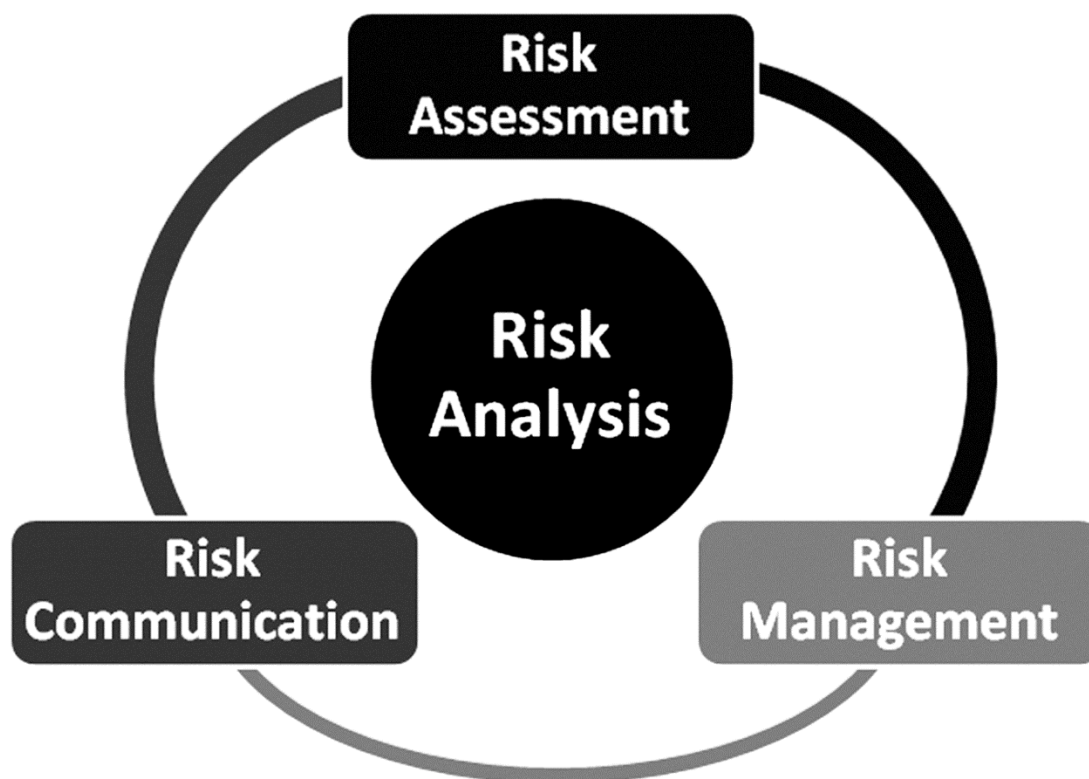


Figure 7 - Diagram of the interactive processes involved in proper risk analysis.

As previously stated, the prevalence of food allergies is increasing and recognition of the importance of food allergy as a public health and food safety issue has improved (Sicherer and Sampson, 2010). The occasional severity of food allergic reactions is evidenced by the number of annual emergency room visits and fatalities, which have served to heighten awareness even further (Bock et al., 2001; Clark et al., 2011). As a consequence, public health authorities and the food industry have developed and implemented strategies to protect the food allergic consumer.

For the food industry, the awareness of food allergies has led to the development and implementation of allergen control plans for manufacturing facilities, improved

labeling approaches as mandated by government authorities, and a proliferation of voluntary advisory statements (e.g. ‘may contain’ and many others) on packages. However, the zero-tolerance policy for allergens used by public health authorities creates practical problems for the food industry and the food allergic consumer because it is impossible to prove that absolutely no allergen residues are present. From an industrial cost-efficiency perspective, the same manufacturing facility has to be used to process multiple products. Shared production facilities and manufacturing equipment for multiple products creates an opportunity for trace residues of an allergenic food to come in contact with another food. Careful production schedules and meticulous cleaning are required when products of similar nature with differing allergen profiles are produced on shared equipment (i.e. ice creams or chocolates) (Taylor and Baumert, 2010; Taylor et al., 2002). When companies are not able to guarantee complete avoidance of cross-contact, an advisory “may contain” statement might be placed on the product label.

The development of better risk assessment approaches for allergenic foods could maintain the safety of foods for food-allergic consumers while expanding food choices. Overall impacts on labeling would be dependent on adoption of these approaches by the food industry and public health authorities. The improved approach is predicated on the existence of safe threshold doses for allergenic foods. Reported clinical observations of confirmed peanut-allergic individuals show that doses of peanut do exist below the exposures at which they will have a reaction (Taylor et al., 2002). The past decade has witnessed an influx of allergen threshold data that has allowed risk assessors to quantitatively adapt the traditional risk analysis approach for use with food allergens. As previously stated, threshold-based risk approaches are viewed favorably by public health

authorities and have been endorsed in consensus conferences (Gendel et al., 2008; Madsen et al., 2009). Similar approaches have long been used for the management of chemical and microbial hazards in food (FDA, 1994, 1995, 2000; Rasekh et al., 2005). A regulatory threshold for peanut and other allergens would reduce the proliferation of advisory labeling. Additionally, regulation would help allergic consumers separate the truly risky products from the products they are safe to consume. While the risk of allergenic foods has been widely recognized, the analysis of the risks (including assessment, management, and communication) has mostly been rudimentary and based upon identification and avoidance.

Risk Assessment

Risk Assessment is the scientific evaluation of known or potential adverse health effects resulting from human exposure to foodborne hazards. The process consists of four steps: hazard identification, hazard characterization, exposure assessment, and risk characterization (FAO/WHO, 2008).

Hazard Identification

Hazard identification is the recognition of a particular agent in foods with known or potential associated health effects (FAO/WHO, 2008). In food allergy, the hazard is a protein (or perhaps carbohydrate) moiety from a specific food that can cause sensitization and allergic reactions on subsequent exposures. Sensitization can occur to multiple proteins within a single food and any of them can be the cause of an allergic reaction (Taylor and Hefle, 2006b). A single protein such as Bet v 1, the major allergen in birch pollen, can cross react with proteins in foods from a number of categories including fresh fruits, vegetables, and legumes (Geroldinger-Simic et al., 2011). Sensitivity to a single

cross-reactive carbohydrate determinant (CCD) can lead to reactions after consumption of multiple foods (i.e. alpha-gal in beef, pork or lamb) (Commins and Platts-Mills, 2009). Clinically, food allergy has been recognized for a long period of time and the first reports of specific oral sensitization to egg appeared 100 years ago (Schloss, 1912; Schofield, 1908). Exposure to the major food allergens are not a risk to the majority of the population, but the food-allergic population must take their avoidance diets seriously as the risk of consumption is potentially life-threatening.

Hazard Characterization

Hazard characterization is the qualitative and/or quantitative evaluation of the nature of the adverse effects. If data are obtainable, a dose-response assessment should be performed (FAO/WHO, 2008). As previously detailed, food-allergic individuals can experience a range of symptoms on exposure to the offending food. Not all allergic reactions are life-threatening, and some food-allergic individuals will never experience a severe reaction. Food allergy symptoms range from very mild, such as itching and flush, to a severe drop in blood pressure and bronchospasm. The severity of an allergic reaction also depends upon the dose of exposure. The MED, or threshold, varies widely across the entire population of individuals allergic to any specific food (Crevel et al., 2010). As detailed in the Interval-Censoring Survival Analysis section, individual food allergen thresholds can be quantitatively ascertained through clinical food challenges, preferably a DBPCFC with objective symptoms as the endpoint. Risk assessors then use the results of these challenges to determine the dose-response curve and population threshold for a particular allergen. Data exist for a number of allergens to conduct quantitative, dose-response based risk assessments of food allergens.

Exposure Assessment

Exposure assessment is the qualitative and/or quantitative evaluation of the likely intake via food and other sources if relevant (FAO/WHO, 2008). For a quantitative risk assessment, two main variables shape the exposure patterns. First, the probability of an allergic consumer purchasing a particular product will determine if there is any exposure. Second, the amount eaten by the individual will influence the outcome of the risk assessment. There is no consumption database available solely for food-allergic consumers. Risk assessors must assume that allergic and non-allergic individuals consume a product at the same rate and their reasons for non-consumption are the same. It is well known that allergic consumers are very brand loyal and shared experiences can lead to avoidance of perceived “risky” products and product categories. However, it has been shown that some allergic consumers will purchase products that have allergen advisory statements (Hefle et al., 2007). While uncertainty exists regarding the consumption patterns of allergic consumers, the only option is to use the consumption patterns of the overall population as a suitable surrogate until dietary surveys are designed specifically for the allergic individual. However, the assumptions involved in that approach must be stated and understood.

There are many ways to estimate consumption patterns and a risk assessor can rely on internal company sales data or population-based dietary intake surveys to estimate the probability of a specific product being purchased. Depending on the company, sales data could include total market size for a product and the market share for the specific product from that company, the average number of packages sold during each transaction, and the estimated time until consumption once the product is in a home.

These data can be useful in a potential recall situation when calculating how many units are in the marketplace for purchase by allergic consumers and how many have already been consumed. Additionally, serving sizes and the likelihood of consuming more or less than a serving can be used to estimate the amount of exposure to a product.

In addition to the internal company data, population based dietary surveys can provide useful information on the consumption patterns of products. The amount of information available will vary depending on the product and country where it is sold. Currently, there are a number of countries with dietary surveys that can help guide risk assessors. For example, the U.S. conducts 2-day, 24-hour dietary recall interviews as part of the National Health and Nutrition Examination Survey (NHANES) and releases the data in 2-year sections. The NHANES interview, like others population surveys, includes demographic, socioeconomic, dietary, and health-related questions. From this data, it is possible to extract consumption data based on product category, age, sex, and a number of other categories if desired. A combination of the 2003-04, 2005-06, and 2007-08 surveys provides over 24,000 individuals with complete dietary recall interviews and more than 750,000 consumption recordings of specific product codes (CDC, 2004, 2006, 2008). During a 24-hour recall, individuals may consume the same product over multiple eating occasions. The risk assessor must choose how to handle repeat exposures in the period of 24 hours and clearly state how consumption was estimated in their final reports.

Risk characterization

Risk characterization is the integration of hazard identification, hazard characterization and exposure assessment into a qualitative and/or quantitative estimation of the adverse effects likely to occur in a given population, with the attendant

uncertainties (FAO/WHO, 2008). There are many options when choosing how to characterize the risk of a food allergen in a product but the three discussed in this chapter will be the NOAEL-based safety assessment method, benchmark dose method, and probabilistic risk assessment.

NOAEL-based safety assessment method

The NOAEL-based safety assessment approach has been widely used in the past for food additives and chemical contaminants. The safety assessment uses NOAELs and LOAELs from animal or human studies, applies an uncertainty factor to set a regulatory level, and compares the product in question to the set level. The standard safety factors used could include 10-fold factors for interspecies differences, intraspecies differences, population bias, and estimation of the NOAEL if only LOAEL data are available (Madsen et al., 2009). Interspecies differences do not apply to food allergies as controlled clinical studies are done in humans. Population bias could occur with small sample populations, but are unlikely in a large dataset from unselected clinical populations. For example, a study combined 750 peanut-allergic individuals from specific European clinics that tested everyone suspected of having a peanut allergy, including those with histories of severe reactions. The peanut thresholds have remained stable with the addition of new populations (Taylor et al., 2013 (In prep); Taylor et al., 2010). Based on objective symptoms, NOAELs are available for the most sensitive individuals for a number of allergens so a safety factor is not required to transition from the LOAEL. The only possible applicable safety factor is the 10-fold difference for intraspecies variation, although it could be argued that intraspecies variation is already taken into account by using an unselected group of individuals with suspected peanut allergy and the reliance

on mild objective symptoms as the endpoint of oral challenges. The lowest NOAEL out of 450 individual thresholds with objective symptoms is 0.1 mg (100 µg) of whole peanut (Taylor et al., 2010). After the 10-fold uncertainty, the potential regulatory level would be 10 µg of whole peanut. A 10 µg regulatory level would equate to 0.2 ppm whole peanut in a 50 g serving. The proposed level could not be consistently attained by the food industry or reliably measured by current testing methods and likely lead to a drastic proliferation of advisory peanut labeling.

The NOAEL-based safety assessment method has been applied for development of hypoallergenic infant formula according to AAP guidelines for a hypoallergenic infant formula study. If no reactions occur in challenges of 29 milk allergic infants there is 95% certainty that 90% of milk-allergic infants will tolerate the formula (essentially the 95% lower confidence interval of the ED₁₀) (American Academy of Pediatrics et al., 2000; Kleinman et al., 1991). Previous food challenge studies have attempted to use similar NOAEL-based approaches to establish safe levels of consumption for allergic consumers. In a study with 30 soy-allergic individuals, Ballmer-Weber et al. (2007) reported a cumulative NOAEL for subjective symptoms of 2 mg soy flour (1.1 mg soy protein) and a NOAEL of 158 mg soy flour (83.7 mg soy protein) for objective symptoms. Thus, there is 95% certainty that 90% of soy-allergic individuals will not have objective reactions to 158 mg soy flour. After application of a 10-fold safety factor, the regulatory level for soy flour would allow 15.8 mg soy flour in a 50 g serving which equates to 316 ppm soy flour. Hefle et al. (2003) used spray-dried whole egg (SDWE) to determine the threshold dose in 39 egg-allergic individuals. The objective NOAEL was 330 µg SDWE (150 µg egg protein). However, subsequent challenges in a similar manner determined a LOAEL

for one individual who reacted to the first dose of 30 µg SDWE (14 µg egg protein). As the individual reacted at the first dose, no NOAEL is available and an additional 10-fold safety factor would be used to estimate the NOAEL from the LOAEL. A 0.3 µg SDWE regulatory level would equate to 0.006 ppm SDWE in a 50 g serving (0.0028 ppm egg protein). The NOAEL-based method in these instances might be argued to endanger a number of soy allergic individuals, while regulatory levels for egg could not be measured by current testing methods. Furthermore, this approach has been used in testing of several food ingredients derived from commonly allergenic food sources. In the case of highly refined peanut oil, 58 peanut-allergic subjects were challenged with no reactions providing 95% confidence that 95% of peanut-allergic individuals would tolerate highly refined peanut oil (Hourihane et al., 1997). Fish gelatin derived from codfish skin has also been tested in cod-allergic subjects and 0 of 29 individuals reacted to ingestion of 3.64 g cumulative dose of fish gelatin (Hansen et al., 2004).

Although this approach has been used in some circumstances with a degree of success, there are many drawbacks to using the NOAEL-based safety assessment approach. The main arguments against the safety assessment approach are that it uses one point from one study and would set regulatory action levels too low to be helpful in some real world applications, e.g. egg. The NOAEL-based safety assessment approach would not benefit the food-allergic consumer, the food industry, or the public health authorities so another method must be used.

Benchmark Dose method

The Benchmark Dose (BD) was developed as a way to fit mathematical models to experimental data for chemicals and carcinogens and as a general improvement over the

NOAEL-based safety assessment (Crump, 1984). The BD is a low but measurable level, usually derived from the lower confidence interval of the statistical distribution. As stated by Crump (1984), “the method can be applied to either “quantal” data in which only the presence or absence of an effect is recorded, or “continuous” data in which the severity of the effect is also noted.” In chemical risk assessment, a dose that will produced a response in 10% of the population is the standard BD level and uncertainty factors can then be applied if necessary. The BD should be within the range of doses observed in the study to avoid extrapolation below the experimental findings and limit strong dependence on a particular mathematical model (Crump, 1984). In the case of food allergy, quantal data from clinical challenges with objective symptoms are analyzed using the Interval-Censoring Survival Analysis technique, as previously described, to obtain a dose-response curve. In food allergy, a BD that would place 10% (BD₁₀) of the food-allergic population at risk is too high and safety factors would likely be applied. Peanut has the most robust allergen dataset available and a BD₀₅ or BD₀₁ could be used, but this level of analysis is not available for all food allergen population thresholds due to the lack of data (Australian Allergen Bureau, 2011; Taylor et al., 2013 (In prep)). Risk management decisions could be made on a level of acceptable risk but first a uniform method for evaluating BD levels and relevant safety factors, if any, must be developed. Once BD levels are set, a Margin of Exposure (MoE) could be evaluated (Madsen et al., 2009). The MoE calculation would utilize the exposure assessment for a particular food, potential allergen contamination levels, and the BD levels for an allergen to try and characterize the risk of a product. However, the risk is still not quantitatively described and multiple

regulatory decisions would be required regarding the appropriate exposure levels to use and the desired MoE.

Quantitative Risk Assessment

Quantitative risk assessment (QRA) was first applied to food allergens in a case study with hazelnut contamination found in nominally hazelnut-free chocolate spreads (Spanjersberg et al., 2007). A second QRA study examined large amounts of milk protein in multiple brands of dark chocolate products and led to a widespread change in labeling practices (Spanjersberg et al., 2010). Although relatively new to food allergens, QRA has been used by microbial and chemical risk assessors for years (EU, 2003b; Kroes et al., 2000; Lammerding and Fazil, 2000; Larsen, 2006; Notermans et al., 1995). As previously stated, consensus has been reached that statistically-based quantitative methodology is the most promising approach for food allergen risk assessment (Gendel et al., 2008; Madsen et al., 2009). Prior to 2009, allergen dose-distribution modeling had been explored but no robust threshold datasets were available for IgE-mediated allergic reactions to any food (Bindslev-Jensen et al., 2002; Crevel et al., 2007). Substantial work has led to large datasets for a number of food allergens including peanut, milk, egg, and hazelnut. Datasets for soy, wheat, cashew, and other allergens have been constructed as well. However, a gap does exist between the number of available subjects in the top four datasets and the rest of the allergens (Australian Allergen Bureau, 2011; Taylor et al., 2013 (In prep)). Kruizinga et al. (2008) noted that a shift in the dose-response curve will have a considerable effect on the results of the QRA. Datasets with a small number of subjects are significantly affected by the addition of individuals at the low or high end of the curve. However, this drawback is not limited to QRA alone as the BD also uses a

dose-response curve and the NOAEL-based method only uses a single point. Additional data at the low end of the dose-response curve could significantly affect the results of all three methods.

Quantitative risk assessments require the most data of any risk characterization, but very little additional data are required to step from a BD approach to a QRA. The main data needed to conduct a QRA include the prevalence of food allergy, allergen dose-response curve, and the allergen intake curve, which consists of consumption data and data on the level of allergen residues present (Figure 8).

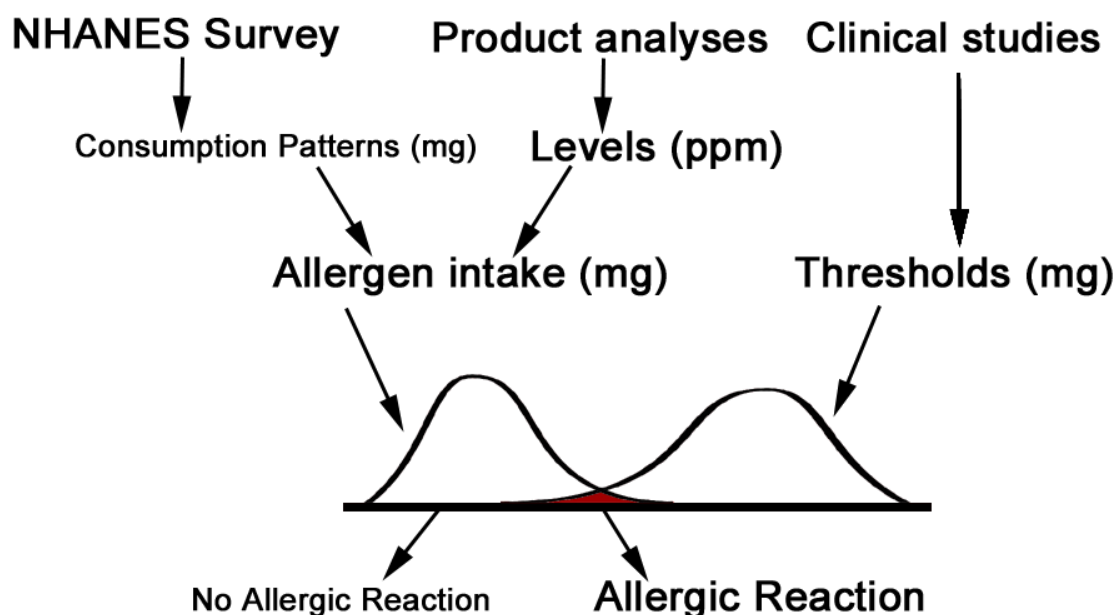


Figure 8 – Probabilistic risk assessment model for food allergens in the U.S., figure adapted from Spanjersberg et al. (2007).

The true prevalence of food allergy is unknown. In a QRA, the prevalence of a specific food allergy is estimated by using published literature as a reference. Once the prevalence of allergy is set, the distribution of allergy in the population is binomial and the simulation will choose if every individual is allergic or non-allergic. If the individual is allergic, a minimal eliciting dose (MED) will be chosen from the statistically fit dose-

response distribution. As stated in the exposure assessment section, population-based dietary intake surveys or internal company data on consumption can be used for an individual product. If no data are available, a determined serving size can be substituted with the likelihood that more than one serving could be consumed. In a QRA, the consumption of a product is binomial, meaning the simulation chooses if purchase and consumption of the product occurs or it does not. The rate of purchase is determined from the relevant population surveys and market share data available to a company. If the product is purchased, the reported individual consumption amounts can be directly sampled from dietary intake surveys to create the consumption input or sampled from a representative statistical distribution. The part per million (ppm or $\mu\text{g/g}$) level of allergen present is determined by laboratory analysis of individual products or through product formulation calculations. Ideally, the risk assessment would be started on a worst-case product formulation scenario and adjusted after laboratory analysis has been completed. The presence of an allergen is a binomial distribution. The simulation will choose based on the percentage of positive samples to determine if the product being consumed has the allergen present or not. If the scenario is worst case or all samples are positive, the presence of allergen will be set to 100%. The ppm levels of allergen present are either directly sampled from the laboratory analysis or chosen from a representative statistical distribution.

The QRA is a Monte Carlo simulation that will randomly sample from each distribution during every run and iteration, match if the individual is allergic, a consumer of the product in question, and if the product contains the allergen to determine if there is a possibility of an allergic reaction. An individual will have a predicted allergic reaction

if the predicted consumed amount and concentration of allergen lead to a dose over the predicted allergen threshold. Risk assessments can use a modified Monte Carlo approach, incorporating the mean and standard error associated with each input variable into a Bayesian framework. Briefly, the Bayesian context of a simulation with 50 runs of 10,000 iterations will be explained using a Log-Normal dose-response curve. As the true population threshold and dose-response curve of each allergen are unknown, the Bayesian analysis will use statistical inference to generate the parameters of the Log-Normal distribution to reflect uncertainty in the true location of each parameter. The confidence intervals around the curve will shrink as more individuals become available for the dose-response curve, thus demonstrating the importance of robust datasets. The end result is that every run will have a new Log-Normal distribution associated with the dose-response curve. The Bayesian framework will create 50 dose-distribution curves that fall within the confidence intervals of the predicted distribution from the interval-censoring procedure. This same process can also be used for the distribution representing consumption amounts and the ppm levels of allergens present.

Additional Bayesian methods can be applied to the binomial prevalence distributions estimating allergic prevalence, presence of allergen in a product, and consumption/purchase of the specific product. Rimbaud et al. (2010) describe the process for fitting a Binomial distribution with a non-informative prior Beta(1,1) distribution. Using the Beta probability density function, the posterior binomial distribution probability can be solved as follows: $p \sim \text{Beta}(1+x, 1+n_1-x)$ with x representing a positive response and n_1 representing the total number of people in the study. Thus, a new binomial probability is chosen for every run of the simulation based on the number of

individuals collected for each input. The probabilities will shift less with each run when more data are available for each input.

When the simulation is run, the stepwise process shown in Figure 9 is done for every iteration to predict if an allergic reaction will occur. The results of the finished simulation can be expressed in multiple ways, but this chapter will discuss the allergic user risk, risk to the allergic population, and risk to the overall population. Please note that the three risk values represent the same number of predicted reactions expressed three different ways. The allergic user risk bears the least number of uncertainties and assumes every individual in the simulation is allergic and consumes the product in question. The allergic population risk assumes every individual is allergic to the allergen in question but only a certain percent of the population consumes the product category. The overall population risk includes every individual, including non-allergic and non-consuming individuals. The three risk values are the same because a non-allergic consumer is never at risk for an allergic reaction. This is different than chemical carcinogen risk assessment where all populations are susceptible but certain groups are more at risk than others. It is critical to understand the risk outputs and present them appropriately to avoid confusion. Quantitative risk assessment is a flexible tool that utilizes the food allergen threshold curves to investigate a wide range of allergen issues. Quantitative methods can be used to determine when to apply advisory labeling, assist with product release or conversely with product recall, and validate clean-in-place measures.

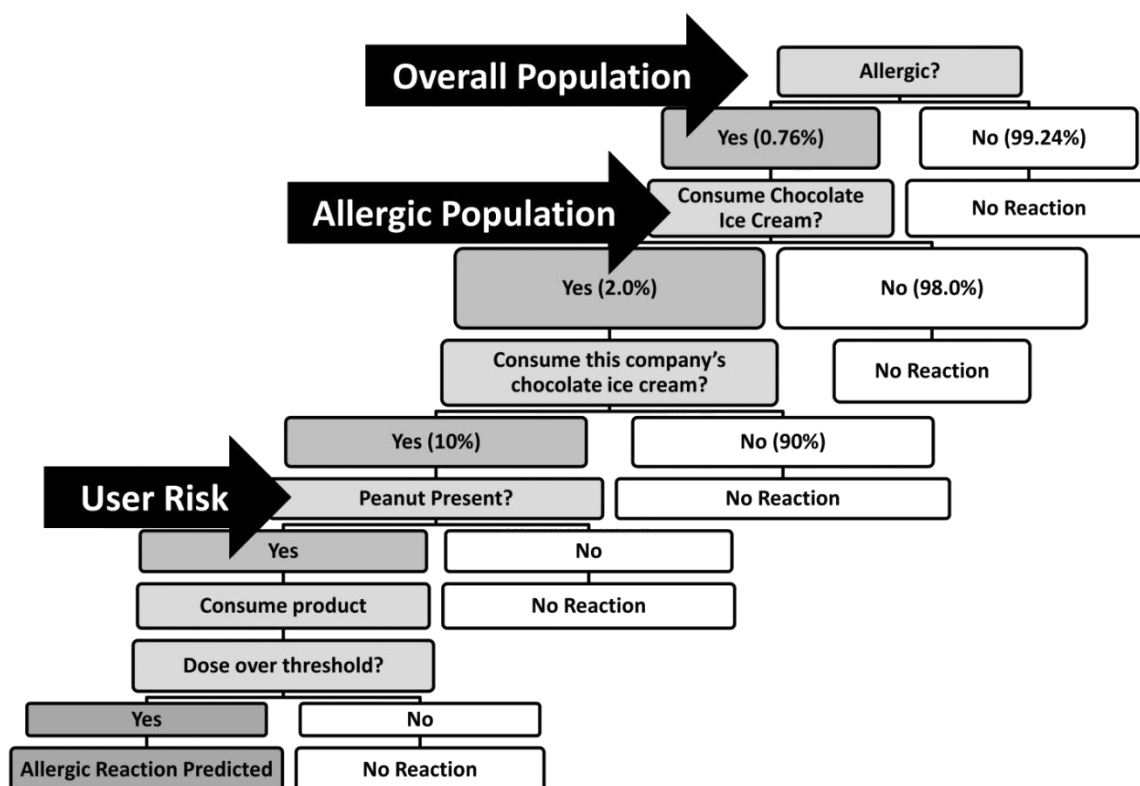


Figure 9 – Quantitative risk assessment step-by-step decision tree during a simulation. Arrows indicate where the allergic user risk, allergic population risk, and overall population risk are calculated from in the simulation.

Risk management

Risk management is defined as the process, distinct from risk assessment, of weighing policy alternatives in consultation with all interested parties. Risk management includes considering risk assessment and other factors relevant for the health protection of consumers and, if needed, selecting appropriate prevention and control options (FAO/WHO, 2008). Risk management decisions should be separate from the risk assessment process to ensure the scientific integrity of the risk assessment and reduce any conflicts of interest. However, interaction between the risk assessors and the risk managers is essential for practical application of any risk management options (FAO/WHO, 2008). Cooperation and collaboration is key when dealing with food allergies as a uniform risk assessment policy would benefit regulatory bodies, academics,

and consulting firms when presenting the results to the risk assessors and risk managers in the food industry. Proper risk management will integrate preliminary risk management activities, evaluation of risk management options, implementation of the risk management decision, and monitoring and review of the process based on the risk assessment (FAO/WHO, 2008).

Preliminary risk management activities

Preliminary risk management activities establish a risk profile to facilitate context and may commission a risk assessment to guide further action. When a risk assessment is needed, the policy should be established by risk managers in advance of risk assessment. Efforts to consult with risk assessors and regulatory bodies before the assessment is done help ensure a complete, unbiased and transparent risk assessment (FAO/WHO, 2008).

The risk assessment should include all assumptions and uncertainties. The responsibility for resolving the impact of uncertainty on the risk management decision lies with the risk manager, not the risk assessors (FAO/WHO, 2008). For food allergens, the form of risk presented (i.e. allergic user risk, allergic population, and overall population) and alternative forms (i.e. predicted reactions from a product) should be clearly defined within the risk assessment. The risk estimate should be reported in a form that is readily understood by risk managers, defensible to regulatory bodies, and easily communicated to the food allergic community

Evaluation of risk management options

Evaluation of risk management options is an overarching process that weighs available options for managing a food safety issue. Decisive factors include available scientific information, a decision on an appropriate level of consumer protection, the

effectiveness of control measures, and a cost-benefit analysis (FAO/WHO, 2008).

Currently, there are no national risk management programs for food allergens. A company is presented with limited options for allergen labeling. When an allergen is in the product formulation, it must be labeled in the ingredient statement and possibly a “contains” statement. If cross-contamination is possible then a risk assessment should be done and a decision must be made between “may contain” advisory labeling or not referencing the allergen on the label. As previously stated, the VITAL expert panel selected a level of 99% consumer protection as the action level for advisory labeling (Australian Allergen Bureau, 2011). When using the VITAL approach, an individual company must use the provided reference dose as a guideline with the evaluation process of their allergen control practices and scrutinize the use of advisory labeling on their products.

Implementation of the risk management decision

Implementation of the risk management decision will involve the application and verification of food safety measures. A HACCP plan is usually included and flexibility is desired, as long as the overall program can be objectively shown to achieve the stated goals (FAO/WHO, 2008). Foods may encounter cross-contamination at any point of the food chain. Shared equipment and storage facilities during the harvesting and transportation of agricultural commodities is a source of contamination for some commonly allergenic foods and is generally exempt from labeling (FDA, 2006; USDA, 2004). Another source of cross-contamination for the food industry is within the processing facility, especially the use of shared equipment when making packaged foods. Supplier auditing is key as there are many levels of suppliers before a food reaches the

end processor and undeclared allergens could enter the food at any location. It is important for a company to have the best information available for the ingredients entering its facility to address any risk of cross-contamination appropriately (Taylor and Baumert, 2010).

Dedicated facilities, processing lines, or specific equipment are not feasible in many commercial situations so proper management of shared equipment and facilities is essential to an allergen control plan (Taylor and Baumert, 2010). The physical separation of allergenic formulations from non-allergen products during manufacturing and the creative use of product scheduling strategies can help reduce the risk of cross-contamination. Effective sanitation of equipment is key when changing back from allergenic to non-allergen products (Taylor and Baumert, 2010). Wet-cleaning methods are effective, but not allowed in many situations. Bakeries and chocolate manufactures must use dry-cleaning methods and have an especially hard time ensuring that all allergens are removed. Analytical tests, like the ELISA method, can be used on site or at an independent laboratory to verify the effectiveness of cleaning procedures. In situations with hard to clean pieces of equipment, it is recommended to test the corners and weld positions for any potential buildup of allergen compared to the smooth surfaces. If an allergen cannot be consistently controlled, advisory labeling may be implemented (Taylor and Baumert, 2010).

Monitoring and review

Monitoring and review is the gathering and analyzing of data to give an overview of food safety and consumer health. If goals are not being achieved, redesign of food safety measures will be needed (FAO/WHO, 2008). Risk management is a continuous

process for a food manufacturer. Monitoring cleaning processes is critical for a company and can be done with easy to use ELISA-based tests (Taylor and Baumert, 2010). In accordance with the U.S. FDA Food Safety Modernization Act (FSMA), a company must “evaluate the hazards (including allergens) that could affect food manufactured, processed, packed, or held by such facility, identify and implement preventive controls to significantly minimize or prevent the occurrence of such hazards and provide assurances that such food is not adulterated, monitor the performance of those controls, and maintain records of this monitoring as a matter of routine practice” (FDA, 2011). As an industry, significant updates will be necessary to document and validate allergen control plans to comply with the regulations outlined by FSMA. However, details on specific validation practices and approved methods are not clearly outlined within the law and companies will need further interpretation from the FDA to ensure compliance. To guarantee the highest levels of protection for the food allergic consumer, new allergen testing methods should be continually evaluated, new scientific knowledge should be reviewed regularly, and reference doses for allergen labeling should be updated as necessary when more data on clinical threshold trials becomes available.

Risk Communication

Risk communication is an interactive process exchanging information and opinion on risk among risk assessors, risk managers, consumers, industry, the academic community and other interested parties (FAO/WHO, 2008). Communication is essential and should continue through the entire risk analysis process. One goal of risk communication is to foster public understanding of the process and to enhance trust and confidence in the safety of the food supply (FAO/WHO, 2008). Thus, dissemination of

information must be done in a way that presents the food allergic consumer with a clear and simple understanding of the process. The main form of risk communication between a food producer and an allergic consumer is the product package/label. Current food allergen regulations mandate labeling of allergenic ingredients to clearly state when an allergenic risk is present to the consumer (Gendel, 2012). A second, less effective form of risk communication is advisory labeling for allergens. While well intended, proliferation of advisory labeling has led to risk taking activities within the food allergic community (FDA, 2006; Hefle et al., 2007). Advisory labeling must be clear, concise, and uniform across the food industry and only adopted when cross-contamination is likely in order to avoid consumer confusion.

Summary

Food allergies affect a small percentage of the population. The prevalence and awareness of IgE-mediated food allergies appear to be increasing in developed countries. Symptoms of a food allergy vary from mild to severe anaphylaxis and death. Avoidance of the allergenic food is the only treatment option but complete success of an avoidance diet is unlikely. Progress has been made on aspects of identifying, characterizing, and detecting food allergens, and more recently on individual food allergen thresholds. Quantitative risk assessment is a flexible tool that utilizes food allergen threshold curves to investigate a wide range of allergen issues, including when to apply advisory labeling, assist with product release or conversely with product recall, and validate the effectiveness of sanitation measures. However, hurdles do remain before regulatory thresholds can be established for food allergens, such as regulatory and consumer agreement on an acceptable risk and consensus on risk communication methods to

demonstrate that a product has undergone a risk assessment. The main objective of this study was to further characterize food allergen population thresholds and develop quantitative risk assessment methods for food allergens in the U.S., in an effort to help reduce the number of severe allergic reactions caused by packaged foods.

References

- Adachi, A., Horikawa, T., Shimizu, H., Sarayama, Y., Ogawa, T., Sjolander, S., Tanaka, A., Moriyama, T., 2009. Soybean β -conglycinin as the main allergen in a patient with food-dependent exercise-induced anaphylaxis by tofu: food processing alters pepsin resistance. *Clin Exp Allergy* 39, 167-173.
- Adachi, M., Kanamori, J., Masuda, T., Yagasaki, K., Kitamura, K., Mikami, B., Utsumi, S., 2003. Crystal structure of soybean 11S globulin: glycinin A3B4 homohexamer. *Proc Natl Acad Sci USA* 100, 7395-7400.
- Adams, F., 1886. *The Genuine works of Hippocrates*. William Wood and Company, New York, NY.
- Altman, D.R., Chiaramonte, L.T., 1996. Public perception of food allergy. *J Allergy Clin Immunol* 97, 1247-1251.
- American Academy of Pediatrics, C.o.N., Baker, S.S., Cochran, W.J., Greer, F.R., Heyman, M.B., Jacobson, M.S., Jaksic, T., Krebs, N.F., Smith, A.E., Yuen, D.E., Dietz, W., Yetley, E., Harris, S.S., Prendergast, A., Grave, G., Hubbard, V.S., Blum-Kemelor, D., Lauer, R.M., Denne, S.C., Kleinman, R., Kanda, P., 2000. Hypoallergenic infant formulas. *Pediatrics* 106, 346-349.
- Amnuaycheewa, P., Gonzalez de Mejia, E., 2010. Purification, characterisation, and quantification of the soy allergen profilin (Gly m 3) in soy products. *Food Chem* 119, 1671-1680.
- Australian Allergen Bureau, 2009. VITAL Q & A for Consumers. <http://www.allergenbureau.net/downloads/vital/VITAL-QandA-for-consumers-v7.pdf> Accessed 03/21/2012.
- Australian Allergen Bureau, 2011. Summary of the VITAL Scientific Expert Panel Recommendations. <http://www.allergenbureau.net/downloads/vital/VSEP-Summary-Report-Oct-2011.pdf>. Accessed 02/12/12, Australia, pp. 1-5.
- Ballmer-Weber, B.K., Holzhauser, T., Scibilia, J., Mittag, D., Zisa, G., Ortolani, C., Osterballe, M., Poulsen, L.K., Vieths, S., Bindslev-Jensen, C., 2007. Clinical characteristics of soybean allergy in Europe: a double-blind, placebo-controlled food challenge study. *J Allergy Clin Immunol* 119, 1489-1496.
- Bando, N., Tsuji, H., Yamanishi, R., Nio, N., Ogawa, T., 1996. Identification of the glycosylation site of a major soybean allergen, Gly m Bd 30K. *Biosci Biotechnol Biochem* 60, 347-348.
- Barać, M.B., Stanojević, S.P., Jovanović, S.T., Pešić, M.B., 2004. Soy protein modification: A review. *Acta Periodica Technologica* 35, 3-16.

- Baur, X., Pau, M., Czuppon, A., Fruhmman, G., 1996. Characterization of soybean allergens causing sensitization of occupationally exposed bakers. *Allergy* 51, 326-330.
- Bazzano, L.A., He, J., Ogden, L.G., Loria, C., Vupputuri, S., Myers, L., Whelton, P.K., 2001. Legume consumption and risk of coronary heart disease in US men and women: NHANES I Epidemiologic Follow-up Study. *Arch Intern Med* 161, 2573.
- Beardslee, T.A., Zeece, M.G., Sarath, G., Markwell, J.P., 2000. Soybean glycinin G1 acidic chain shares IgE epitopes with peanut allergen Ara h 3. *Int Arch Allergy Immunol* 123, 299-307.
- Bernhisel-Broadbent, J., Taylor, S., Sampson, H.A., 1989. Cross-allergenicity in the legume botanical family in children with food hypersensitivity II. Laboratory correlates. *J Allergy Clin Immunol* 84, 701-709.
- Bindslev-Jensen, C., Ballmer-Weber, B.K., Bengtsson, U., Blanco, C., Ebner, C., Hourihane, J., Knulst, A.C., Moneret-Vautrin, D.A., Nekam, K., Niggemann, B., Osterballe, M., Ortolani, C., Ring, J., Schnopp, C., Werfel, T., 2004. Standardization of food challenges in patients with immediate reactions to foods - position paper from the European Academy of Allergology and Clinical Immunology. *Allergy* 59, 690-697.
- Bindslev-Jensen, C., Briggs, D., Osterballe, M., 2002. Can we determine a threshold level for allergenic foods by statistical analysis of published data in the literature? *Allergy* 57, 741-746.
- Blomhoff, R., Carlsen, M.H., Andersen, L.F., Jacobs, D.R., 2006. Health benefits of nuts: potential role of antioxidants. *Br J Nutr* 96, 52-60.
- Blumchen, K., Ulbricht, H., Staden, U., Dobberstein, K., Beschoner, J., de Oliveira, L.C.L., Shreffler, W.G., Sampson, H.A., Niggemann, B., Wahn, U., Beyer, K., 2010. Oral peanut immunotherapy in children with peanut anaphylaxis. *J Allergy Clin Immunol* 126, 83-91.
- Bock, S.A., Muñoz-Furlong, A., Sampson, H.A., 2001. Fatalities due to anaphylactic reactions to foods. *J Allergy Clin Immunol* 107, 191-193.
- Breiteneder, H., Radauer, C., 2004. A classification of plant food allergens. *J Allergy Clin Immunol* 113, 821-830.
- Burks, A., Williams, L.W., Thresher, W., Connaughton, C., Cockrell, G., Helm, R.M., 1992. Allergenicity of peanut and soybean extracts altered by chemical or thermal denaturation in patients with atopic dermatitis and positive food challenges. *J Allergy Clin Immunol* 90, 889-897.
- Caminiti, L., Passalacqua, G., Barberi, S., Vita, D., Barberio, G., De Luca, R., Pajno, G.B., 2009. A new protocol for specific oral tolerance induction in children with IgE-mediated cow's milk allergy. *Allergy Asthma Proc* 30, 443-448.

CDC, 2004. Centers for Disease Control and Prevention (CDC). National Center for Health Statistics (NCHS). National Health and Nutrition Examination Survey Questionnaire (or Examination Protocol, or Laboratory Protocol). Hyattsville, MD: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, http://www.cdc.gov/nchs/nhanes/nhanes2003-2004/nhanes03_04.htm. Accessed 03/01/2013.

CDC, 2006. Centers for Disease Control and Prevention (CDC). National Center for Health Statistics (NCHS). National Health and Nutrition Examination Survey Questionnaire (or Examination Protocol, or Laboratory Protocol). Hyattsville, MD: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, http://www.cdc.gov/nchs/nhanes/nhanes2005-2006/nhanes05_06.htm. Accessed 03/01/2013.

CDC, 2008. Centers for Disease Control and Prevention (CDC). National Center for Health Statistics (NCHS). National Health and Nutrition Examination Survey Questionnaire (or Examination Protocol, or Laboratory Protocol). Hyattsville, MD: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, http://www.cdc.gov/nchs/nhanes/nhanes2007-2008/nhanes07_08.htm. Accessed 03/01/2013.

CFIA, 2011. Allergen Labelling Information for Manufacturers and Importers of Cereal Grain-based Products. Canadian Food Inspection Agency, <http://www.inspection.gc.ca/english/fssa/labeti/allerg/20110520inde.shtml>. Accessed 03/01/2013.

Clark, A.T., Islam, S., King, Y., Deighton, J., Anagnostou, K., Ewan, P.W., 2009. Successful oral tolerance induction in severe peanut allergy. *Allergy* 64, 1218-1220.

Clark, S., Espinola, J., Rudders, S.A., Banerji, A., Camargo, C.A., Jr., 2011. Frequency of US emergency department visits for food-related acute allergic reactions. *J Allergy Clin Immunol* 127, 682-683.

Codina, R., Lockey, R.F., Fernández-Caldas, E., Rama, R., 1997. Purification and characterization of a soybean hull allergen responsible for the Barcelona asthma outbreaks. II. Purification and sequencing of the Gly m 2 allergen. *Clin Exp Allergy* 27, 424-430.

Collett, D., 1993. *Modelling Survival Data in Medical Research*, 2nd ed. Chapman & Hall/CRC Press, Boca Raton, FL.

Commins, S.P., Platts-Mills, T.A., 2009. Anaphylaxis syndromes related to a new mammalian cross-reactive carbohydrate determinant. *J Allergy Clin Immunol* 124, 652-657.

Coombs, R.R.A., Gell, P.G.H., 1975. Classification of allergic reactions responsible for clinical hypersensitivity and disease, in: P.G.H. Gell, R.R.A. Coombs, P.J. Lachmann

(Eds.), *Clinical Aspects of Immunology*, 3rd ed. Blackwell Scientific Publications, Oxford, UK, pp. 761-781.

Crevel, R., Moneret-Vautrin, D.A., Morisset, M., Sheffield, D., Taylor, S.L., Baumert, J.L., 2010. A preliminary analysis of the evolution of peanut thresholds over repeated challenges in a population of consecutive clinic patients. *J Allergy Clin Immunol* 125, AB 84.

Crevel, R.W.R., Briggs, D., Hefle, S.L., Knulst, A.C., Taylor, S.L., 2007. Hazard characterisation in food allergen risk assessment: the application of statistical approaches and the use of clinical data. *Food Chem Toxicol* 45, 691-701.

Crotty, M.P., Taylor, S.L., 2010. Risks associated with foods having advisory milk labeling. *J Allergy Clin Immunol* 125, 935-937.

Crowell, D.N., John, M.E., Russell, D., Amasino, R.M., 1992. Characterization of a stress-induced, developmentally regulated gene family from soybean. *Plant Mol Biol* 18, 459-466.

Crump, K.S., 1984. A new method for determining allowable daily intakes. *Fundam Appl Toxicol* 4, 854-871.

D'Amato, G., Cecchi, L., Bonini, S., Nunes, C., Annesi-Maesano, I., Behrendt, H., Liccardi, G., Popov, T., Van Cauwenberge, P., 2007. Allergenic pollen and pollen allergy in Europe. *Allergy* 62, 976-990.

de Blok, B.M.J., Vlieg-Boerstra, B.J., Oude Elberink, J.N.G., Duiverman, E.J., Dunn-Galvin, A., Hourihane, J.O.B., Cornelisse-Vermaat, J.R., Frewer, L., Mills, C., Dubois, A.E.J., 2007. A framework for measuring the social impact of food allergy across Europe: a EuroPrevall state of the art paper. *Allergy* 62, 733-737.

du Toit, G., Katz, Y., Sasieni, P., Mesher, D., Maleki, S.J., Fisher, H.R., Fox, A.T., Turcanu, V., Amir, T., Zadik-Mnuchin, G., Cohen, A., Livne, I., Lack, G., 2008. Early consumption of peanuts in infancy is associated with a low prevalence of peanut allergy. *J Allergy Clin Immunol* 122, 984-991.

Duke, W., 1934. Soy bean as a possible important source of allergy. *J Allergy* 5, 300-302.

Dunn-Galvin, A., de Blok-Flokstra, B.M.J., Burks, A.W., Dubois, A.E.J., Hourihane, J.O.B., 2008. Food allergy QoL questionnaire for children aged 0 - 12 years: content, construct, and cross-cultural validity. *Clin Exp Allergy* 38, 977-986.

Egbert, W.R., 2004. Isolated Soy Protein, in: K. Liu (Ed.), *Soybeans as Functional Foods and Ingredients*. AOCS Publishing, Urbana, IL, pp. 134-162.

Ellis, M.H., Short, J.A., Heiner, D.C., 1991. Anaphylaxis after ingestion of a recently introduced hydrolyzed whey protein formula. *J Pediatr* 118, 74-77.

Emsley, J., 2011. *Nature's building blocks: an AZ guide to the elements*. Oxford University Press, New York, NY.

Esmailzadeh, A., Azadbakht, L., 2012. Legume consumption is inversely associated with serum concentrations of adhesion molecules and inflammatory biomarkers among Iranian women. *J Nutr* 142, 334-339.

EU, 2003a. European Commission Directive 2003/89/EC of the European Parliament and of the Council of 10 November 2003 amending Directive 2000/13/EC as regards induction of the ingredients present in foodstuffs. *Official journal of the European communities* L308, 15-18.

EU, 2003b. Risk Assessment of Food Borne Bacterial Pathogens: Quantitative Methodology Relevant for Human Exposure Assessment. Scientific Steering Committee, Health and Consumer Protection Directorate-General, European Commission.

FAO, 1995. Report of the FAO Technical Consultation on Food Allergies, Rome, Italy 13-14 November 1995. Food and Agriculture Organization of the United Nations.

FAO/WHO, 2008. Codex Alimentarius Commission procedural manual, 18th ed. Food and Agriculture Organization of the United Nations, Codex Alimentarius Commission, Rome.

FDA, 1994. CPG Sec. 683.100 Action Levels for Aflatoxins in Animal Feeds.

FDA, 1995. CPG Sec. 575.100 Pesticide Residues in Food and Feed - Enforcement Criteria.

FDA, 1999. Food Labeling: Health Claims; Soy Protein and Coronary Heart Disease; Final Rule. *Fed Regist* 64, 57699.

FDA, 2000. Guidance for Industry: Action Levels for Poisonous or Deleterious Substances in Human Food and Animal Feed.
<http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/ChemicalContaminantsandPesticides/ucm077969.htm>. Accessed 03/01/2013.

FDA, 2006. Food Allergen Labeling and Consumer Protection Act of 2004 Public Law 108-282, pp. 1-33.

FDA, 2009. Food Allergies: Reducing the Risks.
<http://www.fda.gov/ForConsumers/ConsumerUpdates/ucm089307.htm> Accessed 10/11/2012.

FDA, 2011. Food Safety Modernization Act Public Law 111–353.

Fiocchi, A., Sarratud, P., Terracciano, L., Vacca, E., Bernardini, R., Fuggetta, D., Ballabio, C., Duranti, M., Magni, C., Restani, P., 2009. Assessment of the tolerance to lupine-enriched pasta in peanut-allergic children. *Clin Exp Allergy* 39, 1045-1051.

Fleischer, D.M., Conover-Walker, M.K., Matsui, E.C., Wood, R.A., 2005. The natural history of tree nut allergy. *J Allergy Clin Immunol* 116, 1087-1093.

Fleischer, D.M., Perry, T.T., Atkins, D., Wood, R.A., Burks, A.W., Jones, S.M., Henning, A.K., Stablein, D., Sampson, H.A., Sicherer, S.H., 2012. Allergic Reactions to Foods in Preschool-Aged Children in a Prospective Observational Food Allergy Study. *Pediatrics* 130, e25-e32.

Ford, L.S., Taylor, S.L., Pacenza, R., Niemann, L.M., Lambrecht, D.M., Sicherer, S.H., 2010. Food allergen advisory labeling and product contamination with egg, milk, and peanut. *J Allergy Clin Immunol* 126, 384-385.

Foucard, T., Yman, I.M., 1999. A study on severe food reactions in Sweden - is soy protein an underestimated cause of food anaphylaxis? *Allergy* 54, 261-265.

Fraser, O., Sumar, S., Jones, M., Sumar, N., 2001. Mechanisms in adverse reactions to foods: a review. *Nutrition & Food Science* 31, 170-178.

Friedman, M., Brandon, D.L., 2001. Nutritional and Health Benefits of Soy Proteins. *J Agric Food Chem* 49, 1069-1086.

FSANZ, 2002. The Australian New Zealand Food Standards Code.
<http://www.foodstandards.gov.au/foodstandards/foodstandardscode.cfm> Accessed 05/03/12.

Fukutomi, Y., Sjölander, S., Nakazawa, T., Borres, M.P., Ishii, T., Nakayama, S., Tanaka, A., Taniguchi, M., Saito, A., Yasueda, H., Nakamura, H., Akiyama, K., 2012. Clinical relevance of IgE to recombinant Gly m 4 in the diagnosis of adult soybean allergy. *J Allergy Clin Immunol* 129, 860-863.e863.

Galvez, A.F., Chen, N., Macasieb, J., de Lumen, B.O., 2001. Chemopreventive Property of a Soybean Peptide (Lunasin) That Binds to Deacetylated Histones and Inhibits Acetylation. *Cancer Res* 61, 7473-7478.

Gendel, S., Buchanan, R., Dennis, S., Acheson, D., Assimon, S.A., Beru, N., Bolger, P., Carlson, D., Carvajal, R., Copp, C., Falci, K., Garber, E., Harden, E., Kane, R., Kvenberg, J., Luccioli, S., Park, D., Raybourne, R., Troxell, T., Vierk, K., Threshold Working Group, 2008. Approaches to establish thresholds for major food allergens and for gluten in food. *J Food Prot* 71, 1043-1088.

Gendel, S.M., 2012. Comparison of international food allergen labeling regulations. *Regul Toxicol Pharmacol* 63, 279-285.

Genta, H., Genta, M., Alvarez, N., Santana, M., 2002. Production and acceptance of a soy candy. *J Food Eng* 53, 199-202.

Geroldinger-Simic, M., Zelniker, T., Aberer, W., Ebner, C., Egger, C., Greiderer, A., Prem, N., Lidholm, J., Ballmer-Weber, B.K., Vieths, S., Bohle, B., 2011. Birch pollen-

related food allergy: clinical aspects and the role of allergen-specific IgE and IgG4 antibodies. *J Allergy Clin Immunol* 127, 616-622.

Goggin, D.E., Mir, G., Smith, W.B., Stuckey, M., Smith, P.M.C., 2008. Proteomic analysis of lupin seed proteins to identify conglutin β as an allergen, Lup an 1. *J Agric Food Chem* 56, 6370-6377.

Gonzalez, R., Varela, J., Carreira, J., Polo, F., 1995. Soybean hydrophobic protein and soybean hull allergy. *Lancet* 346, 48-49.

Graham, P.H., Vance, C.P., 2003. Legumes: importance and constraints to greater use. *Plant Physiol* 131, 872-877.

Guarneri, F., Guarneri, C., Benvenga, S., 2005. Identification of potentially cross-reactive peanut-lupine proteins by computer-assisted search for amino acid sequence homology. *Int Arch Allergy Immunol* 138, 273-277.

Hansen, T.K., Poulsen, L.K., Stahl Skov, P., Hefle, S.L., Hlywka, J.J., Taylor, S.L., Bindslev-Jensen, U., Bindslev-Jensen, C., 2004. A randomized, double-blinded, placebo-controlled oral challenge study to evaluate the allergenicity of commercial, food-grade fish gelatin. *Food Chem Toxicol* 42, 2037-2044.

Havlin, J., Kissel, D., Maddux, L., Claassen, M., Long, J., 1990. Crop rotation and tillage effects on soil organic carbon and nitrogen. *Soil Sci Soc Am J* 54, 448-452.

Hefle, S., Christie, L., Sicherer, S., Althage, K., Burks, A., Sampson, H., Mofidi, S., Noone, S., Michaelis, L., Strobel, S., Hourihane, J., Nordlee, J., Taylor, S., 2003. Threshold dose for egg allergy determined by oral challenge. *J Allergy Clin Immunol* 111, S351.

Hefle, S.L., 1996. The chemistry and biology of food allergens. *Food Technol* 50, 86, 88-92.

Hefle, S.L., Furlong, T.J., Niemann, L., Lemon-Mule, H., Sicherer, S., Taylor, S.L., 2007. Consumer attitudes and risks associated with packaged foods having advisory labeling regarding the presence of peanuts. *J Allergy Clin Immunol* 120, 171-176.

Hefle, S.L., Nordlee, J.A., Taylor, S.L., 1996. Allergenic foods. *Crit Rev Food Sci Nutr* 36, 69S-89S.

Helm, R.M., Cockrell, G., Connaughton, C., Sampson, H.A., Bannon, G.A., Beilinson, V., Livingstone, D., Nielsen, N.C., Burks, A.W., 2000a. A soybean G2 glycinin allergen 1. Identification and characterization. *Int Arch Allergy Immunol* 123, 205-212.

Helm, R.M., Cockrell, G., Connaughton, C., Sampson, H.A., Bannon, G.A., Beilinson, V., Nielsen, N.C., Burks, A.W., 2000b. A soybean G2 glycinin allergen 2. Epitope mapping and three-dimensional modeling. *Int Arch Allergy Immunol* 123, 213-219.

- Helm, R.M., Cockrell, G., Herman, E., Burks, A.W., Sampson, H.A., Bannon, G.A., 1998a. Cellular and molecular characterization of a major soybean allergen. *Int Arch Allergy Immunol* 117, 29-37.
- Helm, R.M., Cockrell, G., Stanley, J.S., Sampson, H.A., Bannon, G.A., Burks, A.W., 1998b. IgE-binding of homologous legume vicilins and glycinins of soybean and peanut allergens. *J Allergy Clin Immunol* 101, S240-S241.
- Herman, E.M., Helm, R.M., Jung, R., Kinney, A.J., 2003. Genetic modification removes an immunodominant allergen from soybean. *Plant Physiol* 132, 36-43.
- Herman, E.M., Melroy, D.L., Buckhout, T.J., 1990. Apparent processing of a soybean oil body protein accompanies the onset of oil mobilization. *Plant Physiol* 94, 341-349.
- Hiemori, M., Bando, N., Ogawa, T., Shimada, H., Tsuji, H., Yamanishi, R., Terao, J., 2000. Occurrence of IgE antibody-recognizing N-linked glycan moiety of a soybean allergen, Gly m Bd 28K. *Int Arch Allergy Immunol* 122, 238-245.
- Hiemori, M., Ito, H., Kimoto, M., Yamashita, H., Nishizawa, K., Maruyama, N., Utsumi, S., Tsuji, H., 2004. Identification of the 23-kDa peptide derived from the precursor of Gly m Bd 28K, a major soybean allergen, as a new allergen. *Biochim Biophys Acta* 1675, 174-183.
- Hofmann, A.M., Scurlock, A.M., Jones, S.M., Palmer, K.P., Lokhnygina, Y., Steele, P.H., Kamilaris, J., Burks, A.W., 2009. Safety of peanut oral immunotherapy protocol in children with peanut allergy. *J Allergy Clin Immunol* 124, 286-291.
- Holzhauser, T., Wackermann, O., Ballmer-Weber, B.K., Bindslev-Jensen, C., Scibilia, J., Perono-Garoffo, L., Utsumi, S., Poulsen, L.K., Vieths, S., 2009. Soybean (*Glycine max*) allergy in Europe: Gly m 5 (β -conglycinin) and Gly m 6 (glycinin) are potential diagnostic markers for severe allergic reactions to soy. *J Allergy Clin Immunol* 123, 452-458.
- Hourihane, J.O.B., Bedwani, S.J., Dean, T.P., Warner, J.O., 1997. Randomised, double blind, crossover challenge study of allergenicity of peanut oils in subjects allergic to peanuts. *Br Med J* 314, 1084-1088.
- Hourihane, J.O.B., Dubois, A.E.J., Flokstra-de Blok, B., Dunn-Galvin, A., 2011. Impact of food challenge tests on children's and parent's health related quality of life: a time series case-control study. *J Allergy Clin Immunol* 127, AB 242.
- Hove, E., King, S., Hill, G., 1978. Composition, protein quality, and toxins of seeds of the grain legumes *Glycine max*, *Lupinus* spp., *Phaseolus* spp. *Pisum sativum*, and *Vicia faba*. *N Z J Agric Res* 21, 457-462.
- Howell, M.D., Kim, B.E., Gao, P., Grant, A.V., Boguniewicz, M., Debenedetto, A., Schneider, L., Beck, L.A., Barnes, K.C., Leung, D.Y., 2007. Cytokine modulation of atopic dermatitis filaggrin skin expression. *J Allergy Clin Immunol* 120, 150-155.

Ibáñez, M., Martinez, M., Sanchez, J., Fernández-Caldas, E., 2003. Legume cross-reactivity. *Allergol Immunopathol* 31, 151-161.

Imamura, T., Kanagawa, Y., Ebisawa, M., 2008. A survey of patients with self-reported severe food allergies in Japan. *Pediatr Allergy Immunol* 19, 270-274.

Ito, K., Sjölander, S., Sato, S., Movérare, R., Tanaka, A., Söderström, L., Borres, M., Poorafshar, M., Ebisawa, M., 2011. IgE to Gly m 5 and Gly m 6 is associated with severe allergic reactions to soybean in Japanese children. *J Allergy Clin Immunol* 128, 673-675.

Japan Consumer Affairs Agency, 2011. FAQs on Labeling System for Foods Containing Allergens. http://www.caa.go.jp/foods/qa/allergy01_qa_en.html#a_c-01. Accessed 10/17/12.

Jenkins Da, K.C.C.A.L.A., et al., 2012. Effect of legumes as part of a low glycemic index diet on glycemic control and cardiovascular risk factors in type 2 diabetes mellitus: A randomized controlled trial. *Arch Intern Med*, 1-8.

Jenkins, J.A., Griffiths-Jones, S., Shewry, P.R., Breiteneder, H., Mills, E.N.C., 2005. Structural relatedness of plant food allergens with specific reference to cross-reactive allergens: an in silico analysis. *J Allergy Clin Immunol* 115, 163-170.

Jones, S.M., Fleischer, D.M., Sicherer, S.H., Wood, R.A., Lindblad, R., Stablein, D., Liu, A.H., Shreffler, W., Leung, D.Y.M., Sampson, H.A., Burks, A.W., 2010. Egg oral immunotherapy (OIT) induces clinical desensitization in a double-blind, placebo-controlled (DBPC) trial in egg allergic children from the consortium of food allergy research (CoFAR). *J Allergy Clin Immunol* 125, AB 357.

Keet, C.A., Wood, R.A., 2007. Food allergy and anaphylaxis. *Immunol Allergy Clin North Am* 27, 193-212.

Kemp, S.F., Lockey, R.F., 1996. Peanut anaphylaxis: an allergist-immunologist's personal experience with food cross-contamination. *J Allergy Clin Immunol* 97, 329.

Kerbach, S., Alldrick, A.J., Crevel, R.W.R., Dömötör, L., DunnGalvin, A., Clare Mills, E.N., Pfaff, S., Poms, R.E., Popping, B., Tömösközi, S., 2009. Managing food allergens in the food supply chain – viewed from different stakeholder perspectives. *Qual Assur Saf Crop* 1, 50-60.

Keshun, L., 1997. Soybeans: chemistry, technology, and utilization. Chapman & Hall, London, UK.

Kim, S.H., Hara, S., Hase, S., Ikenaka, T., Toda, H., Kitamura, K., Kaizuma, N., 1985. Comparative study on amino acid sequences of Kunitz-type soybean trypsin inhibitors, Tia, Tib, and Tic. *J Biochem (Tokyo)* 98, 435-448.

Kindscher, K., 1992. Medicinal wild plants of the prairie. An ethnobotanical guide. University Press of Kansas, Lawrence, KS.

- Kleine-Tebbe, J., Wangorsch, A., Vogel, L., Crowell, D.N., Haustein, U.-F., Vieths, S., 2002. Severe oral allergy syndrome and anaphylactic reactions caused by a Bet v 1-related PR-10 protein in soybean, SAM22. *J Allergy Clin Immunol* 110, 797-804.
- Kleinman, R.E., Bahna, S., Powell, G.F., Sampson, H.A., 1991. Use of infant formulas in infants with cow milk allergy: a review and recommendations. *Pediatr Allergy Immunol* 4, 146-155.
- Koerner, T.B., Cleroux, C., Poirier, C., Cantin, I., Alimkulov, A., Elamparo, H., 2011. Gluten contamination in the Canadian commercial oat supply. *Food Addit Contam* 28, 705-710.
- Koplin, J., Osborne, J., Allen, J., 2010. Prevalence of allergen avoidance advisory statements on packaged processed foods in a supermarket. *Med J Aust* 193, 426-427.
- Kosma, P., Sjölander, S., Landgren, E., Borres, M.P., Hedlin, G., 2011. Severe reactions after the intake of soy drink in birch pollen-allergic children sensitized to Gly m 4. *Acta Paediatr* 100, 305-306.
- Kouzarides, T., 2007. Chromatin modifications and their function. *Cell* 128, 693-705.
- Krishnan, H.B., Kim, W.-S., Jang, S., Kerley, M.S., 2009. All three subunits of soybean β -conglycinin are potential food allergens. *J Agric Food Chem* 57, 938-943.
- Kroes, R., Galli, C., Munro, I., Schilter, B., Tran, L.A., Walker, R., Würtzen, G., 2000. Threshold of toxicological concern for chemical substances present in the diet: A practical tool for assessing the need for toxicity testing. *Food Chem Toxicol* 38, 255-312.
- Kruizinga, A.G., Briggs, D., Crevel, R.W.R., Knulst, A.C., Bosch, L.M.C.v.d., Houben, G.F., 2008. Probabilistic risk assessment model for allergens in food: sensitivity analysis of the minimum eliciting dose and food consumption. *Food Chem Toxicol* 46, 1437-1443.
- Kunitz, M., 1947. Crystalline soybean trypsin inhibitor II. General properties. *J Gen Physiol* 30, 291-310.
- Kwok, K.-C., Liang, H.-H., Niranjana, K., 2002. Mathematical modelling of the heat inactivation of trypsin inhibitors in soymilk at 121–154 °C. *J Sci Food Agric* 82, 243-247.
- Lack, G., 2008. Epidemiologic risks for food allergy. *J Allergy Clin Immunol* 121, 1331-1336.
- Lammerding, A.M., Fazil, A., 2000. Hazard identification and exposure assessment for microbial food safety risk assessment. *Int J Food Microbiol* 58, 147-157.
- Larsen, J.C., 2006. Risk assessment of chemicals in European traditional foods. *Trends Food Sci Technol* 17, 471-481.

- Lawlor, D.W., Lemaire, G., Gastal, F., 2001. Nitrogen, plant growth and crop yield, in: P.J. Lea, J.-F. Morot-Gaudry (Eds.), *Plant nitrogen*. Springer, New York, NY, pp. 343-367.
- Lehmann, K., Schweimer, K., Reese, G., Randow, S., Suhr, M., Becker, W.M., Vieths, S., Rösch, P., 2006. Structure and stability of 2S albumin-type peanut allergens: implications for the severity of peanut allergic reactions. *Biochem J* 395, 463-472.
- Liu, K., 2004a. Soy Isoflavones: Chemistry, Processing Effects, Health Benefits, and Commercial Production, in: K. Liu (Ed.), *Soybeans as Functional Foods and Ingredients*. AOCS Publishing, Urbana, IL, pp. 52-72.
- Liu, K., 2004b. Soybeans as a Powerhouse of Nutrients and Phytochemicals, in: K. Liu (Ed.), *Soybeans as Functional Foods and Ingredients*. AOCS Publishing, Urbana, IL, pp. 1-22.
- Lomer, M.C.E., Parkes, G.C., Sanderson, J.D., 2008. Review article: lactose intolerance in clinical practice – myths and realities. *Aliment Pharmacol Ther* 27, 93-103.
- López-Bellido, L., Fuentes, M., Castillo, J., López-Garrido, F., Fernández, E., 1996. Long-term tillage, crop rotation, and nitrogen fertilizer effects on wheat yield under rainfed Mediterranean conditions. *Agron J* 88, 783-791.
- Madsen, C.B., Hattersley, S., Allen, K.J., Beyer, K., Chan, C.H., Godefroy, S.B., Hodgson, R., Mills, E.N., Munoz-Furlong, A., Schnadt, S., Ward, R., Wickman, M., Crevel, R., 2012. Can we define a tolerable level of risk in food allergy? Report from a EuroPrevall/UK Food Standards Agency workshop. *Clin Exp Allergy* 42, 30-37.
- Madsen, C.B., Hattersley, S., Buck, J., Gendel, S.M., Houben, G.F., Hourihane, J.O.B., Mackie, A., Mills, E.N.C., Norhede, P., Taylor, S.L., Crevel, R.W.R., 2009. Approaches to risk assessment in food allergy: report from a workshop "developing a framework for assessing the risk from allergenic foods". *Food Chem Toxicol* 47, 480-489.
- Maleki, S.J., Chung, S.-Y., Champagne, E., T., Raufman, J.-P., 2000. The effects of roasting on the allergenic properties of peanut proteins. *J Allergy Clin Immunol* 106, 763-768.
- Martinez San Ireneo, M., Ibanez, M.D., Sanchez, J.J., Carnes, J., Fernandez-Caldas, E., 2008. Clinical features of legume allergy in children from a Mediterranean area. *Ann Allergy Asthma Immunol* 101, 179-184.
- Maruyama, N., Fukuda, T., Saka, S., Inui, N., Kotoh, J., Miyagawa, M., Hayashi, M., Sawada, M., Moriyama, T., Utsumi, S., 2003. Molecular and structural analysis of electrophoretic variants of soybean seed storage proteins. *Phytochemistry* 64, 701-708.
- Maruyama, N., Katsube, T., Wada, Y., Oh, M.H., Barba De La Rosa, A.P., Okuda, E., Nakagawa, S., Utsumi, S., 1998. The roles of the N-linked glycans and extension regions

of soybean beta-conglycinin in folding, assembly and structural features. *Eur J Biochem* 258, 854-862.

Mikić, A., Perić, V., Đorđević, V., Srebrić, M., Mihailović, V., 2009. Anti-nutritional factors in some grain legumes. *Biotech Anim Husbandry* 25, 1181-1188.

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

Moneret-Vautrin, D.A., Guérin, L., Kanny, G., Flabbee, J., Frémont, S., Morisset, M., 1999. Cross-allergenicity of peanut and lupine: the risk of lupine allergy in patients allergic to peanuts. *J Allergy Clin Immunol* 104, 883-888.

Morisset, M., Moneret-Vautrin, D.A., Guenard, L., Cuny, J.M., Frentz, P., Hatahet, R., Hanss, C., Beaudoin, E., Petit, N., Kanny, G., 2007. Oral desensitization in children with milk and egg allergies obtains recovery in a significant proportion of cases. A randomized study in 60 children with cow's milk allergy and 90 children with egg allergy. *Eur Ann Allergy Clin Immunol* 39, 12-19.

Moroz, L.A., Yang, W.H., 1980. Kunitz soybean trypsin inhibitor. A specific allergen in food anaphylaxis. *N Engl J Med* 302, 1126-1128.

Morris, J.B., 1997. Special-purpose legume genetic resources conserved for agricultural, industrial, and pharmaceutical use. *Econ Bot* 51, 251-263.

Murphy, K.P., Travers, P., Walport, M., 2008. *Janeway's immunobiology*, 7th ed. Garland Science, Taylor & Francis Group, New York, NY.

Neudecker, P., Lehmann, K., Rösch, P., 2003. Letter to the editor: sequence-specific ¹H, ¹³C and ¹⁵N resonance assignments of SAM22, an allergenic stress-induced protein from soy bean. *J Biomol NMR* 26, 191-192.

Neuman-Sunshine, D.L., Eckman, J.A., Keet, C.A., Matsui, E.C., Peng, R.D., Lenehan, P.J., Wood, R.A., 2012. The Prevalence of Clinical Cross-reactivity of Non-peanut Legumes to Peanut in Patients with Persistent Peanut Allergy. *J Allergy Clin Immunol* 129, AB23.

Niewinski, M.M., 2008. Advances in celiac disease and gluten-free diet. *J Am Diet Assoc* 108, 661-672.

Noimark, L., Gardner, J., Warner, J.O., 2009. Parents' attitudes when purchasing products for children with nut allergy: a UK perspective. *Pediatr Allergy Immunol* 20, 500-504.

North, M.L., Ellis, A.K., 2011. The role of epigenetics in the developmental origins of allergic disease. *Ann Allergy Asthma Immunol* 106, 355-361; quiz 362.

Notermans, S., Gallhoff, G., Zwietering, M.H., Mead, G.C., 1995. The HACCP concept: specification of criteria using quantitative risk assessment. *Food Microbiol* 12, 81-90.

Ogawa, T., 2006. Soy allergy, in: M. Sugano (Ed.), *Soy in health and disease prevention*. CRC Press, Boca Raton, FL, pp. 123-137.

Ogawa, T., Bando, N., Tsuji, H., Nishikawa, K., Kitamura, K., 1995. α -Subunit of β -conglycinin, an allergenic protein recognized by IgE antibodies of soybean sensitive patients with atopic dermatitis. *Biosci Biotechnol Biochem* 59, 831-833.

Ogawa, T., Bando, N., Tsuji, H., Okajima, H., Nishikawa, K., Sasaoka, K., 1991. Investigation of the IgE-binding proteins in soybeans by immunoblotting with the sera of the soybean-sensitive patients with atopic dermatitis. *J Nutr Sci Vitaminol (Tokyo)* 37, 555-565.

Ogawa, T., Samoto, M., Takahashi, K., 2000. Soybean allergens and hypoallergenic soybean products. *J Nutr Sci Vitaminol (Tokyo)* 46, 271-279.

Ogawa, T., Tsuji, H., Bando, N., Kitamura, K., Zhu, Y.-L., Hirano, H., Nishikawa, K., 1993. Identification of the soybean allergenic protein, Gly m Bd 30K, with the soybean seed 34-kDa oil-body-associated protein. *Biosci Biotechnol Biochem* 57, 1030-1033.

Osborne, N., Koplin, J.J., Martin, P.E., Gurrin, L.C., Lowe, A.J., Matheson, M.C., Ponsonby, A.-L., Wake, M., Tang, M.L.K., Dharmage, S.C., Allen, K.J., 2011. Prevalence of challenge-proven IgE-mediated food allergy using population-based sampling and predetermined challenge criteria in infants. *J Allergy Clin Immunol* 127, 668-676.

Pedersen, H., Djurtoft, R., 1989. Antigenic and allergenic properties of acidic and basic peptide chains from glycinin. *Food Agric Immunol* 1, 101-109.

Pele, M., Brohee, M., Anklam, E., van Hengel, A.J., 2007. Peanut and hazelnut traces in cookies and chocolates: relationship between analytical results and declaration of food allergens on product labels. *Food Addit Contam* 24, 1334-1344.

Perry, T.T., Conover Walker, M.K., Pomés, A., Chapman, M.D., Wood, R.A., 2004. Distribution of peanut allergen in the environment. *J Allergy Clin Immunol* 113, 973-976.

Peterson, T.A., Russelle, M.P., 1991. Alfalfa and the nitrogen cycle in the Corn Belt. *J Soil Water Conserv* 46, 229-235.

Pieretti, M.M., Chung, D., Pacenza, R., Slotkin, T., Sicherer, S.H., 2009. Audit of manufactured products: use of allergen advisory labels and identification of labeling ambiguities. *J Allergy Clin Immunol* 124, 337-341.

Pimentel, D., Patzek, T.W., 2005. Ethanol production using corn, switchgrass, and wood; biodiesel production using soybean and sunflower. *Natural resources research* 14, 65-76.

Pisani, P., Parkin, D.M., Bray, F., Ferlay, J., 1999. Estimates of the worldwide mortality from 25 cancers in 1990. *Int J Cancer* 83, 18-29.

Pistiner, M., Gold, D.R., Abdulkerim, H., Hoffman, E., Celedón, J.C., 2008. Birth by cesarean section, allergic rhinitis, and allergic sensitization among children with a parental history of atopy. *J Allergy Clin Immunol* 122, 274-279.

Pomes, A., 2010. Relevant B cell epitopes in allergic disease. *Int Arch Allergy Immunol* 152, 1-11.

Popenoe, H., King, S., Leon, J., Kalinowski, L., 1989. *Lost crops of the Incas*. Washington: National Research Council. National Academy Press.

Prak, K., Nakatani, K., Katsube-Tanaka, T., Adachi, M., Maruyama, N., Utsumi, S., 2005. Structure-function relationships of soybean proglycinins at subunit levels. *J Agric Food Chem* 53, 3650-3657.

Prezioso, D., Denis, L.J., Klocker, H., Sciarra, A., Reis, M., Naber, K., Lobel, B., Pacik, D., Griffiths, K., 2007. Estrogens and aspects of prostate disease. *Int J Urol* 14, 1-16.

Pucar, F., Kagan, R., Lim, H., Clarke, A.E., 2001. Peanut challenge: a retrospective study of 140 patients. *Clin Exp Allergy* 31, 40-46.

Pumphrey, R.S.H., Stanworth, S.J., 1996. The clinical spectrum of anaphylaxis in north-west England. *Clin Exp Allergy* 26, 1364-1370.

Quirce, S., Fernández-Nieto, M., Escudero, C., Cuesta, J., de las Heras, M., Sastre, J., 2006. Bronchial responsiveness to bakery-derived allergens is strongly dependent on specific skin sensitivity. *Allergy* 61, 1202-1208.

Quirce, S., Polo, F., Figueredo, E., González, R., Sastre, J., 2000. Occupational asthma caused by soybean flour in bakers--differences with soybean-induced epidemic asthma. *Clin Exp Allergy* 30, 839-846.

Rabjohn, P., Helm, E.M., Stanley, J.S., West, C.M., Sampson, H.A., Burks, A.W., Bannon, G.A., 1999. Molecular cloning and epitope analysis of the peanut allergen Ara h 3. *J Clin Invest* 103, 535-542.

Radauer, C., Breiteneder, H., 2007. Evolutionary biology of plant food allergens. *J Allergy Clin Immunol* 120, 518-525.

Rasekh, J., Thaler, A.M., Englejohn, D.L., Pihkala, N.H., 2005. Food Safety and Inspection Service Policy for Control of Poultry Contaminated by Digestive Tract Contents: A Review. *J Appl Poultry Res* 14, 603-611.

Renz-Polster, H., David, M., Buist, A., Vollmer, W., O'Connor, E., Frazier, E., Wall, M., 2005. Caesarean section delivery and the risk of allergic disorders in childhood. *Clin Exp Allergy* 35, 1466-1472.

- Rihs, H.-P., Chen, Z., Ruëff, F., Petersen, A., Rozynek, P., Heimann, H., Baur, X., 1999. IgE binding of the recombinant allergen soybean profilin (rGly m 3) is mediated by conformational epitopes. *J Allergy Clin Immunol* 104, 1293-1301.
- Rimbaud, L., Heraud, F., La Vieille, S., Leblanc, J.-C., Crepet, A., 2010. Quantitative risk assessment relating to adventitious presence of allergens in food: a probabilistic model applied to peanut in chocolate. *Risk Anal* 30, 7-19.
- Rona, R.J., Keil, T., Summers, C., Gislason, D., Zuidmeer, L., Sodergren, E., Sigurdardottir, S.T., Lindner, T., Goldhahn, K., Dahlstrom, J., McBride, D., Madsen, C., 2007. The prevalence of food allergy: a meta-analysis. *J Allergy Clin Immunol* 120, 638-646.
- Roychaudhuri, R., Sarath, G., Zeece, M., Markwell, J., 2004. Stability of the allergenic soybean Kunitz trypsin inhibitor. *Biochim Biophys Acta* 1699, 207-212.
- Rubio-Tapia, A., Ludvigsson, J.F., Brantner, T.L., Murray, J.A., Everhart, J.E., 2012. The Prevalence of Celiac Disease in the United States. *Am J Gastroenterol* 107, 1538-1544.
- Sampson, H.A., 2004. Update on food allergy. *J Allergy Clin Immunol* 113, 805-819.
- Sampson, H.A., McCaskill, C.C., 1985. Food hypersensitivity and atopic dermatitis: evaluation of 113 patients. *J Pediatr* 107, 669-675.
- Sampson, H.A., Mendelson, L., Rosen, J.P., 1992. Fatal and near-fatal anaphylactic reactions to food in children and adolescents. *N Engl J Med* 327, 380-384.
- Saylor, J.D., Bahna, S.L., 1991. Anaphylaxis to casein hydrolysate formula. *J Pediatr* 118, 71-74.
- Schloss, O.M., 1912. A case of allergy to common foods. *Am J Dis Child* 3, 342-362.
- Schofield, A.T., 1908. A CASE OF EGG POISONING. *The Lancet* 171, 716.
- Scott, P.T., Pregelj, L., Chen, N., Hadler, J.S., Djordjevic, M.A., Gresshoff, P.M., 2008. *Pongamia pinnata*: an untapped resource for the biofuels industry of the future. *Bioenergy Research* 1, 2-11.
- Sen, M., Kopper, R., Pons, L., Abraham, E.C., Burks, A.W., Bannon, G.A., 2002. Protein structure plays a critical role in peanut allergen stability and may determine immunodominant IgE-binding epitopes. *J Immunol* 169, 882-887.
- Sessa, D.J., Ghantous, P.E., 1987. Chemical inactivation of soybean trypsin inhibitors. *J Am Oil Chem Soc* 64, 1682-1687.
- Shaw, J., Roberts, G., Grimshaw, K., White, S., Hourihane, J., 2008. Lupin allergy in peanut-allergic children and teenagers. *Allergy* 63, 370-373.

Sheth, S.S., Ben-Shoshan, M., Harrington, D.W., Fragapane, J., Soller, L., Joseph, L., St. Pierre, Y., La Vieille, S., Elliott, S.J., Wasserman, S., Alizadehfar, R., Harada, L., Allen, M., Allen, M., Clarke, A.E., 2012. Effect of precautionary statements on the purchasing practices of Canadians directly and indirectly affected by food allergies. *J Allergy Clin Immunol* 129, 1401-1404.

Sicherer, S., Sampson, H.A., Burks, A., 2001. Peanut and soy allergy: a clinical and therapeutic dilemma. *Allergy* 55, 515-521.

Sicherer, S.H., Sampson, H.A., 2010. Food allergy. *J Allergy Clin Immunol* 125, S116-S125.

Skolnick, H.S., Conover Walker, M.K., Barnes-Koerner, C., Sampson, H.A., Burks, W., Wood, R.A., 2001. The natural history of peanut allergy. *J Allergy Clin Immunol* 107, 367-374.

Skripak, J.M., Nash, S.D., Rowley, H., Brereton, N.H., Oh, S., Hamilton, R.G., Matsui, E.C., Burks, A.W., Wood, R.A., 2008. A randomized, double-blind, placebo-controlled study of milk oral immunotherapy for cow's milk allergy. *J Allergy Clin Immunol* 122, 1154-1160.

Sloan, A.E., Powers, M.E., 1986. A perspective on popular perceptions of adverse reactions to foods. *J Allergy Clin Immunol* 78, 127-132.

Smil, V., 1999. Nitrogen in crop production: An account of global flows. *Global Biogeochem Cycles* 13, 647-662.

Sollid, L.M., Khosla, C., 2005. Future therapeutic options for celiac disease. *Nat Clin Pract Gastroenterol Hepatol* 2, 140-147.

Spanjersberg, M., Knulst, A., Kruizinga, A., Van Duijn, G., Houben, G., 2010. Concentrations of undeclared allergens in food products can reach levels that are relevant for public health. *Food Addit Contam* 27, 169-174.

Spanjersberg, M.Q.I., Kruizinga, A.G., Rennen, M.A.J., Houben, G.F., 2007. Risk assessment and food allergy: the probabilistic model applied to allergens. *Food Chem Toxicol* 45, 49-54.

Spergel, J.M., 2006. Nonimmunoglobulin e-mediated immune reactions to foods. *Allergy Asthma Clin Immunol* 2, 78-85.

Strachan, D.P., 2000. Family size, infection and atopy: the first decade of the "hygiene hypothesis". *Thorax* 55 Suppl 1, S2-10.

Strid, J., Hourihane, J., Kimber, I., Callard, R., Strobel, S., 2004. Disruption of the stratum corneum allows potent epicutaneous immunization with protein antigens resulting in a dominant systemic Th2 response. *Eur J Immunol* 34, 2100-2109.

Sudo, N., Sawamura, S.-a., Tanaka, K., Aiba, Y., Kubo, C., Koga, Y., 1997. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J Immunol* 159, 1739-1745.

Taylor, S.L., 1987. Allergic and sensitivity reactions to food components, in: J.N. Hathcock (Ed.), *Nutritional Toxicology*, Volume 2. Academic Press, Inc., Orlando, FL, pp. 173-198.

Taylor, S.L., Baumert, J.L., 2010. Cross-contamination of foods and implications for food allergic patients. *Curr Allergy Asthm Rep* 10, 265-270.

Taylor, S.L., Baumert, J.L., Houben, G.F., Crevel, R.W.R., Brooke-Taylor, S., Allen, K.J., Remington, B.C., Kruizinga, A.G., Sherlock, R., 2013 (In prep). Establishment of reference doses for residues of allergenic foods: Report of the VITAL expert panel. *Food Chem Toxicol*.

Taylor, S.L., Bush, R.K., Busse, W.W., 1986a. Avoidance diets - how selective should we be? *N Engl J Allergy Proc* 7, 527-532.

Taylor, S.L., Crevel, R.W.R., Sheffield, D., Kabourek, J., Baumert, J., 2009. Threshold dose for peanut: risk characterization based upon published results from challenges of peanut-allergic individuals. *Food Chem Toxicol* 47, 1198-1204.

Taylor, S.L., Hefle, S.L., 2001. Food allergies and other food sensitivities. *Food Technol* 55, 68-83.

Taylor, S.L., Hefle, S.L., 2006a. Food allergen labeling in the USA and Europe. *Curr Opin Allergy Clin Immunol* 6, 186-190.

Taylor, S.L., Hefle, S.L., 2006b. Food allergies and intolerances, in: M.E. Shils, M. Shike, A.C. Ross, B. Caballero, R.J. Cousins (Eds.), *Modern Nutrition in Health and Disease*, 10th ed. Lippincott Williams and Wilkins, Baltimore, MD, pp. 1512-1530.

Taylor, S.L., Hefle, S.L., Bindslev-Jensen, C., Atkins, F.M., André, C., Bruijnzeel-Koomen, C., Burks, A.W., Bush, R.K., Ebisawa, M., Eigenmann, P.A., Host, A., Hourihane, J.O.B., Isolauri, E., Hill, D.J., Knulst, A., Lack, G., Sampson, H.A., Moneret-Vautrin, D.A., Rancé, F., Vadas, P.A., Yunginger, J.W., Zeiger, R.S., Salminen, J.W., Madsen, C., Abbott, P., 2004. A consensus protocol for the determination of the threshold doses for allergenic foods: how much is too much? *Clin Exp Allergy* 34, 689-695.

Taylor, S.L., Hefle, S.L., Bindslev-Jensen, C., Bock, S.A., Burks, A.W., Jr., Christie, L., Hill, D.J., Høst, A., Hourihane, J.O.B., Lack, G., Metcalfe, D.D., Moneret-Vautrin, D.A., Vadas, P.A., Rancé, F., Skrypec, D.J., Trautman, T.A., Yman, I.M., Zeiger, R.S., 2002. Factors affecting the determination of threshold doses for allergenic foods: how much is too much? *J Allergy Clin Immunol* 109, 24-30.

Taylor, S.L., Higley, N.A., Bush, R.K., 1986b. Sulfites in foods: uses, analytical methods, residues, fate, exposure assessment, metabolism, toxicity, and hypersensitivity,

in: C.O. Chichester (Ed.), *Advances in Food Research*, Volume 30. Academic Press, Orlando, FL, pp. 1-76.

Taylor, S.L., Lehrer, S.B., 1996. Principles and characteristics of food allergens. *Crit Rev Food Sci Nutr* 36, S91-S118.

Taylor, S.L., Moneret-Vautrin, D.A., Crevel, R.W.R., Sheffield, D., Morisset, M., Dumont, P., Remington, B.C., Baumert, J.L., 2010. Threshold dose for peanut: risk characterization based upon diagnostic oral challenge of a series of 286 peanut-allergic individuals. *Food Chem Toxicol* 48, 814-819.

Taylor, S.L., Nordlee, J.A., Rupnow, J.H., 1989. *Food allergies and sensitivities*. Marcel Dekker, Inc, New York.

Thermo-Fisher-Scientific, 2011. Test Principle ImmunoCAP Specific IgE. <http://www.phadia.com/en-US/Laboratories/Allergy/Products/ImmunoCAP-Assays/ImmunoCAP-Specific-IgE/Test-Principle-ImmunoCAP-Specific-IgE/>. Accessed 10/15/2012.

Thompson, T., Roland Lee, A., Grace, T., 2010. Gluten contamination of grains, seeds, and flours in the United States: a pilot study. *J Am Diet Assoc* 110, 937-940.

Tsuji, H., Bando, N., Hiemori, M., Yamanishi, R., Kimoto, M., Nishikawa, K., Ogawa, T., 1997. Purification and characterization of soybean allergen Gly m Bd 28K. *Biosci Biotechnol Biochem* 61, 942-947.

Untersmayr, E., Jensen-Jarolim, E., 2006. Mechanisms of type I food allergy. *Pharmacol Ther* 112, 787-798.

USDA, 2004. *Grain Inspection Handbook - Book II Grain Grading Procedures - Chapter 13 - Wheat*.

USDA, 2010. 2009 Crop Year is One for the Record Books, USDA Reports. http://www.nass.usda.gov/Newsroom/2010/01_12_2010.asp. Accessed 02/05/2012.

van der Ven, C., Matser, A.M., van den Berg, R.W., 2005. Inactivation of soybean trypsin inhibitors and lipoxygenase by high-pressure processing. *J Agric Food Chem* 53, 1087-1092.

van Reijssen, F.C., Felijs, A., Wauters, E.A.K., Bruijnzeel-Koomen, C.A.F.M., Koppelman, S.J., 1998. T-cell reactivity for a peanut-derived epitope in the skin of a young infant with atopic dermatitis. *J Allergy Clin Immunol* 101, 207-209.

Varshney, P., Jones, S.M., Pons, L., Kulis, M., Steele, P.H., Kemper, A.R., Scurlock, A.M., Perry, T.T., Burks, A.W., 2009. Oral immunotherapy (OIT) induces clinical tolerance in peanut-allergic children. *J Allergy Clin Immunol* 123, S174.

- Vierk, K.A., Koehler, K.M., Fein, S.B., Street, D.A., 2007. Prevalence of self-reported food allergy in American adults and use of food labels. *J Allergy Clin Immunol* 119, 1504-1510.
- Vieths, S., Aulepp, H., Becker, W.-M., Buschmann, L., 1996. Characterization of labile and stable allergens in foods of plant origin. VCH, Weinheim/ New York.
- Vinje, N.E., Namork, E., Lovik, M., 2012. Cross-allergic Reactions to Legumes in Lupin and Fenugreek-Sensitized Mice. *Scand J Immunol* 76, 387-397.
- Weggemans, R.M., Trautwein, E.A., 2003. Relation between soy-associated isoflavones and LDL and HDL cholesterol concentrations in humans: a meta-analysis. *Eur J Clin Nutr* 57, 940-946.
- Wilson, S., Blaschek, K., Gonzalez de Mejia, E., 2005. Allergenic proteins in soybean: processing and reduction of P34 allergenicity. *Nutr Rev* 63, 47-58.
- Wood, R.A., 2003. The natural history of food allergy. *Pediatrics* 111, 1631-1637.
- Xiang, P., Beardslee, T.A., Zeece, M.G., Markwell, J., Sarath, G., 2002. Identification and analysis of a conserved immunoglobulin E-binding epitope in soybean G1a and G2a and peanut Ara h 3 glycinins. *Arch Biochem Biophys* 408, 51-57.
- Yamagiwa, M., Hattori, R., Ito, Y., Yamamoto, S., Kanba, M., Tasaki, T., Ueda, K., Nishizumi, T., 2002. Birch-pollen sensitization in an area without atmospheric birch pollens. *Auris Nasus Larynx* 29, 261-266.
- Yamanishi, R., Huang, T., Tsuji, H., Bando, N., Ogawa, T., 1995. Reduction of the soybean allergenicity by the fermentation with *Bacillus natto*. *Food Sci Technol Int* 1, 14-17.
- Yu, J.W., Kagan, R., Verreault, N., Nicolas, N., Joseph, L., St. Pierre, Y., Clarke, A., 2006. Accidental ingestions in children with peanut allergy. *J Allergy Clin Immunol* 118, 466-472.
- Yunginger, J.W., Nelson, D.R., Squillace, D.L., Jones, R.T., Holley, K.E., Hyma, B.A., Biedrzycki, L., Sweeney, K.G., Sturner, W.Q., Schwartz, L.B., 1991. Laboratory investigation of deaths due to anaphylaxis. *J Forensic Sci* 36, 857-865.
- Yunginger, J.W., Sweeney, K.G., Sturner, W.Q., Giannandrea, L.A., Teigland, J.D., Bray, M., Benson, P.A., York, J.A., Biedrzycki, L., Squillace, D.L., Helm, R.M., 1988. Fatal food-induced anaphylaxis. *J Am Med Assoc* 260, 1450-1452.
- Zeece, M.G., Beardslee, T.A., Markwell, J.P., Sarath, G., 1999. Identification of an IgE-binding region in soybean acidic glycinin G1. *Food Agric Immunol* 11, 83-90.
- Zheng, S., Tian, H., Ma, N., Qin, G., Sun, Z., Yu, C., 2012. Purification and IgE-binding properties of soybean β -conglycinin subunits. *Process Biochem* 47, 2531-2537.

**CHAPTER 2: PRIORITY FOOD ALLERGEN THRESHOLDS AND METHODS
TO IMPROVE CLINICAL FOOD CHALLENGE TRIALS**

Benjamin C. Remington, Joseph L. Baumert, and Steve L. Taylor

Food Allergy Research & Resource Program, Department of Food Science &
Technology, University of Nebraska-Lincoln, Lincoln, NE, USA

Abstract

In the absence of guidance from public health authorities regarding food allergen thresholds, the food industry has implemented the widespread use of various forms of voluntary advisory or precautionary “may contain” labeling in an attempt to manage the risk and protect food-allergic consumers. All stakeholders (regulators, food industry, clinical researchers and food-allergic consumers) agree it is essential to address the current lack of action levels and thresholds for food allergen labeling as the ramped use of advisory labeling and lack of transparency of its use limit food choices and decrease the quality of life of allergic individuals. This study aims to build upon initial work with peanut and develop a global thresholds database for all priority food allergens. Clinical publications and unpublished clinical data were screened for data regarding objective, individual challenge data for priority food allergens. Minimal eliciting doses were found for 13 priority allergens and include over 1800 individuals from published clinical literature or unpublished clinical data. The results of this study show there are sufficient clinical data from food allergic individuals to use for risk assessment purposes and developing regulatory thresholds for several allergenic foods. Allergic populations did not vary when analyzed by age, geographic region, or gender and only slightly varied by study population and challenge material. Expert judgment must be used when developing regulatory thresholds or action levels (reference doses) and evaluating the clinical challenge methods that provide data on relevant food allergic-individuals. In order to benefit all stakeholders, clinical food challenge studies are recommended to start below 1 mg protein from the allergenic food and proceed at a log or semi-log scale dose increases,

depending on the comfort of the physician, until a final discrete dose of 4 – 5 grams of protein is reached.

1. Introduction

Food allergies are a worldwide health concern as they affect an estimated 5 – 10% of children and 3 – 4% of adults in westernized countries (1-3). Food allergen risk assessment and proper labeling are important processes for the food industry, regulatory agencies, and food allergic consumers. Allergic consumers have no choice but to adhere to a strict avoidance diet and carefully read the ingredient labels of the food they eat (4-7). Many developed countries require labeling of the most common allergenic foods (peanuts, milk, eggs, tree nuts, soy, wheat (or cereal grains containing gluten), fish, and crustacean shellfish) and ingredients derived from these foods (8).

Threshold-based risk approaches have long been used for the management of chemical and microbial hazards in food but have not been widely adopted by regulatory agencies in the management of food allergens (9-13). Food allergen thresholds have different meanings to different stakeholders. To the food allergic consumer, their personal threshold or Minimal Eliciting Dose (MED) is the amount of food required to cause an allergic reaction. The population threshold could be the amount of food required to cause a reaction in the most sensitive individual or in a determined percentage of the food allergic population. To the food industry and regulatory bodies, the term threshold could determine how much allergen would trigger a product recall if unlabeled or when to place an advisory statement on the label if allergens are possibly present due to cross-contact. Two countries have attempted to establish regulatory action levels for undeclared allergens in foods. Switzerland has defined an action limit of 1,000 ppm (mg/kg) for

allergens. This limit states that if unavoidable, contamination above 1,000 ppm (0.1%) must be declared as an ingredient, but contamination below 1,000 ppm may be declared if desired (14). Levels of 1000 ppm may provide enough protein (low mg doses) to cause reactions at moderate consumption levels in multiple foods (15). Japan has taken a stricter approach and limited undeclared allergens to 10 ppm protein from the allergenic sources (0.001%) in foods (14). At the present time, the U.S. has not adopted legislation or regulations regarding regulatory thresholds for food allergens and there is no regulatory guidance for trace levels of allergens due to cross-contact. The importance of food allergy as a public health and food safety issue has placed pressure on the food industry and regulatory agencies to implement threshold-based strategies to protect the food allergic consumer.

In the absence of guidance from public health authorities regarding thresholds, the food industry has implemented the widespread use of various forms of voluntary advisory or precautionary “may contain” labeling in an attempt to manage the risk and protect food-allergic consumers. As a result, the quality-of-life of food-allergic consumers has decreased due to the ever decreasing number of food choices available and some are ignoring these advisory statements (5, 16). Additionally, the widespread use of advisory labeling has led to varying advice within the medical community on whether patients should avoid all foods with advisory labeling (17, 18). Regulatory establishment of thresholds could benefit allergic consumers as there would be more transparency in the use of advisory labeling by food industry but they should never be advised to ignore advisory statements on package labels (19). All stakeholders (regulators, food industry, clinical researchers and food-allergic consumers) agreed it is essential to address the

current lack of action levels and thresholds for food allergen labeling, but it is difficult to define and quantify a level of tolerable or accepted risk (20). There is an obvious need for research and scientific advancement in the area of food allergen thresholds.

The FDA Threshold Working Group and others have agreed that allergic population thresholds and a quantitative risk assessment-based approach provides the strongest, most transparent scientific analyses to establish thresholds for the major food allergens (21-23). However, the quantitative (probabilistic) approach has only recently been applied to food allergens (23-26). The FDA Threshold Working Group stated that data available in 2006 were not sufficient to meet the requirements of the quantitative approach and that a research program should be initiated to develop applicable risk assessment tools and to acquire and evaluate the clinical and epidemiological data needed to support the quantitative risk assessment-based approach (21). Recent work by the Food Allergy Research and Resource Program (FARRP) at the University of Nebraska utilized published low dose double-blind, placebo-controlled oral food challenges (DBPCFC) for peanut and additional data from an allergy clinic in Nancy, France to accumulate a total of 450 individual allergic thresholds from DBPCFCs and form a stable population threshold distribution for peanut-allergic individuals (27, 28). This study aims to build upon FARRP's initial work with peanut and develop a global thresholds database for all priority food allergens. Additionally, variations in challenge protocols and the number of subjects used in food allergen threshold studies were examined in order to produce more useful data for all stakeholders. The threshold work conducted by FARRP and TNO in the Netherlands is now being utilized by the Australian Allergen Bureau in the Voluntary Incidental Trace Allergen Labelling (VITAL) program, in which reference dose values

for priority allergens have been developed to aid in risk assessment and precautionary labeling decisions as discussed in more detail in the *Regulatory Thresholds and Uncertainty Factors* section of this chapter.

2. Materials and Methods

Food allergen thresholds

The Food Allergen Labeling and Consumer Protection Act (FALCPA) was passed in 2004 by the U.S. Congress to protect allergic individuals from the ‘major’ food allergens of milk, egg, fish (e.g., bass, flounder, or cod), crustacean shellfish (e.g., crab, lobster, or shrimp), tree nuts (e.g., almonds, pecans, or walnuts), wheat, peanuts, and soybeans by requiring declaration of these allergens on the packaged food label in plain English terms (29). Additional priority allergenic foods within global regulatory frameworks include, but are not limited to: sesame seed, molluscan shellfish, mustard, celery, and lupin (8). Clinical publications were screened for data concerning DBPCFC thresholds for priority food allergens within the global framework. This method was previously described in detail by Taylor et al. (28). Briefly, study inclusion criteria were as follow: allergic by history or other factors, double or single blind PCFC for allergen of interest (open challenge allowed if patient < 3 years of age (30)), data on individual patients, description of a no observed adverse effect level (NOAEL) and/or a lowest observed adverse effect level (LOAEL) (if dosing regimen was provided, the NOAEL could be determined from LOAEL), and objective symptoms at time of reaction. Published studies were supplemented with unpublished clinical data of equal quality, when available. The symptoms at the LOAEL, age, and geographic location of each patient were recorded when possible. Additionally, the challenge material (e.g. whole

peanut, peanut flour, peanut butter, etc) and dosing protocol were recorded for each study. Challenge materials were converted to mg protein to normalize reported doses across studies (Table 1). Multiple centers had slightly different protein contents for a food (e.g. raw whole egg, liquid milk, etc.), in which case a representative, conservative protein value was chosen and listed with its source.

Table 1. Conversion factors used to normalize the threshold challenge material.

Allergen	Conversion to:	Protein Content	mg Protein	mg Food	Reference
Peanut	Whole Peanut	25.0%	1	4.0	Taylor et al. (31) assumptions
Peanut	Defatted Peanut Flour	50.0%	1	2.0	Taylor et al. (31) assumptions
Milk	Liquid Milk	3.3%	1	30.3	USDA - 2% Milk
Milk	NFDM	35.1%	1	2.8	USDA - Instant NFDM
Egg	Raw Whole Egg	12.6%	1	8.0	USDA
Egg	Raw Egg White	10.5%	1	9.5	NEVO
Egg	Cooked Whole Egg	12.6%	1	7.9	USDA
Egg	Cooked Egg White	10.5%	1	9.5	NEVO
Egg	Dried Whole Egg	46.0%	1	2.2	NEVO
Egg	Dried Egg White	81.1%	1	1.2	USDA
Hazelnut	Hazelnut Flour	15.7%	1	6.4	In Wensing et al. (32)
Soy	Whole Soybean	40.0%	1	2.5	Assumptions made by working group
Soy	Soy Flour	53.0%	1	1.9	In Ballmer-Weber et al. (33)
Soy	Soy Milk	3.6%	1	27.8	provided by UMCU
Wheat	Raw or Cooked Wheat Flour	10.0%	1	10.0	NEVO and USDA
Cashew	Cashew Flour	19.0%	1	5.3	NEVO
Mustard	Mustard Seed	26.1%	1	3.8	USDA - S. alba seed
Lupin	Lupin Flour	36.2%	1	2.8	USDA
Lupin	Yellow Lupin Flour	40.0%	1	2.5	In Shaw et al. (34)
Sesame	Crushed Sesame Seeds	17.0%	1	5.9	USDA - Roasted Seed
Sesame	Sesame Seed Flour	30.8%	1	3.2	USDA - High Fat Flour
Shrimp	Whole Cooked Shrimp	22.8%	1	4.4	USDA - Steamed/Boiled
Celery	Raw Celery Root	2.0%	1	50.0	NEVO
Fish	Snapper	26.3%	1	3.8	USDA
Fish	Cooked Codfish	23.0%	1	4.3	NEVO
Fish	Raw Codfish	18.3%	1	5.5	NEVO
Fish	Catfish	18.4%	1	5.4	USDA
Fish	Halibut	19.4%	1	5.2	NEVO
Fish	Tuna	23.7%	1	4.2	provided by UMCU
Fish	Tilapia	17.8%	1	5.6	provided by UMCU

USDA, USDA National Nutrient Database; NEVO, The Dutch Food Composition Table; UMCU, University Medical Center Utrecht.

Data obtained through the literature search were merged with existing food allergen threshold datasets from TNO in order to maximize the number of subjects available for analysis. Datasets were scrutinized on an individual patient basis, with any disparities or duplicates being thoroughly examined. The NOAELs and LOAELs from each individual were modeled using a dose distribution approach as described by Crevel et al. (35). Allergen population threshold curves were generated using an Interval-Censoring Survival Analysis (ICSA) approach (36) and the LIFEREG procedure (SAS v9.2). The ICSA method is appropriate as the exact dose that provokes a reaction in an individual is not known but it is known to fall into a particular interval dependent on the dosing scheme used in the challenge (28). Additionally, ICSA utilizes individuals that react to the first dose (left-censored observations) or those that do not react at all, but whose allergy is nonetheless proven by past history of reaction (right-censored observations). For left-censored individuals, ICSA assigns the NOAEL value as zero and the LOAEL value as the first dose in the oral challenge. For right-censored individuals, the last dose in the oral challenge series is considered the NOAEL value while the LOAEL is considered infinity. No biological evidence exists for the uses of one statistical model over another, so discrete doses (dose given immediately prior to the reaction) and cumulative doses (cumulative of all doses given up to that point) were fit using log-normal, log-logistic, and Weibull distributions for each allergen (35). The ED01, ED05, ED10, and ED50 or doses predicted to provoke a reaction in 1%, 5%, 10%, and 50% of the allergic population, respectively, were extracted from each distribution. Results were analyzed and compared with potential methods available to derive reference doses or

perhaps future regulatory thresholds in an effort to determine how best to utilize food allergen threshold data for food allergen risk assessment.

Where possible, populations (study population, geographic region, age, gender, challenge material) were analyzed non-parametrically using the Generalized Log-Rank Test for interval censored data (37, 38) through the ICSTEST macro (SAS v9.2) (39, 40) to determine if the populations were significantly different. Additionally, the same ICSA data were analyzed after parametric distribution fitting by a second method comparing non-overlapping 84% confidence intervals of modeled distributions as a measure of significance (41). Briefly, 84% confidence intervals of large-sample datasets from the same population have a probability of overlap of 0.95, where 95% confidence intervals have a probability of overlap of 0.995 and are overly conservative. Therefore, 84% confidence intervals that do not overlap are significantly different at $\alpha = 0.05$.

Methods to improve clinical food challenges

Ideal number of subjects in a study

Subjects were randomly selected 1000 times in groups of 10, 20, 30, 50, 100, and 200 from the known peanut threshold database of 450 individuals (27) and analyzed using the LIFEREG procedure. Generated ICSA dose distributions were analyzed using the Generalized Log-Rank Test for interval censored data and by comparing non-overlapping 84% confidence intervals of modeled log-normal distributions as a measure of significance.

Food challenge dose scheme comparisons

Clinical literature was scanned for commonly used food challenge dosing schemes. Representative clinical dose schemes were created for a simulation comparing

individual schemes to the overall known allergen population thresholds. The population thresholds for egg, peanut, and soy flour were used as model allergens due to their respective shifts in population sensitivity. Individual allergen thresholds were generated for 30 or 50 subjects based on the parameters of the egg, peanut, or soy flour threshold curves. Generated thresholds were fit to a selected dose scheme's respective NOAEL and LOAEL values and analyzed using the LIFEREG procedure. Again, Generated ICSA dose distributions were analyzed in nonparametric fashion using the Generalized Log-Rank Test for interval censored data and by comparing non-overlapping 84% confidence intervals as a measure of significance.

3. Results and Discussion

Population thresholds

Minimal eliciting doses were found for 13 priority allergens and over 1800 individuals from published clinical literature or unpublished clinical data. Published and unpublished clinical threshold data are summarized in Tables 2-14. Peanut, milk, egg, and hazelnut have substantially more data available than the other allergens: soybean, wheat, cashew, mustard, lupin, sesame seed, shrimp, celery and fish. Modeling could not be done for celery and fish due to an insufficient number of subjects in the datasets. No threshold data were found for molluscan shellfish or for the priority tree nuts other than hazelnut and cashew (children only).

Peanut

Individual peanut thresholds were obtained for 750 subjects including 489 individuals from published studies and another 261 subjects from unpublished clinical records (Table 2). A clinical trial from Peeters et al. (42) was conducted at a Dutch clinic

and data were included in the previous analysis by Taylor et al. (28). However, the clinic was used to gather subsequent unpublished data, and it was impossible to distinguish between the Peeters et al. (42) subjects and unpublished individuals. Therefore the Peeters' subjects were removed from the published dataset to avoid duplication. The peanut dataset includes 584 children, 99 adults, and 67 individuals of undetermined age (impossible to discern from publication). Of the 750 subjects, 30 were left-censored and 132 were right-censored. The peanut dataset is the strongest of the 11 available allergen datasets due to its number of observations, distribution of thresholds across the dosing spectrum, and acquisition of data from multiple clinical centers. Estimates obtained for both discrete and cumulative doses were considered because they matched closely (data not shown). All three statistical models fit the cumulative data well. The Weibull model provided the most conservative ED estimates but the Weibull model deviated from the actual data and over-estimated the population sensitivity at the lower doses in the distribution. Therefore, in the case of peanut, the log-normal and log-logistic distributions should be used in reference dose determinations (Figure 1A). Within each statistical distribution, 95% confidence intervals of the ED01, ED05, and ED10 do not overlap, illustrating the quality of data in the peanut dataset (Figure 1B). Sufficient threshold data exist for peanut to use the ED01 value from the population threshold to determine a suitable reference dose that can be used for future food allergen risk assessments.

Table 2. Peanut Threshold Data Gleaned From Published and Unpublished DBPCFC Clinical Records.

Allergen	Study	Total No. with Objective Symptoms	Right Censored	Left Censored	Lowest MED† (mg protein)	Highest MED (mg protein)	Population
Peanut	Atkins et al. (43)	2	0	0	1375	2000	Adults
	Hourihane et al. (44)	13	11	0	3.9	88.9	Adults and Children
	Wensing et al. (45)	26	20	0	4.4	44.4	Adults and Children
	Lewis et al. (46)	40	0	3	1.0	3936	Adults and Children
	Flinterman et al. (47)	22	11	0	5.8	2206	Children
	Leung et al. (48)	23	8	1	0.5	1943	Adults and Children
	Oppenheimer et al. (49)	4	0	0	943	7943	Adults and Children
	Nelson et al. (50)	12	0	1	43	3900	Adults
	Nancy (27)*	283	10	7	0.1	2500	Adults and Children
	Anagnostou et al. (51)	18	0	7	1.0	256	Adults and Children
	Clark et al. (52, 53)	5	0	1	1.0	81	Children
	Nicolaou et al. (54)	10	0	2	1	6111	Children
	Blumchen et al. (55)	22	0	2	3.75	492.5	Children
	Wainstein et al. (56)	8	0	0	555	2930	Children
	Patriarca et al. (57)	1	0	0	1637.5	-	Adults
	Unpublished Data**	261	72	6	0.2	4706	Adults and Children
	Total	750	132	30	0.1	7943	

† MED, Minimal Eliciting Dose for objective symptoms in food allergic individuals

* Published only in summarized form

** Wilhelmina Kinderziekenhuis Children's Hospital of the University Medical Center Utrecht; University Medical Center Utrecht (includes (42)); University Medical Center Gronigen

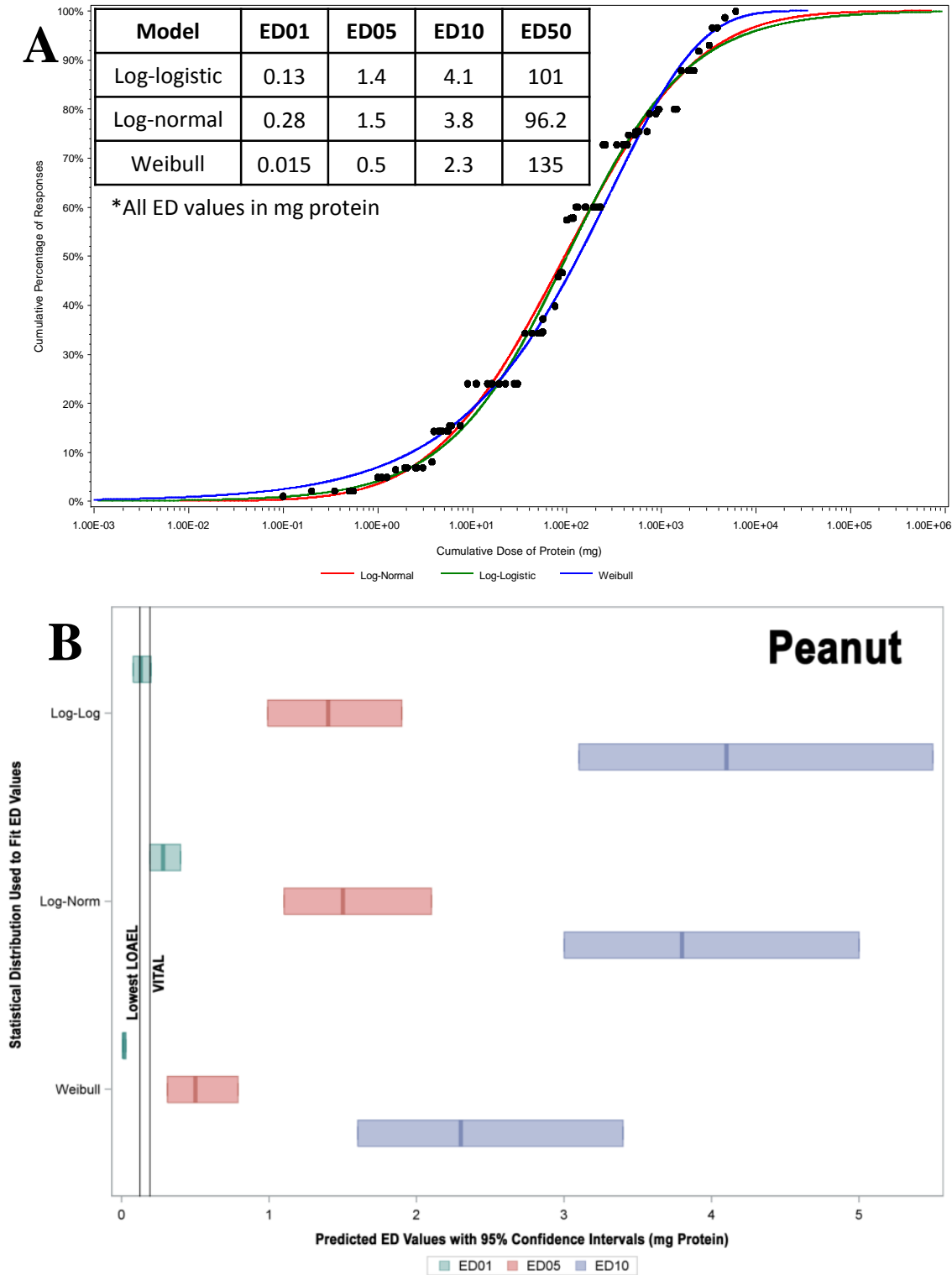


Figure 1. (A) Log-logistic, log-normal, and Weibull probability distribution models of individual peanut thresholds (expressed as mg peanut protein). **(B)** Log-logistic, log-normal, and Weibull ED estimates and corresponding 95% confidence intervals (expressed as mg peanut protein) from the peanut probability distribution models. The most sensitive individual in the dataset and the VITAL reference dose are marked and labeled.

Previously, 185 peanut-allergic individual thresholds were gleaned from clinical literature by Taylor et al. (28). An additional 286 thresholds from a single French medical center in Nancy were added to the dataset in 2010 (27) with no significant differences observed in the ED05 and ED10 by comparison to the previously published dataset. The threshold curves for peanut are stable as demonstrated when Taylor et al. (58) added 300 peanut-allergic individuals to the threshold distribution reported by Taylor et al. (27). No significant differences were found in the ED01, ED05, and ED10 values of distributions based on 750 peanut-allergic individuals versus distributions based on 450 individuals (Figure 2).

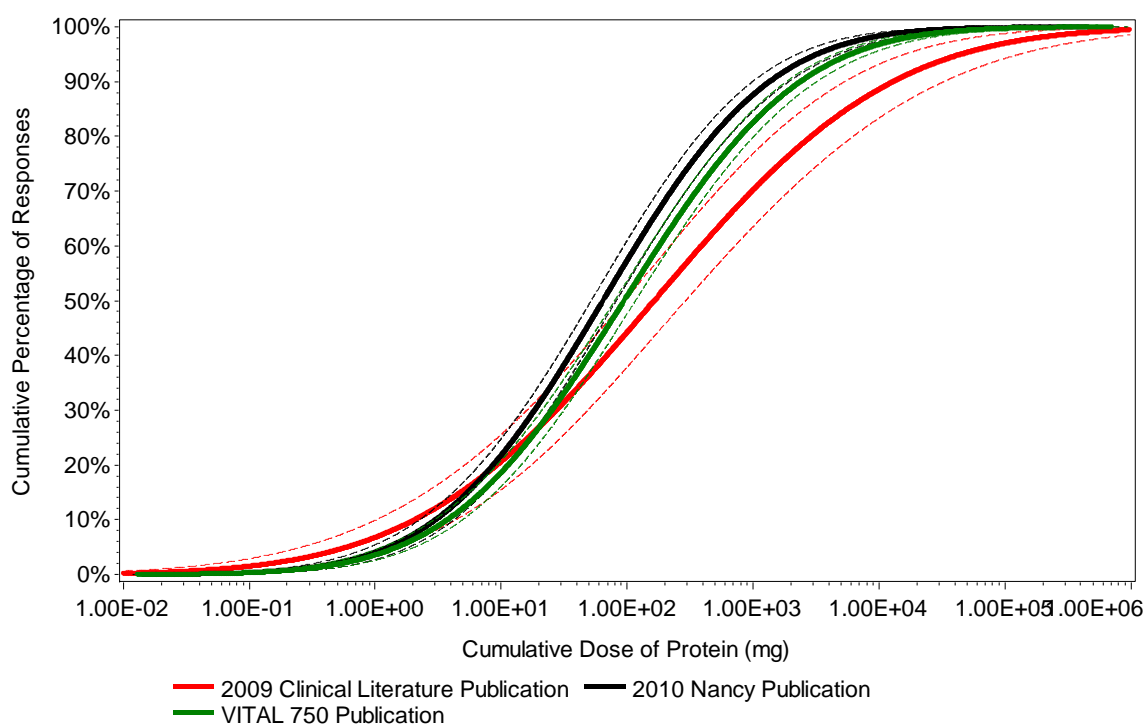


Figure 2. Log-normal probability distribution models of peanut from 3 dataset expansions points (expressed as mg peanut protein). First, 185 individual thresholds were gleaned from clinical literature by Taylor et al. (28). An additional 286 thresholds from a single French medical center were added to the dataset in 2010 (27). Most recently, 300 thresholds were gleaned from newly published clinical literature and medical centers in the Netherlands and combined with the previous datasets (58).

Milk

Individual milk thresholds were obtained for 351 individuals including 222 individuals from published studies and another 129 subjects from unpublished clinical records (Table 3). The milk dataset includes 323 children, 25 adults and 3 individuals of undetermined age. Due to the natural history of milk allergy (approximately 85% of children outgrow their milk allergen by school age (59)), milk-allergic adults are rarely encountered. Of the 351 subjects, 59 were left-censored and 19 were right-censored. Like peanut, the milk dataset was considered to be representative due to a large allergic population, a good distribution of thresholds, and data from multiple clinical centers. One weakness in the milk dataset is the limited number of milk-allergic adults, but the data likely reflect the true age distribution of the milk allergic population. Estimates obtained for both discrete and cumulative doses were considered because they matched closely (data not shown). All three statistical models fit the cumulative data reasonably well but again the Weibull model was not the best fit at the lower doses in the distribution which is important for establishment of reference doses or potential regulatory threshold values that protect the vast majority of food allergic individuals (Figure 3A). Therefore, in the case of milk, the log-normal and log-logistic distributions should be used to determine population thresholds. Within each statistical distribution, 95% confidence intervals of the ED01, ED05, and ED10 do not overlap, in large part due to the quality of data and large number of subjects in the milk dataset (Figure 3B). Sufficient threshold data exist for milk to use the ED01 value from the population threshold to determine a suitable reference dose that can be used for future food allergen risk assessments.

Table 3. Milk Threshold Data Gleaned From Published and Unpublished DBPCFC Clinical

Allergen	Study	Total No. with Objective Symptoms	Right Censored	Left Censored	Lowest MED† (mg protein)	Highest MED (mg protein)	Population
Milk	Staden et al. (60)	3	0	0	13.0	4776	Children
	Norgaard and Bindselev-Jensen (61)	3	0	1	181.5	8250	Adults
	Morisset et al. (62)	3	0	1	3.3	26.4	Adults and Children
	Caminiti et al. (63)	11	0	0	13.2	1465	Children
	Patriarca et al. (64)	8	0	0	3.6	1538	Children
	Morisset et al. (65)	11	0	1	66	6600	Children
	Longo et al. (66)	60	0	9	5.9	52.1	Children
	Skripak et al. (67)	20	0	14	40	1340	Children
	Lam et al. (68)	8	2	0	423	13323	Adults and Children
	Hill et al. (69)	53	0	11	66	6600	Children
	Fiocchi et al. (70)	12	0	5	198	2970	Children
	Baehler et al. (71)	10	0	0	19.3	1371	Children
	Flinterman et al. (72)	11	0	3	180	4500	Children
	Orhan et al. (73)	4	0	0	1815	9240	Children
	Devenney et al. (74)	2	0	1	3.3	184.4	Children
	Host et al. (75)	3	0	2	165	1155	Children
	Unpublished Data *	129	17	11	0.2	15700	Adults and Children
	Total	351	19	59	0.2	15700	

† MED, Minimal Eliciting Dose for objective symptoms in food allergic individuals

* Jeroen Bosch Hospital, The Netherlands; University Medical Center Utrecht; University Medical Center Groningen

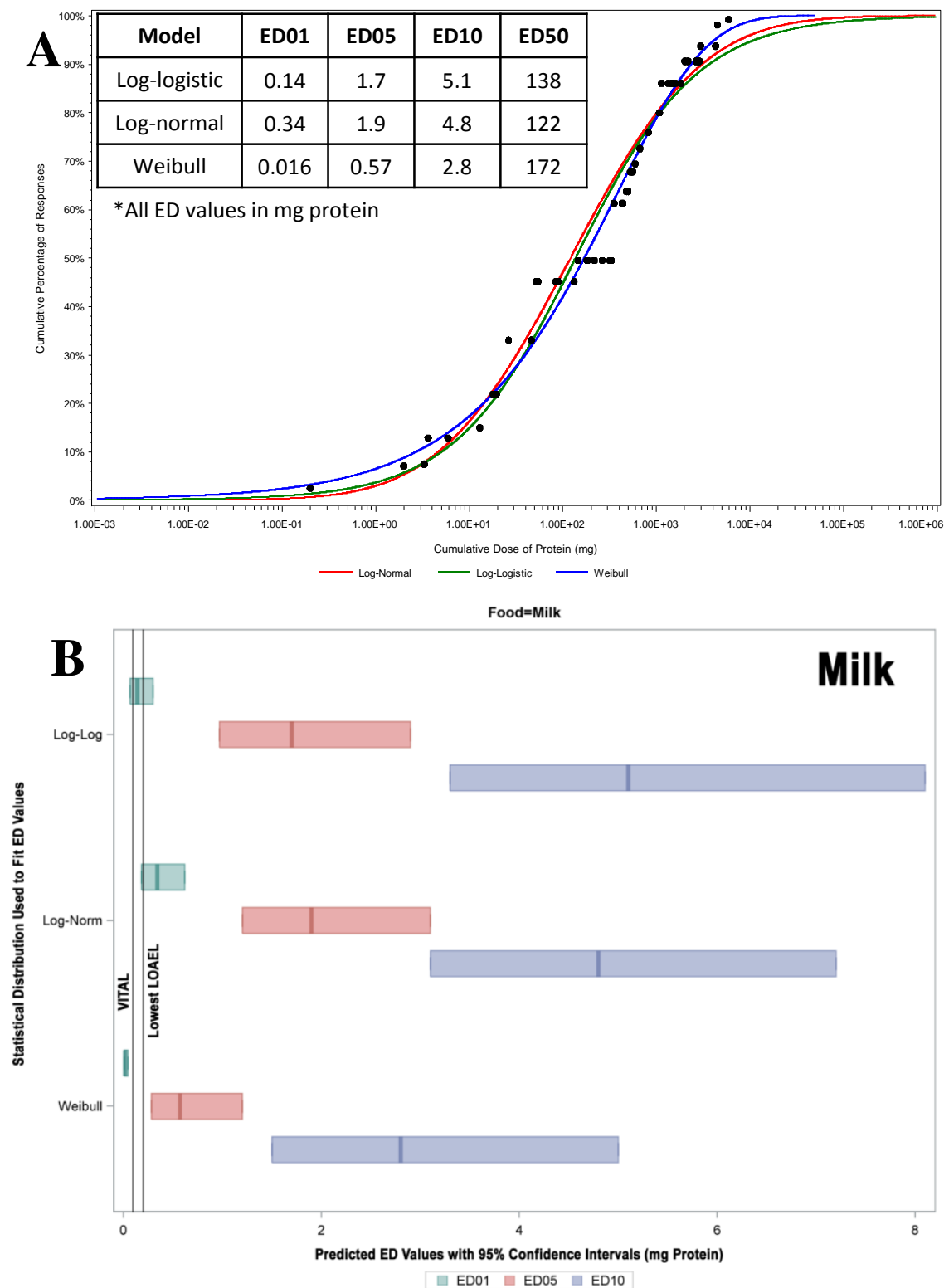


Figure 3. (A) Log-logistic, log-normal, and Weibull probability distribution models of individual milk thresholds (expressed as mg milk protein). (B) Log-logistic, log-normal, and Weibull ED estimates and corresponding 95% confidence intervals (expressed as mg milk protein) from the milk probability distribution models. The most sensitive individual in the dataset and the VITAL reference dose are marked and labeled.

Egg

Individual egg thresholds were obtained for 206 individuals including 110 individuals from published studies and another 96 subjects from unpublished clinical records (Table 4). The egg dataset includes 174 children, 12 adults, and 20 individuals of undetermined age. Again, egg-allergic adults were less prevalent due to the natural history of egg allergy. Of the 206 subjects, 24 were left-censored and 33 were right-censored. The egg dataset was considered to be representative due to the large number of subjects, data from multiple regions, and a good data distribution across the dosing scheme. However, the absence of sufficient data on individual egg thresholds among adults is considered as a small data gap. Estimates obtained for both discrete and cumulative doses were considered (data not shown) and all three statistical models fit the cumulative data reasonably well (Figure 4A). The Weibull model provided the best fit and the most conservative estimates. Therefore, in the case of egg, all three distributions should be considered with an emphasis on the Weibull distribution when developing a reference dose value for use in food allergen risk assessment. For egg, 206 subjects are perhaps marginally sufficient for use of the ED01 for establishment of a reference dose value. The uncertainty in the ED estimates is slightly higher as demonstrated by the overlap of the 95% confidence intervals of the ED05 and ED10 estimates for all distributions (Figure 4B). It is recommended that the ED01 and 95% lower confidence interval of the ED05 values be used in the determination of suitable reference dose values for food allergen risk assessment purposes.

Table 4. Egg Threshold Data Gleaned From Published and Unpublished DBPCFC Clinical Records.

Allergen	Study	Total No. with Objective Symptoms	Right Censored	Left Censored	Lowest MED† (mg protein)	Highest MED (mg protein)	Population
Egg	Staden et al. (60)	3	0	1	5.0	669	Children
	Benhamou et al. (76)	33	0	9	126	4396	Children
	Morisset et al. (62)	20	0	4	0.21	6.8	Adults and Children
	Caffarelli et al. (77)	13	0	0	0.25	6302	Children
	Morisset et al. (65)	15	0	0	6.8	747	Children
	Atkins et al. (43)	1	0	0	-	7686	Adults
	Norgaard and Bindselev-Jensen (61)	7	0	0	0.62	6982	Adults and Children
	Knight et al. (78)	7	0	5	81.1	4461	Children
	Unsel et al. (79)	1	0	0	583	-	Adults
	Orhan et al. (73)	3	0	0	50.3	315	Children
	Eggesbo et al. (80)	7	0	1	126	3900	Children
	Unpublished Data *	96	33	4	0.014	4476	Adults and Children
	Total	206	33	24	0.014	7686	

† MED, Minimal Eliciting Dose for objective symptoms in food allergic individuals

* Food Allergy Research & Resource Program, University of Nebraska – Lincoln; University Medical Center Utrecht; University Medical Center Gronigen

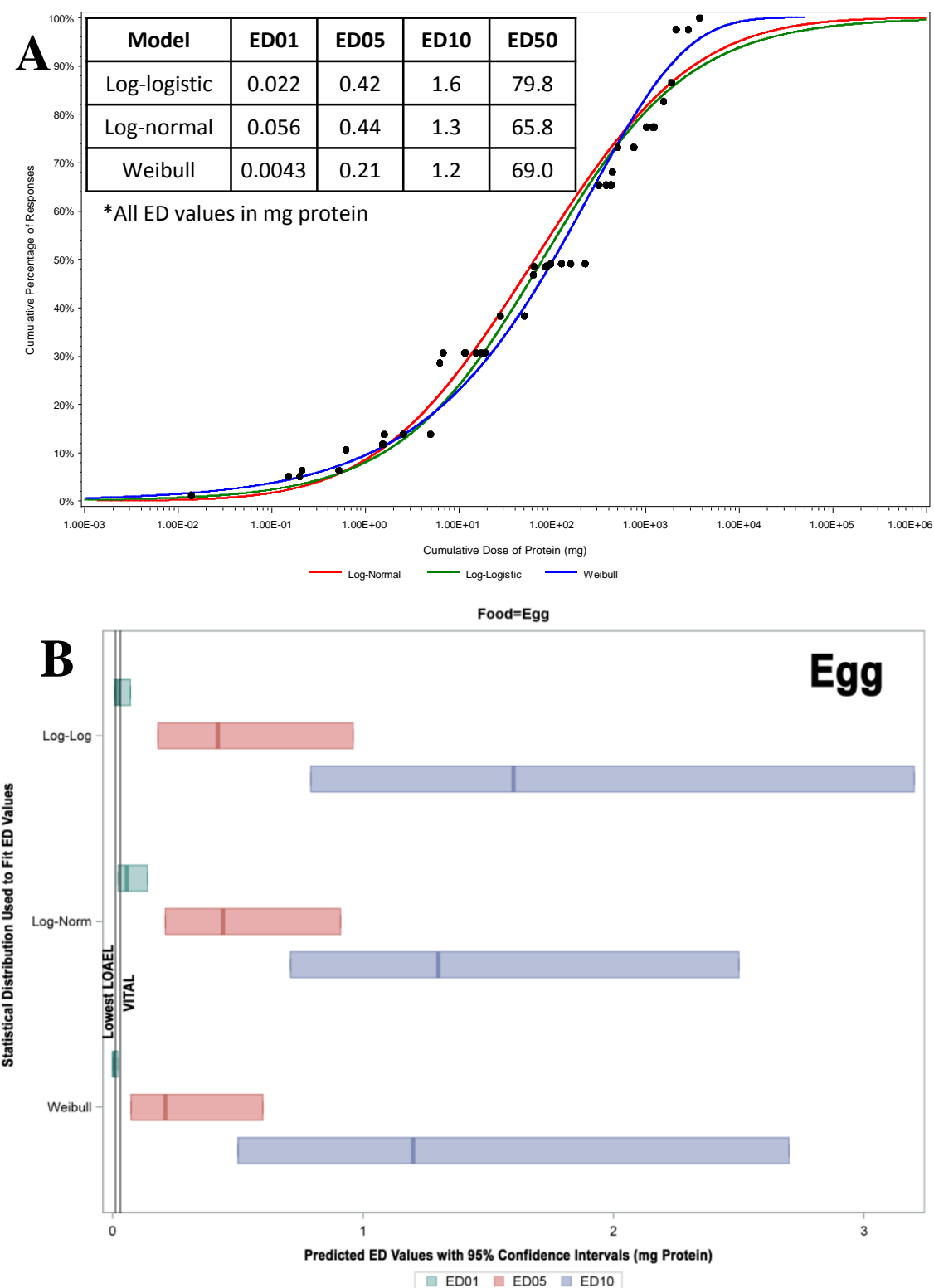


Figure 4. (A) Log-logistic, log-normal, and Weibull probability distribution models of individual egg thresholds (expressed as mg egg protein). (B) Log-logistic, log-normal, and Weibull ED estimates and corresponding 95% confidence intervals (expressed as mg egg protein) from the egg probability distribution models. The most sensitive individual in the dataset and the VITAL reference dose are marked and labeled.

Hazelnut

Individual hazelnut thresholds were obtained for 202 subjects although only 29 individuals were from published studies while the other 173 subjects were from unpublished clinical records (Table 5). The hazelnut dataset includes 61 children and 141 adults. Of the 202 subjects, 4 were left-censored and 67 were right-censored. Overall, the hazelnut dataset was considered to be good, based on the large number of subjects, good distribution of data across the dosing scheme, and multiple clinics providing data. However, the overwhelming majority of data was from the Netherlands and the regional bias could be considered a slight weakness in the dataset. Discrete and cumulative doses were considered because they matched closely (data not shown) and all three distributions should be considered when developing a reference dose value for use in food allergen risk assessment (Figure 5A). For hazelnut, 202 subjects are perhaps marginally sufficient for use of the ED01 as a reference dose for food allergen risk assessment. The uncertainty in the ED estimates is slightly higher than for peanut, milk, and egg, as demonstrated by the overlap of the 95% confidence intervals of the ED05 and ED10 estimates for all distributions (Figure 5B). It is recommended that the ED01 and 95% lower confidence interval of the ED05 values be used suitable reference dose values for food allergen risk assessment purposes.

Table 5. Hazelnut Threshold Data Gleaned From Published and Unpublished DBPCFC Clinical Records.

Allergen	Study	Total No. with Objective Symptoms	Right Censored	Left Censored	Lowest MED† (mg protein)	Highest MED (mg protein)	Population
Hazelnut	Wensing et al. (32)	29	27	1	1.0	1444	Adults
	Unpublished Data *	173	40	3	0.019	2450	Adults and Children
	Total	202	67	4	0.019	2450	

† MED, Minimal Eliciting Dose for objective symptoms in food allergic individuals

*Universitätsmedizin Berlin; University Medical Center Utrecht; University Medical Center Groningen; Wilhelmina Kinderziekenhuis Children's Hospital of the University Medical Center Utrecht (includes (81))

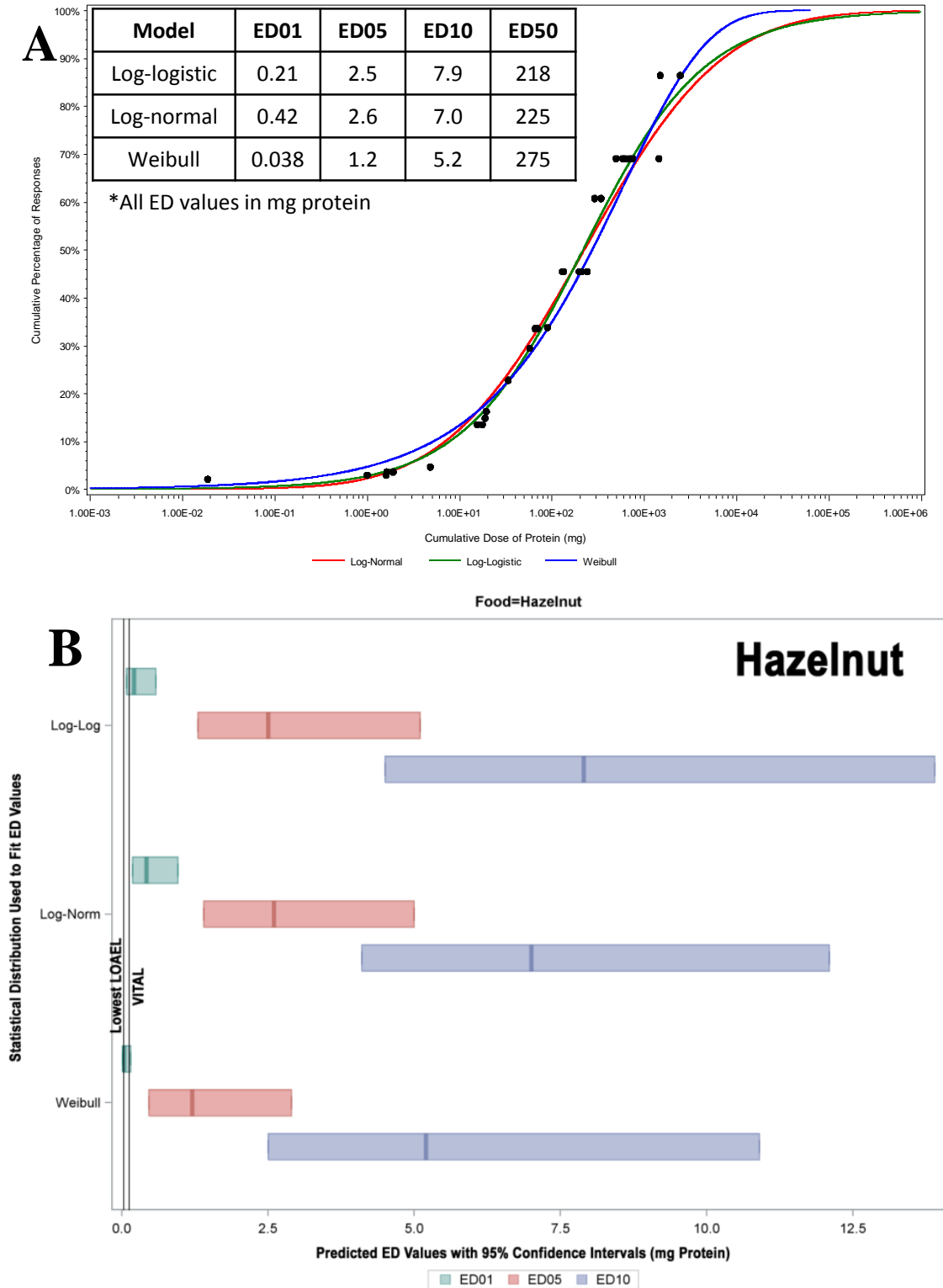


Figure 5. (A) Log-logistic, log-normal, and Weibull probability distribution models of individual hazelnut thresholds (expressed as mg hazelnut protein). **(B)** Log-logistic, log-normal, and Weibull ED estimates and corresponding 95% confidence intervals (expressed as mg hazelnut protein) from the hazelnut probability distribution models. The most sensitive individual in the dataset and the VITAL reference dose are marked and labeled.

Soy flour

Individual soybean thresholds were obtained for 80 subjects including 43 individuals from published studies and 37 subjects from unpublished clinical records (Table 6). The soybean dataset includes 33 children, 25 adults, and 22 individuals of undetermined age. Of the 80 subjects, 6 were left-censored and 28 were right-censored. The soybean dataset was collected from multiple centers but several issues exist with the soybean dataset. First, additional subjects of all ages would increase the confidence of the eliciting dose estimates. Second, the selection of soy-allergic subjects coupled with a specific challenge material may have considerable influence on the estimates. In general, subjects with soy flour challenges have reasonably high individual soybean thresholds whereas subjects with allergic histories to a specific soy milk/protein isolate appear to have much lower individual soybean thresholds. Clearly more clinical research is needed to resolve this issue. However, recent unpublished clinical observations from a Dutch clinic indicate that many of these soy milk-allergic patients can tolerate soy flour and confirm the previous observations that reactivity is associated with certain specific types of soy products. These individuals could be part of a unique, but not unimportant subpopulation that needs to be further studied. Regulators must consider the form(s) of the allergenic ingredient of primary concern, presumably those most likely to fall under the labeling requirements of any future regulatory thresholds that may be established. With regard to cross-contact in manufacturing situations, the most likely forms of soy used would be soy flours, concentrates, and isolates. With that in mind, it is recommended that only individuals allergic to soy flour be used in the establishment of reference doses used for risk assessment purposes. The proposed reference doses would

prove protective for these soy milk-allergic individuals in relation to some forms of soy and they could focus on avoiding soy milk.

Estimates obtained for both discrete and cumulative doses were considered (data not shown) and all three statistical models fit the cumulative data reasonably well (Figure 6A). However, all models had large confidence intervals in which the 95% confidence intervals of the ED05 and ED10 overlapped (Figure 6B). Therefore, in the case of soy flour, all three distributions should be considered when establishing a reference dose value for risk assessment purposes. It is recommended that the 95% lower confidence interval of the ED05 values be used in the determination of reference doses as an insufficient number of soy flour allergic individuals were found to determine a statistically sound ED01 estimate.

Table 6. Soy Threshold Data Gleaned From Published and Unpublished DBPCFC Clinical Records.

Allergen	Study	Total No. with Objective Symptoms	Right Censored	Left Censored	Lowest MED† (mg protein)	Highest MED (mg protein)	Population
Soy	Ballmer-Weber et al. (33)	22	11	0	241	26504	Adults and Children
	Zeiger et al. (82)	9	0	0	365	1260	Children
	Magnolfi et al. (83)	5	1	2	88	3608	Children
	Fiocchi et al. (70)	7	0	1	114	798	Children
	Unpublished Data (Soy Flour)*	8	3	0	11.1	9111	Adults and Children
	Soy Flour Total	51	15	3	11.1	26504	
	Unpublished Data (Soy Milk)*	29	13	3	0.2	7502	Adults and Children

† MED, Minimal Eliciting Dose for objective symptoms in food allergic individuals

* Food Allergy Research & Resource Program, University of Nebraska – Lincoln; Universitätsmedizin Berlin; University Medical Center Utrecht; University Medical Center Groningen

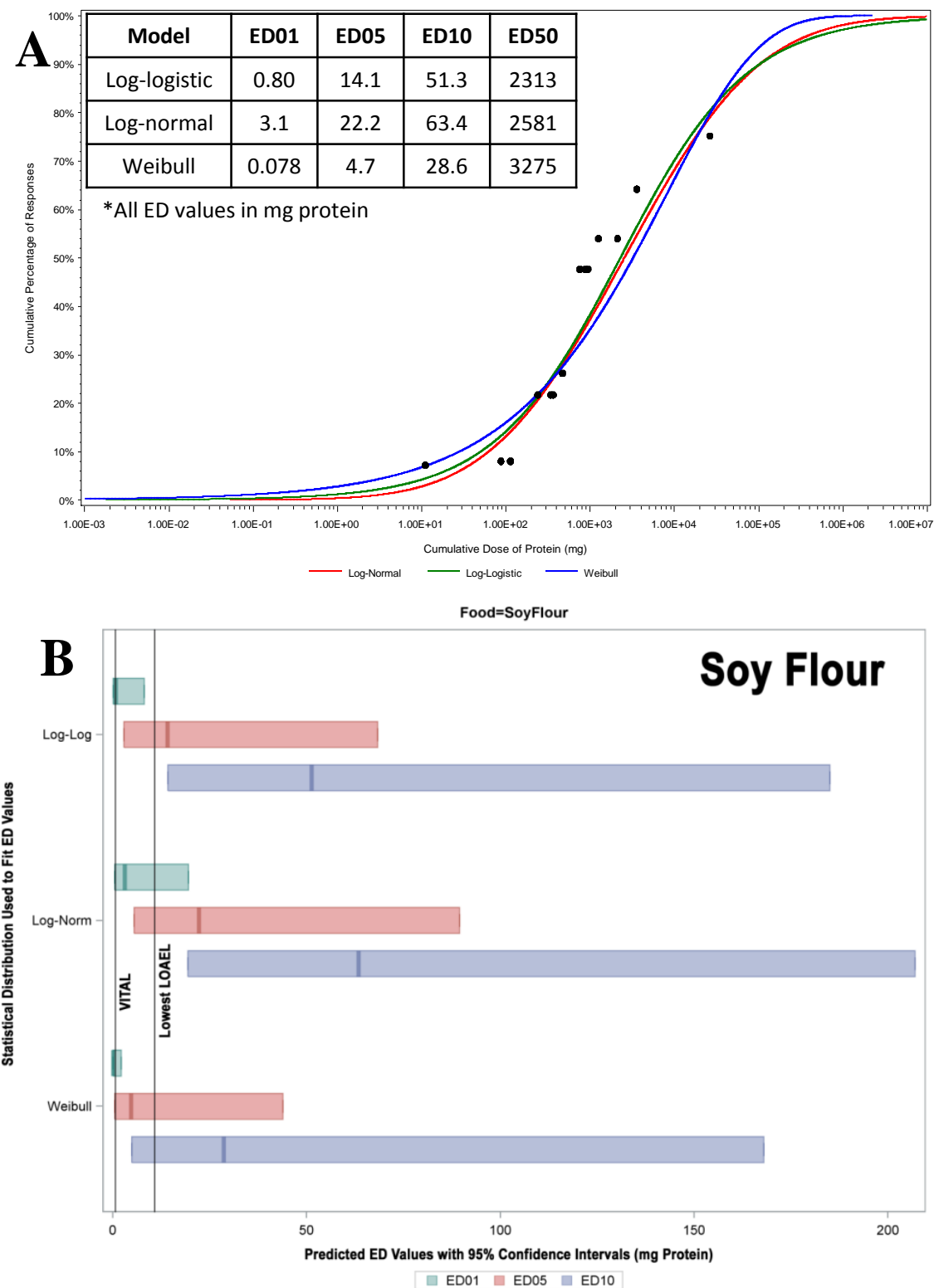


Figure 6. (A) Log-logistic, log-normal, and Weibull probability distribution models of individual soy flour thresholds (expressed as mg soy flour protein). **(B)** Log-logistic, log-normal, and Weibull ED estimates and corresponding 95% confidence intervals (expressed as mg soy flour protein) from the soy flour probability distribution models. The most sensitive individual in the dataset and the VITAL reference dose are marked and labeled.

Wheat

Individual wheat thresholds were obtained for 40 subjects (who had confirmed IgE-mediated allergic reactions to wheat) including 37 individuals from published studies and 3 subjects from unpublished clinical records (Table 7). The wheat dataset includes 28 children and 12 adults. Of the 40 subjects, 5 were left-censored and 1 was right-censored. Overall, the wheat dataset benefited from data collected at multiple centers, but additional data on individual thresholds would strengthen the statistical analysis. Estimates obtained for both discrete and cumulative doses were considered (data not shown) and all three statistical models fit the cumulative data with little difference (Figure 7A). Due to the limited number of wheat-allergic subjects, all models had large confidence intervals in which the 95% confidence intervals of the ED05 and ED10 overlapped (Figure 7B). The 95% lower confidence interval of the ED05 from all 3 models should be considered when determining a reference dose value for use in food allergy risk assessment. The Codex Alimentarius guideline for gluten-free is <20 ppm gluten protein and wheat-allergic consumers would be largely protected by gluten-free foods produced to the Codex specifications.

Table 7. Wheat Threshold Data Gleaned From Published and Unpublished DBPCFC Clinical Records.

Allergen	Study	Total No. with Objective Symptoms	Right Censored	Left Censored	Lowest MED† (mg protein)	Highest MED (mg protein)	Population
Wheat	Ito et al. (84)	21	0	1	2.6	1771	Children
	Pastorello et al. (85)	3	1	1	23.3	93	Children
	Scibilia et al. (86)	13	0	3	10	2500	Adults and Children
	Unpublished Data *	3	0	0	8.6	2209	Children
	Total	40	1	5	2.6	2500	

† MED, Minimal Eliciting Dose for objective symptoms in food allergic individuals

* University Medical Center Gronigen

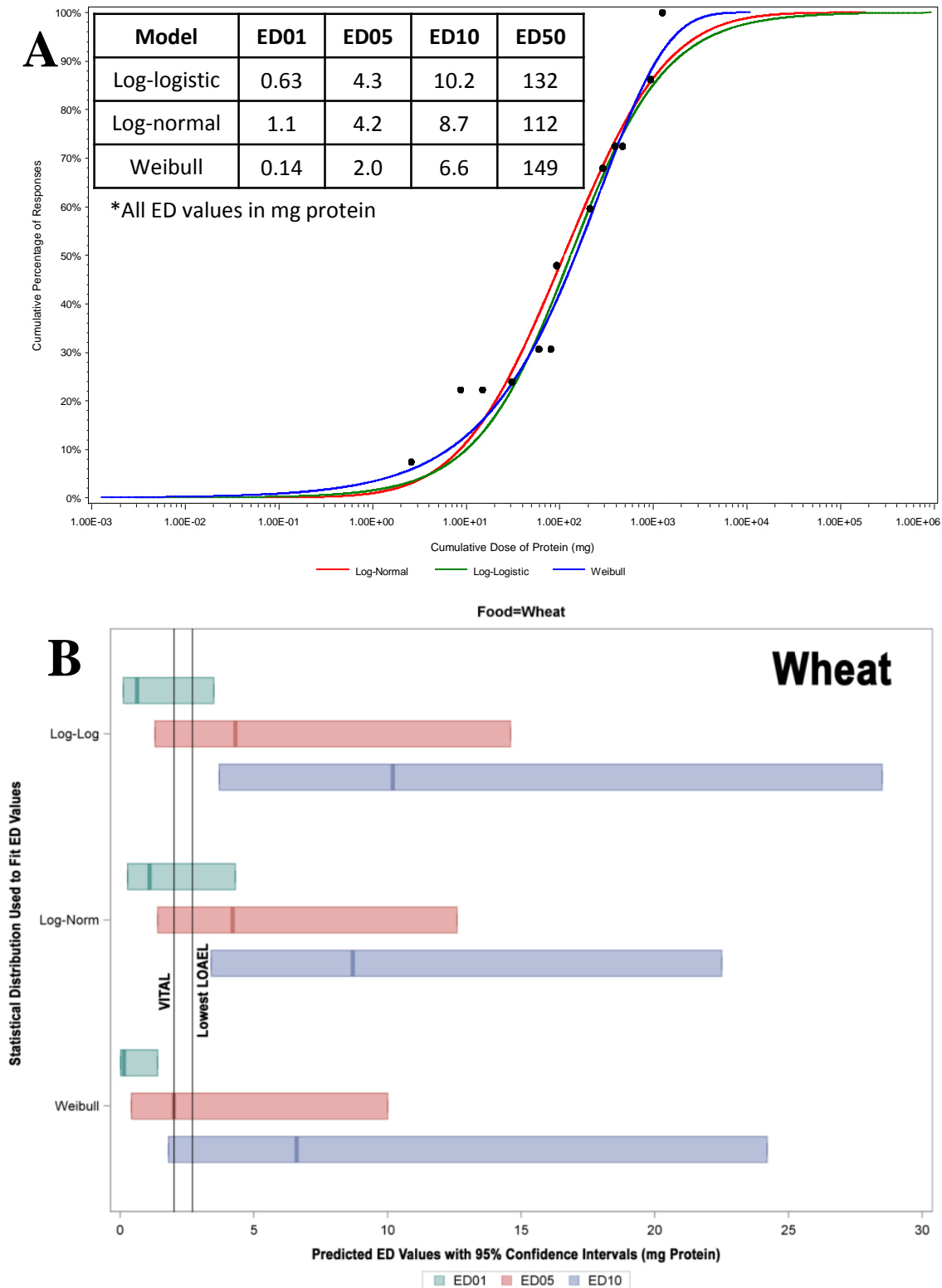


Figure 7. (A) Log-logistic, log-normal, and Weibull probability distribution models of individual wheat thresholds (expressed as mg wheat protein). **(B)** Log-logistic, log-normal, and Weibull ED estimates and corresponding 95% confidence intervals (expressed as mg wheat protein) from the wheat probability distribution models. The most sensitive individual in the dataset and the VITAL reference dose are marked and labeled.

Cashew

Individual cashew thresholds were obtained for 31 subjects; all children from unpublished clinical records at a single Dutch clinic (Table 8). One subject was left-censored and 16 were right-censored. Overall, the cashew dataset was considered to be marginally sufficient for establishment of a reference dose and additional data on adults and children from multiple clinical centers would strengthen the statistical analysis. Similar estimates were obtained for both discrete and cumulative doses (data not shown) and fits for all three distributions should be considered when establishing a reference dose value for use in food allergy risk assessment (Figure 8A). For cashew, the number of subjects is marginal and leads to wide 95% confidence intervals with overlap between the ED01, ED05, and ED10 (Figure 8B). The lower 95% confidence interval of the ED05 is appropriate as the basis of a provisional reference dose for cashew. Another approach that could be considered for the purposes of risk assessment for cashew (and other tree nuts where threshold data is lacking) is to use the established reference dose value for hazelnut until more threshold data is available to strengthen the statistical analysis. This approach would assume that all other tree nuts would result in similar reactivity as hazelnut. Currently there are no published or anecdotal clinical reports to suggest that other tree nuts would be more potent (or severe) than hazelnut.

Table 8. Cashew Threshold Data Gleaned From Unpublished Clinical Records.

Allergen	Study	Total No. with Objective Symptoms	Right Censored	Left Censored	Lowest MED† (mg protein)	Highest MED (mg protein)	Population
Cashew	Unpublished Data	31	16	1	2.3	759	Children
	Total	31	16	1	2.3	759	

† MED, Minimal Eliciting Dose for objective symptoms in food allergic individuals

* University Medical Center Groningen

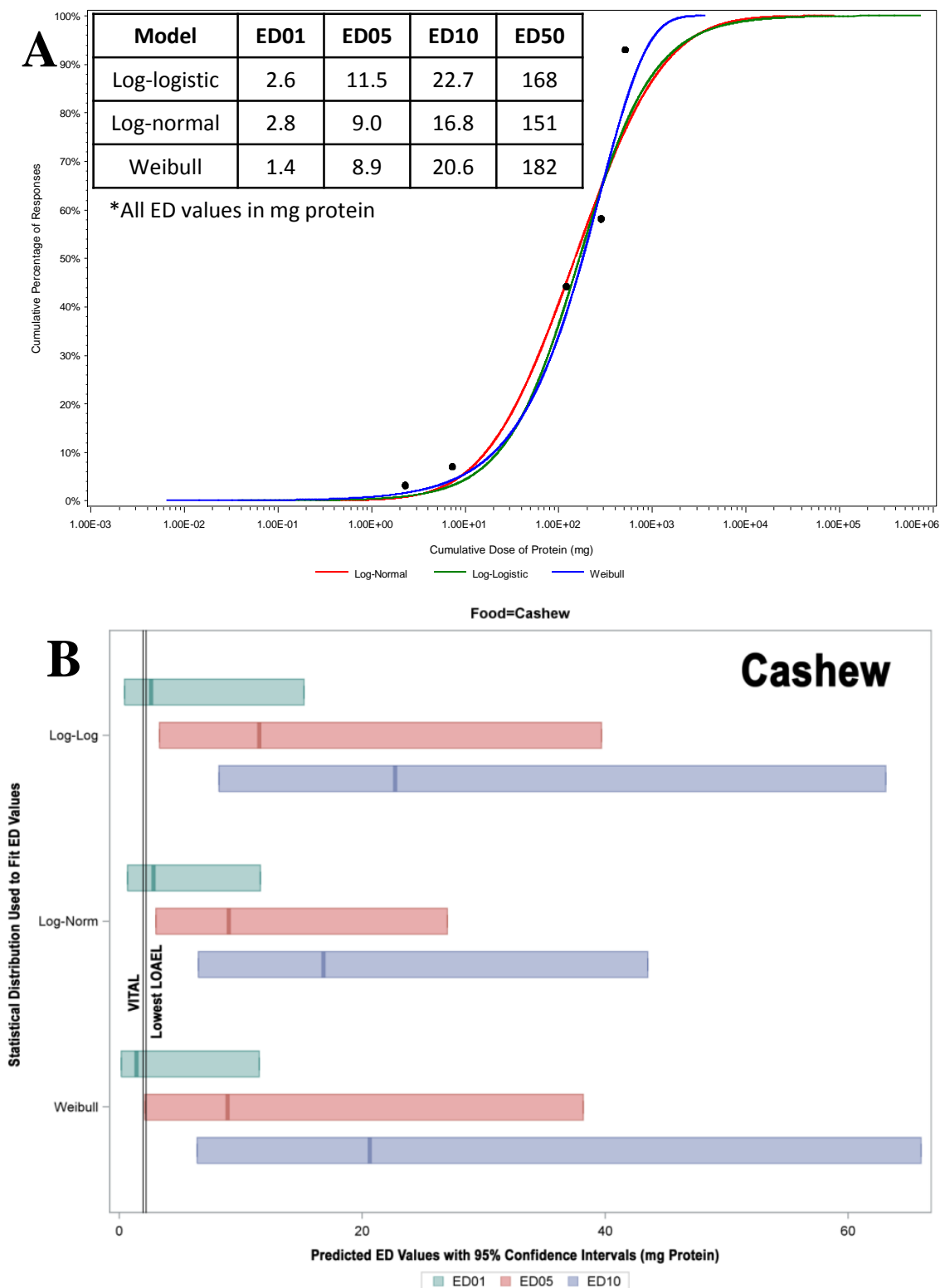


Figure 8. (A) Log-logistic, log-normal, and Weibull probability distribution models of individual cashew thresholds (expressed as mg cashew protein). **(B)** Log-logistic, log-normal, and Weibull ED estimates and corresponding 95% confidence intervals (expressed as mg cashew protein) from the cashew probability distribution models. The most sensitive individual in the dataset and the VITAL reference dose are marked and labeled.

Mustard

Mustard is on the priority list of allergenic foods in the EU and Canada and 33 individual mustard thresholds were obtained from published studies (Table 9). The mustard dataset includes 9 children, 9 adults, and 15 individuals of undetermined age. Of the 33 subjects, 2 were left-censored and 10 were right-censored. Similar estimates were obtained for both discrete and cumulative doses (data not shown). All three distributions should be considered when establishing a reference dose value for mustard (Figure 9A), although the number of subjects is adequate only for determining the lower 95% confidence interval of the ED05, not an ED01. The mustard dataset has data from multiple clinical centers but could benefit from additional data on individual thresholds to tighten the currently overlapping ED confidence intervals (Figure 9B).

Table 9. Mustard Threshold Data Gleaned From Published DBPCFC Clinical Records.

Allergen	Study	Total No. with Objective Symptoms	Right Censored	Left Censored	Lowest MED† (mg protein)	Highest MED (mg protein)	Population
Mustard	Figueroa et al. (87)	14	10	0	11.7	40.9	Adults and Children
	Morisset et al. (88)	4	0	0	3.5	117.4	Children
	Rance et al. (89)	15	0	2	0.26	244	Adults and Children
	Total	33	10	2	0.26	244	

† MED, Minimal Eliciting Dose for objective symptoms in food allergic individuals

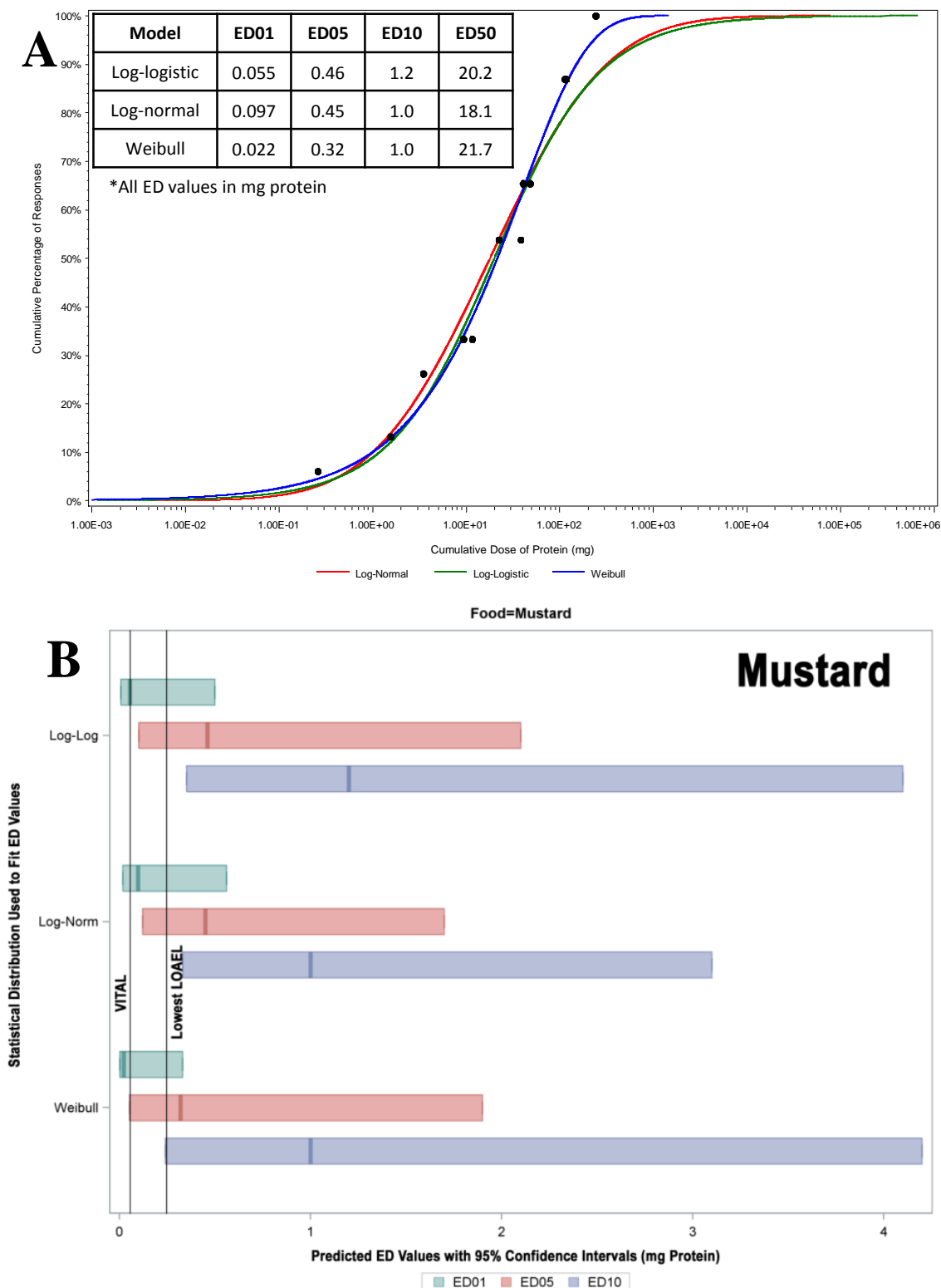


Figure 9. (A) Log-logistic, log-normal, and Weibull probability distribution models of individual mustard thresholds (expressed as mg mustard protein). (B) Log-logistic, log-normal, and Weibull ED estimates and corresponding 95% confidence intervals (expressed as mg mustard protein) from the mustard probability distribution models. The most sensitive individual in the dataset and the VITAL reference dose are marked and labeled.

Lupin

Lupin is on the priority list of allergenic foods in the EU and thresholds were obtained for 24 subjects; 9 individuals from published studies and 15 subjects from unpublished clinical records (Table 10). The lupin dataset includes 9 children and 15 adults. Of the 24 subjects, 2 were left-censored and 7 were right-censored. Overall, the lupin dataset has data from multiple centers, but is heavily weighted towards a single clinic. Additional data on individual thresholds would strengthen the lupin dataset. Discrete and cumulative doses were considered because they matched closely (data not shown). There are a limited number of individual clinical thresholds that are representative of the more sensitive population of lupin allergic individuals (lower end of the population curve) so a data gap for thresholds at lower challenge doses currently exists. The Weibull distribution was the most conservative, however, due to the lack of actual low dose data the Weibull distribution may have provided an over estimation of the overall sensitivity of the lupin allergic population (Figure 10A). Therefore, the emphasis should be placed primarily on the log-normal and log-logistic distributions when establishing a reference dose for lupin. Again, there are overlapping confidence intervals at the ED01, ED05, and ED10 (Figure 10B) and insufficient subjects to base a threshold on the ED01. The lower 95% confidence interval of the ED05 should be considered for establishing a reference dose for lupin that can be utilized for food allergen risk assessment purposes.

Table 10. Lupin Threshold Data Gleaned From Published and Unpublished DBPCFC Clinical Records.

Allergen	Study	Total No. with Objective Symptoms	Right Censored	Left Censored	Lowest MED† (mg protein)	Highest MED (mg protein)	Population
Lupin	Moneret-Vautrin et al. (90)	5	0	0	95.9	362	Children
	Fiocchi et al. (91)	2	0	1	50	3150	Children
	Shaw et al. (34)	2	0	0	111	1111	Children
	Unpublished Data *	15	7	1	36.2	1593	Adults
	Total	24	7	2	36.2	3150	

† MED, Minimal Eliciting Dose for objective symptoms in food allergic individuals

* University Medical Center Utrecht

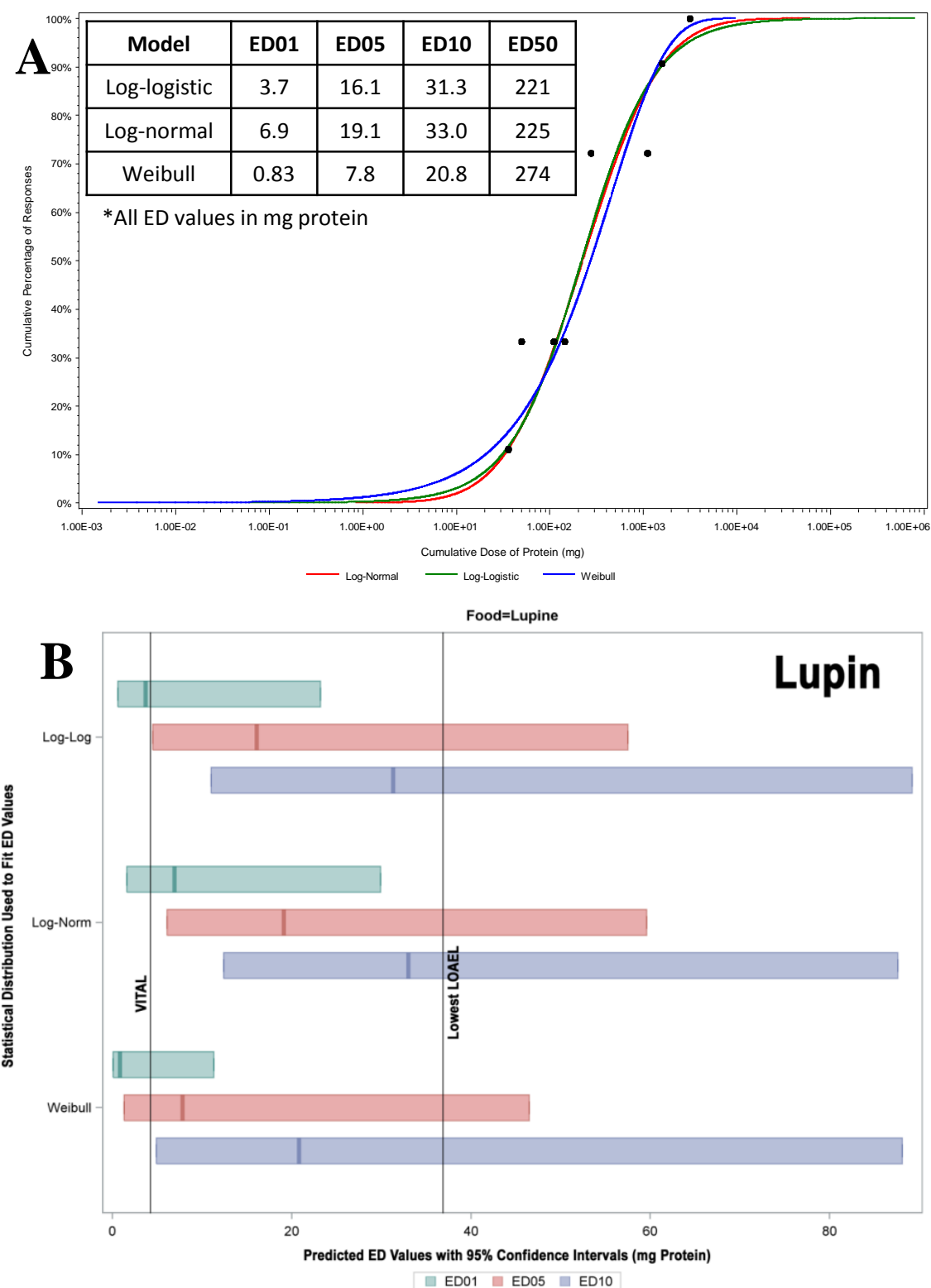


Figure 10. (A) Log-logistic, log-normal, and Weibull probability distribution models of individual lupin thresholds (expressed as mg lupin protein). (B) Log-logistic, log-normal, and Weibull ED estimates and corresponding 95% confidence intervals (expressed as mg lupin protein) from the lupin probability distribution models. The most sensitive individual in the dataset and the VITAL reference dose are marked and labeled.

Sesame seed

Individual sesame seed thresholds were obtained for 21 subjects; all were from published studies (Table 11). The sesame seed dataset includes 6 children, 13 adults, and 2 individuals of undetermined age. The sesame seed dataset was considered as marginally sufficient with most of the data being interval censored, with 2 left- and 1 right-censored individuals. However, all clinical threshold data are from multiple studies at one French allergy center and the addition of individuals from other clinics would strengthen the statistical analysis. Discrete and cumulative distributions were similar (data not shown) and all three models fit the cumulative data well (Figure 11A). In establishment of a reference dose for risk assessment purposes, all three distributions should be considered with a focus on the Weibull model for conservative measures. Again, there are overlapping confidence intervals at the ED01, ED05, and ED10 (Figure 11B) and insufficient subjects to base a threshold on the ED01. The lower 95% confidence interval of the ED05 should be considered for establishment of a reference dose for use in food allergen risk assessment.

Table 11. Sesame Seed Threshold Data Gleaned From Published DBPCFC Clinical Records.

Allergen	Study	Total No. with Objective Symptoms	Right Censored	Left Censored	Lowest MED† (mg protein)	Highest MED (mg protein)	Population
Sesame Seed	Leduc et al. (92)	12	0	1	1.0	1190	Adults and Children
	Morisset et al. (62)	1	0	0	5.1	-	Adults and Children
	Kolopp-Sarda et al. (93)	1	0	0	1209	-	Adults and Children
	Kanny et al. (94)	7	1	1	30.8	3078	Adults and Children
Total		21	1	2	1.0	3078	

† MED, Minimal Eliciting Dose for objective symptoms in food allergic individuals

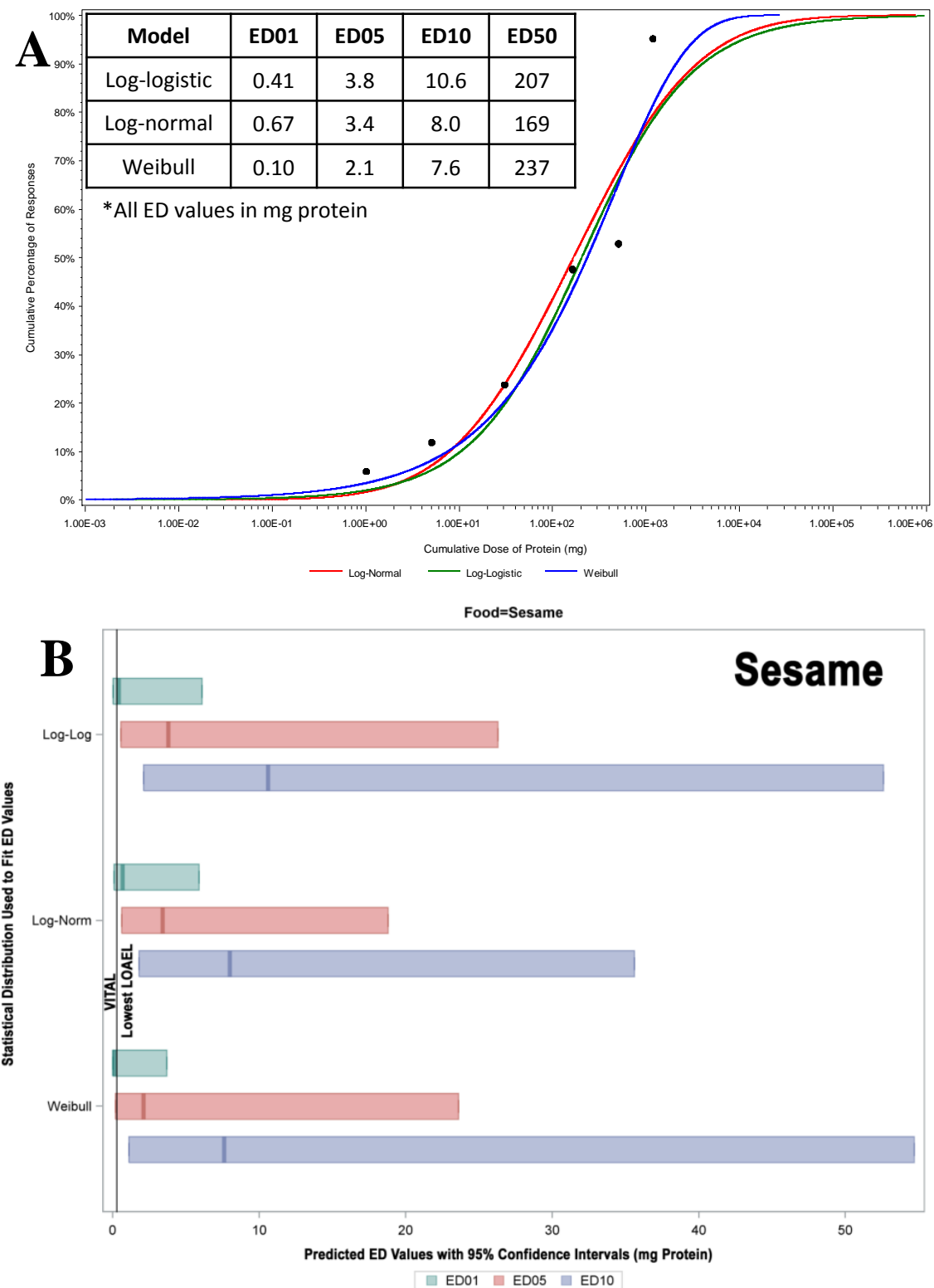


Figure 11. (A) Log-logistic, log-normal, and Weibull probability distribution models of individual sesame seed thresholds (expressed as mg sesame protein). (B) Log-logistic, log-normal, and Weibull ED estimates and corresponding 95% confidence intervals (expressed as mg sesame protein) from the sesame probability distribution models. The most sensitive individual in the dataset and the VITAL reference dose are marked and labeled.

Shrimp

The global priority lists of allergenic foods contains crustacean, but individual threshold data exist only for shrimp. A number of shrimp species exist and it is not known if species-specific differences occur for thresholds. Individual shrimp thresholds were obtained for 48 adults including 25 from published studies and 23 individuals from an unpublished shrimp threshold study (Table 12). Additionally, 54% of individuals were right-censored; none were left-censored. The shrimp dataset was considered as marginally sufficient but could benefit from additional interval-censored data for children and adults. Additionally, a major data gap exists as to whether a threshold dose for shrimp would extend to other crustacea such as crab and lobster. Both discrete and cumulative doses were similar (data not shown) and all three distributions had similar fits (Figure 12A). Again, there are overlapping confidence intervals at the ED01, ED05, and ED10 (Figure 12B) and insufficient subjects to base a threshold on the ED01. The lower 95% confidence interval of the ED05 should be considered for establishment of a reference dose of use in food allergen risk assessment.

Table 12. Shrimp Threshold Data Cleaned From Published and Unpublished DBPCFC Clinical Records.

Allergen	Study	Total No. with Objective Symptoms	Right Censored	Left Censored	Lowest MED† (mg protein)	Highest MED (mg protein)	Population
Shrimp	Atkins et al. (43)	4	0	0	9405	74351	Adults
	Daul et al. (95)	21	12	0	4560	33744	Adults
	FARRP						
	(Unpublished)	23	14	0	2.5	5725	Adults
Total		48	26	0	2.5	74351	

† MED, Minimal Eliciting Dose for objective symptoms in food allergic individuals

* Food Allergy Research & Resource Program, University of Nebraska – Lincoln

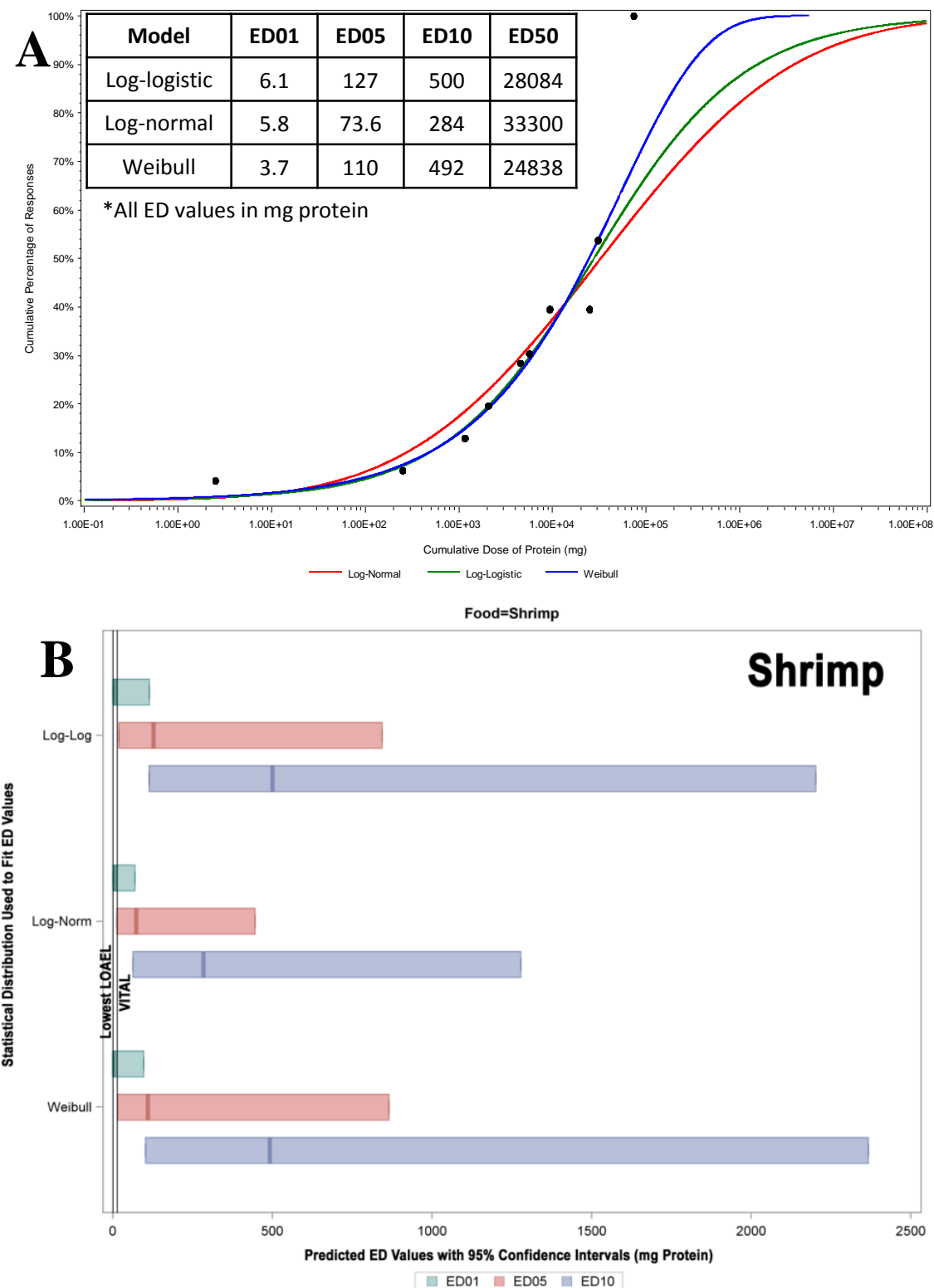


Figure 12. (A) Log-logistic, log-normal, and Weibull probability distribution models of individual shrimp thresholds (expressed as mg shrimp protein). (B) Log-logistic, log-normal, and Weibull ED estimates and corresponding 95% confidence intervals (expressed as mg shrimp protein) from the shrimp probability distribution models. The most sensitive individual in the dataset and the VITAL reference dose are marked and labeled.

Celery & Fish

Celery is on the priority list of allergenic foods in the EU and thresholds were obtained for 39 subjects, including 12 individuals from published studies and 27 subjects from unpublished clinical records (Table 13). The celery dataset includes 27 adults and 12 individuals of undetermined age. Of the 27 subjects, 15 were left-censored and 4 were right-censored. Due to the high number of left-censored individuals, no actual threshold data were collected below what would be the ED30. In its current state, the celery dataset is insufficient to allow an estimate of ED values that could be utilized for establishment of a statistically sound reference dose estimate. Clearly, the dataset will benefit from the addition of more celery-allergic individuals.

Fish is listed as a generic class of allergens on most global allergen lists. Again, species-related differences might occur in threshold estimates, but it is not known to what extent that they influence them. Individual fish thresholds were obtained for 19 subjects including 15 individuals from published studies and 4 subjects from unpublished clinical records (Table 14). The fish dataset includes 18 adults and 1 child challenged to 1 of 6 different species of fish. Of the 19 subjects, 6 were left-censored and 2 were right-censored and no individual threshold data exists from subjects below the predicted ED30. In its current state, the fish dataset is insufficient to allow an estimate of ED values that could be utilized for establishment of a statistically sound reference dose estimate. Clearly, the dataset will benefit from the addition of more fish-allergic children and adults.

Table 13. Celery Threshold Data Gleaned From Published and Unpublished DBPCFC Clinical Records.

Allergen	Study	Total No. with Objective Symptoms	Right Censored	Left Censored	Lowest MED† (mg protein)	Highest MED (mg protein)	Population
Celery	Ballmer-Weber et al. (96)	12	2	2	14	562	Adults and Children
	Unpublished Data *	27	2	13	0.2	1202	Adults
	Total	39	4	15	0.2	1202	
	† MED, Minimal Eliciting Dose for objective symptoms in food allergic individuals						

* Universitätsmedizin Berlin

Table 14. Fish Threshold Data Gleaned From Published and Unpublished DBPCFC Clinical Records.

Allergen	Study	Total No. with Objective Symptoms	Right Censored	Left Censored	Lowest MED† (mg protein)	Highest MED (mg protein)	Population
Fish	Helbling et al. (97)	9	0	4	46.1	1315	Adults and Children
	Hansen and Bindslev-Jensen (98)	6	1	0	10.2	1218	Adults
	Unpublished Data *	4	1	2	1830	16590	Adults
	Total	15	2	6	10.2	16590	

† MED, Minimal Eliciting Dose for objective symptoms in food allergic individuals

* University Medical Center Utrecht

Potency Comparison

Dose distribution models for the 11 available allergens were compared for significant differences in potency between allergens (Figure 13A). Mustard and egg are the most potent allergens at the low end of the dose scheme, ED01 – ED10, while soy flour and shrimp are the least potent allergens. The eliciting dose for allergic reactions to three members of the legume family showed an decreasing potency for peanut > lupin > soy flour. Peanut and milk have comparable potencies across the entire dose scheme. Similarly, hazelnut and sesame seed, as well as lupin and cashew, form comparable potency pairs. Potency measures should be used together with measures of prevalence and severity to determine whether a food should be placed on a priority allergen list. As expected, peanut, milk, and egg are three of the most prevalent and potent food allergens. However, the contrasts between potency and prevalence estimates for mustard (high potency/low prevalence), soy (low potency/high prevalence), and shrimp (low potency/high prevalence) are striking. Figure 13B focuses on the ED10 and 95% confidence interval estimates of each allergen to give a measure of confidence in potency estimates. The broad confidence intervals expressed on cashew, lupin, sesame seed, soy flour, and wheat are similar for estimates of the ED01, ED05, and ED50. Additional clinical data from all allergens would be valuable to increase the statistical confidence in the potency estimates.

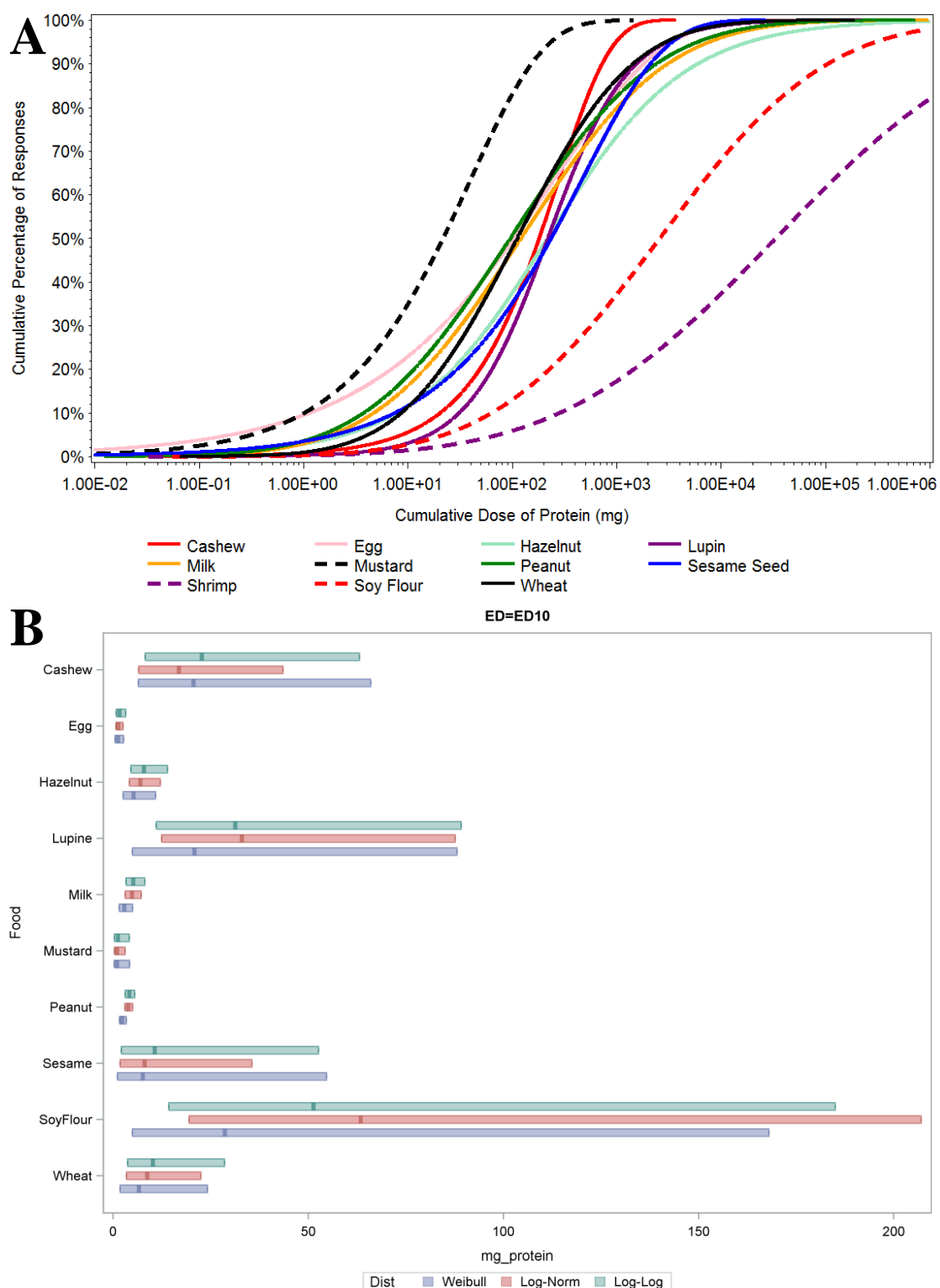


Figure 13. (A) Log-logistic, log-normal, or Weibull probability distribution models of 11 individual allergens (expressed as mg protein). (B) ED10 estimates from log-logistic, log-normal, and Weibull probability distribution models and corresponding 95% confidence intervals (expressed as mg protein) for 10 food allergens. Shrimp is not displayed due to an axis skewing effect from its large ED10 estimate and subsequent 95% confidence intervals.

By age

For all allergens, individual threshold data for children and adults were combined to provide sufficient data for population dose distribution modeling. For this approach to work, it must be assumed that the allergic population thresholds for children and adults do not differ. Where possible, children (less than 18 years of age) and adults were analyzed separately and compared for population differences. Sufficient data for children and adults are available for peanuts and hazelnuts only (Table 15). Other allergens such as cows' milk and eggs, were too skewed towards children as the vast majority of subjects often outgrow their allergies over time leaving a limited number of allergic adults available for potential threshold challenge trials (99).

Peanut-allergic children and adults have comparable ED05 estimates of 1.7 mg and 2.3 mg of peanut protein, respectively (Figure 14A). However, populations of peanut-allergic adults and children were significantly different as determined by the Generalized Log-Rank Test. Examination of the ED05, ED10, and ED50 estimates and their respective confidence intervals revealed similar ED05 and ED10 estimates but a significantly different ED50 in the two populations, thus leading to a significant difference in the overall curves. However, the significant differences at the higher end of the curve are of minimal impact as further risk assessments are most interested in the lower ED estimates to protect the maximum number of allergic individuals.

Hazelnut-allergic children and adults have slightly varied ED05 estimates at 1.2 mg and 4.0 mg hazelnut protein, respectively (Figure 14B). However, populations of hazelnut-allergic adults and children were statistically similar as determined by the Generalized Log-Rank Test and ED confidence interval estimates. Based on the modest

differences between the curves, the combination of the children and adults for each respective allergen seems reasonable. In order to protect the most sensitive populations, data should continue to be collected on adults and children for all allergens in order to determine if differences do exist in the population thresholds.

Table 15. Estimated eliciting doses (ED) for peanut protein and hazelnut protein as affected by age (children were categorized as less than 18 years of age). Significant differences in ED estimates are denoted with different uppercase superscript letters.

Allergen	Region	<u>mg protein</u>		# of Subjects (Rt Cen, Lt Cen)	Distribution
		ED05 (95% CI)	ED10 (95% CI)		
Peanut	Adults (27, 43-45, 49-51, 57) ^b	2.3 (0.6, 9.1) ^A	10.5 (3.6, 30.9) ^A	99 (44, 1)	Weibull
Peanut	Children (27, 44, 45, 47, 51, 53-56) ^{a,c}	1.7 (1.2, 2.3) ^A	4.0 (3.0, 5.2) ^A	584 (79, 25)	LogNormal
Hazelnut	Adults (32) ^{b,e}	4.0 (1.9, 8.1) ^A	10.0 (5.4, 18.5) ^A	153 (57, 2)	LogNormal
Hazelnut	Children ^{a,c}	1.2 (0.3, 5.8) ^A	5.0 (1.2, 17.3) ^A	61 (15,2)	Weibull

a - WKZ –Wilhelmina Kinderziekenhuis Children's Hospital of the University Medical Center Utrecht , The Netherlands

b - UMCU University Medical Center Utrecht, The Netherlands

c -UMCG – University Medical Center Gronigen, The Netherlands

d - JBZ –Joroen Bosch Hospital, The Netherlands

e - Universitätsmedizin Berlin, Germany

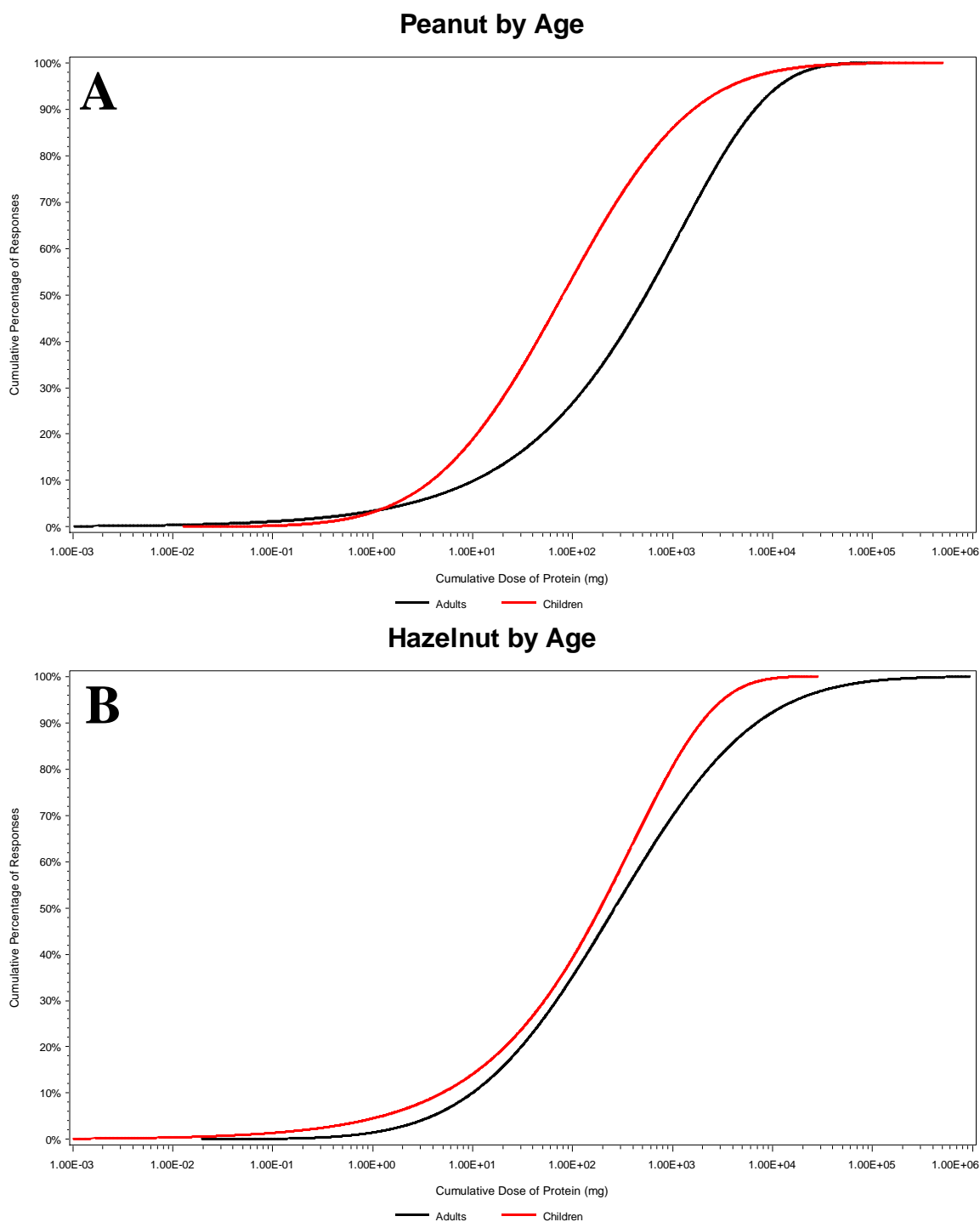


Figure 14. Probability distribution models for individual thresholds (expressed as mg protein) based on the age of the allergic individual at challenge (children were categorized as less than 18 years of age): (A) Peanut, (B) Hazelnut.

By region

For all allergens, individual thresholds from different geographical regions were combined to provide sufficient data for modeling. For this approach to work, it must be assumed that the allergic population thresholds do not differ by geographic region. Where possible, regions were analyzed separately and compared for population differences. The available data needed for a statistically sound analysis limited comparisons by region to the peanut and milk allergens (Table 16).

Populations of peanut-allergic individuals from France, the Netherlands, the U.K., and the U.S. were significantly different as determined by the Generalized Log-Rank Test. Examination of the ED05, ED10, and ED50 estimates and their respective 84% confidence intervals revealed similar ED05 and ED10 estimates for France and the U.S. and the U.S. and the Netherlands, but significantly ED05 and ED10 estimates were found from the U.K. However, a patient selection bias towards sensitive patients may exist in the U.K. peanut dataset, as the majority of subjects came from threshold and immunotherapy studies in which a more sensitive subpopulation of peanut-allergic individuals may be sought for the clinical studies. Significant differences at the higher end of the curve are of minimal impact as further risk assessments are most interested in the lower ED estimates to protect the maximum number of allergic individuals. Inclusion of the more sensitive U.K. population in the overall dataset ensures the maximum number of peanut-allergic individuals are protected in subsequent risk assessments.

Populations of milk-allergic individuals from the Netherlands, Australia, and Italy were significantly different as determined by the Generalized Log-Rank Test. Examination of the ED05, ED10, and ED50 estimates and their respective 84%

confidence intervals revealed similar ED05 and ED10 estimates for the Netherlands and Italy (near 2.0 mg milk protein) but significantly higher estimates for Australia (69.5 mg milk protein) (Figure 15B). The Australian data come from one study (69) where the initial challenges began at 66 - 300 mg of milk protein. It is important to use data from very low dose challenge studies in order to obtain the best estimates of ED values.

Multiple factors including patient selection bias and clinical protocol differences can be mitigated by combining data from various countries and multiple clinics, thus allowing the best ED value to be selected.

Table 16. Estimated eliciting doses (ED) for peanut and milk as affected by the geographic region where the clinical studies were conducted. Significant differences in ED estimates are denoted with different uppercase superscript letters.

Allergen	Geographic Region	mg Protein		# of Subjects (Rt Cen, Lt Cen)	Distribution
		ED05 (95% CI)	ED10 (95% CI)		
Peanut	France (27)	2.0 (1.4, 2.9) ^A	4.0 (2.9, 5.4) ^A	283 (10, 7)	LogNormal
Peanut	Netherlands (45, 47) ^{a,b,c}	4.0 (2.5, 6.6) ^B	10.5 (6.8, 16.1) ^B	309 (103, 6)	LogNormal
Peanut	UK (44, 46, 51, 53, 54)	0.2 (0.09, 0.6) ^C	0.6 (0.3, 1.4) ^C	86 (10, 13)	LogNormal
Peanut	USA (43, 48-50)	4.0 (0.6, 27.7) ^{A,B}	16.3 (3.5, 75.4) ^{A,B}	41 (8, 2)	Weibull
Milk	Australia (69)	69.5 (38.1, 126) ^A	108 (64.2, 182) ^A	53 (0, 11)	LogNormal
Milk	Italy (63, 64, 66, 70)	2.0 (1.1, 3.6) ^B	3.5 (2.1, 5.9) ^B	91 (0, 14)	LogNormal
Milk	Netherlands (68, 72) ^{b,c,d}	1.9 (0.7, 5.4) ^B	8.3 (3.6, 19.1) ^B	148 (19, 14)	Weibull

a - WKZ –Wilhelmina Kinderziekenhuis Children's Hospital of the University Medical Center Utrecht , The Netherlands

b - UMCU University Medical Center Utrecht, The Netherlands

c -UMCG– University Medical Center Gronigen, The Netherlands

d - JBZ –Joroen Bosch Hospital, The Netherlands

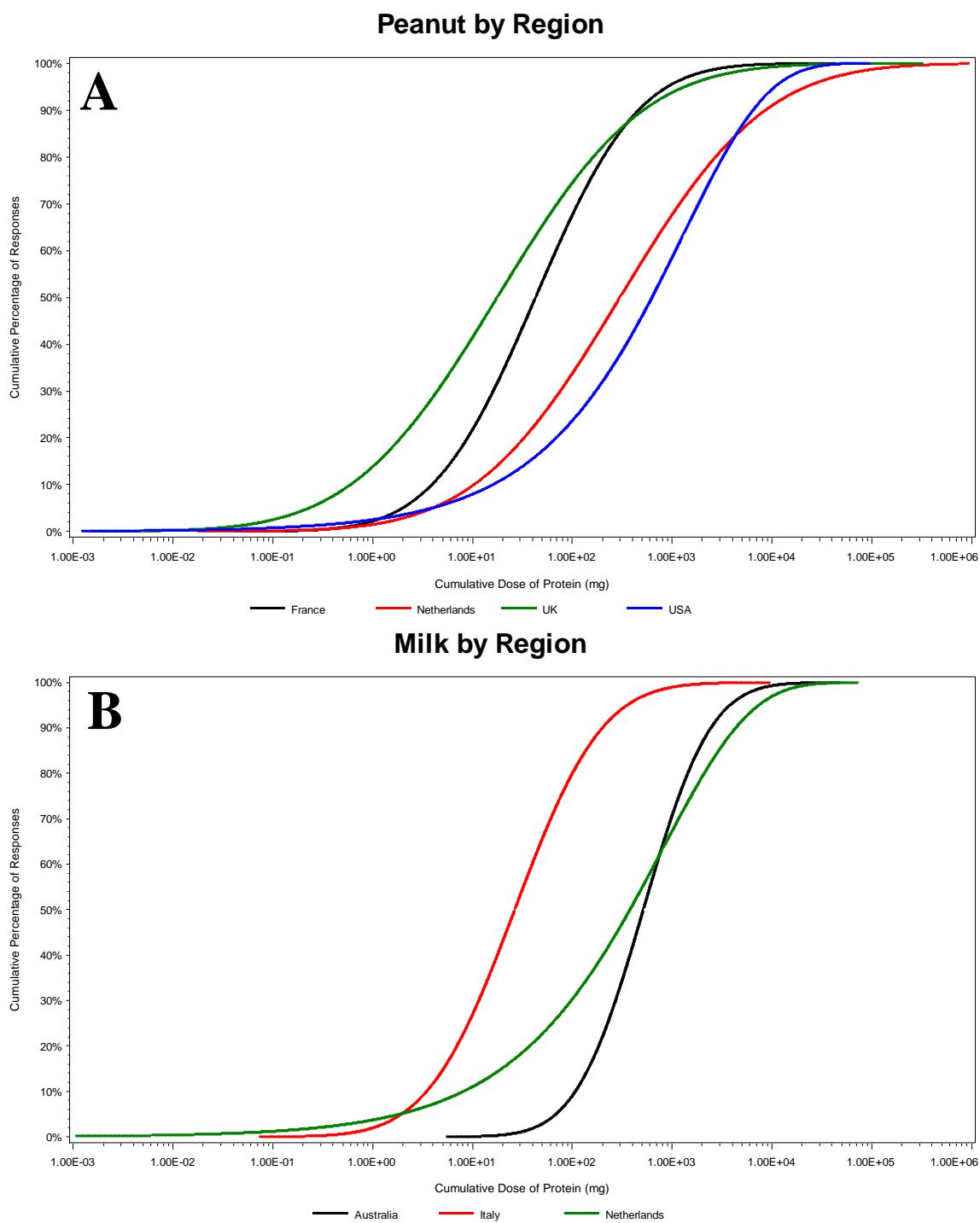


Figure 15. Probability distribution models for individual thresholds (expressed as mg protein) based on the geographic region during challenge: (A) Peanut, (B) Milk.

By challenge material

For all allergens, individuals challenged with different dosing materials were combined to provide sufficient data for modeling. For this approach to work, it must be assumed that the allergic population thresholds do not differ by challenge material. However, recent studies show some egg-allergic children are more tolerant of baked egg than of raw egg (2, 100, 101) and other allergens could exhibit similar differences. Where possible, food challenge materials were analyzed separately and compared for population differences. The available threshold data limited comparisons by challenge materials to the peanut, milk, and egg allergens (Table 17).

Populations of peanut-allergic individuals challenged with pulverized peanut and peanut flour were significantly different as determined by the Generalized Log-Rank Test. Examination of the ED05, ED10, and ED50 estimates and their respective confidence intervals revealed similar ED05 and ED10 estimates but a significantly different ED50 in the two populations (Figure 16A). While statistically significant, differences at the higher end of the curves are of minimal impact to risk assessments concerned with the most sensitive individuals in the population. Populations of milk-allergic individuals challenged with liquid milk and non-fat dry milk were similar as determined by the Generalized Log-Rank Test and ED confidence interval estimates (Figure 16B). Populations of egg-allergic individuals challenged with raw and cooked whole egg were similar as determined by the Generalized Log-Rank Test and ED confidence interval estimates. However, raw egg white was significantly lower than both raw and cooked whole egg as determined by the Generalized Log-Rank Test and ED confidence interval estimates (Figure 16C). Since the ED value is based upon egg

protein, egg white would contain a higher proportion of egg allergens by comparison to egg yolk or whole egg which likely explains the difference. While some egg-allergic patients are known to tolerate baked egg, our study did not find cooked or raw egg to be significantly different from each other. However, of the 88 egg-allergic patients challenged with cooked egg in our study, 38 were challenged with baked egg and the others with boiled or fried eggs. More research is needed to study the differences between raw, boiled or fried, and baked eggs. Additionally, raw eggs are not likely to be found in processed food products and any reference doses or regulatory threshold doses used for purposes of food allergen risk assessments for egg should consider industry standards for processing and the most likely form of egg to be present in cross-contact scenarios. This analysis also shows that normalization of data on protein basis is a reasonable approach.

Table 17. Estimated eliciting doses (ED) for peanut, milk, and egg as affected by challenge dose material. Significant differences in ED estimates are denoted with different uppercase superscript letters.

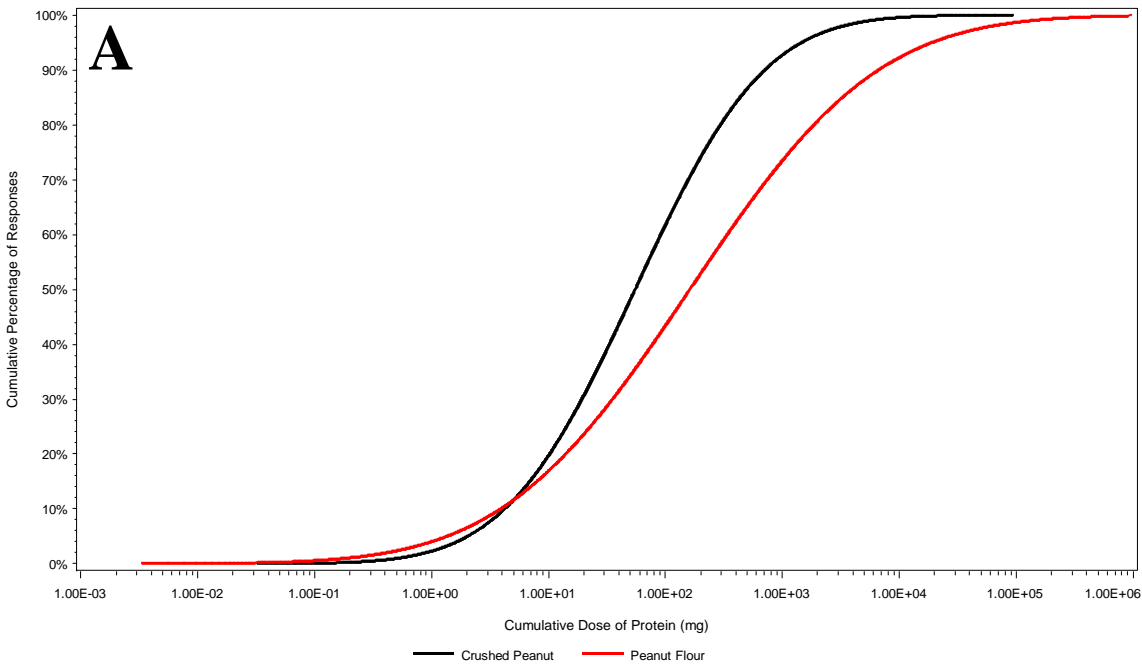
Allergen	Dose Material	mg Protein		# of Subjects (Rt Cen, Lt Cen)	Distribution
		ED05 (95% CI)	ED10 (95% CI)		
Peanut	Crushed Peanut (27, 43, 45, 55-57)	2.1 (1.5, 2.9) ^A	4.3 (3.2, 5.8) ^A	342 (30, 9)	LogNormal
Peanut	Peanut Flour (44, 47-51, 53, 54) ^{a,b,c}	1.4 (0.9, 2.2) ^A	4.0 (2.6, 6.0) ^A	408 (101, 21)	LogNormal
Milk	Liquid Cow's Milk (60-66, 69, 70, 73-75) ^{c,d}	1.9 (1.1, 3.1) ^A	4.6 (2.9, 7.1) ^A	287 (13, 41)	LogNormal
Milk	Nonfat Dry Milk (67, 68, 71, 72)	2.7 (0.7, 10.4) ^A	6.0 (1.9, 19.3) ^A	49 (2, 17)	LogNormal
Egg	Cooked Whole Egg (73, 76, 80) ^{b,c}	4.9 (2.1, 11.5) ^A	11.1 (5.3, 23.4) ^A	88 (14, 13)	LogNormal
Egg	Raw Whole Egg (43, 60, 61, 76)	3.4 (0.6, 20.1) ^A	8.6 (1.8, 41.3) ^A	22 (0, 1)	LogNormal
Egg	Raw Egg White (62, 65)	0.2 (0.05, 0.6) ^B	0.4 (0.1, 1.1) ^B	35 (0, 4)	LogNormal

a - WKZ – Wilhelmina Kinderziekenhuis Children's Hospital of the University Medical Center Utrecht, The Netherlands

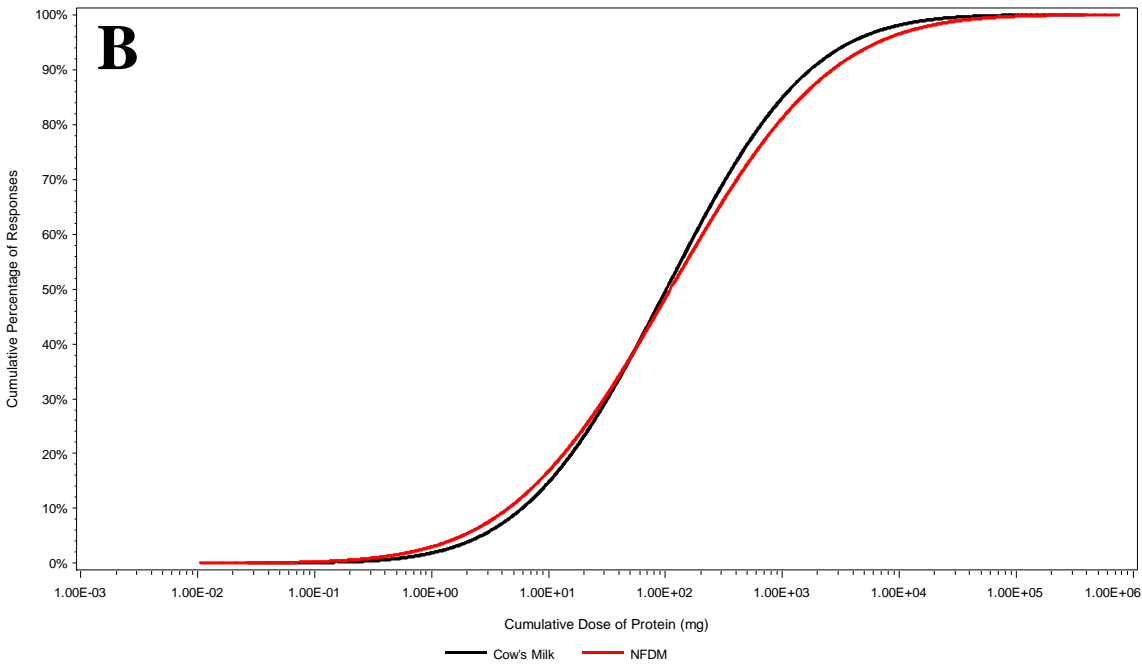
b - UMCU University Medical Center Utrecht, The Netherlands

c -UMCG – University Medical Center Gronigen, The Netherlands

Peanut by Dose Material



Milk by Dose Material



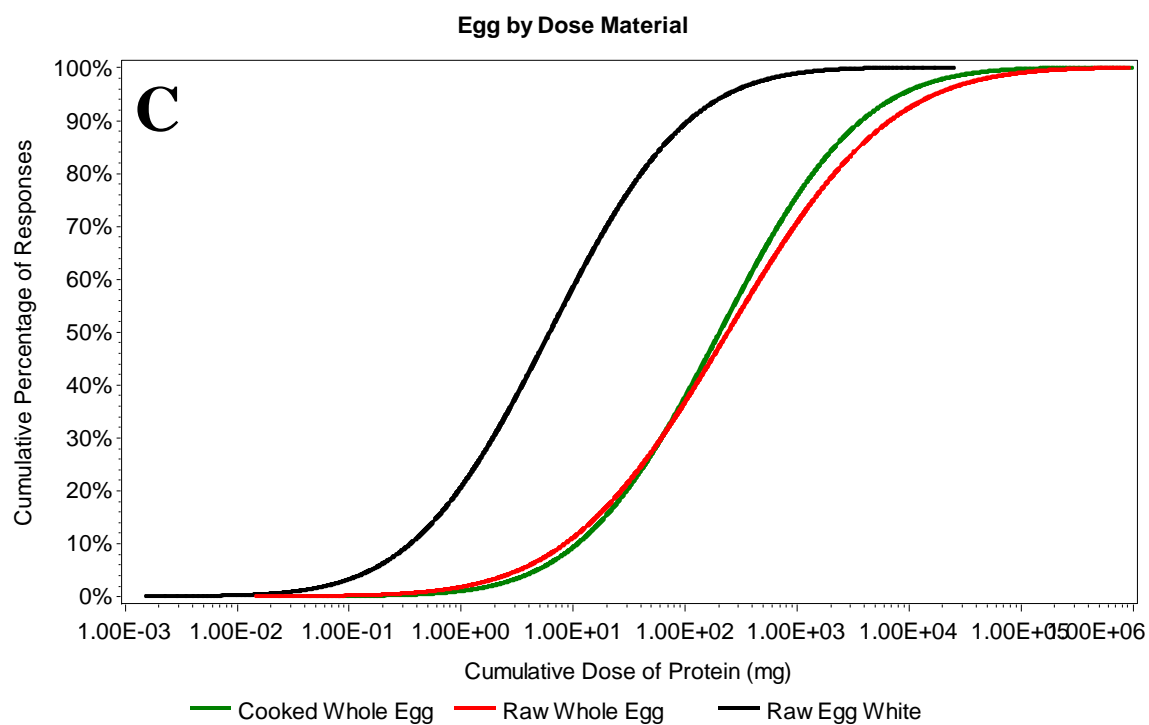


Figure 16. Probability distribution models for individual thresholds (expressed as mg protein) based on the dose material given at challenge: (A) Peanut, (B) Milk, (C) Egg.

By study population

For all allergens, individual thresholds from study patient populations were combined to provide sufficient data for modeling. For this approach to work, it must be assumed that the allergic population thresholds do not differ and no patient selection bias exists by study type. The quality of the resulting ED values is dependent upon the representativeness of the patient population. Where possible, study types (diagnostic, threshold, and immunotherapy) were analyzed separately and compared for population differences. The available threshold data limited comparisons by study type to the peanut and milk allergens (Table 18).

Populations of peanut-allergic individuals from diagnostic, threshold, and immunotherapy trials were significantly different as determined by the Generalized Log-Rank Test. Examination of the ED05, ED10, and ED50 estimates and their respective 84% confidence intervals revealed similar ED05 and ED10 estimates for diagnostic and threshold challenges and for threshold and immunotherapy challenges. However, ED05 and ED10 estimates for immunotherapy trials were significantly lower than those for diagnostic challenges (Figure 17A). This analysis indicates that a selection bias towards more sensitive peanut-allergic individuals for immunotherapy trials exists but a dramatic difference in the overall ED values was not observed. Populations of milk-allergic individuals from diagnostic and immunotherapy trials were significantly different as determined by the Generalized Log-Rank Test. Examination of the ED05, ED10, and ED50 estimates and their respective 84% confidence intervals revealed similar ED05 estimates for diagnostic and immunotherapy challenges, but the difference between the ED10 levels of immunotherapy and diagnostic groups is more evident for milk (2.6 and

13.0 mg of milk protein) than it was for peanut (Figure 17B). This example shows inclusion of the immunotherapy subjects leads to a more conservative estimate of ED values for establishment of reference doses or regulatory thresholds and only serves to protect more milk-allergic individuals.

Table 18. Estimated eliciting doses (ED) for peanut and milk as affected by study population. Significant differences in ED estimates are denoted with different uppercase superscript letters.

Allergen	Type of Study	mg Protein		# of Subjects (Rt Cen, Lt Cen)	Distribution
		ED05 (95% CI)	ED10 (95% CI)		
Peanut	Diagnostic (27, 43, 54, 56) ^{a,b,c}	2.0 (1.5, 2.7) ^A	4.6 (3.5, 6.1) ^A	564 (82, 15)	LogNormal
Peanut	Threshold (44-47)	0.9 (0.3, 2.7) ^{A,B}	3.0 (1.2, 7.6) ^{A,B}	101 (41, 3)	LogNormal
Peanut	Immunotherapy (48-51, 53, 55, 57)	0.4 (0.1, 1.3) ^B	1.2 (0.4, 3.4) ^B	85 (8, 12)	LogNormal
Milk	Diagnostic (61, 68-74) ^{b,c,d}	3.6 (1.7, 7.5) ^A	13.0 (7.2, 23.7) ^A	232 (19, 32)	Weibull
Milk	Immunotherapy (60, 63-67)	1.3 (0.7, 2.6) ^A	2.6 (1.5, 4.7) ^B	113 (0, 24)	LogNormal

a - WKZ –Wilhelmina Kinderziekenhuis Children’s Hospital of the University Medical Center Utrecht , The Netherlands

b - UMCU University Medical Center Utrecht, The Netherlands

c -UMCG– University Medical Center Gronigen, The Netherlands

d - JBZ –Joroen Bosch Hospital, The Netherlands

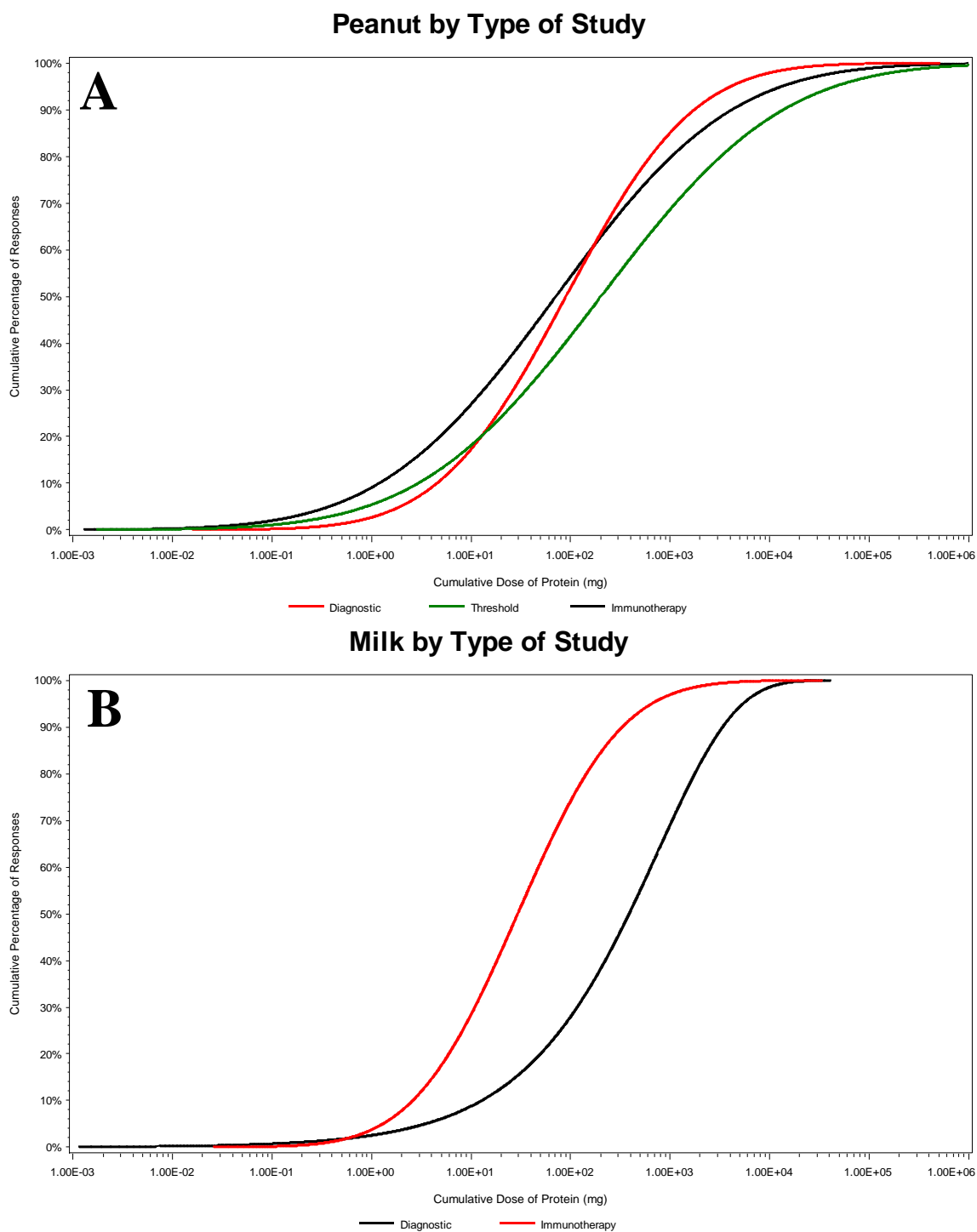


Figure 17. Probability distribution models for individual thresholds (expressed as mg protein) based on the study population: (A) Peanut, (B) Milk.

By gender

For all allergens, individual threshold data for males and females were combined to provide sufficient data for population dose distribution modeling. For this approach to work, it must be assumed that the allergic population thresholds for males and females do not differ. Where possible, males and females were analyzed separately and compared for population differences. Sufficient data for children and adults are available for peanuts (data not shown). Populations of peanut-allergic males and females were statistically similar as determined by the Generalized Log-Rank Test and ED confidence interval estimates. Based on the extreme similarity between the curves, the combination of the males and females for each respective allergen seems reasonable.

Regulatory thresholds and uncertainty factors

Switzerland and Japan have set regulatory limits as to the levels of undeclared food allergens in packaged foods but national, risk-based thresholds for food allergens have not been established. At the present time, there is no regulatory guidance for trace levels of allergens due to cross-contact which essentially establishes a zero threshold situation in which food industry is left with significant challenges to reach an unachievable level of allergenic residue. The Allergen Bureau of Australia developed the Voluntary Incidental Trace Allergen Labelling (VITAL) program in 2007 as a guide to limit advisory labeling relating to the unintended presence of allergens. Initially, VITAL established very conservative action levels that were expressed as concentrations (ppm protein from the allergenic source, mg/kg) in a 5 g serving. Recently, the VITAL program was updated with new, less conservative Reference Doses expressed as mg protein in an effort to limit advisory labeling to situations where a significant risk exists.

The Reference Doses were derived by an international expert panel which utilized quantitative risk assessment methods and the data presented previously in this chapter. For the most part, these data were not available at the time of establishment of the initial VITAL grid. Recent evidence demonstrates a lower proportion of severe reactions occurring at lower doses of egg, milk, wheat, or soy, but more research is needed to confirm these results (102). The expert panel agreed that where sufficient data were available, the ED01 based on objective (observable) reactions should form the basis of the reference dose and protect at least 99% of individuals allergic to the particular food. While the ED01 minimizes the probability of a severe reaction, the possibility cannot be excluded. When data were insufficient for ED01 estimation, the expert panel decided to use the lower 95% confidence interval of the ED05 which would likely protect 97 – 99% of the affected population. The choice of the 99% level leads to the optimal public health outcome in that the large majority of the allergic population is protected and the food industry has mg doses of residual allergenic protein that can be operationally achieved and also have a positive impact on the extent of, and compliance with advisory labeling. The expert panel derived Reference Doses for peanut, milk, egg, and hazelnut based on the ED01 and the lower 95% confidence interval of the ED05 for soy flour, wheat, cashew, mustard, lupin, sesame seed, and shrimp (Table 19, top row).

Table 19. Comparison of potential methods for deriving a regulatory threshold for individual allergenic foods. Methods displayed include the safety assessment-based approach (lowest reactor), the benchmark approach (and accompanying uncertainty estimates), and the VITAL approach (a hybrid benchmark and probabilistic risk approach). All mg values are expressed in mg protein.

	Peanut	Milk	Egg	Hazelnut	Soy Flour
VITAL Numbers	0.2 mg protein	0.1 mg protein	0.03 mg protein	0.1 mg protein	1 mg protein
Based On	ED01 of LogNormal and LogLogistic distributions of adults and children, discrete and cumulative	ED01 of LogNormal and LogLogistic distributions of adults and children, discrete and cumulative	ED01 and 95% LCI of the ED05 values of the Weibull and other distributions of adults and children, discrete and cumulative	ED01 and 95% LCI of the ED05 values of the LogLogistic and other distributions of adults and children, discrete and cumulative	95% LCI of the ED05 values of the LogNormal and other distributions of adults and children, discrete and cumulative
	Peanut	Milk	Egg	Hazelnut	Soy Flour
Benchmark	LogNormal Cumulative	LogNormal Cumulative	LogLogistic Cumulative	LogLogistic Cumulative	LogLogistic Discrete
ED01	0.28 mg	0.34 mg	0.022 mg	0.21 mg	0.27 mg
U3	0.093	0.113	0.0073	0.07	0.09
U5	0.056	0.068	0.0044	0.042	0.054
U10	0.028	0.034	0.0022	0.021	0.027
ED05	1.5 mg	1.9 mg	0.42 mg	2.5 mg	5.6 mg
U3	0.5	0.63	0.14	0.83	1.87
U5	0.3	0.38	0.084	0.5	1.12
U10	0.15	0.19	0.042	0.25	0.56
ED05 LC	1.1 mg	1.2 mg	0.18 mg	1.3 mg	1.0 mg
U3	0.37	0.4	0.06	0.43	0.3
U5	0.22	0.24	0.036	0.26	0.2
U10	0.11	0.12	0.018	0.13	0.1
ED10	3.8 mg	4.8 mg	1.6 mg	7.9 mg	22.4 mg
U3	1.27	1.6	0.53	2.63	7.47
U5	0.76	0.96	0.32	1.58	4.48
U10	0.38	0.48	0.16	0.79	2.24
ED10 LC	3 mg	3.1 mg	0.79 mg	4.5 mg	5.6 mg
U3	1	1.03	0.263	1.5	1.87
U5	0.6	0.62	0.158	0.9	1.12
U10	0.3	0.31	0.079	0.45	0.56
	Peanut	Milk	Egg	Hazelnut	Soy Flour
Lowest Reactor					
NOAEL	0.025 mg	.	.	0.0017 mg	1.1 mg
LOAEL	0.1 mg	0.2 mg	0.0138 mg	0.0187 mg	11.1 mg
Based On	NOAEL	LOAEL	LOAEL	NOAEL	NOAEL
U10/100	10	100	100	10	10
mg with U	0.0025 mg	0.002 mg	0.000138 mg	0.00017 mg	0.11 mg
	Peanut	Milk	Egg	Hazelnut	Soy Flour
ED10 → VITAL					
ED10	3.8 mg (Log Normal Cumulative)	4.8 mg (Log Normal Cumulative)	1.6 mg (LogLogistic Cumulative)	7.9 mg (LogLogistic Cumulative)	22.4 mg (LogLogistic Discrete)
VITAL	0.2 mg	0.1 mg	0.03 mg	0.1 mg	1 mg
U Needed	19	48	53.3	79	22.4
	Peanut	Milk	Egg	Hazelnut	Soy Flour
ED10 → ED01					
ED10	3.8 mg	4.8 mg	1.6 mg	7.9 mg	22.4 mg
ED01	0.28 mg	0.34 mg	0.022 mg	0.21 mg	0.27 mg
U Needed	4	14	73	38	83

	Wheat	Cashew	Mustard	Lupin	Sesame	Shrimp
VITAL Numbers	1 mg protein	2 mg protein	0.05 mg protein	4 mg protein	0.2 mg protein	10 mg protein
Based On	95% LCI of the ED05 values derived from all three distributions of adults and children, discrete and cumulative	95% LCI of the ED05 values derived from all three distributions of children (provisional due to lack of adults), discrete and cumulative	95% lower confidence interval of the ED05 values derived from all three distributions of adults and children, discrete and cumulative	95% LCI of the ED05 values derived from the LogNormal and LogLogistic distributions of adults and children, discrete and cumulative	95% LCI of the ED05 values derived from all three distributions of adults and children, discrete and cumulative	95% LCI of the ED05 values derived from all three distributions of adults, discrete and cumulative
	Wheat	Cashew	Mustard	Lupin	Sesame	Shrimp
Benchmark	LogNormal Cumulative	Weibull Cumulative	Weibull Cumulative	LogLogistic Cumulative	Weibull Cumulative	LogNormal Cumulative
ED01	1.1 mg	1.4 mg	0.022 mg	3.7 mg	0.1 mg	5.8 mg
U3	0.37	0.47	0.0073	1.23	0.03	1.93
U5	0.22	0.28	0.0044	0.74	0.02	1.16
U10	0.11	0.14	0.0022	0.37	0.01	0.58
ED05	4.2 mg	8.9 mg	0.32 mg	16.1 mg	2.1 mg	73.6 mg
U3	1.4	2.97	0.107	5.37	0.7	24.53
U5	0.84	1.78	0.064	3.22	0.42	14.72
U10	0.42	0.89	0.032	1.61	0.21	7.36
ED05 LC	1.4 mg	2.1 mg	0.052 mg	4.5 mg	0.18 mg	12.1 mg
U3	0.47	0.7	0.0173	1.5	0.06	4.03
U5	0.28	0.42	0.0104	0.9	0.036	2.42
U10	0.14	0.21	0.0052	0.45	0.018	1.21
ED10	8.7 mg	20.6 mg	1 mg	31.3 mg	7.6 mg	284 mg
U3	2.9	6.87	0.3	10.4	2.53	94.7
U5	1.74	4.12	0.2	6.26	1.52	56.8
U10	0.87	2.06	0.1	3.13	0.76	28.4
ED10 LC	3.4 mg	6.4 mg	0.24 mg	11 mg	1.1 mg	63.1 mg
U3	1.13	2.13	0.08	3.7	0.37	21.03
U5	0.68	1.28	0.048	2.2	0.22	12.62
U10	0.34	0.64	0.024	1.1	0.11	6.31
	Wheat	Cashew	Mustard	Lupin	Sesame	Shrimp
Lowest Reactor						
NOAEL	0.253 mg
LOAEL	2.6 mg	2.3 mg	0.2608 mg	36.2 mg	1.02 mg	2.53 mg
Based On	LOAEL	LOAEL	LOAEL	LOAEL	LOAEL	NOAEL
U10/100	100	100	100	100	100	10
mg with U	0.026 mg	0.023 mg	0.002608 mg	0.362 mg	0.0102 mg	0.0253 mg
	Wheat	Cashew	Mustard	Lupin	Sesame	Shrimp
ED10 → VITAL						
ED10	8.7 mg (LogNormal Cumulative)	20.6 mg (Weibull Cumulative)	1 mg (Weibull Cumulative)	31.3 mg (LogLogistic Cumulative)	7.6 mg (Weibull Cumulative)	284 mg (LogNormal Cumulative)
VITAL	1 mg	2 mg	0.05 mg	4 mg	0.2 mg	10 mg
U Needed	8.7	10.3	20	7.825	38	28.4
	Wheat	Cashew	Mustard	Lupin	Sesame	Shrimp
ED10 → ED01						
ED10	8.7 mg	20.6 mg	1 mg	31.3 mg	7.6 mg	284 mg
ED01	1.1 mg	1.4 mg	0.022 mg	3.7 mg	0.1 mg	5.8 mg
U Needed	8	15	41	8	84	49

However, the lack of a standardized, scientific method for deriving the Reference Doses across all allergens is one criticism of the VITAL expert panel approach. Standard methods are described for deriving the dose distributions from interval censored data, but as there is no biological factor to discern between models, expert judgment was used when deciding between log-logistic, log-normal, and Weibull models that fit the actual clinical threshold data in similar fashion. For example, instead of selecting one model as

the best fit and basis for the reference dose, slight differences in the predicted ED values could lead to multiple models contributing to the final, averaged reference dose. It could be argued that a second panel might not reach the same conclusions. In an effort to standardize the selection of a reference dose, the other frameworks outlined by the US Food and Drug Administration (FDA) Threshold Working Group (analytical methods-based, statutorily derived, safety assessment-based, and risk assessment-based) were investigated (21). The analytical methods and statutorily derived approaches are not applicable as they do not utilize individual food challenge data. The safety assessment-based method determines a “safe” level using the No Observed Adverse Effect Level (NOAEL) from human challenge studies and applies an appropriate Uncertainty Factor (UF) applied to account for knowledge gaps. Typically, uncertainty factors of 10 are applied for differences in sensitivity between animals and human, for inter-individual variation among humans, and in cases where the LOAEL is used instead of the NOAEL. The animal-to-human UF is not relevant as all food challenge tests were done in humans. The most sensitive allergic individual is listed and proper UF are applied for all 11 allergens with VITAL Reference Doses under the “Lowest Reactor” section (Table 19). The safety assessment approach does not consider the entire food allergic population or the probable health risk that would be predicted at these exposure levels. Results from this method of risk assessment are extremely conservative and in many cases, produce mg levels that would be below the limit of detection for current analytical methods for detection.

The benchmark risk assessment-based approach has been used for chemical risks in foods and was the starting point for the VITAL expert panel’s decision making

process. The benchmark dose of the ED01, ED05, ED05 lower 95% confidence interval, ED10, and ED10 lower 95% confidence interval are presented in Table 19 with an accompanying UF of 3, 5, and 10. To complete this method, a statistical model must be chosen for each allergen by expert judgment, with consideration of simple statistical (AIC) and visual fits at the most sensitive end of the distributions. After UF application, the VITAL Reference Doses for peanut, milk, egg, and hazelnut (based on ED01) align closest with the UF of 5 applied to the lower 95% confidence interval of the ED05. All other Reference Doses (based on ED05 lower 95% confidence interval) aligned well with the UF of 5 applied to the lower 95% confidence interval of the ED10. However, not all allergen dose distribution curves have the same slope, as demonstrated by the UF needed to reach the VITAL reference dose from the predicted ED10. This point is further illustrated by the calculated UF needed to reach the ED01 from the predicted ED10 (Table 19, bottom 2 sections). A standard UF based on a standard ED would be over conservative in some cases and overly risky in others. The decision on the optimal method to determine regulatory thresholds is not straightforward and although not an easy task, the resulting thresholds should be transparent and easily defensible.

Methods to improve food challenges

Importance of number of subjects in a study

Subjects randomly selected from the previously published Taylor et al. (27) peanut threshold database of 450 individuals and generated ICSA dose distributions were analyzed using the Generalized Log-Rank Test for interval censored data (Table 20). The probability that a randomly selected population was significantly different than the 450 population decreased as the number of random subjects increased. Similar results were

found when comparing the 84% confidence intervals of modeled log-normal distributions as an indicator of significant differences (Table 21). Both tests predict a less than 2% chance of 200 individuals being different than the known 450 peanut-allergic individuals. However, the collection of data from 200 well characterized allergic subjects would be quite difficult for some allergenic foods especially for single clinics. Individual clinics and DBPCFC studies do not have the time, money, or patient population to collect 200 data points in rapid succession. If a clinic was able to challenge 30 individuals, it is predicted to have a 5 – 10% chance of finding significantly different results than the Taylor et al. (27) data. The two statistical tests do not mirror one another completely, as the 84% confidence interval test found a higher number of populations to be different when fewer subjects were selected. These differences should be investigated with additional studies.

Table 20. Probability of the randomly selected subject population nonparametric distribution being significantly different than Taylor et al. (27) 450 dataset, alpha = 0.05.

<u>No. of random subjects</u>	<u>Generalized Log-Rank Test I (Zhao & Sun (38))</u>	<u>Generalized Log-Rank Test II (Sun, Zhao & Zhao (37))</u>
10	4.3%	4.2%
20	3.2%	3.6%
30	4.6%	4.5%
50	3.4%	3.7%
100	2.2%	2.3%
200	1.6%	1.8%

Table 21. Chance of the randomly selected subject log-normal distribution being significantly different than Taylor et al. (27) 450 dataset at selected ED values, 84% CI used (alpha = 0.05).

<u>No. of random subjects</u>	<u>ED01</u>	<u>ED05</u>	<u>ED10</u>	<u>ED50</u>
10	16.2%	16.3%	16.5%	13.9%
20	14.8%	14.4%	13.8%	10.4%
30	10.3%	10.4%	10.0%	9.2%
50	7.5%	7.9%	8.2%	7.0%
100	5.5%	4.7%	5.0%	3.4%
200	1.9%	1.5%	1.9%	1.6%

Figure 18 shows the predicted log-normal ED10 values of the randomly selected subject populations with the Taylor et al. (27) log-normal ED10 of 3.1 mg peanut protein indicated in red. The maximum predicted ED10 value with 10 subjects was 110 mg peanut protein, 35 times higher than the 450 dataset. With 30 subjects, the maximum ED10 was 9 times higher than the 450 dataset. By 100 and 200 subjects, the maximum ED10 estimates were 3 and 2 times higher, respectively, than the 450 dataset. These results show that a clinical population with fewer subjects is more likely to significantly overestimate the ED10 of an allergic population and subsequently put a higher proportion of allergic individuals at risk if that ED10 from an individual study alone is used in risk management decisions. However, the cost of clinical studies and limited size of allergic populations do not always allow for 100 or 200 subjects to be tested. Single center studies with 30 allergic individuals challenged by a recommended dosing scheme (discussed below) would have ~90% certainty that the ED values predicted were statistically similar to the true population values and the use of additional uncertainty factors could provide reasonable data for provisional risk assessments.

Populations of 30 or 50 subjects were randomly generated 1000 times from the soy flour, peanut, and egg distribution models and fit to 10 different dosing schemes selected from or based on published clinical dosing schemes.

[illegible]

Results of the Generalized Log-Rank Tests indicate that schemes 2, 3, 4, 6, 9, and 10 are more likely to produce populations that are significantly different than the base distribution models for egg, peanut, and soy flour (Table 23). Scheme 6 is particularly different due to its extreme dosage increase to end the challenge. Analysis of the 84% confidence intervals revealed that schemes 2, 5, and 10 produced significantly different results than the other schemes tested (data not shown). Schemes 2, 5, and 10 challenge doses start significantly higher or end significantly lower than other schemes tested and these results indicate the importance of a challenge protocol that covers the entire dose distribution curve. Such approaches generate fewer left- and/or right-censored individuals.

Table 23 - Probability of the dose scheme creating a nonparametric dose distribution from randomly selected subjects that is significantly different than the VITAL egg, peanut, and soy flour population models. Generalized Log-Rank Test I & II alpha = 0.05.

	Egg 30		Egg 50		Peanut 30		Peanut 50		Soy 30		Soy 50	
	I	II	I	II	I	II	I	II	I	II	I	II
Scheme 1	2.8%	3.4%	Scheme 1	3.3%	Scheme 1	3.8%	Scheme 1	2.4%	Scheme 1	0.6%	Scheme 1	0.5%
Scheme 2	0.3%	2.1%	Scheme 2	0.0%	Scheme 2	8.2%	Scheme 2	4.0%	Scheme 2	1.4%	Scheme 2	0.8%
Scheme 3	3.7%	4.9%	Scheme 3	3.8%	Scheme 3	7.9%	Scheme 3	6.2%	Scheme 3	1.7%	Scheme 3	0.7%
Scheme 4	3.7%	4.9%	Scheme 4	3.8%	Scheme 4	7.7%	Scheme 4	6.2%	Scheme 4	1.7%	Scheme 4	0.7%
Scheme 5	1.8%	0.9%	Scheme 5	0.7%	Scheme 5	0.2%	Scheme 5	1.8%	Scheme 5	4.3%	Scheme 5	1.1%
Scheme 6	0.9%	5.1%	Scheme 6	0.5%	Scheme 6	26.7%	Scheme 6	7.7%	Scheme 6	1.1%	Scheme 6	1.0%
Scheme 7	0.1%	1.1%	Scheme 7	0.0%	Scheme 7	4.2%	Scheme 7	0.4%	Scheme 7	0.0%	Scheme 7	0.0%
Scheme 8	3.1%	4.0%	Scheme 8	2.3%	Scheme 8	4.1%	Scheme 8	5.1%	Scheme 8	3.9%	Scheme 8	2.7%
Scheme 9	3.1%	4.5%	Scheme 9	2.4%	Scheme 9	7.1%	Scheme 9	5.7%	Scheme 9	0.9%	Scheme 9	0.1%
Scheme 10	2.9%	5.0%	Scheme 10	2.4%	Scheme 10	9.9%	Scheme 10	4.3%	Scheme 10	2.3%	Scheme 10	1.2%

Limitations in the statistical tests are demonstrated by scheme 7, which is recommended by both statistical methods but could cause severe reactions in a clinical setting with its extreme dose escalations. Additional limitations are illustrated Figure 19, in which all 3 examples would be considered statistically similar to the original population but schemes 2 and 5 do not visually fit the dose distribution. Expert judgment must be used to interpret all results and clinical reaction implications and to select the optimal dose scheme. Recommended challenge protocols would follow a log or semi-log scale increase in protein doses and cover the entire range of dose distributions, similar to schemes 1, 4, 8, or 9.

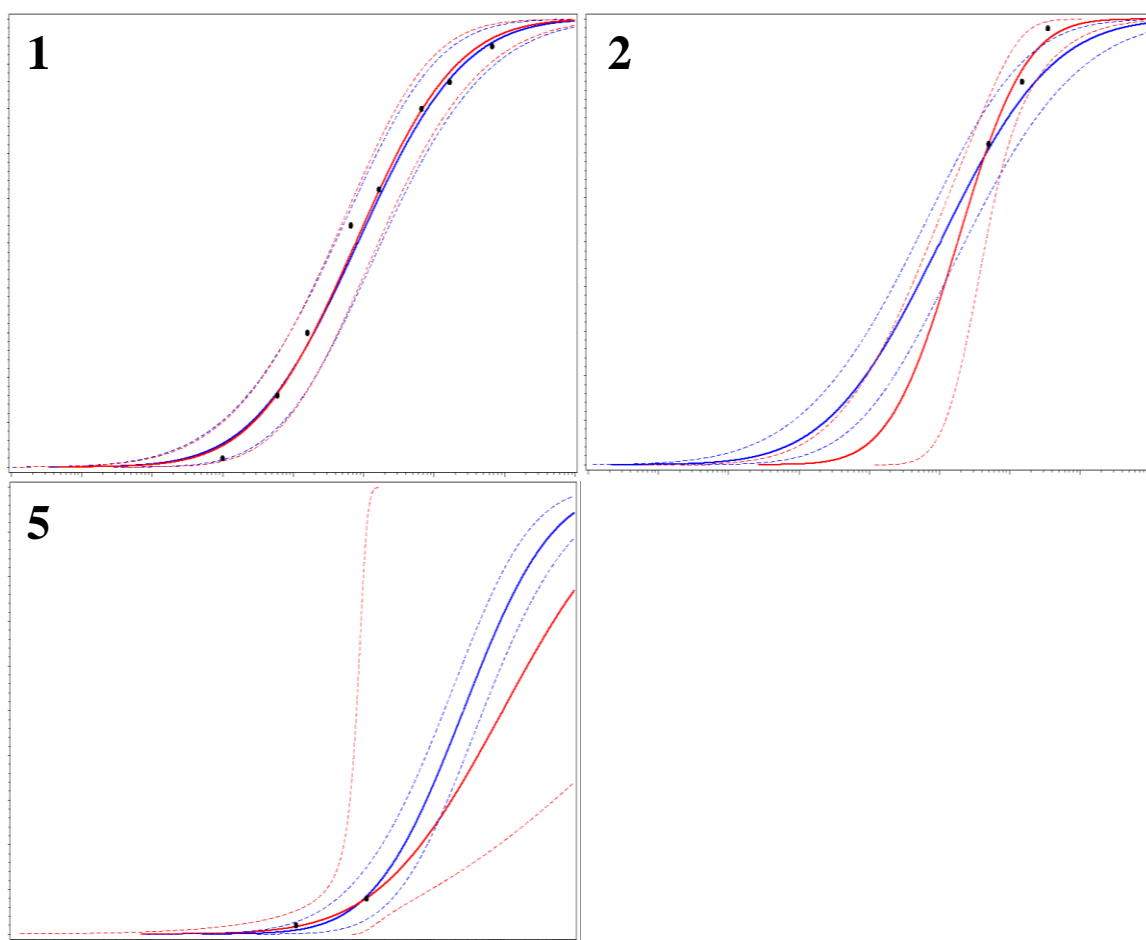


Figure 19. Visual depiction of scheme fits. Selected schemes include protocols 1, 2, and 5. Due to large confidence intervals in schemes 2 and 5, all dose distribution curves displayed (red) are not statistically different than the original VITAL datasets (blue).

4. Conclusion

The results of this study show that sufficient clinical data from food allergic individuals exist to use for risk assessment purposes and perhaps to develop regulatory thresholds for several allergenic foods. However, data collection must continue on all allergens, especially the priority tree nuts such almond and walnut where little or no data currently exist. As noted by others, the data in this study strengthen the notion that statistical risk assessment methods provide the best and the most transparent approach to developing regulatory thresholds. Allergic populations did not vary when analyzed by age, geographic region, or gender and only slightly varied by study population and challenge material. Due to limited global data, it would serve all stakeholders to continue to combine and merge datasets from different studies in an effort to mitigate biases from an individual study and enhance the overall dataset. Different approaches exist to derive a reference dose for use in risk assessments and comparisons demonstrated that a traditional toxicological benchmark-UF approach and the VITAL approach arrive at essentially the same suggested reference doses.

Scientifically sound clinical challenge dosing protocols would benefit all stakeholders. The low dose log or semi-log dosing protocols, recommended by others for its clinical safety and efficiency, were proven to be as sound as any other dose scheme available. Dose schemes are recommended to start below 1 mg protein from the allergenic food source and proceed at a log or semi-log scale, depending on the comfort of the physician, until a final discrete dose of 4 – 5 grams of protein is reached. As with the threshold datasets, expert judgment must be used when developing a dose scheme to

ensure the safety of subjects involved and the usefulness of subsequent challenge data for risk assessors.

References

1. Sicherer SH, Sampson HA. Food allergy. *Journal of Allergy and Clinical Immunology* 2010;**125**(2, Supplement 2):S116-S125.
2. Osborne N, Koplin JJ, Martin PE, Gurrin LC, Lowe AJ, Matheson MC, et al. Prevalence of challenge-proven IgE-mediated food allergy using population-based sampling and predetermined challenge criteria in infants. *Journal of Allergy and Clinical Immunology* 2011;**127**:668-676.
3. Rona RJ, Keil T, Summers C, Gislason D, Zuidmeer L, Sodergren E, et al. The prevalence of food allergy: a meta-analysis. *Journal of Allergy and Clinical Immunology* 2007;**120**:638-646.
4. Taylor SL, Bush RK, Busse WW. Avoidance diets - how selective should we be? *New England and Regional Allergy Proceedings* 1986;**7**(6):527-532.
5. Hefle SL, Furlong TJ, Niemann L, Lemon-Mule H, Sicherer S, Taylor SL. Consumer attitudes and risks associated with packaged foods having advisory labeling regarding the presence of peanuts. *Journal of Allergy and Clinical Immunology* 2007;**120**:171-176.
6. Pieretti MM, Chung D, Pacenza R, Slotkin T, Sicherer SH. Audit of manufactured products: use of allergen advisory labels and identification of labeling ambiguities. *Journal of Allergy and Clinical Immunology* 2009;**124**:337-341.
7. Yu JW, Kagan R, Verreault N, Nicolas N, Joseph L, St. Pierre Y, et al. Accidental ingestions in children with peanut allergy. *Journal of Allergy and Clinical Immunology* 2006;**118**:466-472.
8. Gendel SM. Comparison of international food allergen labeling regulations. *Regulatory Toxicology and Pharmacology* 2012;**63**(2):279-285.
9. EU. Risk Assessment of Food Borne Bacterial Pathogens: Quantitative Methodology Relevant for Human Exposure Assessment: Scientific Steering Committee, Health and Consumer Protection Directorate-General, European Commission; 2003.
10. Kroes R, Galli C, Munro I, Schilter B, Tran LA, Walker R, et al. Threshold of toxicological concern for chemical substances present in the diet: A practical tool for assessing the need for toxicity testing. *Food and Chemical Toxicology* 2000;**38**(2-3):255-312.
11. Lammerding AM, Fazil A. Hazard identification and exposure assessment for microbial food safety risk assessment. *International Journal of Food Microbiology* 2000;**58**(3):147-157.
12. Larsen JC. Risk assessment of chemicals in European traditional foods. *Trends in Food Science & Technology* 2006;**17**(9):471-481.

13. Notermans S, Gallhoff G, Zwietering MH, Mead GC. The HACCP concept: specification of criteria using quantitative risk assessment. *Food Microbiology* 1995;**12**(0):81-90.
14. Kerbach S, Aldrick AJ, Crevel RWR, Dömötör L, DunnGalvin A, Clare Mills EN, et al. Managing food allergens in the food supply chain – viewed from different stakeholder perspectives. *Quality Assurance and Safety of Crops & Foods* 2009;**1**(1):50-60.
15. Taylor SL, Hefle SL, Bindslev-Jensen C, Atkins FM, André C, Bruijnzeel-Koomen C, et al. A consensus protocol for the determination of the threshold doses for allergenic foods: how much is too much? *Clinical and Experimental Allergy* 2004;**34**:689-695.
16. Hourihane JOB, Dubois AEJ, Flokstra-de Blok B, Dunn-Galvin A. Impact of food challenge tests on children's and parent's health related quality of life: a time series case-control study. *Journal of Allergy and Clinical Immunology* 2011;**127**(Suppl):AB 242.
17. Koplin J, Osborne J, Allen J. Prevalence of allergen avoidance advisory statements on packaged processed foods in a supermarket. *The Medical Journal of Australia* 2010;**193**(7):426-427.
18. Vierk KA, Koehler KM, Fein SB, Street DA. Prevalence of self-reported food allergy in American adults and use of food labels. *Journal of Allergy and Clinical Immunology* 2007;**119**(6):1504-1510.
19. Taylor SL, Hefle SL. Food allergen labeling in the USA and Europe. *Current Opinion in Allergy and Clinical Immunology* 2006;**6**:186-190.
20. Madsen CB, Hattersley S, Allen KJ, Beyer K, Chan CH, Godefroy SB, et al. Can we define a tolerable level of risk in food allergy? Report from a EuroPrevall/UK Food Standards Agency workshop. *Clin Exp Allergy* 2012;**42**(1):30-37.
21. Gendel S, Buchanan R, Dennis S, Acheson D, Assimon SA, Beru N, et al. Approaches to establish thresholds for major food allergens and for gluten in food. *Journal of Food Protection* 2008;**71**:1043-1088.
22. Madsen CB, Hattersley S, Buck J, Gendel SM, Houben GF, Hourihane JOB, et al. Approaches to risk assessment in food allergy: report from a workshop "developing a framework for assessing the risk from allergenic foods". *Food and Chemical Toxicology* 2009;**47**:480-489.
23. Spanjersberg MQI, Kruizinga AG, Rennen MAJ, Houben GF. Risk assessment and food allergy: the probabilistic model applied to allergens. *Food and Chemical Toxicology* 2007;**45**:49-54.
24. Kruizinga AG, Briggs D, Crevel RWR, Knulst AC, Bosch LMCvd, Houben GF. Probabilistic risk assessment model for allergens in food: sensitivity analysis of the

minimum eliciting dose and food consumption. *Food and Chemical Toxicology* 2008;**46**(5):1437-1443.

25. Rimbaud L, Heraud F, La Vieille S, Leblanc J-C, Crepet A. Quantitative risk assessment relating to adventitious presence of allergens in food: a probabilistic model applied to peanut in chocolate. *Risk Analysis* 2010;**30**(1):7-19.

26. Spanjersberg M, Knulst A, Kruizinga A, Van Duijn G, Houben G. Concentrations of undeclared allergens in food products can reach levels that are relevant for public health. *Food Additives and Contaminants* 2010;**27**(02):169-174.

27. Taylor SL, Moneret-Vautrin DA, Crevel RWR, Sheffield D, Morisset M, Dumont P, et al. Threshold dose for peanut: risk characterization based upon diagnostic oral challenge of a series of 286 peanut-allergic individuals. *Food and Chemical Toxicology* 2010;**48**:814-819.

28. Taylor SL, Crevel RWR, Sheffield D, Kabourek J, Baumert J. Threshold dose for peanut: risk characterization based upon published results from challenges of peanut-allergic individuals. *Food and Chemical Toxicology* 2009;**47**:1198-1204.

29. FDA. Food Allergen Labeling and Consumer Protection Act of 2004 Public Law 108-282; 2006 2006, July.

30. Bindslev-Jensen C, Ballmer-Weber B, Bengtsson U, Blanco C, Ebner C, Hourihane J, et al. Standardization of food challenges in patients with immediate reactions to foods—position paper from the European Academy of Allergology and Clinical Immunology. *Allergy* 2004;**59**(7):690-697.

31. Taylor SL, Hefle SL, Bindslev-Jensen C, Bock SA, Burks AW, Jr., Christie L, et al. Factors affecting the determination of threshold doses for allergenic foods: how much is too much? *Journal of Allergy and Clinical Immunology* 2002;**109**:24-30.

32. Wensing M, Penninks AH, Hefle SL, Akkerdaas JH, van Ree R, Koppelman SJ, et al. The range of minimum provoking doses in hazelnut-allergic patients as determined by double-blind, placebo-controlled food challenges. *Clinical and Experimental Allergy* 2002;**32**:1757-1762.

33. Ballmer-Weber BK, Holzhauser T, Scibilia J, Mittag D, Zisa G, Ortolani C, et al. Clinical characteristics of soybean allergy in Europe: a double-blind, placebo-controlled food challenge study. *Journal of Allergy and Clinical Immunology* 2007;**119**:1489-1496.

34. Shaw J, Roberts G, Grimshaw K, White S, Hourihane J. Lupin allergy in peanut-allergic children and teenagers. *Allergy* 2008;**63**:370-373.

35. Crevel RWR, Briggs D, Hefle SL, Knulst AC, Taylor SL. Hazard characterisation in food allergen risk assessment: the application of statistical approaches and the use of clinical data. *Food and Chemical Toxicology* 2007;**45**:691-701.

36. Collett D. Modelling Survival Data in Medical Research. 2nd ed. Boca Raton, FL: Chapman & Hall/CRC Press; 1993.
37. Sun J, Zhao Q, Zhao X. Generalized Log-Rank Tests for Interval-Censored Failure Time Data. *Scandinavian journal of statistics* 2005;**32**(1):49-57.
38. Zhao Q, Sun J. Generalized log-rank test for mixed interval-censored failure time data. *Statistics in medicine* 2004;**23**(10):1621-1629.
39. So Y, Johnston G, Kim S. Analyzing Interval-Censored Survival Data with SAS Software. In: Proceedings of the SAS Global Forum 2010 Conference, Cary NC: SAS Institute Inc; 2010: Citeseer; 2010.
40. SAS. Sample 24980: Nonparametric estimation and comparison of survival curves from interval-censored data. <http://support.sas.com/kb/24/980.html>. Accessed 11/02/2012. In; 2012.
41. Payton ME, Greenstone MH, Schenker N. Overlapping confidence intervals or standard error intervals: what do they mean in terms of statistical significance? *J Insect Sci* 2003;**3**:34.
42. Peeters KABM, Koppelman SJ, van Hoffen E, van der Tas CWH, den Hartog Jager CF, Penninks AH, et al. Does skin prick test reactivity to purified allergens correlate with clinical severity of peanut allergy? *Clinical and Experimental Allergy* 2007;**37**:108-115.
43. Atkins FM, Steinberg SS, Metcalfe DD. Evaluation of immediate adverse reactions to foods in adult patients II. A detailed analysis of reaction patterns during oral food challenge. *Journal of Allergy and Clinical Immunology* 1985;**75**(3):356-363.
44. Hourihane JOB, Kilburn SA, Nordlee JA, Hefle SL, Taylor SL, Warner JO. An evaluation of the sensitivity of subjects with peanut allergy to very low doses of peanut protein: a randomized, double-blind, placebo-controlled food challenge study. *Journal of Allergy and Clinical Immunology* 1997;**100**(5):596-600.
45. Wensing M, Penninks AH, Hefle SL, Koppelman SJ, Bruijnzeel-Koomen CAFM, Knulst AC. The distribution of individual threshold doses eliciting allergic reactions in a population with peanut allergy. *Journal of Allergy and Clinical Immunology* 2002;**110**:915-920.
46. Lewis SA, Grimshaw KEC, Warner JO, Hourihane JOB. The promiscuity of immunoglobulin E binding to peanut allergens, as determined by Western blotting, correlates with the severity of clinical symptoms. *Clinical and Experimental Allergy* 2005;**35**:767-773.
47. Flinterman AE, Pasmans SG, Hoekstra MO, Meijer Y, van Hoffen E, Knol EF, et al. Determination of no-observed-adverse-effect levels and eliciting doses in a

representative group of peanut-sensitized children. *Journal of Allergy and Clinical Immunology* 2006;**117**:448-454.

48. Leung DYM, Sampson HA, Yunginger JW, Burks AW, Jr., Schneider LC, Wortel CH, et al. Effect of anti-IgE therapy in patients with peanut allergy. *New England Journal of Medicine* 2003;**348**:986-993.

49. Oppenheimer JJ, Nelson HS, Bock SA, Christensen F, Leung DYM. Treatment of peanut allergy with rush immunotherapy. *Journal of Allergy and Clinical Immunology* 1992;**90**(2):256-262.

50. Nelson HS, Lahr J, Rule R, Bock A, Leung D. Treatment of anaphylactic sensitivity to peanuts by immunotherapy with injections of aqueous peanut extract. *Journal of Allergy and Clinical Immunology* 1997;**99**(6):744-751.

51. Anagnostou K, Islam S, King Y, Deighton J, Clark AT, Ewan PW. Induction of tolerance in severe peanut allergy. *Clinical & Experimental Allergy* 2009;**39**(12):1954.

52. Clark AT, King Y, Islam S, Anagnostou K, Deighton J, Ewan PW. The study of tolerance to oral peanut (STOP). *Clinical & Experimental Allergy* 2008;**38**(12):1995.

53. Clark AT, Islam S, King Y, Deighton J, Anagnostou K, Ewan PW. Successful oral tolerance induction in severe peanut allergy. *Allergy* 2009;**64**:1218-1220.

54. Nicolaou N, Poorafshar M, Murray C, Simpson A, Winell H, Kerry G, et al. Allergy or tolerance in children sensitized to peanut: prevalence and differentiation using component-resolved diagnostics. *Journal of Allergy and Clinical Immunology* 2010;**125**:191-197.

55. Blumchen K, Ulbricht H, Staden U, Dobberstein K, Beschoner J, de Oliveira LCL, et al. Oral peanut immunotherapy in children with peanut anaphylaxis. *Journal of Allergy and Clinical Immunology* 2010;**126**:83-91.

56. Wainstein BK, Studdert J, Ziegler M, Ziegler JB. Prediction of anaphylaxis during peanut food challenge: usefulness of the peanut skin prick test (SPT) and specific IgE level. *Pediatric Allergy and Immunology* 2010;**21**:603-611.

57. Patriarca G, Nucera E, Pollastrini E, De Pasquale T, Lombardo C, Buonomo A, et al. Oral rush desensitization in peanut allergy: a case report. *Digestive Diseases and Sciences* 2006;**51**:471-473.

58. Taylor SL, Baumert JL, Houben GF, Crevel RWR, Brooke-Taylor S, Allen KJ, et al. Establishment of reference doses for residues of allergenic foods: Report of the VITAL expert panel. *Food and Chemical Toxicology* 2013 (In prep).

59. Sicherer SH, Teuber S. Current approach to the diagnosis and management of adverse reactions to foods. *Journal of Allergy and Clinical Immunology* 2004;**114**(5):1146-1150.

60. Staden U, Rolinck-Werninghaus C, Brewe F, Wahn U, Niggemann B, Beyer K. Specific oral tolerance induction in food allergy in children: efficacy and clinical patterns of reaction. *Allergy* 2007;**62**:1261-1269.
61. Norgaard A, Bindslev-Jensen C. Egg and milk allergy in adults: diagnosis and characterization. *Allergy* 1992;**47**(5):503-509.
62. Morisset M, Moneret-Vautrin DA, Kanny G, Guénard L, Beaudouin E, Flabbee J, et al. Thresholds of clinical reactivity to milk, egg, peanut and sesame in immunoglobulin E-dependent allergies: evaluation by double-blind or single-blind placebo-controlled oral challenges. *Clinical and Experimental Allergy* 2003;**33**:1046-1051.
63. Caminiti L, Passalacqua G, Barberi S, Vita D, Barberio G, De Luca R, et al. A new protocol for specific oral tolerance induction in children with IgE-mediated cow's milk allergy. *Allergy and Asthma Proceedings* 2009;**30**:443-448.
64. Patriarca G, Buonomo A, Roncallo C, Del Ninno M, Pollastrini E, Milani A, et al. Oral desensitisation in cow milk allergy: immunological findings. *International Journal of Immunopathology and Pharmacology* 2002;**15**:53-58.
65. Morisset M, Moneret-Vautrin DA, Guenard L, Cuny JM, Frentz P, Hatahet R, et al. Oral desensitization in children with milk and egg allergies obtains recovery in a significant proportion of cases. A randomized study in 60 children with cow's milk allergy and 90 children with egg allergy. *European Annals of Allergy and Clinical Immunology* 2007;**39**:12-19.
66. Longo G, Barbi E, Berti I, Meneghetti R, Pittalis A, Ronfani L, et al. Specific oral tolerance induction in children with very severe cow's milk-induced reactions. *Journal of Allergy and Clinical Immunology* 2008;**121**:343-347.
67. Skripak JM, Nash SD, Rowley H, Brereton NH, Oh S, Hamilton RG, et al. A randomized, double-blind, placebo-controlled study of milk oral immunotherapy for cow's milk allergy. *Journal of Allergy and Clinical Immunology* 2008;**122**:1154-1160.
68. Lam HY, van Hoffen E, Michelsen A, Guikers K, van der Tas CHW, Bruijnzeel-Koomen CAFM, et al. Cow's milk allergy in adults is rare but severe: both casein and whey proteins are involved. *Clinical and Experimental Allergy* 2008;**38**:995-1002.
69. Hill DJ, Ford RPK, Shelton MJ, Hosking CS. A study of 100 infants and young children with cow's milk allergy. *Clinical Reviews in Allergy* 1984;**2**:125-142.
70. Fiocchi A, Travaini M, D'Auria E, Banderali G, Bernardo L, Riva E. Tolerance to a rice hydrolysate formula in children allergic to cow's milk and soy. *Clinical and Experimental Allergy* 2003;**33**:1576-1580.
71. Baehler P, Chad Z, Gurbindo C, Bonin AP, Bouthillier L, Seidman EG. Distinct patterns of cow's milk allergy in infancy defined by prolonged, two-stage double-blind,

placebo-controlled food challenges. *Clinical and Experimental Allergy* 1996;**26**(3):254-261.

72. Flinterman AE, Knulst AC, Meijer Y, Bruijnzeel-Koomen CAFM, Pasmans SGMA. Acute allergic reactions in children with AEDS after prolonged cow's milk elimination diets. *Allergy* 2006;**61**:370-374.

73. Orhan F, Karakas T, Cakir M, Aksoy A, Baki A, Gedik Y. Prevalence of immunoglobulin E-mediated food allergy in 6-9-year-old urban schoolchildren in the eastern Black Sea region of Turkey. *Clinical and Experimental Allergy* 2009;**39**:1027-1035.

74. Devenney I, Norrman G, Oldæus G, Strömberg L, Fälth-Magnusson K. A new model for low-dose food challenge in children with allergy to milk or egg. *Acta Paediatrica* 2006;**95**:1133-1139.

75. Høst A, Samuelsson E-G. Allergic reactions to raw, pasteurized, and homogenized/ pasteurized cow milk: a comparison. *Allergy* 1988;**43**:113-118.

76. Benhamou AH, Zamora SA, Eigenmann PA. Correlation between specific immunoglobulin E levels and the severity of reactions in egg allergic patients. *Pediatric Allergy and Immunology* 2008;**19**:173-179.

77. Caffarelli C, Cavagni G, Giordano S, Stapane I, Rossi C. Relationship between oral challenges with previously uningested egg and egg-specific IgE antibodies and skin prick tests in infants with food allergy. *Journal of Allergy and Clinical Immunology* 1995;**95**(6):1215-1220.

78. Knight AK, Shreffler WG, Sampson HA, Sicherer SH, Noone S, Mofidi S, et al. Skin prick test to egg white provides additional diagnostic utility to serum egg white-specific IgE antibody concentration in children. *Journal of Allergy and Clinical Immunology* 2006;**117**:842-847.

79. Unsel M, Sin AZ, Ardeniz O, Erdem N, Ersoy R, Gulbahar O, et al. New onset egg allergy in an adult. *Journal of Investigational Allergology and Clinical Immunology* 2007;**17**:55-58.

80. Eggesbø M, Botten G, Halvorsen R, Magnus P. The prevalence of allergy to egg: a population-based study in young children. *Allergy* 2001;**56**:403-411.

81. Flinterman AE, Hoekstra MO, Meijer Y, van Ree R, Akkerdaas JH, Bruijnzeel-Koomen CA, et al. Clinical reactivity to hazelnut in children: association with sensitization to birch pollen or nuts? *Journal of Allergy and Clinical Immunology* 2006;**118**:1186-1189.

82. Zeiger RS, Sampson HA, Bock SA, Burks AW, Jr., Harden K, Noone S, et al. Soy allergy in infants and children with IgE-associated cow's milk allergy. *Journal of Pediatrics* 1999;**134**(5):614-622.

83. Magnolfi CF, Zani G, Lacava L, Patria MF, Bardare M. Soy allergy in atopic children. *Annals of Allergy, Asthma, and Immunology* 1996;**77**(3):197-201.
84. Ito K, Futamura M, Borres MP, Takaoka Y, Dahlström J, Sakamoto T, et al. IgE antibodies to ω -5 gliadin associate with immediate symptoms on oral wheat challenge in Japanese children. *Allergy* 2008;**63**:1536-1542.
85. Pastorello EA, Farioli L, Conti A, Pravettoni V, Bonomi S, Iametti S, et al. Wheat IgE-mediated food allergy in European patients: α -amylase inhibitors, lipid transfer proteins and low-molecular-weight glutenins. Allergenic molecules recognized by double-blind, placebo-controlled food challenge. *International Archives of Allergy and Immunology* 2007;**144**:10-22.
86. Scibilia J, Pastorello EA, Zisa G, Ottolenghi A, Bindslev-Jensen C, Pravettoni V, et al. Wheat allergy: a double-blind, placebo-controlled study in adults. *Journal of Allergy and Clinical Immunology* 2006;**117**:433-439.
87. Figueroa J, Blanco C, Dumpierrez AG, Almeida L, Ortega N, Castillo R, et al. Mustard allergy confirmed by double-blind placebo-controlled food challenges: clinical features and cross-reactivity with mugwort pollen and plant-derived foods. *Allergy* 2005;**60**:48-55.
88. Morisset M, Moneret-Vautrin DA, Maadi F, Frémont S, Guénard L, Croizier A, et al. Prospective study of mustard allergy: first study with double-blind placebo-controlled food challenge trials (24 cases). *Allergy* 2003;**58**:295-299.
89. Rancé F, Dutau G, Abbai M. Mustard allergy in children. *Allergy* 2000;**55**:496-500.
90. Moneret-Vautrin DA, Guérin L, Kanny G, Flabbee J, Frémont S, Morisset M. Cross-allergenicity of peanut and lupine: the risk of lupine allergy in patients allergic to peanuts. *Journal of Allergy and Clinical Immunology* 1999;**104**(4):883-888.
91. Fiocchi A, Sarratud P, Terracciano L, Vacca E, Bernardini R, Fuggetta D, et al. Assessment of the tolerance to lupine-enriched pasta in peanut-allergic children. *Clinical and Experimental Allergy* 2009;**39**:1045-1051.
92. Leduc V, Moneret-Vautrin DA, Tzen JTC, Morisset M, Guerin L, Kanny G. Identification of oleosins as major allergens in sesame seed allergic patients. *Allergy* 2006;**61**:349-356.
93. Kolopp-Sarda MN, Moneret-Vautrin DA, Gobert B, Kanny G, Brodschii M, Bene MC, et al. Specific humoral immune responses in 12 cases of food sensitization to sesame seed. *Clinical and Experimental Allergy* 1998;**27**(11):1285-1291.
94. Kanny G, De Hauteclocque C, Moneret-Vautrin DA. Sesame seed and sesame seed oil contain masked allergens of growing importance. *Allergy* 1996;**51**(12):952-957.

95. Daul CB, Morgan JE, Hughes J, Lehrer SB. Provocation-challenge studies in shrimp-sensitive individuals. *Journal of Allergy and Clinical Immunology* 1988;**81**(6):1180-1186.
96. Ballmer-Weber BK, Vieths S, Lüttkopf D, Heuschmann P, Wüthrich B. Celery allergy confirmed by double-blind, placebo-controlled food challenge: a clinical study in 32 subjects with a history of adverse reactions to celery root. *Journal of Allergy and Clinical Immunology* 2000;**106**:373-378.
97. Helbling A, Haydel R, Jr., McCants ML, Musmand JJ, El-Dahr J, Lehrer SB. Fish allergy: is cross-reactivity among fish species relevant? Double-blind placebo-controlled food challenge studies of fish allergic adults. *Annals of Allergy, Asthma, and Immunology* 1999;**83**:517-523.
98. Hansen TK, Bindslev-Jensen C. Codfish allergy in adults: identification and diagnosis. *Allergy* 1992;**47**(6):610-617.
99. Wood RA. The natural history of food allergy. *Pediatrics* 2003;**111**(Suppl):1631-1637.
100. Des Roches A, Nguyen M, Paradis L, Primeau MN, Singer S. Tolerance to cooked egg in an egg allergic population. *Allergy* 2006;**61**:900-901.
101. Eigenmann PA. Anaphylactic reactions to raw eggs after negative challenges with cooked eggs. *Journal of Allergy and Clinical Immunology* 2000;**105**:587-589.
102. Rolinck-Werninghaus C, Niggemann B, Grabenhenrich L, Wahn U, Beyer K. Outcome of oral food challenges in children in relation to symptom-eliciting allergen dose and allergen-specific IgE. *Allergy* 2012;**67**(7):951-957.

CHAPTER 3: QUANTITATIVE RISK ASSESSMENT OF FOODS CONTAINING PEANUT ADVISORY LABELING

Benjamin C. Remington^a, Joseph L. Baumert^a, David B. Marx^b, and Steve L. Taylor^{a*}

^a Food Allergy Research & Resource Program, Department of Food Science & Technology, University of Nebraska-Lincoln, Lincoln, NE, USA

^b Department of Statistics, University of Nebraska-Lincoln, Lincoln, NE, USA

***Corresponding author**

Steve L. Taylor
Food Allergy Research and Resource Program
255 Food Industry Complex
University of Nebraska-Lincoln
Lincoln, NE 68593-0919

Telephone: +1-402-472-2833

Fax: +1-402-472-5307

E-mail: staylor2@unl.edu

Abbreviations

Double-blind, placebo-controlled oral food challenge (DBPCFC)
Food Allergy & Anaphylaxis Network (FAAN)
Food Allergen Labeling and Consumer Protection Act (FALCPA)
United States Food and Drug Administration (FDA)
Lowest Observed Adverse Effect Level (LOAEL)
National Health and Nutrition Examination Survey (NHANES)
No Observed Adverse Effect Level (NOAEL)

Abstract

Foods with advisory labeling (i.e. “may contain”) continue to be prevalent and may be increasingly ignored by allergic consumers. We sought to determine the residual levels of peanut in various packaged foods bearing advisory labeling, compare similar data from 2005 and 2009, and determine any potential risk for peanut-allergic consumers. Of 186 food products bearing advisory statements regarding peanut or 16 products that had peanut listed as a minor ingredient (last three ingredients on statement), 16 (8.6%) and 6 (37.5%) contained detectable levels of peanut (>2.5 ppm whole peanut). Peanut was detected at similar rates and levels in 54 identical products tested in both 2005 and 2009. Although detectable levels of peanut protein were lower among all products tested in 2009, peanut-allergic consumers may still be at risk by consuming these products. Since the nutrition bar category contained the highest levels of peanut among products with advisory labeling, an additional market survey was conducted with 399 products including 120 nutrition bars with peanut listed as an ingredient, 44 products that listed peanut as a minor ingredient, 15 products declared as peanut-free, 49 products with no mention of peanut, 159 products with advisory statements for peanuts, 7 products with advisory statements for nuts, and 5 products declaring peanuts as a minor ingredient and also bearing an advisory statement. Of 215 nutrition bars with peanuts as a minor ingredient and/or an advisory statement for peanuts, 53 products (24.6%) tested positive for peanut (3.1 – 44,000 ppm) compared to 2 of 49 (4%) products with no mention of peanuts on the label. Probabilistic risk assessment showed the risk of a reaction to peanut allergic consumers from advisory labeled nutrition bars was significant but brand-dependent. Peanut advisory labeling may be overused on some nutrition bars but

prudently used on others. The probabilistic approach could provide the food industry with a quantitative method to assist with determining when advisory labeling is most appropriate.

Keywords: Peanut, Allergy, Labeling, Risk assessment, Probabilistic, Quantitative

1. Introduction

Peanut is one of the most common allergenic foods with a prevalence of 0.6 – 2.9% (Osborne et al., 2011; Rona et al., 2007; Sicherer et al., 2010; Sicherer and Sampson, 2010). Peanut allergies are potentially life-threatening and the most common cause of food-allergy fatalities in the United States (Bock et al., 2007; Keet and Wood, 2007; Rona et al., 2007). Peanut allergic consumers must adhere to a strict avoidance diet and carefully examine ingredient labels (Hefle et al., 2007; Pieretti et al., 2009; Taylor et al., 1986; Yu et al., 2006). The presence of peanut in mislabeled or unlabeled packaged products has led to allergic reactions in consumers relying on clear and accurate ingredient statements (Kemp and Lockey, 1996; Yu et al., 2006).

In the U.S., the Food Allergen Labeling and Consumer Protection Act (FALCPA) that protects allergic individuals from unclear or unlabeled products was passed in 2004 and became effective January 1, 2006 (FDA, 2006). Similar international allergen labeling laws have been implemented in 18 other national regulatory frameworks to address the issue of improved labeling of allergens in food (Gendel, 2012). For public health authorities, the primary strategies have been to develop lists of priority allergenic foods and enact regulations to assure that any ingredients derived from these foods are declared on the labels of packaged foods (Gendel, 2012). FALCPA requires that

companies must declare ingredients derived from the major allergenic foods including peanuts. Labeling of peanut is required by all 19 international regulatory frameworks that address allergen labeling (Gendel, 2012). However, advisory labeling for allergens is voluntarily used by the food industry and not directly regulated or addressed by FALCPA or most similar international regulations. The U.S. Food, Drug, and Cosmetics Act does require that label statements be “truthful and not misleading” (Chapter II, Sec. 201). Since advisory label statements are voluntary, a variety of advisory labels are used which can cause confusion and lead to weighted opinions of differing label statements. In a report by the U.S. Food and Drug Administration (FDA), both allergic and non-allergic consumers indicate that shorter “may contain” advisory labels are more likely to contain peanuts or other listed allergens. Additionally, both allergic and non-allergic consumers were more likely to serve a product with a longer “shared facility” or “shared equipment” statement to an allergic individual than a product with a shorter “may contain” label (FDA, 2006). Allergic consumers are more likely to avoid products that state they “May contain” or were “Manufactured on the same/shared equipment” than products that state they were “Manufactured in a facility that also processes/uses” (Hefle et al., 2007). However, peanut may be present in products that contain any form of advisory label for peanut (Hefle et al., 2007; Pele et al., 2007).

A U.S. supermarket survey found 17% of products contain an advisory label statement for food allergens. The wording of such statements was split evenly among products as 38% had “May contain”, 33% had “Same/shared equipment”, and 29% had “Shared facility” labels. Certain categories, such as chocolate candy, cookies, and baking mixes, had the highest prevalence of advisory statement usage with 40-54% of the

products having an advisory label (Pieretti et al., 2009). While the use of advisory labeling is high, Hefle et al. (2007) found in a 2005 survey that only 7.3% of products with peanut advisory statements tested had detectable levels of peanut. Consumer avoidance of advisory labeled products has decreased and the prevalence of detectable peanut is low, but a risk of an allergic reaction still exists when consuming advisory labeled products (Hefle et al., 2007).

The current study surveyed package foods with an advisory label for peanut for the presence of peanut. Special attention was paid to the difference between foods with peanut advisory labeling and peanut labeled as a minor ingredient. Comparisons to a similar study conducted in 2005 by Hefle et al. (2007) were done and the potential risk to the peanut allergic consumer was determined through the use of probabilistic risk assessment. Monte Carlo simulations have become popular in the quantitative assessment of microbial and chemical risks (EU, 2003; Kroes et al., 2000; Lammerding and Fazil, 2000; Larsen, 2006; Notermans et al., 1995) but their use with food allergens is a recent development. Probabilistic risk assessment of food allergens was introduced by TNO in the Netherlands and used to investigate the risk associated with undeclared hazelnut in chocolate spreads (Spanjersberg et al., 2007). Additional research conducted by the same group, and others in France, has shown the robust capabilities of the probabilistic models and expanded the concept to products with advisory labels for milk and peanut (Kruizinga et al., 2008; Rimbaud et al., 2010; Spanjersberg et al., 2010). To date, probabilistic risk assessment of food allergens based on packaged products found in the U.S. has not been done. This study aims to assess the risk to a peanut allergic consumer who intentionally purchases packaged food products with advisory labeling for peanut.

2. Methods and Materials

2.1. Peanut Advisory Labeling Studies

2.1.1. 2009 Packaged food samples

A total of 202 packaged food products bearing advisory statements regarding peanut (186) or products that had peanut listed as a minor ingredient (last three ingredients on statement; 16) were purchased in Lincoln, Nebraska, USA. Products were categorized as baked goods/mixes, baking ingredients, candy/confectionery, cereals/cereal bars, frozen desserts, instant meals, nutritional/meal bars, or snack foods. Two different lot numbers of each product were obtained leading to a total of 404 samples. Fifty-four products purchased in the 2005 study (Hefle et al., 2007) were available to purchase again in 2009 for comparison.

2.1.2. Peanut analysis

A representative sample from each package was homogenized and then analyzed for the presence of peanut using a commercial enzyme-linked immunosorbent assay (Peanut Veratox®, Neogen) with a lower limit of quantification of 2.5 parts per million (ppm, µg/g) whole peanut (0.63 ppm peanut protein). Samples were prepared and analyzed according to instructions provided with the kit.

2.1.3. 2010 Nutrition bar market survey

Due to the level of peanut in nutrition bars with advisory labeling in the 2005 and 2009 surveys, an in depth survey of nutrition bars available in Lincoln, Nebraska, USA was conducted. Products were categorized by the nature of the declaration of peanut on the labeling including contains peanut, minor ingredient, advisory statement for peanut, unique advisory labeling, declaration of peanut free, and no mention of peanut on the

label. A total of 399 nutrition bars were recorded and 279 were purchased for testing including 49 with minor ingredient declaration, 166 with advisory labeling statements, 15 declared peanut free, and 49 with no mention of peanut. Products stated to contain peanut were not tested. A single lot number of each nutrition bar was tested in this study.

2.2. Quantitative Approach to Risk Assessment

The probabilistic risk assessment model for food allergens was described by Spanjersberg et al. (2007) and Kruizinga et al. (2008). Briefly, data inputs for prevalence of food allergy, allergen thresholds, consumption patterns, and product test results can be fit to statistical distributions for use in a Monte Carlo simulation. The Monte Carlo program will randomly sample from each distribution during every run and iteration, match if the individual is allergic, a consumer of the type of product, and if the product contains peanut to determine if there is a possibility of an allergic reaction. An individual will have a predicted allergic reaction if the predicted consumed amount and concentration of peanut lead to a dose over the predicted allergen threshold for that individual. The current risk assessment uses a modified Monte Carlo approach, incorporating the mean and standard error associated with each input into a Bayesian framework to better estimate the confidence of the risk of an allergic reaction from consumption of nutrition bars with advisory labeling for peanut. The simulation scheme can be seen in Figure 1.

For the probabilistic risk assessment model, the prevalence of peanut allergy was taken from the study of Sicherer et al. (2010) that estimates peanut and tree nut allergy in the U.S.. This telephone survey data documents that 103 of 13,534 subjects or 0.76% were allergic to peanut. By nature, peanut allergy is a binomial distribution with yes/no

responses as individuals are either allergic or nonallergic. Using the Bayesian framework, Rimbaud et al. (2010) describe the process for fitting a binomial distribution with a non-informative prior $Beta(1,1)$ distribution. The posterior binomial distribution can be solved as follows: $p \sim Beta(1+x, 1+n_1-x)$ with x representing a positive response and n_1 representing the total number of people in the study. The allergic prevalence data are fit as follows $p \sim Beta(104, 13432)$. The beta distribution provides a new allergic prevalence probability estimate for the binomial distribution for every run in the simulation. In turn, the binomial distribution decides if an individual is allergic or not for every iteration in the simulation. The probabilities estimating allergic prevalence, peanut present in the nutrition bar, allergic shoppers purchasing advisory labeled products, and nutrition bar consumption are all binomial distributions, and the method shown by Rimbaud et al. (2010) was used to fit these inputs with prior beta distributions throughout the study.

The distribution of individual threshold doses from DBPCFC of 450 peanut-allergic individuals as based on objective symptoms was taken from Taylor et al. (2010). The No Observed Adverse Effect Levels (NOAELs) and Lowest Observed Adverse Effect Levels (LOAELs) from each individual were used, as previously described (Taylor et al., 2009), to fit a posterior Log-Normal probability distribution function for the threshold dose expressed as mg whole peanut. The Bayesian context uses statistical inference to generate the intercept and scale parameters of the Log-Normal distribution and assumes a prior normal distribution for each using their respective mean and standard error estimates from the LIFEREG procedure in SAS. These measures help reflect uncertainty in the true location of each parameter and can be used similarly with consumption and contamination distributions.

Four variables shape the consumption input distributions, the probability of a nutrition bar containing advisory labeling for peanut, the probability of the allergic shopper purchasing advisory labeled products, the probability of eating nutrition bars, and the amount eaten. The 2010 nutrition bar survey determined the probability of nutrition bars containing advisory labeling for peanut. The probability was set to a non-informative prior $Beta(1,1)$ distribution with a binomial posterior distribution. Surveys conducted at the Food Allergy & Anaphylaxis Network (FAAN) patient conferences were used to estimate how many allergic consumers buy products with advisory labeling on the package (Hefle et al., 2007). The probability of allergic shoppers purchasing advisory labeled products was conservatively estimated at 40% (258 of 645), the number parents with food allergic children from the survey indicating that they would possibly purchase products with a “same facility” label. This probability was fit to a non-informative prior $Beta(1,1)$ distribution with a binomial posterior distribution.

Consumption data for nutrition bars were extracted from the U.S. National Health and Nutrition Examination Survey (NHANES) using a combination of the 2003-04, 2005-06, and 2007-08 surveys (CDC, 2004, 2006, 2008). Only individuals completing both of the 24 hour dietary recall interviews were included in the dataset. Within the NHANES data, USDA food codes specific to high protein nutrition and meal replacement bars, PowerBar™, and Snickers™ Marathon bars (41435110, 53541200, 53544450, 91780010, and 91781010) were used to create the nutrition bar product category. Individuals were considered consumers if they ate nutrition bars during one of the two recall days. For individuals consuming multiple nutrition bars in a 24 hour period, the intakes were summed to a daily consumption level. As there is no

consumption database available solely for allergic consumers, the assumption was made that allergic and nonallergic individuals consume nutrition bars at the same rate and their reasons for nonconsumption are the same.

From the NHANES survey, the probability of consuming nutrition bars was estimated and fit to a non-informative prior $Beta(1,1)$ distribution with a binomial posterior distribution. Two simulations were run with differing consumption inputs. First, the simulation randomly selected a value from the individual consumption amounts in NHANES to represent consumption. A second, repeated simulation fit the range of consumption levels to a Log-Normal distribution and simulated a value expressed as the consumed amount. Inputs for the intercept and scale properties of the posterior Log-Normal distribution were assumed to follow prior normal distributions.

Nutrition bar products from all packaged food surveys conducted in 2005, 2009, and 2010 provided a total of 197 unique brand and flavor combinations with advisory labeling for peanut. As one lot number of each brand/flavor combination was tested in 2010 and two lot numbers were testing in 2005 and 2009, each lot number was treated as an individual sample for a total of 270 tested products. The probability of peanut being present was fit to a prior Beta distribution with a binomial posterior distribution. Sample concentrations of the positive products were expressed two ways. The first simulation used a randomly selected ppm result from the laboratory analysis data. The second, repeat simulation fit the laboratory analysis range of ppm results to a Log-Logistic distribution and simulated a value expressed as ppm whole peanut. Inputs for the intercept and scale properties of the posterior Log-Logistic distribution were assumed to follow prior normal distributions.

2.3. Software and statistics

All statistical tests were done in SAS (version 9.2). The χ^2 test with a P value of less than 0.05 being significant was used to compare the frequency of positives from all 2005 and 2009 products, products purchased in both 2005 and 2009, and nutrition bars against all other products with minor ingredient labeling. Fisher's Exact Test with a P value of less than 0.05 being significant was used to compare the frequency of positives between advisory labeled products and products with no mention of peanut on the label in the 2010 nutrition bar survey due to an expected cell count of less than 5. For probabilistic modeling, a Monte Carlo sampling technique was performed with 50 runs of 100,000 iterations for each simulation. The stepwise method of the simulation can be seen in Figure 2.

3. Results

3.1. Peanut advisory labeling study

Peanut was detected in at least one lot in 8.6% (16/186) of products with peanut advisory statements and 37.5% (6/16) of products with peanut listed as a minor ingredient (Table 1). In the advisory labeled products, 3 of 46 products with “may contain” statements, 9 of 65 products with “shared equipment” statements, 2 of 69 products with “shared facility” statements, and 2 of 6 products with unique advisory labels had detectable levels of peanut in one or both lots. These results are similar to the 2005 survey conducted by Hefle et al. (2007) that found detectable peanut in 7.3% of advisory labeled products from the same categories ($P= 0.64$). Detectable peanut was found in the advisory labeled nutrition/meal bars (6/24), candy/confectionary (4/32), baking ingredients (2/16), cereal/cereal bars (2/20), snack foods (1/25), and baked goods/mixes

(1/43) categories. No detectable peanut was found in the advisory labeled frozen desserts products (0/9) or the instant meals (0/17).

The 16 advisory labeled products with detectable levels of peanut had levels ranging from 3 to 510 ppm. Inconsistencies were seen as only 7 of 16 products had both lots tested positive for detectable peanut. Detectable peanut in the 6 products with minor ingredient labeling ranged from 5 to 69,000 ppm. Both lots tested positive for peanut in 5 of 6 cases.

Of 54 products tested in both the 2005 and 2009 advisory labeling surveys, peanut was detected in 13.0% (7/54) and 16.7% (9/54) of similar products in 2005 and 2009, respectively. These percentages were not significantly different even though FALCPA was implemented in 2006 ($P=0.59$).

3.2. Nutrition Bar Market Survey

Of 399 different nutrition bars, peanut is listed on 84% of labels, 42.4% (169/399) contain peanut in the ingredient statement and 41.6% (166/399) have an advisory label for peanut. A small number of nutrition bars, 3.8% (15/399), claim to be peanut free and 12.2% (49/399) have no declaration of peanut on the label. Detailed category breakdowns and ppm ranges can be found in Table 2. Among nutrition bars with minor ingredient labeling, 34 of 44 tested positive for peanut. Products contained up to 44,000 ppm whole peanut with 9 products over 1,100 ppm, 9 between 50 – 650 ppm, and 16 between 3.5 – 11 ppm. Five nutrition bars had peanut listed both as a minor ingredient and had an advisory statement indicating they were processed in the same facility with peanut; all tested positive at a range of 17 – 49,000 ppm. Of 159 nutrition bars with advisory labeling, 12 contained peanut with one 26,000 ppm sample, 4 samples between 70 – 150

ppm, and 7 samples between 3 – 40 ppm. Seven nutrition bars were labeled “may contain nuts” and 2 of 7 samples tested positive for peanut at 2.8 and 16.2 ppm. No detectable peanut was found in 15 products with various peanut-free statements including “peanut free facility”, “nut free facility, peanut free”, or “nut free”. Two of 49 nutrition bars with no mention of peanut on the label tested positive at 13 and 1,260 ppm. No significant difference was found in the frequency of detectable peanut in products with advisory labeling for peanut (12/159) and products that had no mention of peanut on the label (2/49) ($P=0.20$).

3.3. Quantitative Risk Assessment

The 2010 nutrition bar survey determined 41.6% (166/399) of nutrition bars have an advisory label for peanut. From the combined labeling surveys, 11.1% of nutrition bars with advisory labels for peanut were found to contain detectable levels of peanut residues at concentrations ranging from 2.5 to 26,000 ppm whole peanut. Two of the 30 positive samples (6.7%) contained peanut at 26,000 and 4,000 ppm whole peanut. Five samples (16.7%) had 131 – 510 ppm whole peanut present. Four samples (13.3%) were between 70 – 87 ppm whole peanut and 4 more samples (13.3%) were within 27 – 39 ppm whole peanut. The remaining 15 samples (50%) contained between 2.5 and 18 ppm whole peanut. From the NHANES survey, the probability of consuming nutrition bars was estimated to be 0.82% (201 of 24,621), including 35 individuals consuming nutrition bars both days. The average consumption of nutrition bars was 64 g per day, close to a common serving size for a single nutrition bar (Table 3). The 90th percentile intake was 130 g (2 nutrition bars). The 99th percentile intake was 204 g (3 nutrition bars) and the maximum amount consumed was 272 g (4 nutrition bars) in one day. Probabilistic risk

assessment modeling allows quantification of the risk faced by peanut-allergic consumers from consumption of advisory labeled nutrition bars.

The simulation input parameters and results are listed in Table 4. Simulation risk results are presented in three ways including the allergic user risk, the risk to the peanut allergic population, and the risk to the overall population. These three risk values represent the same number of predicted reactions expressed three different ways. The allergic user risk assumes every individual is allergic to peanut and consumes advisory labeled nutrition bars. The predicted number of reactions is 5.5 – 8.1 per 1,000 allergic users. To estimate the risk for the peanut allergic population, all individuals are allergic to peanut, 0.82% consume nutrition bars, 40% are assumed purchase advisory labeled nutrition bars, and 42% of nutrition bars contain advisory labeling for peanut. The predicted number of reactions is 0.8 – 1.1 per 100,000 peanut allergic individuals. To estimate the risk to the overall population, the inputs include that 0.76% of people are allergic to peanut, 0.82% consume nutrition bars, 40% are assumed purchase advisory labeled nutrition bars, and 42% of nutrition bars contain advisory labeling for peanut. The predicted number of reactions due to nutrition bars with advisory labeling for peanut is 5.8 – 8.5 per 100,000,000 people in the U.S. No differences were noted between using simulation inputs from the log-normal consumption and concentration distributions versus using a random selection of actual consumption levels and concentrations taken from the actual data sets.

The number of allergic reactions in peanut-allergic consumers can be predicted on a per day basis by utilizing the number of predicted peanut advisory labeled nutrition bars consumed in the U.S. per day, the prevalence of peanut allergy, and the allergic user risk.

The number of peanut advisory labeled nutrition bars consumed can be calculated with the equation below:

# consumed overall per day	=	U.S. population	×	Nutrition bar consumption probability	×	Probability of nutrition bar with advisory labeling for peanut
1.03 million nutrition bars	=	300 million	×	0.0082	×	0.42

The number of nutrition bars with advisory labeling for peanut consumed is used to estimate the number of allergic individuals purchasing nutrition bars with advisory labeling for peanut on a daily basis as shown in the equation below:

# consumed by allergic individuals	=	Consumed overall	×	Prevalence of peanut allergy	×	Probability of purchasing advisory labeled products
3,131 advisory labeled nutrition bars consumed by peanut allergic per day	=	1.03 million	×	0.0076	×	0.40

The number of expected allergic reactions per day can then be calculated by multiplying the number of peanut advisory labeled nutrition bars consumed by allergic individuals by the risk to the allergic user population in the equation below:

# of reactions	=	# Consumed by allergic individuals	×	Allergic user risk
27 predicted reactions per day	=	3,131	×	0.0085

The predicted reactions were heavily influenced by the two samples with the highest concentration of peanut (26,000 ppm and 4,000 ppm). These samples each account for 3.3% of the positive samples but are respectively responsible for ~35% and ~22% of predicted reactions. Still, nutrition bars with lower concentrations are also a risk

as products with levels of less than 100 ppm whole peanut led to 15 – 25% of the predicted reactions. Roughly 50% of the predicted reactions occurred at consumption amounts of 65 g or less, a common nutrition bar serving size. Approximately 10% of the reactions involved predicted consumption values over 130 g or 2 or more nutrition bars and ~1% involved predicted consumption values were over 204 g. The simulation predicts that 70 – 80% of the reactions have a dose over of 10 mg whole peanut, ~85% of the reactions have a dose over 5 mg whole peanut, and ~99.5% of the reactions have a dose over the most sensitive recorded individual threshold of 0.4 mg whole peanut as observed in Taylor et al. (2010). These results indicate that consuming nutrition bars with advisory labels for peanut would present a significant health risk to the peanut allergic community. When the 26,000 ppm and 4,000 ppm samples are removed from the simulation, the number of predicted reactions was reduced by 50%, but 23% of the predicted reactions occurred at a dose of more than 20 mg whole peanut. The lower concentrations of peanut in advisory labeled nutrition bars would still present a significant health risk to the peanut allergic community.

When using the Log-Normal and Log-Logistic curves, the simulation can predict unrealistic values for the consumption and ppm values at the extreme high and low ends of the curve. For instance, consumption values up to 563 g or 8 to 10 nutrition bars were predicted to cause a reaction in one simulation. Additionally, concentration values as low as 0.0000007 ppm peanut and higher than one million parts per million peanut (clearly impossible) were predicted by the Log-Logistic curve. However, these extreme values are statistically expected when repeating the simulation with a large number of iterations. A simulation was run using the Log-Logistic curve with the lower and upper extremes of

the concentration values capped at 2.5 to 26,000 ppm respectively. No significant changes in the results were seen when comparing the unbound simulations to the simulations with concentration values capped at 2.5 to 26,000 ppm whole peanut.

4. Discussion

Detectable levels of peanut were found in 8.6% (16/186) of packaged products during the 2009 advisory labeling survey. Results were not significantly different from a similar study conducted in 2005 before FALCPA fully went into effect. As found in the 2005 study, nutrition/meal bars and candy/confectionary products tested in 2009 were most likely to contain peanut among products with advisory labels. Our results indicate that 25% (6/24) of advisory labeled nutrition bars contained peanut in 2009, as compared to 14% (4/28) in 2005. However, the expanded 2010 market survey for nutrition bars found peanut in only 7.5% (12/159) of advisory labeled nutrition bars. A lower prevalence of peanut in the 2010 survey may be attributed to testing of only one lot number compared to two in previous surveys. The lack of positive samples does not necessarily mean the labels are misleading since only one or two lot numbers and a single sample from each were tested. However, the criteria used for advisory labeling within the food industry varies and the probability of detectable peanut can range from high to nearly nonexistent. Advisory labeling leads to consumer frustration and confusion and may lead to a false sense of security. Allergic consumers are most likely to avoid products that state “May contain” an allergen on the label by comparison to products with labels stating “Manufactured on shared equipment” or “Manufactured in a facility that also uses/processes” allergens, but similar levels of peanut were found in all three advisory labels. In 2009, products with “shared equipment” labels had the highest levels

of detectable peanut, but such products had the lowest levels in 2005. The products with “shared facility” labels had the highest levels in 2005 but had the lowest prevalence of positive tests in 2009. Based on such inconsistencies and the presence of detectable peanut in 8.6% of advisory labeled products, peanut-allergic individuals should be advised to avoid such products regardless of the wording of the advisory statement.

In some instances, minor ingredient declarations for peanut have been used by food companies as a stronger deterrent than advisory labeling. In 2005, 33% (7/21) products with minor ingredient statements had detectable levels of peanut. No change was found in 2009 with 37.5% (6/16) containing detectable peanut. Nutrition bars with minor ingredient labeling had a significantly higher rate of detectable peanut with 79.7% (47/59) positive during the combined 2005, 2009, and 2010 surveys compared to a combination of all other products with minor ingredient labeling with 20.3% (13/64) testing positive ($P < 0.0001$). While, these surveys comprise a small sample of all products with minor ingredient statements for peanut, such products demonstrate a significant risk to peanut allergic consumers. It is strongly recommended that peanut allergic consumers avoid products with peanut listed as a minor ingredient.

It is clear that many products with advisory labeling for peanut contain over 0.4 mg whole peanut, the individual threshold for an objective allergic reaction of the most sensitive peanut-allergic patients (Taylor et al., 2010), but how large of a health risk do they present? The ED05 and ED10, or doses predicted to provoke a reaction in 5% and 10% of the peanut-allergic population, were 5.2 mg and 12.3 mg whole peanut respectively (Taylor et al., 2010). Of predicted reactions in the simulation, 70 – 80% have a dose over of 10 mg whole peanut and ~85% have a dose over 5 mg whole peanut.

Probabilistic risk assessment indicates that nutrition bars with advisory labels for peanut constitute a serious risk for many peanut-allergic individuals. This risk assessment predicts that 27 allergic reactions should be occurring in the U.S. each day from ingestion of nutrition bars. Removal of samples with 26,000 or 4,000 ppm from the risk assessment results in 13 predicted allergic reactions per day. No evidence exists to suggest that the prevalence of allergic reactions to nutrition bars with advisory labeling is so high. The simulation seems to greatly overestimate the number of reactions per day in the U.S. due to a number of conservative input estimates, including the number of peanut allergic consumers purchasing nutrition bars and more specifically advisory labeled nutrition bars. The current simulation does not account for consumer preference for products absent of advisory labeling. However unlikely, it is assumed that 40% of allergic individuals will purchase an advisory labeled nutrition bar at the same rate as a product absent of advisory labeling. Additionally, mild reactions to these products may go unnoticed or unreported by peanut-allergic individuals. Still, advisory labeling for peanut is justified on many nutrition bars. The overlap of predicted exposure doses with confirmed DBPCFC peanut thresholds is sufficient to create a probable risk.

Conversely, the choice to consume products with no mention of peanut on the label will not remove all risk of an allergic reaction in peanut-allergic individuals. Two nutrition bars from the 2010 survey that contained detectable levels of peanut (12.6 and 1260 ppm respectively) did not list peanut on the label. While two data points are not enough to run a probabilistic simulation, the large serving size of nutrition bars demonstrates that a risk does exist for peanut allergic consumers of these 2 products. Nutrition bars labeled as peanut free or produced in a peanut free facility did not contain

detectable peanut. While only 15 peanut free samples were tested, these results are encouraging for peanut allergic individuals and suggest a safer choice for peanut allergic individuals who want to consume nutrition bars.

The only strategy to avoid an allergic reaction is avoidance of the food. Current food allergen regulations allow for ambiguous advisory statements to be placed on a label and more consumers are beginning to ignore these labels (Hefle et al., 2007). This study demonstrates that a risk is present to peanut-allergic consumers of nutrition bars with and without advisory labeling for peanut. Quantitative risk assessment gives risk assessors a flexible tool to help inform decisions on labeling, product release, and product recalls. A full quantitative risk assessment was not necessary to see the risk in nutrition bars with advisory labeling for peanut, but it provides justification for an advisory label beyond that provided by a simple deterministic approach. In cases with lower concentrations of peanut, such as the baking ingredient category in Table 1, quantitative risk assessment could detail the low risk of a product in realistic consumption scenarios and advocate the removal of advisory labeling from the product. The use of quantitative risk assessments by the food industry to make decisions about advisory labeling would help clarify the current situation, expand choices for allergic consumers, and reduce the potential for allergic reactions caused by packaged foods by making advisory labels more meaningful.

References

- Bock, S.A., Muñoz-Furlong, A., Sampson, H.A., 2007. Further fatalities caused by anaphylactic reactions to food, 2001-2006. *J Allergy Clin Immunol* 119, 1016-1018.
- CDC, 2004. Centers for Disease Control and Prevention (CDC). National Center for Health Statistics (NCHS). National Health and Nutrition Examination Survey Questionnaire (or Examination Protocol, or Laboratory Protocol). Hyattsville, MD: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, http://www.cdc.gov/nchs/nhanes/nhanes2003-2004/nhanes03_04.htm. Accessed 03/01/2013.
- CDC, 2006. Centers for Disease Control and Prevention (CDC). National Center for Health Statistics (NCHS). National Health and Nutrition Examination Survey Questionnaire (or Examination Protocol, or Laboratory Protocol). Hyattsville, MD: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, http://www.cdc.gov/nchs/nhanes/nhanes2005-2006/nhanes05_06.htm. Accessed 03/01/2013.
- CDC, 2008. Centers for Disease Control and Prevention (CDC). National Center for Health Statistics (NCHS). National Health and Nutrition Examination Survey Questionnaire (or Examination Protocol, or Laboratory Protocol). Hyattsville, MD: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, http://www.cdc.gov/nchs/nhanes/nhanes2007-2008/nhanes07_08.htm. Accessed 03/01/2013.
- EU, 2003. Risk Assessment of Food Borne Bacterial Pathogens: Quantitative Methodology Relevant for Human Exposure Assessment. Scientific Steering Committee, Health and Consumer Protection Directorate-General, European Commission.
- FDA, 2006. Food Allergen Labeling and Consumer Protection Act of 2004 Public Law 108-282, pp. 1-33.
- Gendel, S.M., 2012. Comparison of international food allergen labeling regulations. *Regul Toxicol Pharmacol* 63, 279-285.
- Hefle, S.L., Furlong, T.J., Niemann, L., Lemon-Mule, H., Sicherer, S., Taylor, S.L., 2007. Consumer attitudes and risks associated with packaged foods having advisory labeling regarding the presence of peanuts. *J Allergy Clin Immunol* 120, 171-176.
- Keet, C.A., Wood, R.A., 2007. Food allergy and anaphylaxis. *Immunol Allergy Clin North Am* 27, 193-212.
- Kemp, S.F., Lockey, R.F., 1996. Peanut anaphylaxis: an allergist-immunologist's personal experience with food cross-contamination. *J Allergy Clin Immunol* 97, 329.

- Kroes, R., Galli, C., Munro, I., Schilter, B., Tran, L.A., Walker, R., Würtzen, G., 2000. Threshold of toxicological concern for chemical substances present in the diet: A practical tool for assessing the need for toxicity testing. *Food Chem Toxicol* 38, 255-312.
- Kruizinga, A.G., Briggs, D., Crevel, R.W.R., Knulst, A.C., Bosch, L.M.C.v.d., Houben, G.F., 2008. Probabilistic risk assessment model for allergens in food: sensitivity analysis of the minimum eliciting dose and food consumption. *Food Chem Toxicol* 46, 1437-1443.
- Lammerding, A.M., Fazil, A., 2000. Hazard identification and exposure assessment for microbial food safety risk assessment. *Int J Food Microbiol* 58, 147-157.
- Larsen, J.C., 2006. Risk assessment of chemicals in European traditional foods. *Trends Food Sci Technol* 17, 471-481.
- Notermans, S., Gallhoff, G., Zwietering, M.H., Mead, G.C., 1995. The HACCP concept: specification of criteria using quantitative risk assessment. *Food Microbiol* 12, 81-90.
- Osborne, N., Koplin, J.J., Martin, P.E., Gurrin, L.C., Lowe, A.J., Matheson, M.C., Ponsonby, A.-L., Wake, M., Tang, M.L.K., Dharmage, S.C., Allen, K.J., 2011. Prevalence of challenge-proven IgE-mediated food allergy using population-based sampling and predetermined challenge criteria in infants. *J Allergy Clin Immunol* 127, 668-676.
- Pele, M., Brohee, M., Anklam, E., van Hengel, A.J., 2007. Peanut and hazelnut traces in cookies and chocolates: relationship between analytical results and declaration of food allergens on product labels. *Food Addit Contam* 24, 1334-1344.
- Pieretti, M.M., Chung, D., Pacenza, R., Slotkin, T., Sicherer, S.H., 2009. Audit of manufactured products: use of allergen advisory labels and identification of labeling ambiguities. *J Allergy Clin Immunol* 124, 337-341.
- Rimbaud, L., Heraud, F., La Vieille, S., Leblanc, J.-C., Crepet, A., 2010. Quantitative risk assessment relating to adventitious presence of allergens in food: a probabilistic model applied to peanut in chocolate. *Risk Anal* 30, 7-19.
- Rona, R.J., Keil, T., Summers, C., Gislason, D., Zuidmeer, L., Sodergren, E., Sigurdardottir, S.T., Lindner, T., Goldhahn, K., Dahlstrom, J., McBride, D., Madsen, C., 2007. The prevalence of food allergy: a meta-analysis. *J Allergy Clin Immunol* 120, 638-646.
- Sicherer, S.H., Muñoz-Furlong, A., Godbold, J.H., Sampson, H.A., 2010. US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. *J Allergy Clin Immunol* 125, 1322-1326.
- Sicherer, S.H., Sampson, H.A., 2010. Food allergy. *J Allergy Clin Immunol* 125, S116-S125.

- Spanjersberg, M., Knulst, A., Kruizinga, A., Van Duijn, G., Houben, G., 2010. Concentrations of undeclared allergens in food products can reach levels that are relevant for public health. *Food Addit Contam* 27, 169-174.
- Spanjersberg, M.Q.I., Kruizinga, A.G., Rennen, M.A.J., Houben, G.F., 2007. Risk assessment and food allergy: the probabilistic model applied to allergens. *Food Chem Toxicol* 45, 49-54.
- Taylor, S.L., Bush, R.K., Busse, W.W., 1986. Avoidance diets - how selective should we be? *N Engl J Allergy Proc* 7, 527-532.
- Taylor, S.L., Crevel, R.W.R., Sheffield, D., Kabourek, J., Baumert, J., 2009. Threshold dose for peanut: risk characterization based upon published results from challenges of peanut-allergic individuals. *Food Chem Toxicol* 47, 1198-1204.
- Taylor, S.L., Moneret-Vautrin, D.A., Crevel, R.W.R., Sheffield, D., Morisset, M., Dumont, P., Remington, B.C., Baumert, J.L., 2010. Threshold dose for peanut: risk characterization based upon diagnostic oral challenge of a series of 286 peanut-allergic individuals. *Food Chem Toxicol* 48, 814-819.
- Yu, J.W., Kagan, R., Verreault, N., Nicolas, N., Joseph, L., St. Pierre, Y., Clarke, A., 2006. Accidental ingestions in children with peanut allergy. *J Allergy Clin Immunol* 118, 466-472.

Tables and Figures

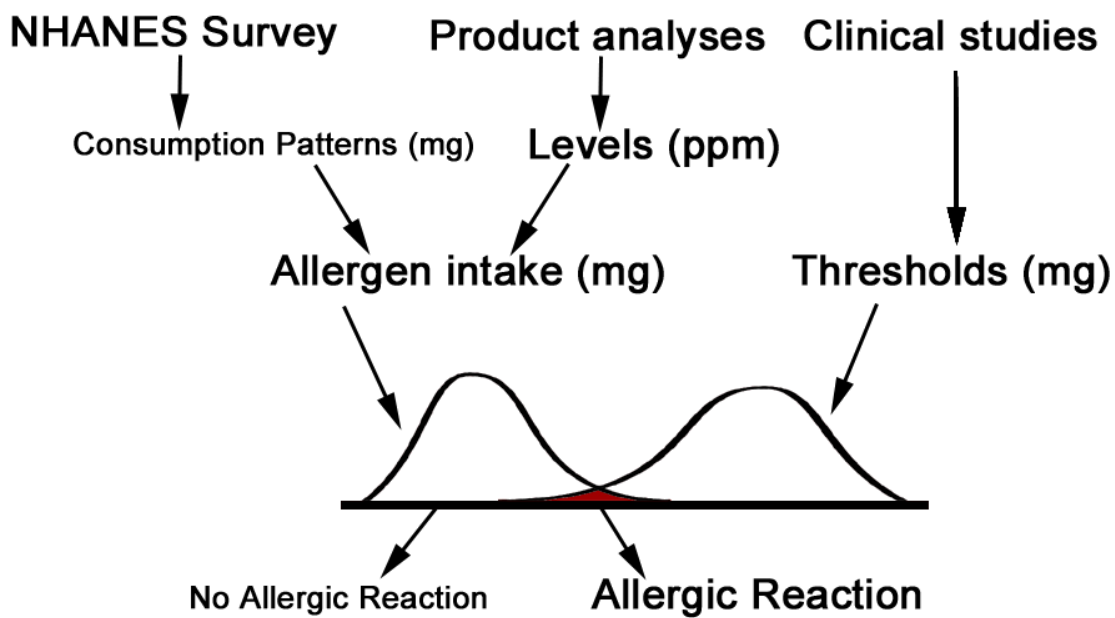


Figure 1. Probabilistic risk assessment model for food allergens in the U.S., figure adapted from Spanjersberg et al. (2007).

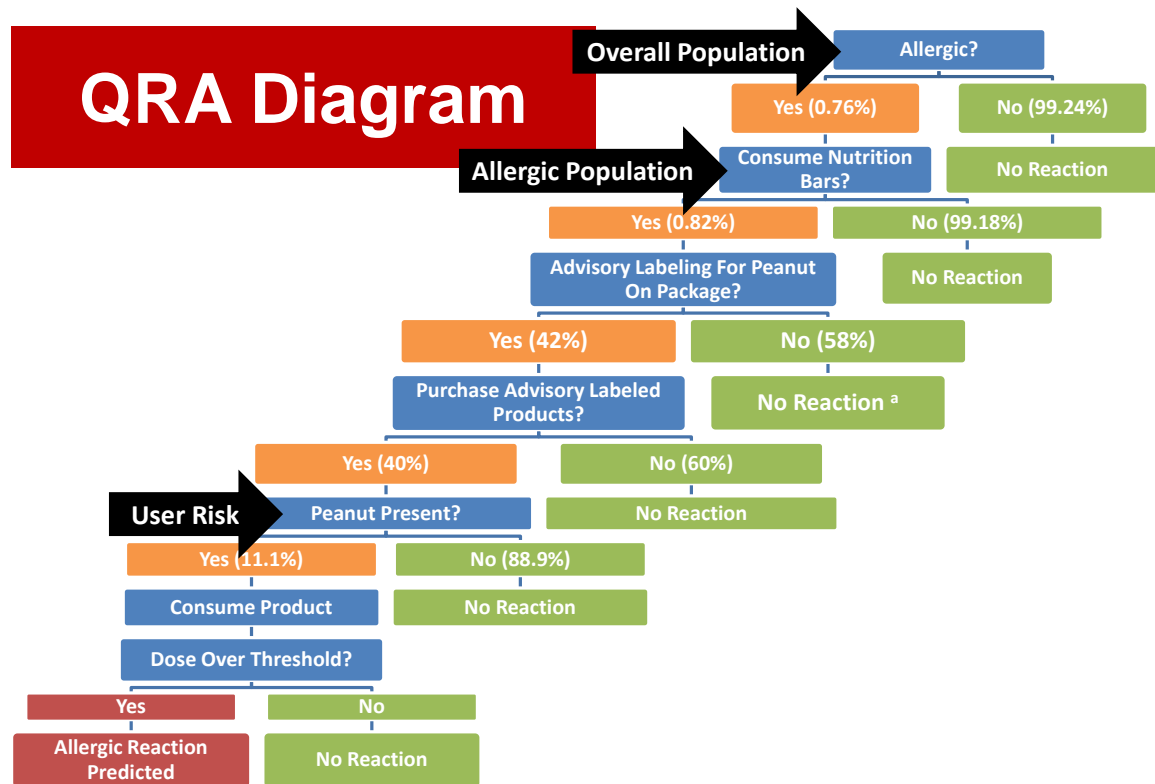


Figure 2. Stepwise progression of the Monte Carlo Simulation.

Consumers that follow advisory labeling warnings and avoid nutrition bars with advisory labeling for peanut are still at risk for an allergic reaction, as nutrition bars absent of peanut on the label have been shown to contain detectable levels of peanut.

Table 1. Concentration and serving-size doses of peanut in packaged foods bearing allergy advisory statements for peanut (2009 survey).

Product category	"May contain" labeling			"Shared equipment" labeling			"Shared facility" labeling			Other unique labeling			Total	Minor ingredient labeling		
	No.*	Conc†	Dose‡	No.	Conc	Dose	No.	Conc	Dose	No.	Conc	Dose	No.	No.	Conc	Dose
Baked goods/ mixes	14 (0)	-	-	14 (1)	8 ppm, BLQ	0.2 mg, <0.08 mg	15 (0)	-	-	-	-	-	43 (1)	-	-	-
Baking ingredients	4 (1)	3 ppm, BLQ	0.1 mg, <0.07 mg	6 (1)	9 ppm, 11 ppm	0.3 mg, 0.3 mg	6 (0)	-	-	-	-	-	16 (2)	-	-	-
Candy/ confectionary	8 (1)	15 ppm, BLQ	0.8 mg, <0.1 mg	13 (3)	24 ppm, BLQ	0.8 mg, <0.1 mg	10 (0)	-	-	1 (0)	-	-	32 (4)	2 (1)	93 ppm, BLQ	1.7 mg, <0.05 mg
		-	-		3 ppm, BLQ	0.1 mg, <0.1 mg		-	-		-	-		-	-	-
		-	-		5 ppm, BLQ	0.2 mg, <0.1 mg		-	-		-	-		-	-	-
Cereal/ cereal bars	5 (0)	-	-	8 (0)	-	-	5 (0)	-	-	2 (2)	97 ppm, 19 ppm	3.4 mg, 0.7 mg	20 (2)	8 (2)	11 ppm, 27 ppm	0.3 mg, 0.6 mg
		-	-		-	-		-	-		20 ppm, 19 ppm	0.6 mg, 0.5 mg		-	32 ppm, 32 ppm	1.1 mg, 1.1 mg
Frozen desserts	5 (0)	-	-	2 (0)	-	-	2 (0)	-	-	-	-	-	9 (0)	-	-	-
Instant meals	-	-	-	2 (0)	-	-	15 (0)	-	-	-	-	-	17 (0)	-	-	-
Nutrition/ meal bars	7 (1)	4 ppm, BLQ	0.2 mg, <0.1 mg	7 (3)	17 ppm, 32 ppm	0.7 mg, 1.3 mg	9 (2)	4 ppm, BLQ	0.2 mg, <0.1 mg	1 (0)	-	-	24 (6)	3 (3)	86 ppm, 121 ppm	6.7 mg, 9.4 mg
		-	-		510 ppm, 11 ppm	20.4 mg, 0.4 mg		4 ppm, 29 ppm	0.2 mg, 1.2 mg		-	-		-	5 ppm, 5 ppm	0.3 mg, 0.3 mg
		-	-		3 ppm, 131 ppm	0.1 mg, 5.2 mg		-	-		-	-		-	69000 ppm, 56000 ppm	3795 mg, 3080 mg
Snack foods	3 (0)	-	-	13 (1)	3 ppm, BLQ	0.1 mg, <0.1 mg	7 (0)	-	-	2 (0)	-	-	25 (1)	3 (0)	-	-
Total	46 (3)			65 (9)			69 (2)			6 (2)			186 (16)	16 (6)		

Table 2. Concentration of peanut in packaged nutrition bars in 2010 market survey.

Nutrition Bars	No.	No. Tested	No. Positive	PPM Range
Contains Peanut	104	0	-	-
Minor Ingredient	44	44	34	3.6 - 44,000
Advisory Label	159	159	12	3.1 - 26,000
<i>May Contain</i>	50	50	1	3.1
<i>Shared Equipment</i>	34	34	4	87 - 26,000
<i>Shared Facility</i>	75	75	7	5.6 - 70
Unique Label	28	13	8	2.8 – 49,000
<i>Contains Peanut + Advisory Statement^a</i>	16	0	-	-
<i>Minor Ingredient + Advisory Statement</i>	5	5	5	17.8 - 49,000
<i>May Contain Nuts^b</i>	7	7	2	2.8 - 16.2
Peanut Free Label	15	15	0	-
No Mention of Peanut	49	49	2	13 - 1,260
Total	399	279	55	

^a Not tested because they contain peanut and have an advisory label

^b "May Contain Nuts" or nut advisory label statements not specifically mentioning peanut

Table 3. Consumption of nutrition bars using NHANES 2003 – 2008 foodcodes.

Age Group	Sex	Percent of Population Consuming Nutrition Bars	Avg. Daily consumption (g)	Std. Deviation	90 th Percentile Consumption	Maximum Consumption	No. of Consumers	No. of People in Age Group
Total Population		0.82	64.7	38.9	130	272	201	24621
Infants		0.04	8.5		8.5	8.5	1	2751
Children		0.22	59	12.9	85.1	85.1	9	4077
Teenagers	Female	0.61	52.1	12.3	75	100	15	2479
Teenagers	Male	0.69	59.5	11.4	68	130	17	2449
Adults	Female	1.48	62.2	39.9	130	204	100	6752
Adults	Male	0.97	71	46.9	136	272	59	6113

Table 4. Inputs and results of the probabilistic modeling approach to risk assessment of nutrition bars with advisory labeling for peanut. All nutrition bars in the simulation are assumed to contain advisory labeling for peanut.

Parameter Inputs	Average Value (%)	Distribution Shape	Source of Data
Prevalence of Peanut Allergy	0.76	Beta	Sicherer et al. (2010)
Allergic Peanut Threshold		Log-Normal	Taylor et al. (2010)
Probability of purchasing advisory labeled products	40	Beta	Hefle et al. (2007)
Consumption Probability	0.82	Beta	NHANES Database
Amount Eaten		Log-Normal	NHANES Database
Presence of Peanut Probability	11.1	Beta	Labeling Surveys
Level of Peanut Present		Log-Logistic	Labeling Surveys
Presence of Advisory Labeling for Peanut	41.6	Beta	Labeling Surveys
Simulation Results		Mean	Std. Dev.
Reaction Probability in Allergic User Population		5.5 – 8.1 per 1,000	1.5 – 2.0
Reaction Probability in Peanut Allergic Population		0.8 – 1.1 per 100,000	0.2 – 0.3
Reaction Probability in Overall Population		5.8 – 8.5 per 100,000,000	2.0 – 2.3

CHAPTER 4: SOY IN WHEAT – CONTAMINATION LEVELS AND RISK

ASSESSMENT

Benjamin C. Remington^a, Joseph L. Baumert^a, David B. Marx^b, Barbara J. Petersen^c, and
Steve L. Taylor^{a*}

^aFood Allergy Research & Resource Program, Department of Food Science & Technology, University of Nebraska-Lincoln, Lincoln, NE, USA

^bDepartment of Statistics, University of Nebraska-Lincoln, Lincoln, NE, USA

^cExponent, Inc., Center for Chemical Regulation and Food Safety, Washington, DC, USA

*Corresponding author.

Fax: 1-402-472-5307

E-mail address: staylor2@unl.edu (S. L. Taylor)

Abstract

In the United States, food ingredients derived from allergenic sources must be clearly labeled on a package. However, no requirement exists to declare the presence of raw agricultural commodities due to agricultural commodity comingling. Clinical reports of allergic reactions to undeclared soy in wheat based products are rare and uncertainty exists regarding the degree of risk posed by wheat based products that are comingled with soy. Wheat flours available at local grocery stores were surveyed for the presence of soy and the potential risk to soy-allergic consumers of these products is determined. Soybean residues were found in 22 of 35 (62.8%) samples representing all forms of wheat flour. Detectable soy was found in wheat flours at concentrations of 3 – 443 ppm soy flour (1.6 – 236 ppm soy protein). Conservative probabilistic risk assessments predict a risk of allergic reaction among the most sensitive soy-allergic individuals of 2.8 ± 2.0 per 1,000 soy-allergic user eating occasions of foods containing wheat flour. Given the low level of predicted risk and the lack of evidence for allergic reactions among soy-allergic consumers to wheat based products, the avoidance of wheat based products by soy-allergic consumers does not appear to be necessary. However, diminution of this low risk remains a desirable goal. Accordingly, the distribution of soy residues during the wheat milling process was examined to determine the effect of milling on the amount of soy in various milling fractions. Experimental milling (6 flour streams, bran, shorts) and stream separation was conducted with spiked soy in wheat samples. Soy was detected in all 8 streams; 53% of the soy is separated in the bran or shorts and 47% of the soy enters one of the 6 flour streams. Stream separation was not enough to produce soy-free wheat flour

and additional cleaning measures will be needed to remove soy before wheat milling begins to obtain soy-free wheat flour.

1. Introduction

Recent studies estimate that 4 – 10% of children and 3 – 4% of adults are affected by food-induced allergic reactions (Branum and Lukacs, 2009; Osborne et al., 2011; Rona et al., 2007; Sicherer and Sampson, 2010). Soy is one of the most common allergenic foods in children, and soy allergy can be severe (Sicherer and Sampson, 2010). Soy-allergic consumers are advised to adhere to a strict avoidance diet and carefully read ingredient labels of all foods (Hefle et al., 2007; Pieretti et al., 2009; Taylor et al., 1986). The Food Allergen Labeling and Consumer Protection Act (FALCPA) was passed in 2004 by the United States Congress to protect allergic individuals from unclear or unlabeled products and became effective January 1, 2006 (FDA, 2006). Although ingredients derived from commonly allergenic sources must be labeled in clear terms when added to food, raw agricultural commodities are exempt from FALCPA (FDA, 2006). Due to the nature of agricultural production in the United States and worldwide, raw agricultural commodities are often comingled with other agricultural commodities during harvest, transport, and storage with shared equipment and facilities. The United States Department of Agriculture (USDA) Grain Inspection Handbook allows up to 10% of other grains with established standards to be present in wheat (USDA, 2004). Other grains with established standards include barley, canola, corn, flaxseed, oats, rye, sorghum, soybeans, sunflower seed, and triticale (USDA, 2004). The 10% level equates to 100,000 parts per million (ppm) or 100,000 mg/kg ($\mu\text{g/g}$) of other grains and would cause visual contamination within containers of wheat. The economics of buying and

selling wheat have kept commodity comingling well below these allowed limits as food processors demand a cleaner, higher grade of wheat. However, the extent of the risk from commodity comingling to allergic consumers has not been investigated.

The issue of soy in other grains has become an issue for food safety inspection as highlighted by numerous food alerts within the European Union Rapid Alert System for Food and Feed (RASFF) and the Canadian Food Inspection Agency (CFIA). The Food Safety Authority of Ireland has issued multiple food alerts for the presence of soy in wheat and corn based products (white bread flour, flour and corn tortillas, corn chips, and a batter mix) (RASFF Reference 2011.0015; 2011.0019; 2011.0022; 2011.0023; 2011.0215). The CFIA also issued a food recall in a wheat-based cereal due to undeclared soy (Reference Number: 6848). Despite the lack of consumer complaints associated with this cereal, products were recalled. In all likelihood, this cereal product has been produced for years with similar levels of soy. While levels of soy and other grains are known to occur in grain-based products, few studies have reported the levels of allergenic contaminants in agricultural commodities or finished products manufactured from these commodities. One exception is gluten, in large part due to “gluten-free” labeling, with North American levels of gluten contamination in other grains reported by multiple studies. Thompson et al. (2010) reported gluten contamination in 9 of 22 (41%) inherently gluten-free grain samples with levels up to 2,925 ppm gluten. In a study by Health Canada, Koerner et al. (2011) reported 117 of 133 (88%) retail oat products contained levels up to 3784 ppm gluten. However, only one oat variety with a “gluten-free” label was tested and it was consistently below the limit of quantitation for gluten. Recent IgE-mediated allergic reactions due to commodity contamination of wheat have

led the CFIA to encourage manufacturers and importers of grain-based products to inform consumers and transition towards the inclusion of precautionary labeling (a 'may contain wheat' statement) on their products containing cereal grains, such as oats or barley, to indicate the potential presence of wheat at low levels (CFIA, 2011).

Conversely, Health Canada has released guidance stating exposure to soy in grain-based foods is not likely to represent a health risk for soy-allergic individuals and advised the industry not to use advisory labeling for soy (Health Canada, 2013). Other countries have attempted to establish action levels for undeclared allergens including cross contamination of other grains in products and commodities. Switzerland has defined an action limit of 1,000 ppm for allergens. This limit states that if unavoidable, contamination above 1,000 ppm must be declared as an ingredient, but contamination below 1,000 ppm may be declared if desired (Kerbach et al., 2009). Levels of 1000 ppm may provide enough protein (low mg doses) to cause reactions at moderate consumption levels in multiple foods (Taylor et al., 2004). Japan has taken a stricter approach and limited undeclared allergens including commodities to 10 ppm in foods (Kerbach et al., 2009). Commodity grain shipments may be expected to exceed this Japanese limit but with unknown frequency.

While the actual levels of other grains in wheat are much lower than the 10% allowed by the USDA Handbook, allergic individuals could still be at risk by consuming these products. This study surveyed wheat flours available at local grocery stores for the presence of soy. The resultant potential risk to soy-allergic consumers of these products is determined. Additionally, experimental milling of wheat flour and subsequent stream analysis was done with wheat samples spiked with soy to determine the distribution of

soy through the milling process and into the various milling streams. The goal was to observe if current size separation cleaning practices could produce soy-free wheat through stream separation.

2. Materials and Methods

2.1. Wheat flour market survey

A total of 35 wheat flour products were purchased in 2010 from grocery stores in Lincoln, Nebraska, USA. Products included all-purpose, whole wheat, white wheat, bread, and pastry flours. A representative sample from each package was homogenized and then analyzed for the presence of soybean using a commercial enzyme-linked immunosorbent assay (Neogen Veratox[®] Soy Allergen kit) with a lower limit of quantification of 2.5 parts per million soy flour (ppm, $\mu\text{g/g}$). Samples were prepared and analyzed according to instructions provided with the kit. Results were converted to ppm soy protein for quantitative risk assessment by assuming that soy flour contains 53% protein (Ballmer-Weber et al., 2007).

2.2. Quantitative risk assessment of soy in commercially available wheat flours

The probabilistic risk assessment model for food allergens utilizes the Monte Carlo simulation to quantitatively estimate the risk of a specific product for a food allergic population (Kruizinga et al., 2008; Spanjersberg et al., 2007). Briefly, data inputs for prevalence of soy allergy, soy thresholds, consumption patterns, and the concentrations of soy in flour samples can be fitted to statistical distributions for use in a Monte Carlo simulation. Allergic reactions are predicted if the estimated consumed amount and concentration of soy in wheat lead to an estimated dose over the predicted soy threshold for that individual. The modified Monte Carlo approach for risk analysis

incorporates the mean and standard error associated with each input into a Bayesian framework to better estimate the confidence of the risk of allergic reaction as demonstrated by Rimbaud et al. (2010). The simulation scheme can be seen in Figure 1.

The distribution of individual threshold doses from double-blind, placebo-controlled food challenges (DBPCFC) of 43 soy-allergic individuals as based on objective symptoms was taken from Remington et al. (2013 (In prep)). The lowest-observed-adverse-effect-levels (LOAELs) and/or no-observed-adverse-effect-levels (NOAELs) from each individual were used, as previously described (Taylor et al., 2009), to fit posterior Log-Normal, Log-Logistic, and Weibull probability distribution functions for the threshold dose expressed as mg soy protein. Simple fit statistics (AIC, AICC, BIC) and the visual fit were similar for all three distributions. No biological basis exists upon which to select any one of the distributions over the others; the Log-Normal distribution was chosen for use in all probabilistic risk assessments. The Bayesian context uses statistical inference to generate the intercept and scale parameters of the Log-Normal distribution to help reflect uncertainty in the true location of each parameter.

The presence of soy residues in wheat flour resulting from commodity comingling was used to assess the risk to soy-allergic consumers across a broad spectrum of wheat flour-based foods. Consumption of wheat flour was based on individuals in the United States and extracted from the 2003-2006 National Health and Nutrition Examination Surveys (NHANES) of the U.S. Department of Agriculture (USDA). Individuals that did not complete both days of the survey were excluded from the analysis. Wheat flour consumption was estimated using the Foods Analysis and Residue Evaluation (FARE™) program from Exponent® with an eating occasion being defined as

one hour of consumption. Conservatively, this study estimated that one hour of consumption was equal to one eating occasion. The recipes in FARE™ are based on the recipes provided by USDA with modifications to analyze individual components of foods. Consumption of wheat flour has two inputs, the probability of consuming wheat flour and the amount eaten in one hour. Wheat flour consumption is a binomial distribution with a yes/no response as individuals are consumers or non-consumers. Using the Bayesian framework, Rimbaud et al. (2010) describe the process for fitting a Binomial distribution with a non-informative prior Beta(1,1) distribution. The posterior Binomial distribution can be solved as follows: $p \sim \text{Beta}(1+x, 1+n_1-x)$ with x representing a positive response and n_1 representing the total number of people in the study. The Beta distribution provides a new wheat flour consumption probability estimate for the Binomial distribution for every run in the simulation. In turn, the Binomial distribution determines if an individual is a wheat flour consumer or not for every iteration in the simulation. The amount of wheat flour consumed is fit to a Weibull distribution with statistical inference used to generate the scale and shape parameters as part of the Bayesian framework.

Chocolate chip cookies were used as a model food for risk assessment of a single food category. Consumption of chocolate chip cookies was based on the 2003-2006 NHANES survey. Chocolate chip cookies are estimated to be 20% wheat flour by weight after analyzing the weights of ingredients in popular chocolate chip cookie recipes from the USDA National Nutrient Database. Since no consumption database exists solely for allergic consumers, the assumption was made that allergic and non-allergic individuals

consume wheat flour and chocolate chip cookies at the same rate and their reasons for non-consumption are the same.

Results from the market survey were used to estimate the prevalence and concentration of soy in commercially available wheat flours. The prevalence of soy in wheat flours is a binomial distribution. The simulated concentrations of soy were randomly selected from the ELISA analytical results for every consumption event. The dose of soy per consumption event was determined using the concentrations of soy in wheat flour and the amount of wheat flour consumed.

2.3. Stream analysis during the milling process of clean wheat seed spiked with soy

Soy-free, hard red winter wheat seed was obtained from the University of Nebraska-Lincoln Department of Agronomy and Horticulture courtesy of Dr. Stephen Baenziger. Soybeans were broken into pieces of soy smaller than $\frac{1}{2}$ of a soybean using a coffee grinder. The smaller pieces represented soy likely to pass through physical screens during the wheat cleaning/screening process and enter into the flour mill. Soybean was spiked into wheat seed by weight at a level of 1000 ppm (mg/kg). Ten individual spikes were weighed into tempering buckets for repeated milling runs (1998 g wheat with 2 g broken soybean). The spiked samples were tempered to a moisture content of 16% from an initial moisture content of 11% to prepare for milling and to reflect commercial conditions.

Samples were milled on a Bühler Experimental Mill (Model MLU-202) according to the American Association of Cereal Chemists (AACC) International Method 26-21.02 for hard wheat. A diagram of the experimental mill displays 3 break cycles and 3 reduction cycles for the wheat to potentially pass through (Figure 2). Each break or

reduction is followed by a sifter to separate the sample into the appropriate streams. Screen sizes and stream allocations can be found in Table 1. Screens ranged in size from 160 – 600 microns with particles less than 160 microns entering a finished flour stream. The cleanout milling method was used instead of continuous milling to reduce potential cross-contamination of soy into subsequent samples. Briefly, during cleaning, the mill was brushed and tapped down after each spiked sample to remove flour adhering to pieces inside the mill. After brushing, 500 g of tempered soy-free wheat was milled as a buffer between samples and upon completion the mill was brushed and tapped down a second time. All spiked samples were milled after the clean-buffer-clean cycle. All 8 milling streams were collected, weighed for mass balance, and analyzed for the presence of soybean using a commercial enzyme-linked immunosorbent assay (Neogen Veratox[®] Soy Flour kit) with a lower limit of quantification of 2.5 parts per million soy flour (ppm, µg/g). Results were converted to ppm soy protein for comparison to the wheat flour market survey assuming soy flour is 53% protein (Ballmer-Weber et al., 2007).

2.4. Software and statistics

All statistical tests were done in SAS (version 9.2). For the probabilistic modeling, a Monte Carlo sampling technique was performed (50 runs; 10,000 iterations). For every iteration during the simulation, an individual is selected and determined if allergic to soybean. If allergic, the simulation chooses if they consume wheat flour. If both allergic and consumers of wheat flour, the amount of wheat flour consumed is multiplied by the concentration level of soybean in the product to estimate the mg dose of soybean protein that was eaten. The dose produced is then matched against the predicted allergic threshold value for that individual to predict if an allergic reaction will occur.

Streams from the experimental milling procedure were analyzed as part of a randomized complete block design (RCBD) to determine if soy was separated into each stream of flour at a significantly different rate.

3. Results

3.1. Wheat flour market survey

Soybean residues were found in all forms of wheat flour with 22 of 35 (62.8%) of flours containing detectable soy (Table 2) at concentrations of 3 – 443 ppm soy flour (1.6 – 236 ppm soy protein). An average serving size for wheat flour (30 g) would contain 0.05 – 7.1 mg soy protein. Due to the small sample size for each wheat flour category, differences in frequency and levels of soy could not be deemed significant.

3.2. Risk assessment of soy in commercially available wheat flours

On a daily basis, wheat flour is consumed by 97.1% of the total U.S. population, and greater than 99.3% in the population 3 years of age and up (Table 3). Average wheat flour consumption was 34 ± 36 g or slightly more than a suggested serving of wheat flour per eating occasion. Chocolate chip cookies are consumed by 11.7% of the U.S. population (Table 4). The average chocolate chip cookie consumption amount was 29 ± 29 g (1 – 2) cookies per day or 5.8 ± 5.8 g wheat flour based on the amount of wheat flour in the chocolate chip cookie recipe.

Simulation risk results are presented as the allergic user risk (Table 5). The allergic user risk assumes every individual is allergic to soybean and consumes wheat flour or chocolate chip cookies. Two simulations were run for wheat flour, first using the range of soy concentrations detected in wheat flour and second with the maximum amount of 10% soy allowed in wheat by the USDA Grain Inspection Handbook (USDA,

2004). The predicted number of reactions is 2.8 ± 2.0 per 1,000 soy-allergic user eating occasions for concentrations of soy found during the wheat flour survey. If the maximum amount of soy allowed was milled into wheat flour, the predicted number of reactions would be 23.4 ± 4.9 per 100 soy-allergic user eating occasions. The predicted number of allergic reactions from chocolate chip cookies is 6.6 ± 6.9 per 10,000 soy-allergic user eating occasions.

The number of allergic reactions in soy-allergic consumers can be predicted on a per day basis if it is assumed that a soy-allergic individual consumes wheat flour only once per day. The number of eating occasions is found by multiplying the total population, the probability of wheat flour consumption, and the probability of a soy-allergic individual. In the U.S., the prevalence of soy allergy is estimated at 0.35% (Sicherer and Sampson, 2010). The minimum number of eating occasions per day is predicted below:

Minimum eating occasions per day	=	U.S. population	×	Wheat consumption probability	×	Prevalence of soy allergy
1.02 million wheat flour consumption occasions	=	300 million	×	0.971	×	0.0035

The number of eating occasions is multiplied by the soy-allergic user risk to predict the number of allergic reactions per day. The number of predicted reactions can be calculated with the equation below:

Predicted reactions per day from soy in wheat	=	Minimum eating occasions per day	×	Soy-allergic user risk
2,850 objective reactions per day	=	1.02 million wheat flour consumptions	×	0.0028

None of the reactions were predicted to occur at doses of soy greater than 88 mg soy protein, the lowest LOAEL found in the 43 DBPCFCs for soy (Magnolfi et al., 1996). The Log-Normal ED₀₅, or dose predicted to provoke a reaction in 5% of the soy-allergic population is 24.5 mg soy protein, with a lower 95% confidence interval of 5.5 mg soy protein (Remington et al., 2013 (In prep)); 41% of predicted reactions had doses greater than 5.5 mg soy protein. The suggested Reference Dose to guide advisory labeling for soy flour is 1.0 mg protein in the VITAL program from the Allergy Bureau of Australia (Taylor et al., 2013 (In prep)); 83% of predicted reactions had doses greater than 1.0 mg soy protein (Figure 3A). The same methods can be used to calculate 81 predicted allergic reactions per day due to chocolate chip cookie consumption. No reactions were predicted to occur at doses over 88 mg soy protein, 1.5% of predicted reactions occurred at doses greater than 5.5 mg soy protein, and 34% of predicted reactions occurred at doses greater than 1.0 mg soy protein (Figure 3B).

3.3. Stream analysis during the milling process of clean wheat seed spiked with soy

An average mass recovery of $96.2 \pm 0.5\%$ was achieved after milling. An average soy recovery of $130 \pm 20\%$ was observed with the Veratox[®] Soy Flour ELISA kit. Streams were separated into 3 break flours, 3 reduction flours, the bran, and the shorts with soy being detected in all samples (Table 6). Separate streams contained detectable soy at 28 – 5235 ppm soy flour (15 – 2775 ppm soy protein) with a significant difference in the concentration of soy within the final flour streams ($P < 0.001$). The 1st and 2nd break flours had significantly less soy present than other streams. Shorts had the highest concentration of soy, followed by 3rd break and 3rd reduction flours. After mass adjustment, separate streams of wheat contained 4 – 1097 mg soy flour (2.1 – 581 mg soy

protein). In mass units, shorts contained the highest levels of soy followed by 1st reduction flour, bran, and 2nd reduction flour. The 1st and 2nd break flours contained significantly less soy than other streams. By weight, 53% of the soy is separated in the bran or shorts and 47% of the soy enters one of the 6 flour streams.

4. Discussion

Detectable levels of soy were found in 62.8% (22/35) of wheat flours during the market survey. All types of wheat flours contained detectable soy proteins. Cake and pastry flours are made from soft wheat due to ideal texture properties imparted by a high starch and low protein content. Soybeans and soft wheat share many growing regions in the US and it could be hypothesized that a higher soy content would be expected in soft wheat flours due to the proximity of soy in the production process. In this survey, the 3 pastry flours contained low levels of soy in comparison to harder wheat flours, but these results could be attributed to the small number of samples. Further studies with larger numbers of samples could be of use to investigate the effects of regional differences on concentrations of soy in different wheat flours and to determine if different types of wheat flours have significantly different levels of detectable soy.

Soy protein was found at concentrations up to 236 ppm in wheat flour, a dose of 7.1 mg soy protein per 30 g serving. No published soy challenges have reported an objective allergic reaction from doses at or below 7.1 mg soy protein. Despite this observation, the quantitative risk assessment indicates that the user risk from soy comingling with wheat flour is rather substantial predicting 2850 reactions per day among soy-allergic consumers in the U.S. alone. Since no published reports exist of reactions that might be attributable to soy comingling with wheat flour, clinical allergists

are either overlooking all of these cases (unlikely if 2850 occur per day in the U.S.) or the quantitative risk assessment is overstating the actual risk. A thorough examination of the inputs to the quantitative risk assessment suggests that are several factors that could result in an overestimation.

Probably owing to the comparatively low prevalence of soy allergy, the current investigation was only able to find published individual threshold data from DBPCFC soy flour challenges for 43 soy-allergic individuals (Remington et al., 2013 (In prep)). Peanut allergy is far more prevalent. The published dataset contains thresholds from 450 peanut-allergic individuals (Taylor et al., 2010). Populations with a low number of individuals have wider confidence intervals and can be significantly affected by the addition of data points at the low or high end of the threshold dose distribution. Probabilistic risk assessment is still an option with fewer subjects, but the predicted threshold distribution will vary widely within the Bayesian context. Additional clinical data from soy challenges would be valuable to increase the statistical confidence in the population threshold estimates. The study populations for three of the studies used (Fiocchi et al., 2003; Magnolfi et al., 1996; Zeiger et al., 1999) involved infants, while the remaining study (Ballmer-Weber et al., 2007) assessed an older patient population (Remington et al., 2013 (In prep)). Supplementary data on older children and adults would enrich the current threshold dataset. The most sensitive objective NOAEL found in the clinical literature for soy challenges was 83.7 mg soy protein (Ballmer-Weber et al., 2007), while the most sensitive objective LOAEL was 88 mg soy protein (Magnolfi et al., 1996). The Log-Normal ED_{05} , is 24.5 mg soy protein, with a lower 95% confidence interval of 5.5 mg soy protein (Remington et al., 2013 (In prep)). By comparison, a

NOAEL-based risk assessment would utilize a 10-fold safety factor to convert the LOAEL of 88 mg soy protein to a NOAEL equivalent of 8.8 mg soy protein. If a benchmark dose risk assessment were used, a reference dose comparable to the lower 95% confidence interval of the ED₀₅ would be selected from a the Log-Normal, Log-Logistic, or Weibull distributions. Larger threshold datasets, such as peanut, will have general agreement between the lowest eliciting dose, ED₀₁ and/or 95% LCI of the ED₀₅, and the VITAL reference dose. A smaller dataset, such as soybean, yields a conservative estimate of the ED₀₅ that varies greatly from the lowest eliciting dose.

A NOAEL-based worst case scenario utilizing the p₉₀ consumption of 73 g wheat flour with a soy protein concentration of 236 ppm contains a dose of 17.2 mg soy protein. The risk of an allergic reaction to the most sensitive soy-allergic individuals could not be ruled out. Probabilistic risk assessments have a number of measurements to eliminate the uncertainty found in NOAEL-based risk assessments but they still predict a risk of allergic reactions due to soy in wheat flour. If soy-allergic individuals consume wheat based products once a day, our simulation predicts an estimated 2,850 objective allergic reactions per day due to commodity contamination of soy in wheat. Obviously, reactions of this number would be reported or observed on a national scale. Additional factors also contribute to the overestimation of the risk to soy-allergic consumers due to soy in wheat. The estimated prevalence of soy allergy of 0.35% (Sicherer and Sampson, 2010) is likely too high. Historically, estimates of soy allergy prevalence have been based on perceived allergy in pediatric populations (Luccioli et al., 2008; Zuidmeer et al., 2008). Perception of adverse reactions to food far outnumbers the true prevalence of food allergy (Rona et al., 2007; Zuidmeer et al., 2008). Studies estimating the prevalence of soy allergy based

on the rate of sensitization to soy through skin prick tests are also based on pediatric populations. These studies will overestimate the prevalence of soy allergy as it is well known that sensitization alone does not equate to true food allergy (Sicherer and Sampson, 2010; Zuidmeer et al., 2008). Oral challenges found 0.7% of children up to age 14 were allergic to soy milk (Roehr et al., 2004). However, many soy patients reactive to soy milk and other less processed forms of soy can tolerate further cooked or processed soy products (Mittag et al., 2004). Finally, an estimated 70% of children will outgrow their food allergies (Eggleson, 1987). All of these factors combined indicate a prevalence of soy allergy in the overall population is closer to 0.1%.

The datasets for soy contamination in wheat flour and for consumption of wheat flour-based foods are reasonably good. However, the nature of probabilistic simulations predicted multiple reactions with individual consumption amounts greater than the 99th percentile estimate for intake and ranged from 150 – 240 g wheat flour (35 – 58 mg soy protein), 5 – 8 suggested servings of wheat flour in one sitting. More realistic consumption amounts of a highly contaminated wheat flour can produce doses of soy protein close to the lower 95% confidence interval of the ED₀₅ from the threshold curve, 41% of predicted reactions had doses greater than 5.5 mg soy protein. No dose was predicted to be above 60 mg soy protein. The probabilistic risk assessment of chocolate chip cookies predicts 81 soy-allergic reactions per day due to cross contamination of soy in wheat. The simulation estimates 29 of the 81 reactions occur after consumption of 1 cookie (29 g), the average consumption amount in the U.S. Additionally, 34 reactions were predicted after consumption of 2 cookies, 8 reactions after consuming 3 cookies, 8 reactions after consuming 4 cookies, and 2 reactions after consumption amounts greater

than 4 cookies. No reaction was predicted with a dose over 25 mg soy protein (105 g wheat flour, 18 cookies). Certainly, this predicted number of reactions to chocolate chip cookies have not been observed or reported among soy-allergic consumers. The predicted risk from chocolate chip cookies is likely an overestimate of the actual risk for the same reasons as outlined for overall wheat flour consumption. While the quantitative risk assessment predicts a level of risk from soy comingling with wheat, that level of risk is exceedingly small (2.8 ± 2.0 per 1,000 soy-allergic user eating occasions for consumption of wheat flour based upon the analysis of wheat flour in our survey) and not evident from reported clinical observations. In DBPCFC, experienced clinicians are recording relatively mild transitory objective reactions occurring at specific doses; these become the LOAELs. Perhaps some mild reactions are indeed occurring from the consumption of wheat based products by the soy-allergic population but they are so mild and short-lived that affected individuals do not complain. In our opinion, the vast majority of soy-allergic individuals should not be advised to avoid foods with wheat flour, although the possibility exists that a small number of highly sensitive soy-allergic consumers do exist who could be at risk from such exposures.

However, the level of predicted risk (23.4 ± 4.9 per 100 soy-allergic user eating occasions) occurring if wheat flour contained the maximum level of soy contamination (10%) allowed by USDA grain standards is 100-fold higher. Perhaps consideration should be given to lowering these allowable levels of soy comingling to assure protection of soy-allergic consumers. From our survey of wheat flour, the milling industry appears able to achieve a much lower level of soy contamination from comingling.

Does the current wheat flour milling process allow production of flour streams with little or no soy contamination? Throughout the wheat cleaning process, size exclusion screening will remove mature soybeans and a number of splits. Immature soybeans along with smaller splits and pieces will proceed with the wheat through size exclusion screenings and be milled into wheat flour. In commercial milling, wheat and soy are broken and milled into 20-40 main streams of flour, bran and shorts. During the experimental mill study (6 flour streams, bran, shorts), soy was detected in all 8 streams produced. The experimental milling process reduced the amount of soy in flour streams, with 53% of the soy separated into the bran or shorts and 47% of the soy entering one of the 6 flour streams. The bran and shorts fractions are removed from white flour, thus reducing the amount of soy entering the commercial product. Whole wheat flours contain the bran and shorts and will include 100% of the soy that enters the milling process. There was a significant difference in the concentrations of soy within each stream, but no stream could be considered soy-free. Stream separation alone is not enough to create soy-free wheat. Additionally, single streams are not available for purchase by consumers. Milled flour streams are mixed at different ratios to achieve specified protein contents, ash ratios, and final properties for customers of different retail products (cake flour, pastry flour, bread flour, all-purpose flour, quick-mixing flour, etc). Flours with different stream ratios have different dough qualities, baking properties, etc. Additional wheat cleaning methods are being explored to remove soy from wheat in the cleaning house but currently there are no solutions to completely remove soy from wheat once they have been comingled.

The only strategy to prevent an allergic reaction is avoidance of the food. Current food allergen regulations allow for commodity contamination of agricultural products to go unlabeled. Our conservative risk assessment shows the most sensitive soy-allergic individuals could have mild, objective reactions after consuming large amounts wheat flour or wheat based products. Clinical reports of allergic reactions attributable to soy contamination of wheat flour do not match with the predicted level of risk by the risk analysis, indicating that the risk assessment model is very conservative and that mild reactions, if they are actually occurring, are going unreported. Consequently, no changes should be considered to labeling laws regarding commodity comingling and specifically soy in wheat.

5. Acknowledgements

We thank Glen Weaver, Scott Baker, and ConAgra Mills for their assistance with the experimental milling of wheat flour and subsequent stream analysis portion of this project.

References

- Ballmer-Weber, B.K., Holzhauser, T., Scibilia, J., Mittag, D., Zisa, G., Ortolani, C., Osterballe, M., Poulsen, L.K., Vieths, S., Bindslev-Jensen, C., 2007. Clinical characteristics of soybean allergy in Europe: a double-blind, placebo-controlled food challenge study. *J Allergy Clin Immunol* 119, 1489-1496.
- Branum, A.M., Lukacs, S.L., 2009. Food allergy among children in the United States. *Pediatrics* 124, 1-7.
- CFIA, 2011. Allergen Labelling Information for Manufacturers and Importers of Cereal Grain-based Products. Canadian Food Inspection Agency, <http://www.inspection.gc.ca/english/fssa/labeti/allerg/20110520inde.shtml>. Accessed 03/01/2013.
- Eggleston, P.A., 1987. Prospective studies in the natural history of food allergy. *Ann Allergy* 59, 179-182.
- FDA, 2006. Food Allergen Labeling and Consumer Protection Act of 2004 Public Law 108-282, pp. 1-33.
- Fiocchi, A., Travaini, M., D'Auria, E., Banderali, G., Bernardo, L., Riva, E., 2003. Tolerance to a rice hydrolysate formula in children allergic to cow's milk and soy. *Clin Exp Allergy* 33, 1576-1580.
- Health Canada, 2013. Information for Canadians with Soy Allergy. Health Canada, <http://www.hc-sc.gc.ca/fn-an/securit/allerg/fa-aa/soy-soya-eng.php>. Accessed 03/01/2013.
- Hefle, S.L., Furlong, T.J., Niemann, L., Lemon-Mule, H., Sicherer, S., Taylor, S.L., 2007. Consumer attitudes and risks associated with packaged foods having advisory labeling regarding the presence of peanuts. *J Allergy Clin Immunol* 120, 171-176.
- Kerbach, S., Alldrick, A.J., Crevel, R.W.R., Dömötör, L., DunnGalvin, A., Clare Mills, E.N., Pfaff, S., Poms, R.E., Popping, B., Tömösközi, S., 2009. Managing food allergens in the food supply chain – viewed from different stakeholder perspectives. *Qual Assur Saf Crop* 1, 50-60.
- Koerner, T.B., Cleroux, C., Poirier, C., Cantin, I., Alimkulov, A., Elamparo, H., 2011. Gluten contamination in the Canadian commercial oat supply. *Food Addit Contam* 28, 705-710.
- Kruizinga, A.G., Briggs, D., Crevel, R.W.R., Knulst, A.C., Bosch, L.M.C.v.d., Houben, G.F., 2008. Probabilistic risk assessment model for allergens in food: sensitivity analysis of the minimum eliciting dose and food consumption. *Food Chem Toxicol* 46, 1437-1443.

- Luccioli, S., Ross, M., Labiner-Wolfe, J., Fein, S.B., 2008. Maternally reported food allergies and other food-related health problems in infants: characteristics and associated factors. *Pediatrics* 122 Suppl 2, S105-112.
- Magnolfi, C.F., Zani, G., Lacava, L., Patria, M.F., Bardare, M., 1996. Soy allergy in atopic children. *Ann Allergy Asthma Immunol* 77, 197-201.
- Mittag, D., Vieths, S., Vogel, L., Becker, W.-M., Rihs, H.-P., Helbling, A., Wüthrich, B., Ballmer-Weber, B.K., 2004. Soybean allergy in patients allergic to birch pollen: clinical investigation and molecular characterization of allergens. *J Allergy Clin Immunol* 113, 148-154.
- Osborne, N., Koplin, J.J., Martin, P.E., Gurrin, L.C., Lowe, A.J., Matheson, M.C., Ponsonby, A.-L., Wake, M., Tang, M.L.K., Dharmage, S.C., Allen, K.J., 2011. Prevalence of challenge-proven IgE-mediated food allergy using population-based sampling and predetermined challenge criteria in infants. *J Allergy Clin Immunol* 127, 668-676.
- Pieretti, M.M., Chung, D., Pacenza, R., Slotkin, T., Sicherer, S.H., 2009. Audit of manufactured products: use of allergen advisory labels and identification of labeling ambiguities. *J Allergy Clin Immunol* 124, 337-341.
- Remington, B.C., Baumert, J.L., Kabourek, J., Kruizinga, A.G., Houben, G.F., Taylor, S.L., 2013 (In prep). Threshold dose for soybean: risk characterization based upon published results from challenges of soybean-allergic individuals. *Food Chem Toxicol*.
- Rimbaud, L., Heraud, F., La Vieille, S., Leblanc, J.-C., Crepet, A., 2010. Quantitative risk assessment relating to adventitious presence of allergens in food: a probabilistic model applied to peanut in chocolate. *Risk Anal* 30, 7-19.
- Roehr, C.C., Edenharter, G., Reimann, S., Ehlers, I., Worm, M., Zuberbier, T., Niggemann, B., 2004. Food allergy and non-allergic food hypersensitivity in children and adolescents. *Clin Exp Allergy* 34, 1534-1541.
- Rona, R.J., Keil, T., Summers, C., Gislason, D., Zuidmeer, L., Sodergren, E., Sigurdardottir, S.T., Lindner, T., Goldhahn, K., Dahlstrom, J., McBride, D., Madsen, C., 2007. The prevalence of food allergy: a meta-analysis. *J Allergy Clin Immunol* 120, 638-646.
- Sicherer, S.H., Sampson, H.A., 2010. Food allergy. *J Allergy Clin Immunol* 125, S116-S125.
- Spanjersberg, M.Q.I., Kruizinga, A.G., Rennen, M.A.J., Houben, G.F., 2007. Risk assessment and food allergy: the probabilistic model applied to allergens. *Food Chem Toxicol* 45, 49-54.
- Taylor, S.L., Baumert, J.L., Houben, G.F., Crevel, R.W.R., Brooke-Taylor, S., Allen, K.J., Remington, B.C., Kruizinga, A.G., Sherlock, R., 2013 (In prep). Establishment of

reference doses for residues of allergenic foods: Report of the VITAL expert panel. *Food Chem Toxicol*.

Taylor, S.L., Bush, R.K., Busse, W.W., 1986. Avoidance diets - how selective should we be? *N Engl Reg Allergy Proc* 7, 527-532.

Taylor, S.L., Crevel, R.W.R., Sheffield, D., Kabourek, J., Baumert, J., 2009. Threshold dose for peanut: risk characterization based upon published results from challenges of peanut-allergic individuals. *Food Chem Toxicol* 47, 1198-1204.

Taylor, S.L., Hefle, S.L., Bindslev-Jensen, C., Atkins, F.M., André, C., Bruijnzeel-Koomen, C., Burks, A.W., Bush, R.K., Ebisawa, M., Eigenmann, P.A., Host, A., Hourihane, J.O.B., Isolauri, E., Hill, D.J., Knulst, A., Lack, G., Sampson, H.A., Moneret-Vautrin, D.A., Rancé, F., Vadas, P.A., Yunginger, J.W., Zeiger, R.S., Salminen, J.W., Madsen, C., Abbott, P., 2004. A consensus protocol for the determination of the threshold doses for allergenic foods: how much is too much? *Clin Exp Allergy* 34, 689-695.

Taylor, S.L., Moneret-Vautrin, D.A., Crevel, R.W.R., Sheffield, D., Morisset, M., Dumont, P., Remington, B.C., Baumert, J.L., 2010. Threshold dose for peanut: risk characterization based upon diagnostic oral challenge of a series of 286 peanut-allergic individuals. *Food Chem Toxicol* 48, 814-819.

Thompson, T., Roland Lee, A., Grace, T., 2010. Gluten contamination of grains, seeds, and flours in the United States: a pilot study. *J Am Diet Assoc* 110, 937-940.

USDA, 2004. Grain Inspection Handbook - Book II Grain Grading Procedures - Chapter 13 - Wheat.

Zeiger, R.S., Sampson, H.A., Bock, S.A., Burks, A.W., Jr., Harden, K., Noone, S., Martin, D., Leung, S., Wilson, G., 1999. Soy allergy in infants and children with IgE-associated cow's milk allergy. *J Pediatr* 134, 614-622.

Zuidmeer, L., Goldhahn, K., Rona, R.J., Gislason, D., Madsen, C., Summers, C., Sodergren, E., Dahlstrom, J., Lindner, T., Sigurdardottir, S.T., McBride, D., Keil, T., 2008. The prevalence of plant food allergies: a systematic review. *J Allergy Clin Immunol* 121, 1210-1218.

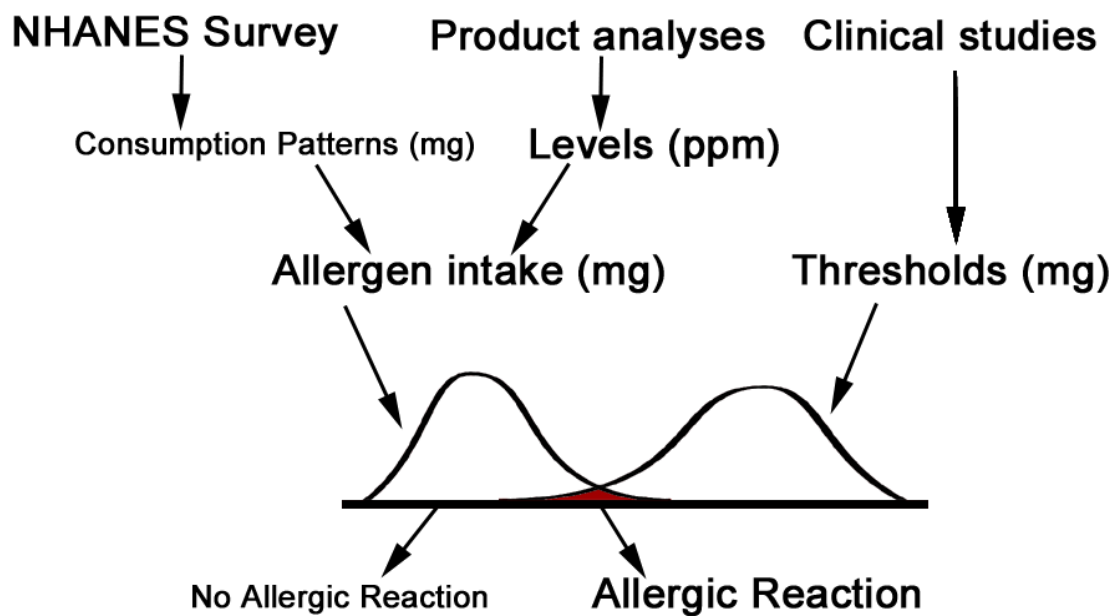
Figures and Tables

Figure 1 –Probabilistic risk assessment model for food allergens in the United States, figure adapted from Spanjersberg et al. (2007).

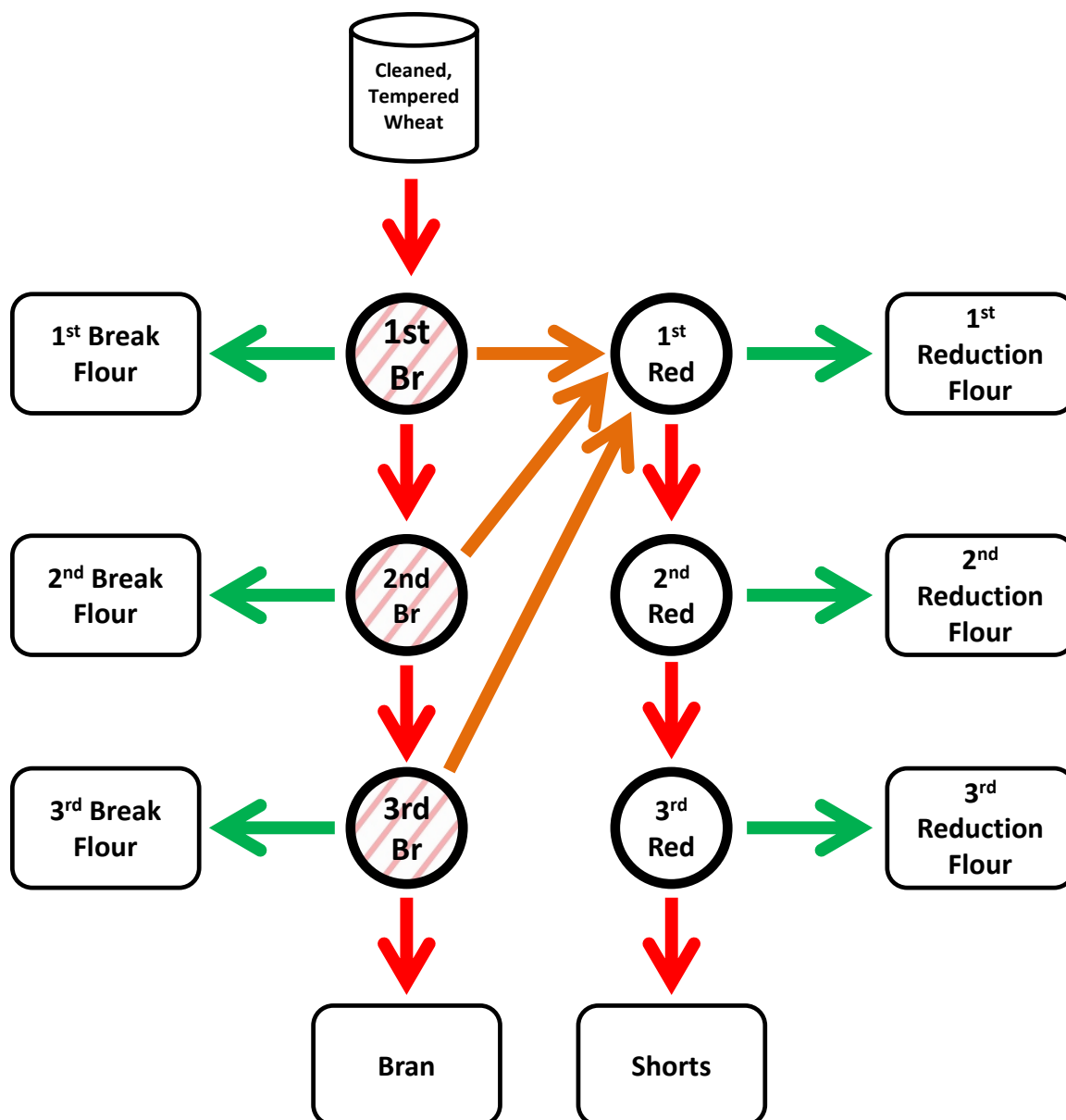


Figure 2 – Diagram of the Bühler Experimental Mill (Model MLU-202). The “Br” break cycles are denoted with red lines to represent corrugated rollers. Each break or reduction is accompanied by a sifter. Particles < 160 microns in size entered a flour stream.

Table 1 – Micron screen sizes and destinations for higher flour yield with hard wheat. All particles smaller than screen 1 will pass through to be separated by screen 2.

Break/Reduction	Screen 1	> Screen 1 to:	Screen 2	> 160 to:	< 160 to:
1 st Break	600 ^A	2 nd Break	160 ^D	1 st Reduction	1 st Break Flour
2 nd Break	475 ^B	3 rd Break	160	1 st Reduction	2 nd Break Flour
3 rd Break	475	Bran	160	1 st Reduction	3 rd Break Flour
1 st Reduction	600	Shorts	160 ^E	2 nd Reduction	1 st Reduction Flour
2 nd Reduction	275 ^C	Shorts	160 ^E	3 rd Reduction	2 nd Reduction Flour
3 rd Reduction			160 ^E	Shorts	3 rd Reduction Flour

^A 600 - opening of 600 microns (0.0236")

^B 475 - opening of 475 microns (0.0187")

^C 275 - opening of 275 microns (0.0108")

^D 160 - opening of 160 microns (0.0063")

^E 1st, 2nd, and 3rd reductions have two 160 micron screens present for efficient flow through

Table 2 – Concentration of soybean in packaged wheat flours.

Wheat Flours	No. Tested	No. Positive	PPM Soy Protein
All Purpose Flour	13	10	1.6 – 236
Bread Flour	4	2	7.3 – 152
Pastry Flour	3	2	5.0 – 7.1
White Wheat Flour	4	2	23 – 70
Whole Wheat Flour	10	5	2.1 – 128
Unique	1	1	39
Total	35	22 (62.8%)	1.6 – 236

Table 3 – Consumption of wheat flour in the United States per eating occasion (defined as one hour time frame) as determined by the FARE™ program from Exponent®.

Age	Percent User	Mean (g)	Std. Dev. (g)	P90 (g)	Max (g)
Total Population	97.1%	34	36	73	906
Infants	78.8%	14	10	32	167
Children	99.9%	28	24	60	446
Teenagers	99.7%	42	31	87	906
Adults	99.3%	36	44	75	701

Table 4 – Consumption of chocolate chip cookies per day in the United States based on the 2003-2006 NHANES database.

Age	Percent User	Mean (g)	Std. Dev. (g)	P90 (g)	Max (g)
Total Population	11.7%	29	29	56	510
Infants	8.0%	14	8	30	71
Children	16.2%	26	18	45	126
Teenagers	16.1%	29	20	53	315
Adults	9.0%	31	40	60	510

Table 5 – Simulation results

Product	Consumption Pattern	Level of Soy Present (ppm)	Soy-Allergic User Risk (%)	SD
Wheat Flour	Total Population	1.6 - 236 ppm soy protein	0.28	0.20
Wheat Flour	Total Population	40,000 ppm soy protein	23.4	4.9
Chocolate Chip Cookies	Total Population	1.6 - 236 ppm soy protein	0.066	0.069

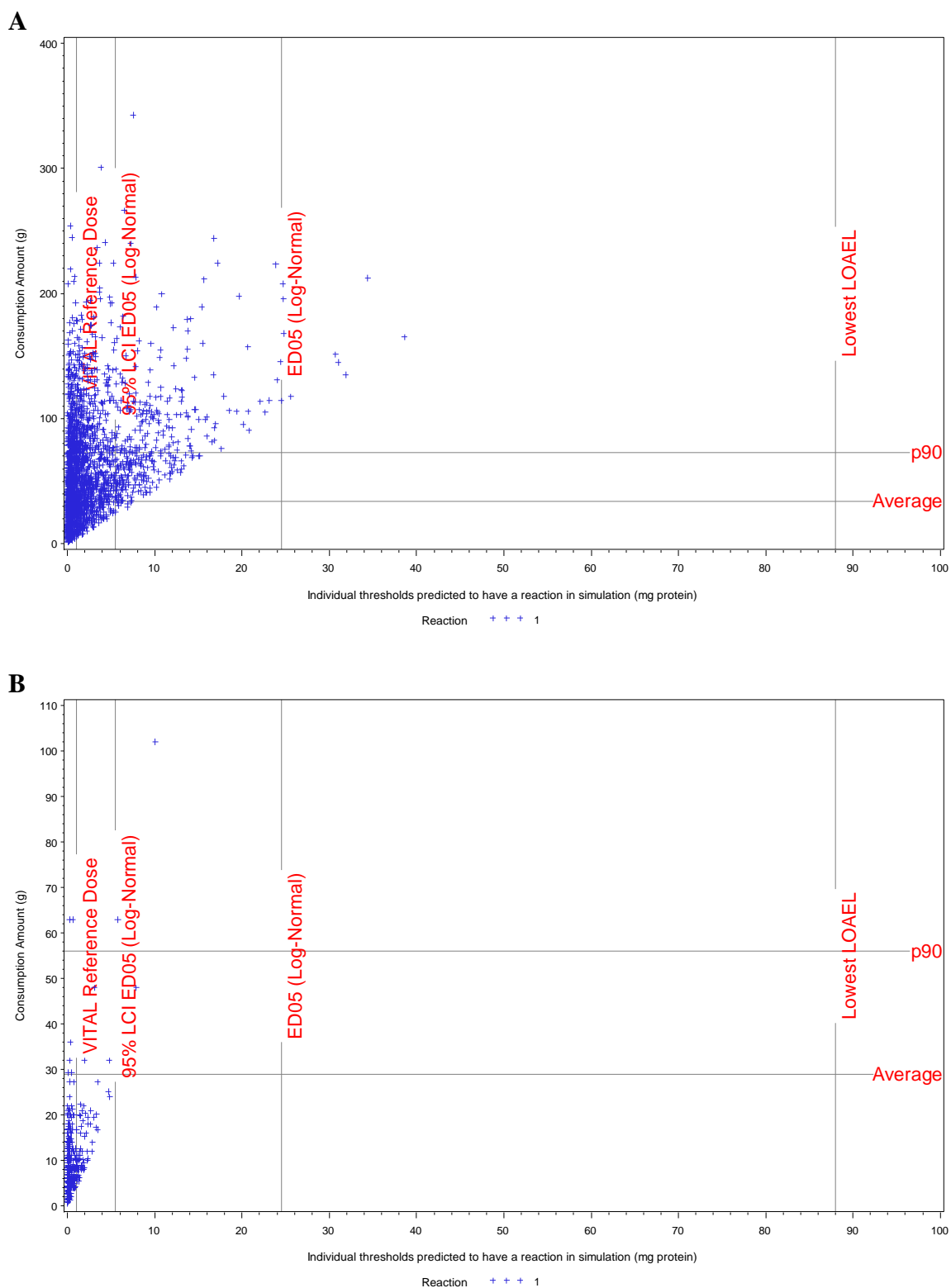


Figure 3 – Thresholds of predicted reactions expressed as mg protein with the predicted consumption for each reaction: (A) Soy in Wheat Simulation, (B) Chocolate Chip Cookie Simulation. Relevant reference doses and threshold levels are labeled.

Table 6 – Stream analysis of milled wheat samples in ppm soy flour with mass balance calculated in mg soy. Significant differences within streams or mg soy are denoted with different superscript letters.

	Total fraction weight (g)	Percent total wheat flour	Average PPM soy protein	Percent total soy	Average mg soy protein
1st Break Flour	114 ± 3 g	6 ± 0%	15 ± 3 ppm ^A	0.1 ± 0.0%	2 ± 0.4 mg ^A
2nd Break Flour	227 ± 8 g	11 ± 0%	63 ± 14 ppm ^A	1 ± 0%	16 ± 4 mg ^A
3rd Break Flour	55 ± 1 g	3 ± 0%	1120 ± 195 ppm ^D	6 ± 2%	88 ± 15 mg ^B
1st Reduction Flour	727 ± 11 g	36 ± 1%	329 ± 58 ppm ^B	17 ± 3%	247 ± 41 mg ^E
2nd Reduction Flour	296 ± 19 g	15 ± 1%	615 ± 183 ppm ^C	14 ± 3%	196 ± 59 mg ^D
3rd Reduction Flour	82 ± 10 g	4 ± 0%	1240 ± 256 ppm ^D	9 ± 3%	131 ± 27 mg ^C
Bran	352 ± 10 g	17 ± 1%	564 ± 244 ppm ^C	18 ± 11%	213 ± 93 mg ^{D,E}
Shorts	186 ± 9 g	9 ± 0%	2780 ± 704 ppm ^E	35 ± 11%	581 ± 149 mg ^F

CHAPTER 5: QUANTITATIVE ANALYSIS OF INDIVIDUAL SOY

ALLERGENS IN PROCESSED SOY PRODUCTS

Benjamin C. Remington^a, Joseph L. Baumert^a, Renu Nandakumar^b, and Steve L. Taylor^a

^a Food Allergy Research & Resource Program, Department of Food Science & Technology, University of Nebraska-Lincoln, Lincoln, NE, USA

^b Proteomics and Metabolomics Core Facility, Department of Biochemistry, University of Nebraska-Lincoln, Lincoln, NE, USA

Abstract

The food industry utilizes different varieties of soy flours and protein products for a number of purposes in a wide range of available products. The traditional methods to modify the functional properties of soy products include adjustment of the protein content, heat denaturation, full or partial protein hydrolysis, and pH adjustment. Newer methods include jet-cooking/flash cooling, high pressure treatment, and different solvent extractions. Due to the widespread use of soy, it is necessary to understand the effects that these treatments have on the different proteins found in soybeans, including the allergens, with respect to protein identification and IgE binding capabilities. In this study, allergens were quantified in all major forms of soy available to the food industry and IgE binding studies using sera from soy-allergic individuals were conducted on these same products. The results from this study show that LC-MS/MS is a viable method to identify the majority of allergenic soy proteins. Fourteen known allergens were identified in the soy product samples, including all subunits of Gly m 5 (β -conglycinin) and Gly m 6 (glycinin), the Kunitz trypsin inhibitor, Gly m Bd 28K, and Gly m Bd 30K. Additional method refinements or different techniques might be necessary to detect low abundance proteins such as Gly m 3 and 4. IgE binding patterns could not be clearly correlated to clinical histories and symptoms as patients with similar histories could exhibit intense binding or no binding at all. However, when IgE binding was present, the relative amount of an allergen in a sample correlated positively to the intensity of IgE binding to proteins at the expected molecular weight.

1. Introduction

Soybeans are a staple food in many cultures and play a large role in the diet of food-producing animals (Barać et al., 2004; Friedman and Brandon, 2001). Studies estimate that 4 – 10% of children and 3 – 4% of adults are affected by food-induced allergic reactions (Branum and Lukacs, 2009; Osborne et al., 2011; Rona et al., 2007; Sicherer and Sampson, 2010). Soy is one of the most common allergenic foods in children, and soy allergy can be severe (Sicherer and Sampson, 2010). Soy-allergic consumers are advised to adhere to a strict avoidance diet and carefully read ingredient labels of all foods (Hefle et al., 2007; Pieretti et al., 2009; Taylor et al., 1986).

A typical dry soybean is composed of 40% protein, 20% oil, 35% carbohydrates, and 5% ash (Liu, 2004). Previous research quantified 8 soybean allergens in varieties of unprocessed soybeans using tandem mass spectrometry-based multiple reaction monitoring (MRM) (Houston et al., 2011). Gly m 3 and Gly m 4 were not detected but have respectively been found by others to be present at 0.6 – 0.8% and 0.01 – 0.15% of total soy protein (Amnuaycheewa and Gonzalez de Mejia, 2010; Houston et al., 2011; Julka et al., 2012; Mittag et al., 2004). Natural variability exists in allergen expression among soy varieties (Houston et al., 2011; Nordlee, 1995), but environmental effects have been shown to affect protein expression patterns with greater significance than breeding differences (Stevenson et al., 2012). Different varieties of soy can be grown to contain up to 48% protein, but the easiest way to obtain higher protein levels is to process the bean into defatted soy flour, soy protein concentrate (SPC), or soy protein isolate (SPI). Most soy flours are made by grinding dehulled and defatted soy flakes, containing at least 50% protein (dry basis), and are traditionally used as an ingredient in the baking

industry. Soy protein concentrates (SPC) are made by removing the soluble sugars from the defatted flake with an aqueous alcohol extraction, contain at least 65% protein, and are used to bind water while adding protein and textural characteristics to many different products. Soy protein isolates (SPI) have the soluble and insoluble carbohydrates removed from the defatted flake, contain at least 90% protein, and are used for gelation, emulsification, water binding, viscosity, foaming, and whipping (Egbert, 2004). Soy flours, SPC, and SPI are used in many different products ranging from protein shakes to soups, baked products, meats, and cheeses. Soy ingredients can be used for protein fortification, but other applications include improving texture, gelation, emulsification, water binding, viscosity, foaming, and whipping. Soy sauce and hydrolyzed vegetable protein are used for flavor enhancement. The traditional methods to modify the functional properties of soy products include adjustment of the protein content, heat denaturation, full or partial protein hydrolysis, and pH adjustment. Newer methods include jet-cooking/flash cooling, high pressure treatment, and different solvent extractions (Egbert, 2004). Due to the widespread use of soy, it is necessary to understand the effects that these treatments have on the different proteins found in soybeans, including the allergens, with respect to protein identification and IgE binding capabilities.

Serum IgE binding to at least 16 soy proteins has been shown in soy-sensitive individuals with atopic dermatitis (Ogawa et al., 1991). There are six proteins in soy recognized as allergens by the International Union of Immunological Societies (IUIS) including two soy hull proteins (Gly m 1 and Gly m 2), profilin (Gly m 3), a stress induced, pathogenesis-related starvation associated message protein (Gly m 4), β -conglycinin (Gly m 5), and glycinin (Gly m 6). In addition to the official IUIS allergens,

multiple other proteins, not recognized by the IUIS, have been shown to cause reactions or bind IgE from soy-allergic individuals. These proteins include the vacuolar protein P34 or Gly m Bd 30 K, Gly m Bd 28 K, the Kunitz trypsin inhibitor, and lectin. More research is needed to investigate the production processes of soy milks (SM), SPIs, and other soy products to help understand how processing affects the detectable protein levels and IgE binding characteristics of allergens present. In this study, allergens were quantified in all major forms of soy available to the food industry including full-fat flours, defatted flours, soy protein concentrates, soy protein isolates, and multiple soy milks and drinks. Additionally, samples associated with reactions specifically associated with Gly m 4-sensitive patients in the United States and Europe were analyzed to investigate if a difference in protein levels and IgE binding patterns is present in the final products.

2. Methods and Materials

Soy products used in study

Sixteen soy protein ingredients or drink products were acquired for allergen characterization by liquid chromatography-tandem mass spectrometry (LC-MS/MS) relative protein quantification and human IgE binding studies (Table 1). Samples believed to be representative of the range of soy proteins used in the food industry were provided by a large soy protein ingredient supplier. Additional samples implicated in soy-allergic reactions were provided by clinical allergists or purchased from local stores in Lincoln, Nebraska, U.S.

Table 1. Soy products acquired for allergen evaluation.

Category	Product Name	Description	Info From	Acquired Through
Defatted Soy Flour	SF1	Minimally dry heat processed and most nearly resembles the native defatted fraction of raw soybeans.	Product information sheet	Soy protein supplier
	SF2	Moderately dry heat treated with its greatest use in bakery and cereal applications.	Product information sheet	Soy protein supplier
	SF3	Fully dry heat treated and used in cookies, crackers, cereals, beverages, calf milk replacers, and fermentation media.	Product information sheet	Soy protein supplier
Full Fat Soy Flour	SF4	Stone ground, not heat treated. Germ, bran and natural oils are fully preserved.	Product website	Retail Purchase
Soy Protein Concentrate	SPC1	A traditional alcohol washed SPC manufactured to remove soluble sugars and reduce the anti-nutritional factors.	Product information sheet	Soy protein supplier
	SPC2	Manufactured by an alcohol wash with additional (unknown) steps and an acid precipitation.	Product information sheet	Soy protein supplier
	SPC3	A water-washed soy protein concentrate with a low flavor profile and high protein solubility.	Product information sheet	Soy protein supplier
Soy Protein Isolate	SPI1	A SPI with a pH drop. It is a medium- to low-viscosity protein that is dispersible in water or other liquid systems.	Product information sheet	Soy protein supplier
	SPI2	A hydrolyzed SPI. It is specially processed for applications where a very low-viscosity protein is desired.	Product information sheet	Soy protein supplier
	SPI3	A specially processed SPI product used for its non-water binding properties. It is used in the manufacture of natural soy cheese.	Product information sheet	Soy protein supplier
	SPI4	A functional product with no modifications. It is a soluble, dispersible product developed for use in food systems where a highly functional protein is required.	Product information sheet	Soy protein supplier
Soy Milk	8 th Continent	Manufactured with an unknown SPI as its protein source. Specific production methods are unknown. Purchased for comparison to Alpro™ Soya.	Ingredient List	Retail Purchase
	SILK® Original	Manufactured in the U.S. and made with filtered whole soybeans. Implicated in U.S. reactions to soy and purchased for comparison to Alpro™ Soya.	Ingredient List	Retail Purchase
	Alpro™ Soya	Manufactured in Europe, made with filtered whole soybeans, and implicated in Gly m 4 related reactions.	Ingredient List	Allergist Provided
Other Products	Almased®	A SPI/cow's milk/honey nutritional drink that has been implicated in Gly m 4 related reactions. It originated in Europe and is sold in the U.S. as well. The SPI used in Almased is produced through cold-pressed separation of the oils from the soy flakes, protein extraction at pH 8-8.5, neutralization, and spray drying without an acid precipitation of the proteins (Kleine-Tebbe et al., 2002; Mittag et al., 2004). There is an additional modification step, however, that is confidential information and not released by the manufacturer.	(Kleine-Tebbe et al., 2002; Mittag et al., 2004)	Retail Purchase
	Soybean Powder (SBP)	A specially designed product for use in dairy systems. This product is made from de-hulled organic whole soybeans which are finely ground in a high-temperature aqueous environment and then spray dried.	Product information sheet	Soy protein supplier

Relative Quantification of Soy Allergens using Mass Spectrometry

Protein Digestion

Soy product samples were dissolved and acetone precipitated before digestion. Briefly, 500µg of protein was precipitated by cold acetone (-20°C) added (80 µL) at four times the largest sample volume in a polypropylene tube. Samples were vigorously stirred, incubated for 60 minutes at -20°C, and centrifuged for ten minutes at 13,000 x g. The supernatant was decanted and the protein pellet was dried at room temperature for 30 minutes. The protein pellet was reconstituted in 20 µL of 100 mM ammonium bicarbonate (AMBC) and 6M urea. Proteins were reduced with 1 µL of 15% dithiothreitol (w/v) in 100 mM AMBC at 25°C for 1 hour. Alkylation of proteins was achieved with the addition of 20 µL of 3.6% iodoacetamide in 100 mM AMBC and incubation for 1 hour in the dark at room temperature. Leftover alkylating agent was consumed by the addition of 4 µL of 15% dithiothreitol (w/v) in 100 mM AMBC and incubation for 45 minutes at room temperature. Urea concentrations were diluted to less than 1M through buffer exchange. Briefly, samples were transferred to Amicon® Ultra 3K centrifugal filter concentrators (UFC500396, Millipore, Billerica, MA) and buffer exchange done with 200µL of 50 mM AMBC. Samples were centrifuged for 14,000 x g for 10 min and the flow through buffer was discarded. The washing process was repeated 4 times. Exchanged samples are collected into clean microfuge tubes by reverse centrifugation at 1,000 x g for 2 minutes. Samples were then subjected to a 2-step multi-enzyme digestion. Sequencing grade endoproteinase Glu-C from *Staphylococcus aureus* (1µg/µL in 50 mM AMBC) (P6181, Sigma-Aldrich, St. Louis, MO) was added at a 1:50 enzyme to protein ratio (10 µL per sample) and incubated for 3-6 hours at 25°C.

Sequencing grade trypsin from porcine pancreas (1 µg/µL in 50 mM AMBC) (T6567, Sigma-Aldrich, St. Louis, MO) was added at a 1:20 enzyme to protein ratio (25 µL per sample) and digested overnight at 37°C.

LC-MS/MS analysis

LC-MS/MS was performed with an Ultimate® 3000 Dionex MDLC system (Dionex Corporation, USA) integrated with a nanospray source and LCQ Fleet Ion Trap mass spectrometer (ThermoFinnigan, USA). LC-MS/MS included on-line sample pre-concentration and desalting using a monolithic C₁₈ trap column (Pep Map, 300 µm I.D x 5mm, 100 Å, 5 µm, Dionex). Samples were loaded onto the monolithic trap column at a flow rate of 40 µl/min. The desalted peptides were eluted and separated on a C₁₈ Pep Map column (75 µm I.D x 15 cm, 3 µm, 100 Å, New Objective, USA) by applying an acetonitrile (ACN) gradient (ACN plus 0.1% formic acid, 90 minute gradient at a flow rate of 300 nl/min) and were introduced into the mass spectrometer using the nano spray source. The LCQ Fleet mass spectrometer was operated with the following parameters: nano spray voltage, 2.0 kV; heated capillary temperature, 200°C; full scan m/z range, 400-2,000). The mass spectrometer was operated in data dependent mode with 4 MS/MS spectra for every full scan, 5 microscans averaged for full scans and MS/MS scans, a 3 m/z isolation width for MS/MS isolations, and 35% collision energy for collision-induced dissociation.

Database analysis

The MS/MS spectra were searched against the *Glycine max* protein sequence database using MASCOT (Version 2.2 Matrix Science, London, UK). Database search criteria were as follows: enzyme: endoproteinase Glu-C/Trypsin, missed cleavages: 2;

mass: monoisotopic; fixed modification: carbamidomethyl (C); variable modification: oxidation (M); peptide tolerance: 2Da; MS/MS fragment ion tolerance: 1Da. Probability assessment of peptide assignments and protein identifications were accomplished by Scaffold (Scaffold 3.0 Proteome Software Inc., Portland, OR). Criteria for protein identification included detection of at least 1 unique identified peptide and a peptide and protein probability score of ≥ 90 . Relative quantitation of the proteins was done based on the label-free method of spectral counting using the normalized spectral counts for each protein.

IgE binding studies with soy sensitive subjects

Human Sera

Serum samples were collected from consenting individuals with a positive clinical food challenge to soybean or a convincing history of allergic reactions to soybean. All sera were collected under Institutional Review Board oversight at their respective clinical institutions. Sera from 31 soybean-allergic individuals and one control subject without soybean allergy were used in this study (Table 2). The allergic patients utilized in this study had soybean specific IgE level ranging from <0.35 -34.8 kU/L as measured by ImmunoCAP[®]. Representative sera from 8 individuals were chosen to represent all 32 sera due to their characteristic binding patterns (Table 3). The control subject used in this study has no reported food allergies.

Table 2. Sera from 31 soybean-allergic individuals and one control subject without soybean allergy used to investigate IgE binding patterns to a spectrum of soy ingredients and products.

Subject	Soy CAP	Gly m 4 CAP	Food Challenge	Soy Milk Reactor	Soy Flour Reactor	Peanut Allergic
DDB	34.8		No	Unknown	Yes	No
ACG	3.18		No	Unknown	Yes	No
JENB	<0.35		No	Unknown	Yes	Unknown
MA	<0.35		No	Unknown	Yes	Unknown
HS	0.6		No	Unknown	Yes	No
JB	<0.35		No	Unknown	Yes	Unknown
JL 007	1.22		No	Unknown	Yes	Yes
AB 008			No	Unknown	Yes	Yes
SP902	0.78	17.6	Yes	Yes	No	Yes
SP903	1.55	0	Yes	No	Yes	Yes
SP904	8.96	5.11	Yes	No	Yes	Yes
SP905	4.55	0.44	Yes	Yes	No	No
SP906	1.43	0.5	Yes	No	Yes	No
SP907	<0.35	26.2	Yes	Yes	No	Yes
SP908	<0.35		Yes	Yes	No	No
SP909	9.23	>100	Yes	Yes	No	Yes
219	14.1		No	Yes	No	Unknown
791			No	Yes	No	Unknown
450	4.4		No	Yes	No	Unknown
681	0.35	9.81	Yes	Yes	No	No
422	7.65	0.13	No	Yes	No	Yes
42	<0.35	16.2	No	Yes	No	Yes
48	<0.35	14.9	No	Yes	No	No
925	0.35	3.62	No	Yes	No	No
339	0.35	5.69	No	Yes	No	Yes
893	<0.35	5.02	No	Yes	No	No
668	0.9	11.4	No	Yes	No	No
155	0.6	20.6	No	Yes	No	Yes
138	<0.35	5.79	No	Yes	No	Yes
32	0.35	28.8	Yes	Yes	No	No
579	<0.35	14.6	No	Yes	No	No
Control 1	<0.35			No	No	No

Table 3. Representative soy-allergic sera used in the IgE binding study.

Patient number	Age/ Gender	Reaction History	Soy CAP (kU/L)	Gly m 4 CAP	Gly m 5 CAP	Gly m 6 CAP	Bet v 1 ISAC	Bet v 2 ISAC	Gly m 4 ISAC	Gly m 5 ISAC	Gly m 6 ISAC	Peanut Allergic	Reasoning for Inclusion
668	50/F	Anaphylactic History	0.9	11.4	<0.35	<0.35	29.8	6.5	2.2	0	0	No	Clear History of Allergy to Soy Milk
422	19/F	Anaphylactic History	7.7	<0.35	4.8	7.8	0	0	0	2.8	7.4	Yes	Clear History of Allergy to Soy Milk
681	78/M	Food Challenge	0.35	9.8	<0.35	<0.35	47.9	0	1.6	0	0	No	Challenge Pos. History to Soy Milk
SP902	22/F	Food Challenge	0.8	17.6			41.5	0	0	0	0	Yes	Neg. Soy Flour Challenge, Pos. Soy Milk Challenge
SP909	61/M	Food Challenge	9.2	>100			124.9	0	66.0	0	0	Yes	Neg. Soy Flour Challenge, Pos. Soy Milk Challenge
SP903	27/F	Food Challenge	1.6	0			0	0	0	0	2.8	Yes	Pos. Soy Flour Challenge
ACG	32/F	Clear Mild Reaction History	3.18				180.0	0	9.4	0	0	No	Pos. Soy CAP
893	44/M	Clear Mild Reaction History	<0.35	5.0	<0.35	<0.35	15.7	0	0.6	0	0	No	Neg. Soy CAP
Control 1	58/F	None	<0.35				0	0	0	0	0	No	Control Sera

Extraction of soy proteins

Soluble proteins from the obtained soy samples were extracted 1:10 (w/v) in 0.01 M phosphate buffered saline (PBS; 0.002 M NaH₂PO₄, 0.008 M Na₂HPO₄, 0.85% NaCl, pH 7.4) overnight with gentle rocking at room temperature. Extracts were centrifuged for 30 minutes at 4100 × g in a tabletop centrifuge at 10°C. The supernatant was aliquoted in 1 mL intervals and stored at -20°C. The protein content of the extracts was determined by the micro BCA protein method (Smith et al., 1985) (23252, Pierce Biotechnology, Inc., Rockford, IL).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein separation by SDS-PAGE was done with a Bio-Rad Mini-Protein[®] Tetracell electrophoresis unit (Bio-Rad Laboratories, Hercules, CA). For non-reduced samples, 25 µg each respective soy sample were boiled for 5 minutes in Laemmli sample buffer and separated on a 12% Mini-PROTEAN[®] TGX[™] Tris-HCl precast gel (Bio-Rad Laboratories, Hercules, CA) at 200V (constant voltage) for 30 minutes. For reduced samples, 25 µg of each sample were boiled in Laemmli sample buffer containing 5.4% dithiothreitol (w/v) and separated on a 12% Mini-PROTEAN[®] TGX[™] Tris-HCl precast gel (Bio-Rad Laboratories, Hercules, CA) at 200V (constant voltage) for 30 minutes. Protein stained gels were fixed in a solution of 60% trichloroacetic acid and 17.5% 5-sulfosalicylic acid (F7264-500ML, Sigma-Aldrich, St. Louis, MO), stained with Coomassie Brilliant Blue R-250 Staining Solution (161-0436, Bio-Rad Laboratories, Hercules, CA), and destained with Coomassie Brilliant Blue R-250 Destaining Solution (161-0438, Bio-Rad Laboratories, Hercules, CA). Gel images were captured using a

Kodak Gel Logic 440 Imaging System (Eastman Kodak, Rochester, NY) equipped with Carestream Molecular Imaging software (v5.0.2.30, Carestream Health, Rochester, NY).

Immunoblotting procedures

As described above, 25 µg of protein from each sample was separated by SDS-PAGE. After electrophoresis, the proteins were transferred onto a polyvinyl difluoride (PVDF) membrane using the Trans-Blot[®] Turbo[™] Transfer Pack Midi format (170-4147, Bio-Rad Laboratories, Hercules, CA) and Trans-Blot[®] Turbo[™] Transfer System at 25V (limit voltage), 2.5A (constant amperage) for 10 minutes. The membrane was then blocked by incubation with 0.01 M PBS, pH 7.4 containing 0.05% Tween 20 (PBS-T) and 5% nonfat dry milk (NFDM) for 2 hours at room temperature. Individual human sera were diluted 1:10, in 2.5% NFDM in PBS-T, blocked for 1 hour, and then incubated with the blocked membrane overnight at room temperature. Unbound antibody was removed from the membranes by washing with PBS-T, four repetitions, 2 minutes each with vigorous shaking. Bound IgE was detected using monoclonal horseradish peroxidase (HRP) conjugated anti-human IgE (9160-05, clone B3102E8, SouthernBiotech, Birmingham, AL), diluted 1:1000 with 2.5% NFDM in PBS-T. Unbound secondary antibody was removed from the membranes by washing with PBS-T, four repetitions, 2 minutes each with vigorous shaking. Supersignal West Dura Extended Duration substrate (34076, Pierce, Rockford, IL, USA) was used for detection. Membrane images were captured using a Kodak Gel Logic 440 Imaging System (Eastman Kodak, Rochester, NY) equipped with Carestream Molecular Imaging software (v5.0.2.30, Carestream Health, Rochester, NY).

3. Results and Discussion

LC-MS/MS results

Endoproteinase Glu-C and trypsin digested proteins were analyzed by LC-MS/MS. A single sample of each soy product was digested and analyzed in a single replication. A MASCOT search of the *Glycine max* protein database assigned over 600 proteins with at least 1 unique identified peptide and a peptide and protein probability score of ≥ 90 . Approximately 66% of identified proteins are “putative uncharacterized proteins” and 33% are known proteins. Fourteen known allergens were identified in the soy product samples, including all subunits of Gly m 5 (β -conglycinin) and Gly m 6 (glycinin), the Kunitz trypsin inhibitor, Gly m Bd 28K, and Gly m Bd 30K (Table 2). Additionally, profilin can be identified in one sample, SF3, when the peptide probability threshold is lowered to >0 and the protein probability score is lowered to ≥ 20 . Gly m 4 was not identified by relative quantification. Figure 1 shows the normalized spectral counts for each allergen. The subunits of seed storage proteins Gly m 5 and 6 accounted for the majority of the 10 most abundant proteins, allergens and non-allergens included, in all tested products. The G1 subunit of Gly m 6 is the most abundant protein in all 16 product samples. The G2 subunit of Gly m 6 is the 2nd most abundant protein in 13 of 16 product samples, 3rd most abundant in 1 of 16 samples, and 4th most abundant in 1 of 16 samples. The α -subunit of Gly m 5 is the 2nd most abundant protein in 2 of 16 product samples, 3rd most abundant protein in 13 of 16 samples, and 4th most abundant in 1 of 16 samples. Eleven allergens were identified at least 13 products. However, proteins less frequently identified included Gly m Bd 28K and Kunitz trypsin inhibitor (KTI) B, in 6 products and Gly m 6 subunit G3, in 9 products.

Table 4 – Allergenic proteins of interest in soybean during relative quantification by LC-MS/MS.

Allergen		Number of Products Identified In (Out of 16)	Accession Number
Gly m 3		1	3021373
Gly m 4		0	134194
Gly m 5	α subunit	16	Q948X9
	α' subunit	14	Q4LER6
	β subunit	15	P25974
Gly m 6	G1	16	P04776
	G2	16	18609
	G3	9	18639
	G4	16	Q9S9D0
	G5	16	P93707
Kunitz Trypsin Inhibitor Isoforms	KTI A	13	18770
	KTI B	6	P01071
	KTI 1	14	P25272
	KTI 2	15	P25273
Gly m Bd 28K		6	187766751
Gly m Bd 30K		16	O64458

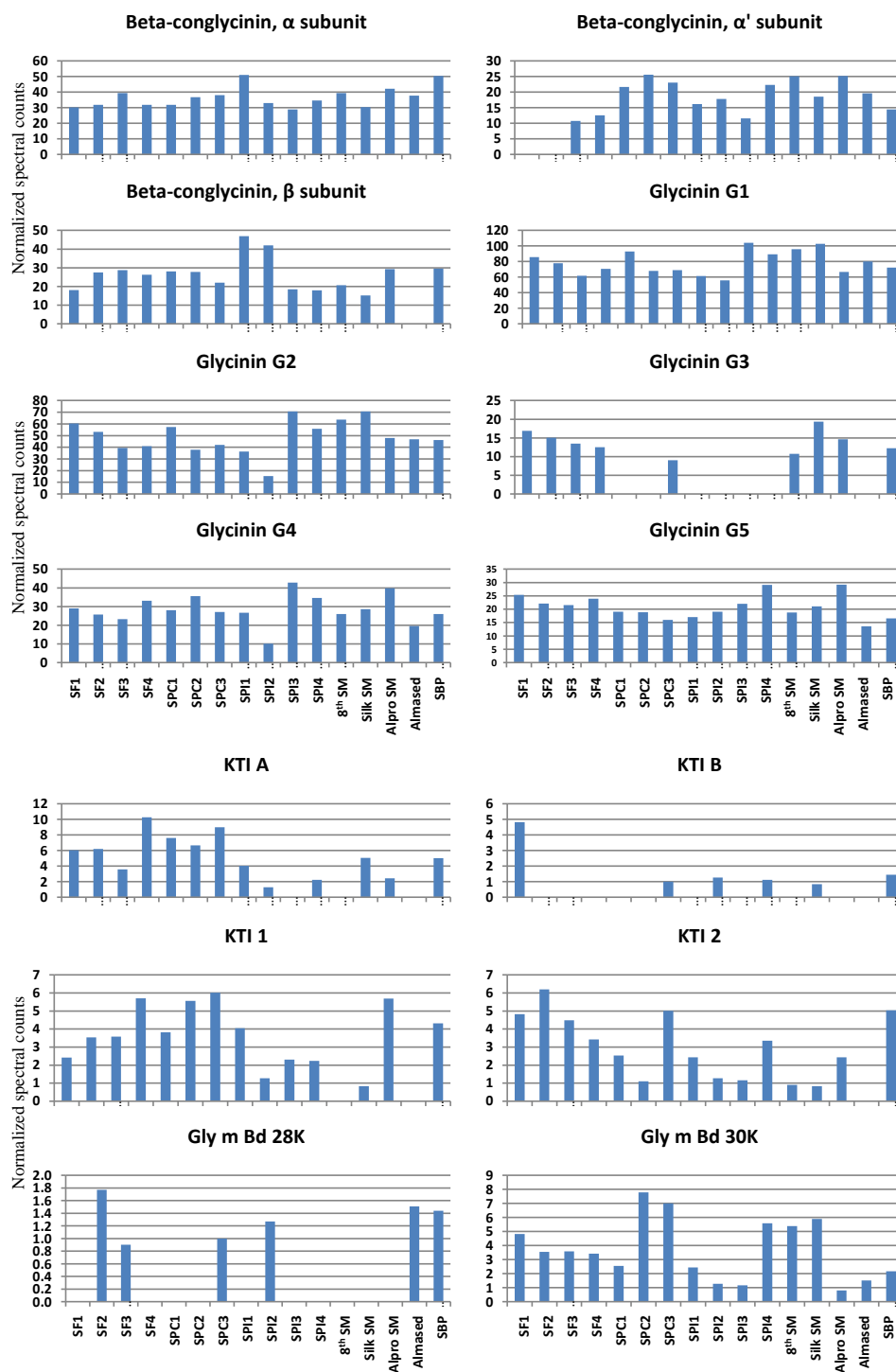


Figure 1 – Relative quantitation of 14 soy allergens in 16 soy ingredients and products by LC-MS/MS after endoproteinase Glu-C/Trypsin digestion. Criteria for protein identification included detection of at least 1 unique identified peptide and a peptide and protein probability score of ≥ 90 . Relative quantitation of the proteins was done based on the label-free method of spectral counting using the normalized spectral counts for each protein.

Profiles of individual soy ingredients and products show slight variations in frequency and abundance of individual proteins identified (Figure 2). Defatting and varying heat treatments did not significantly affect the proteins expressed in available soy flours. Additionally, the processing involved in creating the soybean powder did not alter the protein profile from the less processed soy flours. Glycinin G3 was identified in all of the soy flours, soy milks, and soybean powder, but not in the 4 SPIs tested. Additionally, glycinin G3 was not identified in the alcohol washed SPCs 1 and 2, but was identified in the water washed SPC3. Gly m Bd 28K is the least abundant identified allergen. Similar to glycinin G3, Gly m Bd 28K was not identified in the alcohol washed SPCs but was identified in the water washed SPC. Gly m Bd 28K was not identified in less processed soy flours, SFs 1 and 4, but was identified in high heat treated samples, SFs 2 and 3.

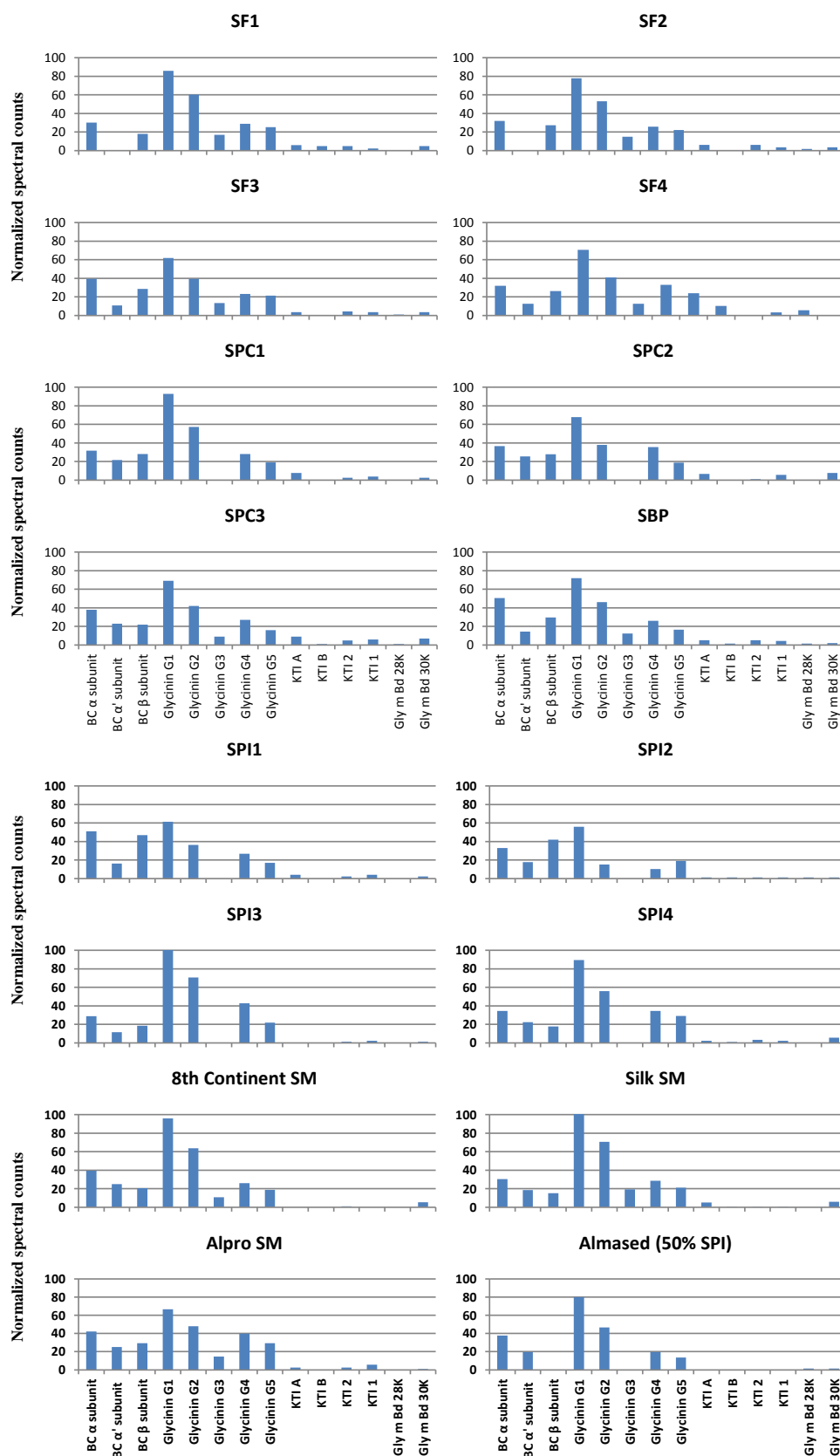


Figure 2 – Relative quantitation of soy allergens grouped by individual products analyzed with LC-MS/MS after endoproteinase Glu-C/Trypsin digestion. Criteria for protein identification included detection of at least 1 unique identified peptide and a peptide and protein probability score of ≥ 90 . Relative quantitation of the proteins was done based on the label-free method of spectral counting using the normalized spectral counts for each protein.

IgE binding results

Figure 3 shows the protein profile of the soy products and ingredients tested under non-reducing and reducing conditions. The heavy banding present from 50-75 kDa in the non-reduced gels represents the subunits of Gly m 5 and 6. Reduced gels still contain the major Gly m 5 subunits near 70 kDa, but the subunits of Gly m 6 have separated into acidic and basic components clustered around 35 kDa. Hydrolysis has a distinct effect on the protein profile of select soy ingredients known to be subjected to the hydrolysis process (SPI 2 and 3). All soy milks have protein profiles similar to whole soy or minimally processed soy. Almased, a mix of specially processed SPI (50%), cow's milk, and honey, has a distinct protein profile but the effects can be partially attributed to the dilution of soy protein due to cow's milk and honey present in the sample. Brown rice was included as negative control sample. Navy bean was included as a 2nd negative control sample and as an indicator of possible cross-reactive carbohydrate (CCD) epitopes bound by allergic individuals (Panda, 2012).

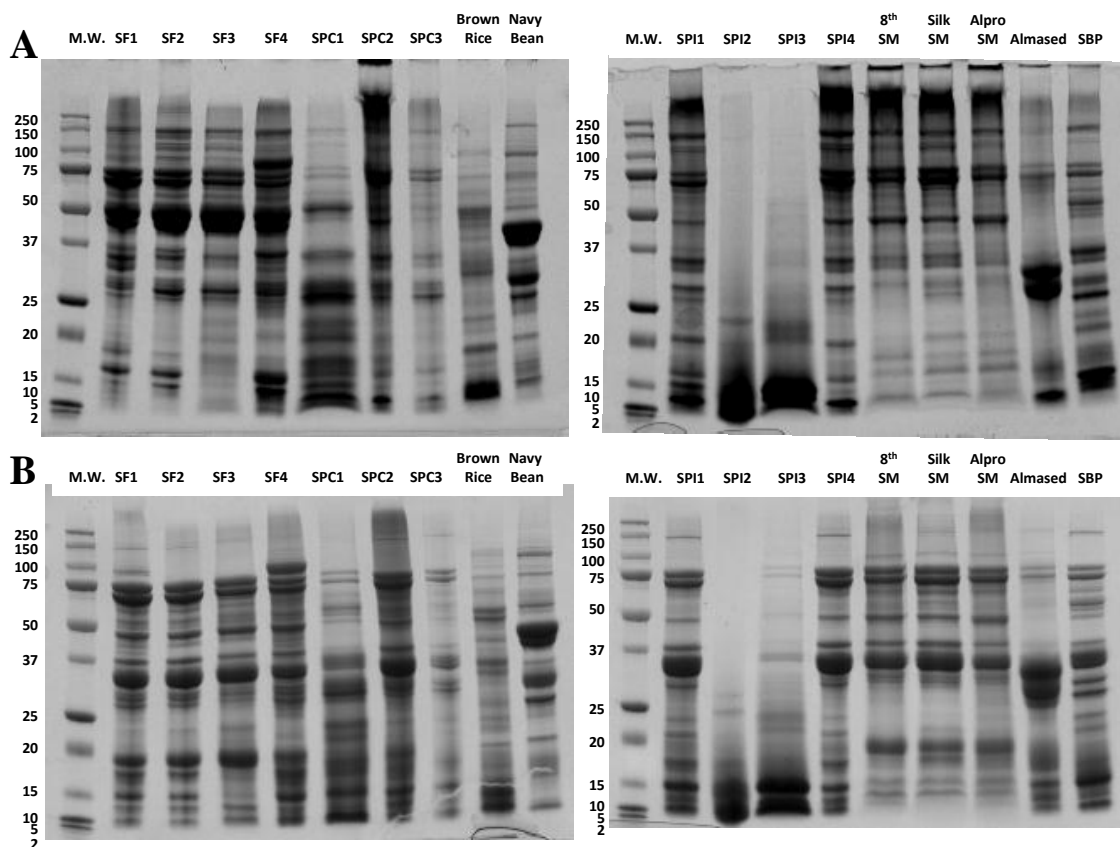


Figure 3 – SDS-PAGE gels of soy products under non-reducing (A) and reducing (B) conditions. Sample gels (25 µg extract protein per lane) were run under SDS-PAGE (200V, constant voltage) non-reducing and reducing conditions, fixed, and then stained using Coomassie Brilliant Blue R-250. After destain, gel images were captured using a Kodak Gel Logic 440 Imaging System.

Soy-allergic sera were divided into multiple categories for analysis of binding patterns. Categories included an anaphylactic history to soy, a positive food challenge to soy, or a clear history of mild reaction to soy. Challenge positive individuals were further split into soy flour and soy milk reactors. Each group had individual serum IgE that bound strongly to representative soy samples, while others showed weak or no binding at all. The majority of sera showed binding to navy bean near 35 kDa, indicative of CCD epitopes in soy (Panda, 2012). The 8 soybean-allergic sera in Figures 4-11 were selected from the 31 available soybean-allergic sera for their representative binding patterns.

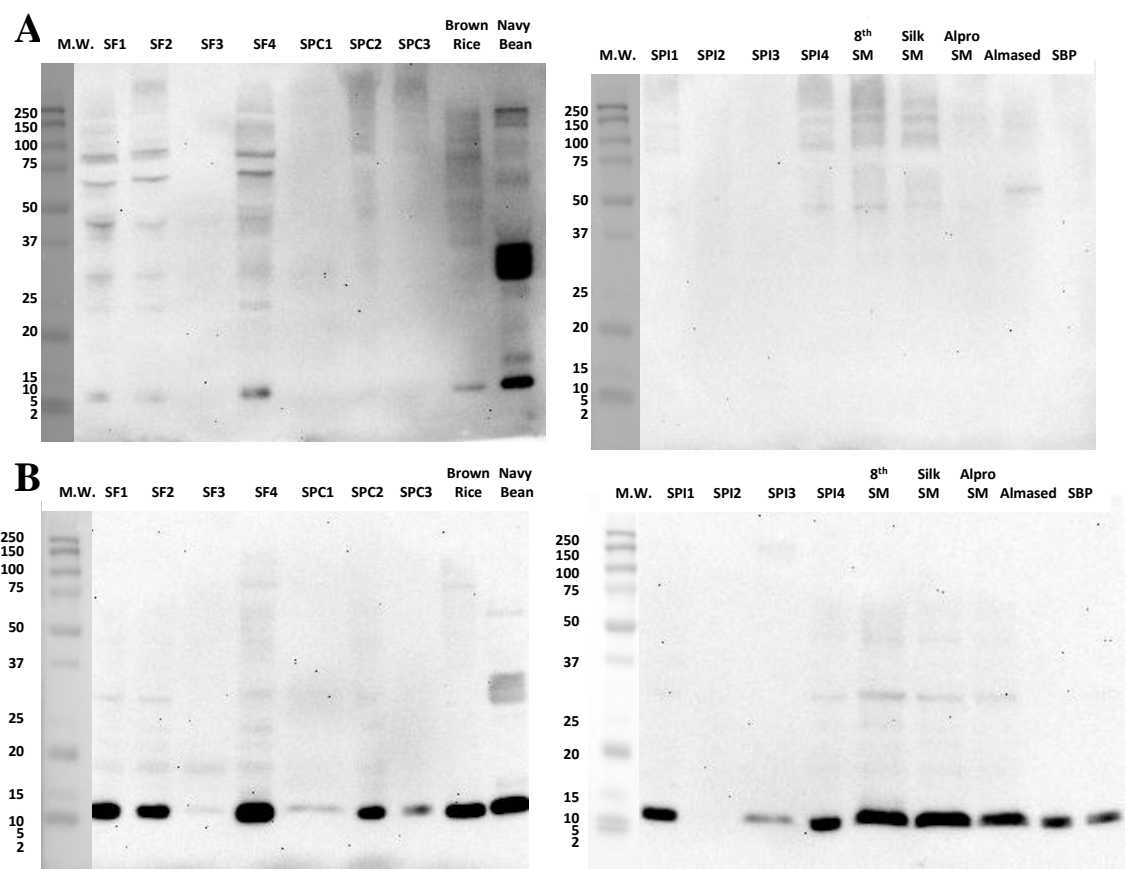


Figure 4 – Serum 668 IgE immunoblot of soy products under non-reducing (A) and reducing (B) conditions. Sample gels (25 µg extract protein per lane) were run under SDS-PAGE non-reducing and reducing conditions and transferred to PVDF membranes. Membranes were blocked in PBS-T with 5% NFDM followed by incubation with serum diluted 1:10. Bound IgE was detected using monoclonal HRP conjugated anti-human IgE diluted 1:1000 in 2.5% NFDM in PBS-T. Chemiluminescent substrate was used for detection and membrane images were captured using a Kodak Gel Logic 440 Imaging System.

Patients 668 and 422 had a history of clear allergic reactions to soy milk and provided sera 668 and 422 (Figure 4 and 5). Serum 668, which had a history of anaphylaxis to soy milk, had low CAP and ISAC scores to Gly m 5 and 6 but had moderate responses to Gly m 4. Low CAP scores for Gly m 5 and 6 correlated to light binding to a number of high and low molecular weight proteins in non-reduced IgE immunoblots (Figure 4). Reducing conditions induced small changes to the binding profile, such as reduction in binding to high molecular weight proteins and an intensified binding to a 12-14 kDa protein. Binding of serum 668 intensified to the 12-14 kDa

protein in both navy bean and brown rice on reduced gels. There is a distinct lack of binding to the 12-14 kDa protein in 4 hydrolyzed and/or highly heat processed soy ingredients (SF3, SPC1, SPIs 2 and 3). One serum (SP905) shared the binding profile of patient 668 to this protein in soy, brown rice, and navy bean. Five sera (DDB, SP902, SP909, 138, 925) bound to a ~15 kDa protein in soy products but not brown rice or navy bean. One serum (SP906) bound strongly to this protein in soy products and brown rice, but not in navy bean. Three sera (ACG, 42, 579) bound to this protein in soy and navy bean, but not brown rice. Four sera (JL 007, 32, 48, SP904) bound to the ~15 kDa protein in rice and/or navy bean, but not soy products. Gly m 3 (14 kDa) has known analogs in rice (Accession No. Q5VMJ3) and navy bean (Accession No. P49231) with high sequence conservation and secondary structure prediction between the 3 proteins (PRALINE multiple sequence alignment, data not shown). Additionally, Gly m 4 (17 kDa) has analogs in rice (Accession No. NP_001049857) and navy bean (Accession No. CAA65727) with moderate sequence conservation but high similarity in secondary structure prediction (PRALINE multiple sequence alignment, data not shown). It is impossible to distinguish between Gly m 3 and 4 based on the data available in the current results. Additional 1-D inhibition binding studies, 2-D binding studies, or basophil histamine release studies could help distinguish if the binding near 15 kDa is to Gly m 3 or 4 and if the binding is clinically relevant. However, since these patients are not reportedly allergic to brown rice or navy bean, the clinical relevance of the binding to these proteins in brown rice and navy bean is questionable. Since patient 668 had a history of an anaphylactic reaction to soy milk, the binding to this protein in soy product extracts may be of greater significance but this requires further evaluation.

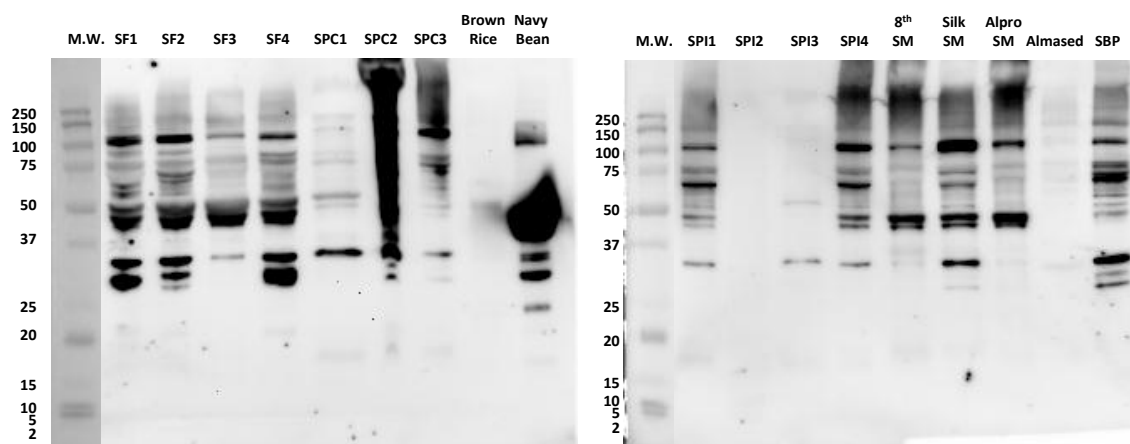


Figure 5 – Serum 422 IgE immunoblot of soy products under non-reducing conditions. Sample gels (25 µg extract protein per lane) were run under SDS-PAGE non-reducing conditions and transferred to PVDF membranes. Membranes were blocked in PBS-T with 5% NFDM followed by incubation with serum diluted 1:10. Bound IgE was detected using monoclonal HRP conjugated anti-human IgE diluted 1:1000 in 2.5% NFDM in PBS-T. Chemiluminescent substrate was used for detection and membrane images were captured using a Kodak Gel Logic 440 Imaging System.

Patient 422 had a history of anaphylaxis to soy milk and is allergic to peanuts.

The serum from this patient (Serum 422) had moderate CAP and ISAC scores to Gly m 5 and 6 but had no response to Gly m 4. Higher CAP and ISAC scores for Gly m 5 and 6 correlated to strong binding to a number of high molecular weight proteins in non-reduced IgE immunoblots (Figure 5). Binding was strong for both the soy flours and soy milks. Again, there was lack of or diminished binding to hydrolyzed SPI, but this may not be clinically significant. Patients 668 and 422 both had a history of anaphylaxis to soy milk, but have very different binding profiles. Patient 668 seemingly reacts to Gly m 4 while patient 422 seemingly reacts to Gly m 5 and 6. While these sera 668 and 422 have binding to a broad range of proteins, sera from 3 other patients (155, 339, 925) with a positive soy CAP and a mild or anaphylactic reaction history had little to no binding to soy proteins under non-reducing conditions (data not shown).

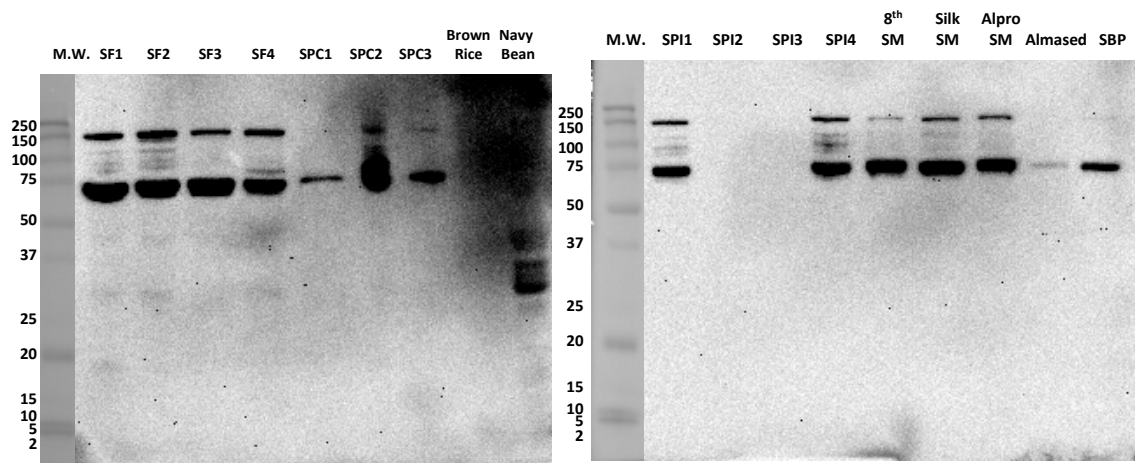


Figure 6 – Serum 681 IgE immunoblot of soy products under non-reducing conditions. Sample gels (25 μ g extract protein per lane) were run under SDS-PAGE non-reducing conditions and transferred to PVDF membranes. Membranes were blocked in PBS-T with 5% NFDM followed by incubation with serum diluted 1:10. Bound IgE was detected using monoclonal HRP conjugated anti-human IgE diluted 1:1000 in 2.5% NFDM in PBS-T. Chemiluminescent substrate was used for detection and membrane images were captured using a Kodak Gel Logic 440 Imaging System.

Patient 681 had a history of positive food challenge to soy milk. Moderate CAP scores to Gly m 4 were observed with serum 681 but the overall soy CAP was low. Curiously, strong binding is present to high molecular weight proteins in the majority of soy products (Figure 6). A low soy CAP did not correlate to low binding to high molecular weights in this serum. Little, if any, binding was noted to proteins in the 17kDa region where Gly m 4 would be expected. The serum from patient 32 had a similar history and CAP scores and showed very weak binding to high molecular weight proteins in soy flour and soy milk (data not shown).

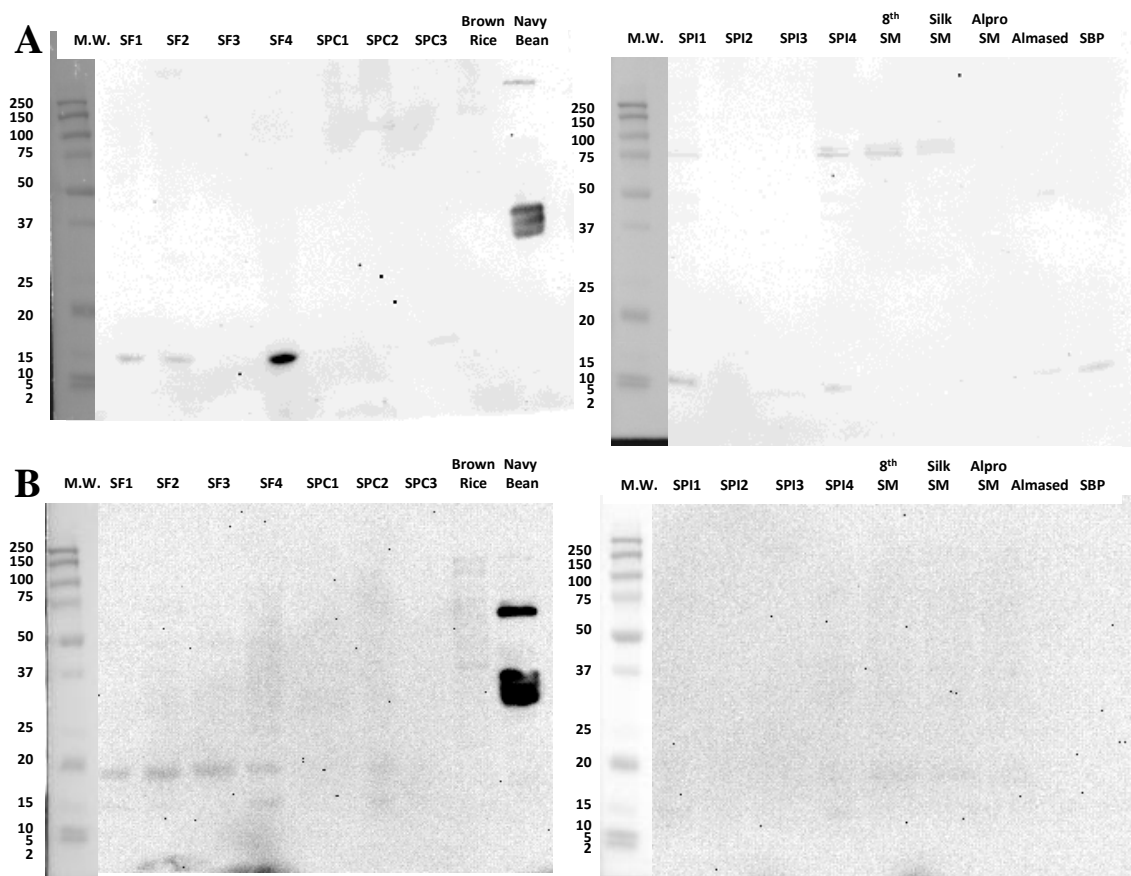


Figure 7 – Serum SP902 IgE immunoblot of soy products under non-reducing (A) and reducing (B) conditions. Sample gels (25 µg extract protein per lane) were run under SDS-PAGE non-reducing and reducing conditions and transferred to PVDF membranes. Membranes were blocked in PBS-T with 5% NFDM followed by incubation with serum diluted 1:10. Bound IgE was detected using monoclonal HRP conjugated anti-human IgE diluted 1:1000 in 2.5% NFDM in PBS-T. Chemiluminescent substrate was used for detection and membrane images were captured using a Kodak Gel Logic 440 Imaging System.

Patient SP902 had a positive challenge to soy milk after passing a soy flour challenge. This individual had low a soy CAP but a high CAP for Gly m 4. The low soy CAP scores are reflected in minimal binding observed with serum SP902 in both the non-reduced and reduced IgE immunoblots (Figure 7). Binding was observed to a ~15 kDa protein in the non-reduced blots and the protein could be Gly m 3 or 4 based on molecular weight. The relatively weak binding observed in these gels is consistent with the low amount of these proteins determined by LC/MS-MS. No pattern can be found for

individuals with this clinical history as Figure 8 represents IgE binding from an individual with similar manifestations of soy allergy.

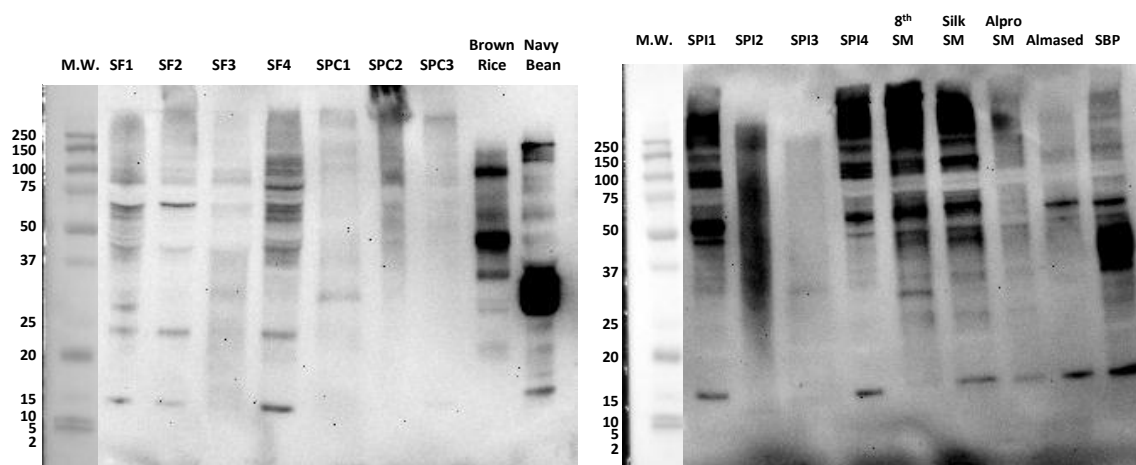


Figure 8 – Serum SP909 IgE immunoblot of soy products under non-reducing conditions. Sample gels (25 µg extract protein per lane) were run under SDS-PAGE non-reducing conditions and transferred to PVDF membranes. Membranes were blocked in PBS-T with 5% NFDm followed by incubation with serum diluted 1:10. Bound IgE was detected using monoclonal HRP conjugated anti-human IgE diluted 1:1000 in 2.5% NFDm in PBS-T. Chemiluminescent substrate was used for detection and membrane images were captured using a Kodak Gel Logic 440 Imaging System.

Similarly, patient SP909 had a positive challenge to soy milk after passing a soy flour challenge. This individual had a moderate soy CAP score and an extremely high Gly m 4 CAP (>100). Strong binding was observed to a number of high molecular weight proteins in soy milks and isolates, including the hydrolyzed SPI2, but this same intensity was not observed in soy flours and concentrates (Figure 8). Binding to a ~15 kDa protein was observed in 9 soy products and this protein could be Gly m 3 or 4, but is most likely Gly m 4 due to this individual's extremely high CAP to Gly m 4. Although this serum is unique with its strong binding to the ~15 kDa protein, strong binding to higher molecular weight proteins in soy milks and protein isolates was observed with one other serum (SP905) that had a positive challenge to soy milk after passing a soy flour challenge.

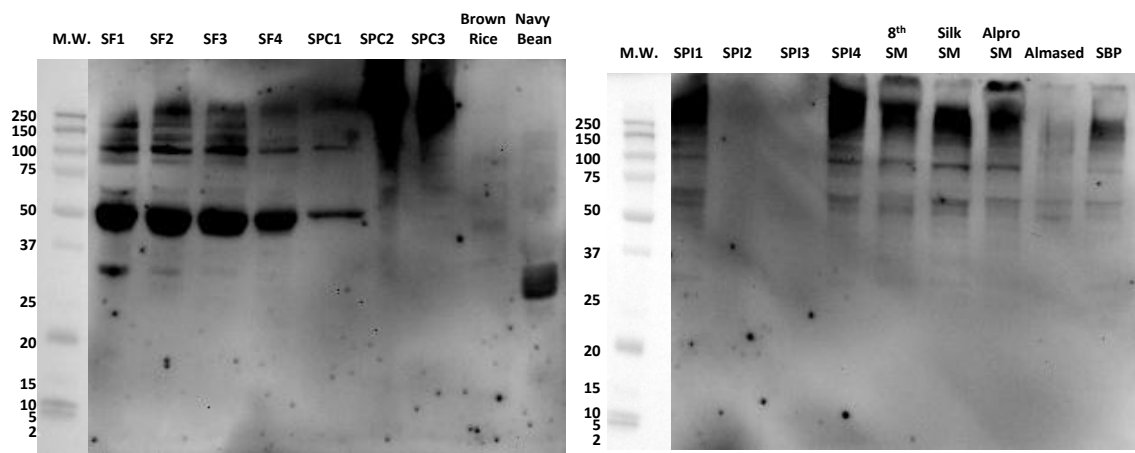


Figure 9 – Serum SP903 IgE immunoblot of soy products under non-reducing conditions. Sample gels (25 µg extract protein per lane) were run under SDS-PAGE non-reducing conditions and transferred to PVDF membranes. Membranes were blocked in PBS-T with 5% NFDm followed by incubation with serum diluted 1:10. Bound IgE was detected using monoclonal HRP conjugated anti-human IgE diluted 1:1000 in 2.5% NFDm in PBS-T. Chemiluminescent substrate was used for detection and membrane images were captured using a Kodak Gel Logic 440 Imaging System.

Patient SP903 had a positive food challenge to soy flour. A low soy CAP was obtained but serum SP903 displayed strong IgE binding to high molecular weight proteins in all soy products except the hydrolyzed protein isolate (Figure 9). A low soy CAP did not correlate to low binding to high molecular weights in the immunoblots using this serum. Two additional individuals (SP904, SP906) with a positive food challenge to soy flour, 1 individual with a history of OAS to soy (791), and 2 individuals with unknown histories but positive soy CAPs (219, 450) shared similar binding profiles.

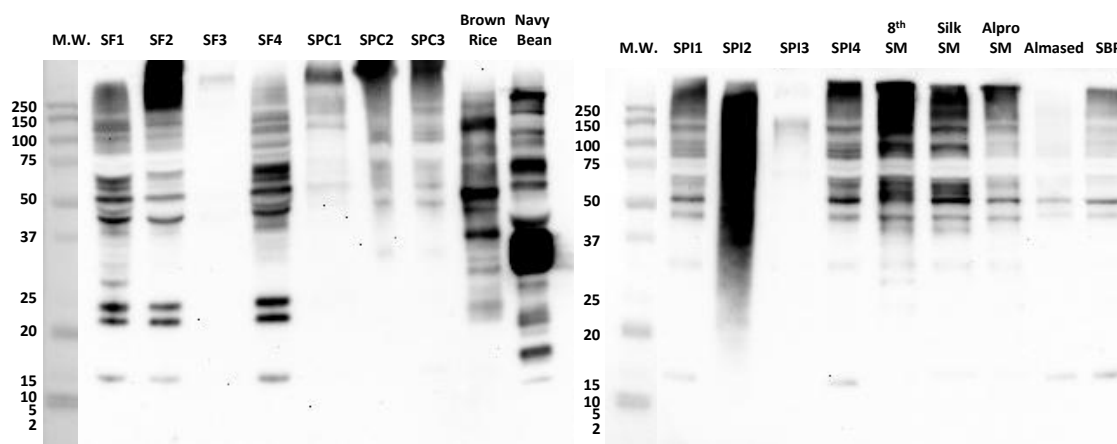


Figure 10 – Serum ACG IgE immunoblot of soy products under non-reducing conditions. Sample gels (25 µg extract protein per lane) were run under SDS-PAGE non-reducing conditions and transferred to PVDF membranes. Membranes were blocked in PBS-T with 5% NFDm followed by incubation with serum diluted 1:10. Bound IgE was detected using monoclonal HRP conjugated anti-human IgE diluted 1:1000 in 2.5% NFDm in PBS-T. Chemiluminescent substrate was used for detection and membrane images were captured using a Kodak Gel Logic 440 Imaging System.

Patient ACG had a positive soy CAP, a positive ISAC to Gly m 4, and a history of respiratory symptoms after soy ingestion. IgE binding with serum ACG was observed to high molecular weight proteins of nearly all soy products, but not to the highly heat treated SF3 (Figure 10). Serum ACG had an extremely high ISAC score to Bet v 1, a moderate score to Gly m 4, and a negative ISAC result to Bet v 2 indicating the binding to the ~15 kDa protein is likely to Gly m 4. Four other individuals from the same study as patient ACG had positive soy CAP scores and/or a history of anaphylaxis. One anaphylactic individual (DDB) shared a similar binding profile as patient ACG, but the other 3 patients (HS, JL 007, AB 008) had little or no binding to high molecular weight proteins and very little IgE binding to soy overall.

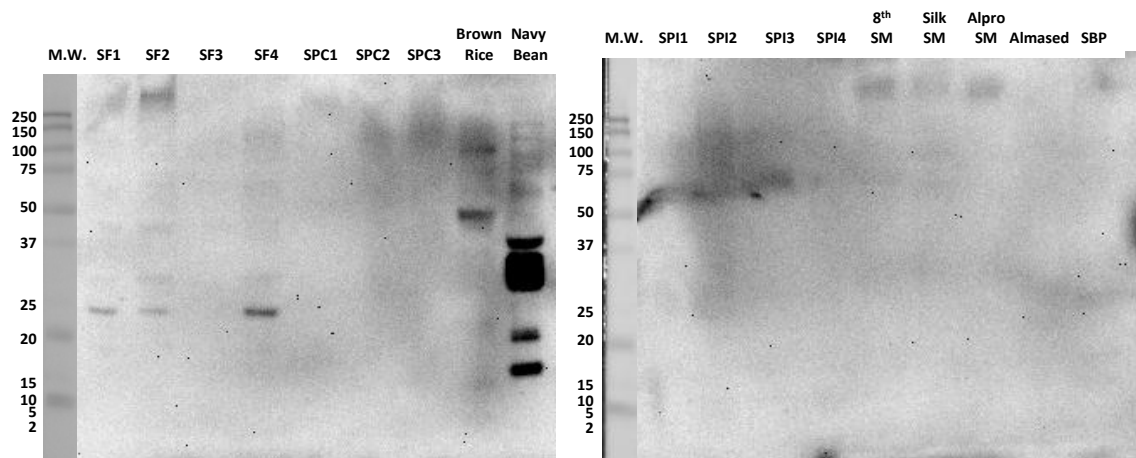


Figure 11 – Serum 893 IgE immunoblot of soy products under non-reducing conditions. Sample gels (25 µg extract protein per lane) were run under SDS-PAGE non-reducing conditions and transferred to PVDF membranes. Membranes were blocked in PBS-T with 5% NFDM followed by incubation with serum diluted 1:10. Bound IgE was detected using monoclonal HRP conjugated anti-human IgE diluted 1:1000 in 2.5% NFDM in PBS-T. Chemiluminescent substrate was used for detection and membrane images were captured using a Kodak Gel Logic 440 Imaging System.

Patient 893 had a clear history of mild reactions to soy and a positive Gly m 4 CAP but a negative soy CAP score. Serum 893 displayed minimal IgE binding to soy products (Figure 11). Eight other subjects with negative soy CAP scores and various allergic histories had similar binding profiles of weak IgE reactivity to one or a few proteins or no IgE binding at all (JENB, JB, MA, SP907, SP908, 42, 48, 579). However, serum from patient 138 had an anaphylactic history and negative soy CAP score but had binding similar to Figure 4A. Again, no observable IgE binding pattern was found in sera from subjects with negative soy CAP scores.

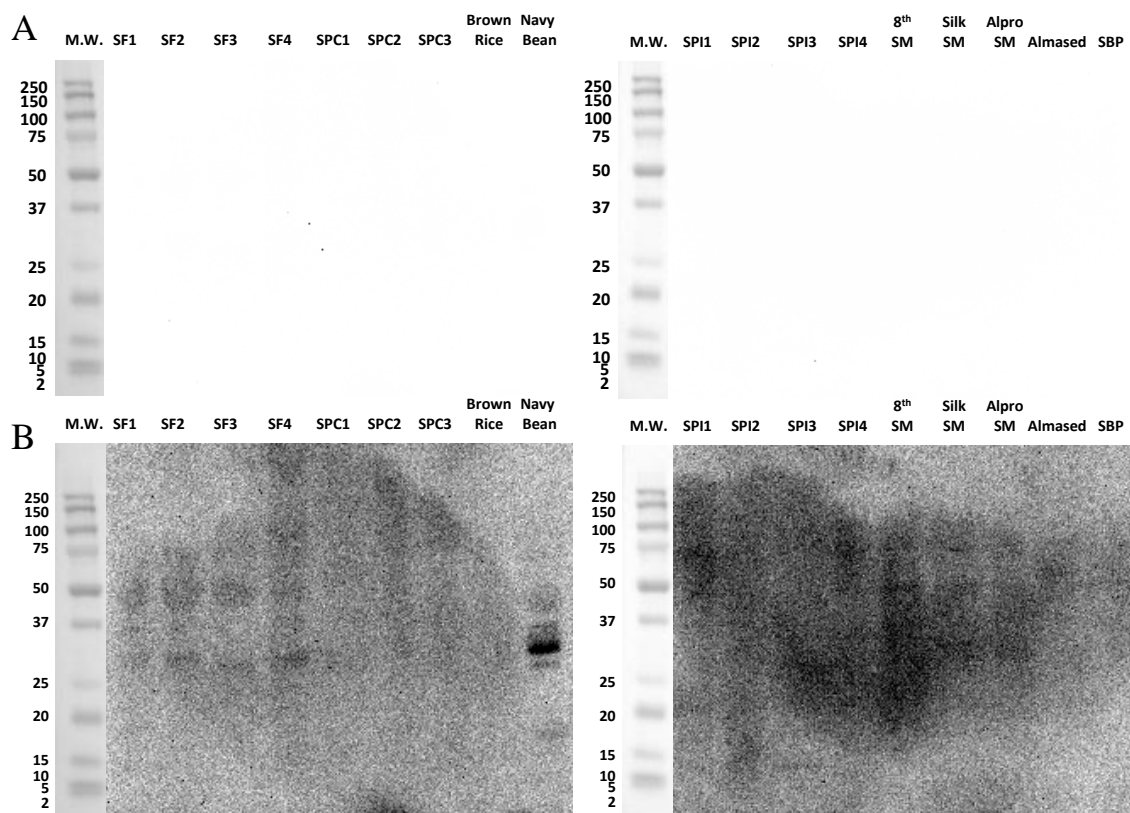


Figure 12 – Control serum 1 IgE immunoblot of soy products under non-reducing conditions with low (A) and extreme (B) image contrast. Sample gels (25 µg extract protein per lane) were run under SDS-PAGE non-reducing conditions and transferred to PVDF membranes. Membranes were blocked in PBS-T with 5% NFDM followed by incubation with serum diluted 1:10. Bound IgE was detected using monoclonal HRP conjugated anti-human IgE diluted 1:1000 in 2.5% NFDM in PBS-T. Chemiluminescent substrate was used for detection and membrane images were captured using a Kodak Gel Logic 440 Imaging System.

The control serum 1 used in this study was from an individual with no known food allergies. On extreme image contrast, binding could be observed to navy bean (likely CCD reactivity) and very weak binding to a number of soy proteins (Figure 12).

LC-MS/MS relative quantitation successfully identified the presence of 14 known soy allergens in a wide variety of soy products. In the majority of soy-allergic subjects, IgE binding studies showed strong binding to abundant allergens and weaker binding to less abundant allergens. Gly m 3 is a 14 kDa hydrophilic, heat-labile protein. Gly m 3 was identified in only 1 of 16 products during our study. However, others have shown soy profilin to be estimated at <1% of soluble protein (Amnuaycheewa and Gonzalez de

Mejia, 2010), so the lack of detection by LC-MS/MS could be due to its comparatively low abundance. Profilin detected in soy milks ranged from 4.37 ± 0.14 to 7.24 ± 0.30 mg/g protein (0.4 – 0.7%), while in fermented products profilin ranged from 1.67 ± 0.02 to 5.47 ± 0.02 mg/g protein (0.2 – 0.5%) (Amnuaycheewa and Gonzalez de Mejia, 2010) which correlates well with our findings. A recombinant form of profilin (rGly m 3) was shown to bind IgE from 69% soy-allergic sera tested (Rihs et al., 1999). In the current study, 11 of 31 (35%) subjects bound to a protein of ~15 kDa in size, most likely Gly m 3 or 4. The soy allergen Gly m 4 was identified as a cross-reactive protein with Bet v 1, a major birch pollen allergen and a cause of reactions in individuals cross-reactive to both birch pollen and soy (Kleine-Tebbe et al., 2002). However, rGly m 3 is cross-reactive with Bet v 2, birch pollen profilin, and previous studies have shown a number of soy milk reactive individuals sensitized to rGly m 3 but not rGly m 4, suggesting that Gly m 3 is also involved in cross-reactions between birch pollen and soy (Mittag et al., 2004; Rihs et al., 1999). Soy milk is a potentially hazardous product to Gly m 3 sensitive individuals as the food matrix of soy milk affects the thermal stability of profilin (Amnuaycheewa and Gonzalez de Mejia, 2010). IgE binding to rGly m 3 depends on the availability of the full length protein in its original conformational structure as no IgE binding was observed to profilin fragments (Rihs et al., 1999). Pasteurization of soy milk does not alter the conformational structure of Gly m 3 and boiling is necessary to reduce conformational epitopes (Amnuaycheewa and Gonzalez de Mejia, 2010). While a concern in less processed foods, Gly m 3 is not considered a major allergen in boiled or highly processed soy products.

Gly m 4 is a 17 kDa stress induced, pathogenesis-related starvation associated message protein (SAM22). Gly m 4 is found in the roots and leaves of maturing plants and can be induced by wounding or stressing young leaves (Crowell et al., 1992). Gly m 4 related reactions have been reported in individuals consuming raw soy sprouts and a low processed soy protein isolate (Mittag et al., 2004). Additionally, reactions to tofu, soy milk, and a soy pudding been reported in Gly m 4 sensitive individuals but many could tolerate cooked or highly processed soy products (Mittag et al., 2004). Gly m 4 was not found during relative quantitation by mass spectrometry. Published data estimate Gly m 4 at 0.036 – 0.061 % total seed weight or, assuming a protein content of 40%, 0.09 – 0.15% total soy protein (Julka et al., 2012), thus the lack of detection is likely due to the low abundance. However, Gly m 4 was detected through immunoblotting with Gly m 4 specific rabbit antibodies, with the highest reactivity to SF4, the stone ground full fat soy flour (data not shown). The 11 individuals that bound to the ~15 kDa protein, binding was strongest in SF4, the stone ground full fat soy flour. While the individuals in our study may be sensitized to Gly m 4, the protein is present in such low abundance that a number of individuals with a positive Gly m 4 CAP score do not show binding at ~15 kDa of immunoblots.

Gly m 5, or β -conglycinin, makes up 30% of the total seed proteins and is densely packed into trimers composed of three glycosylated subunits, α (67 kDa), α' (71 kDa), and β (50 kDa) (Holzhauser et al., 2009; Maruyama et al., 1998). Across all tested products, the current study identified Gly m 5 as 18.4% of the total normalized spectral counts by LC-MS/MS and found binding to Gly m 5 in 18 of 31 (58%) soy-allergic individuals, with proteins of molecular weight equal to all 3 subunits being recognized in

the study. These findings are in line with European and Japanese IgE binding studies that respectively found 43% and 100% recognition of Gly m 5 by soy-allergic sera (Holzhauser et al., 2009; Ito et al., 2011). The 3 subunits of Gly m 5 are among the most abundant proteins in the 16 studied soy products.

Gly m 6, or glycinin, makes up 40% of the total seed protein, is a hexameric protein, and each subunit (G1, G2, G3, G4, G5) has at least one basic and acidic subunit linked by a disulfide bond (Holzhauser et al., 2009; Prak et al., 2005). Five major subunits range from 52 – 61 kDa. IgE binding studies respectively found 36% and 100% recognition of Gly m 6 by soy-allergic sera (Holzhauser et al., 2009; Ito et al., 2011). All five subunits are known to react with IgE (Holzhauser et al., 2009). Across all tested products, the current study identified Gly m 6 subunits as 45.7% of the total normalized spectral counts by LC-MS/MS and found 15 of 31 (48%) of soy-allergic subjects had IgE binding to Gly m 6, with proteins at molecular weights for all subunits demonstrating binding. As previously noted, binding at ~15 kDa could be due to Gly m 3 or 4. Additional data from Zeece et al. (1999) found the acidic chain of glycinin G1 (A1a) to have a single IgE-binding fragment of approximately 15 kDa. While a number of subjects are sensitized to Gly m 4, more specific binding studies would need to be conducted to determine if any binding at ~15 kDa was due to the A1a fragment. Houston et al. (2011) identified glycinin G3 at low quantities in all tested varieties and although present through relative quantitation, it was present at significantly lower concentrations in certain specific soybean varieties. The current study did not identify glycinin G3 in 7 of 16 soy ingredients and products, indicating that processing or varietal genetic differences lowered the concentration of glycinin G3 below the LC-MS/MS detection limit and

confirmed its status as a lower abundance protein. Two subunits of Gly m 6, glycinins G1 and G2, have high sequence similarity with peanut Ara h 3 and the acidic chains of A1a and A2 share IgE binding epitope regions with Ara h 3 (Beardslee et al., 2000; Rabjohn et al., 1999; Xiang et al., 2002). Glycinins G1 and G2 are the two most abundant proteins in soybean as shown by mass spectrometry. The high abundance of these shared epitopes could in part explain the cross-reactivity found between peanut and soy in some allergic individuals.

Both Gly m 5 and 6 can bind IgE through linear and conformational epitopes (Helm et al., 2000; Ogawa et al., 1995; Zeece et al., 1999). Gly m 5 and 6 are stable proteins and potent allergens due to the combination of linear and conformational epitopes and their structural resistance to denaturation from tight packing and disulfide bonds. Accordingly, intense binding was observed in our study between 50 and 70 kDa under non-reducing conditions. Previous studies have found a higher prevalence of soybean specific IgE to Gly m 5 or 6 in individuals with severe reactions when compared to mild or moderate reactors, or those only sensitized to soy but not clinically allergic (Holzhauser et al., 2009; Ito et al., 2011). The current study found no difference in the prevalence of IgE to Gly m 5 or 6 based on reaction histories, 4 of 7 (57%) with anaphylactic histories versus 8 of 17 (47%) with mild or moderate reaction profiles.

Soy Kunitz trypsin inhibitor (KTI) is an inhalant allergen with a molecular weight of 20 kDa and an isoelectric point of 4.5 that represents 4-7% of the total extractable protein in soy. The protein is tightly packed, not glycosylated, and trypsin inhibition is achieved through reversible binding of KTI to the trypsin enzyme (Barać et al., 2004; Baur et al., 1996; Friedman and Brandon, 2001; Kunitz, 1947; Mikić et al., 2009; Quirce

et al., 2006). There are three major isoforms of KTI, A, B, and C, with only one amino acid difference between A and C and eight amino acid differences between A and B (Kim et al., 1985). Isoforms A and B were respectively found in 13 and 6 products; isoform C was not able to be distinguished from isoform A due similar sequences. Precursors to the trypsin inhibitor, KTI1 and KTI2, were respectively found in 14 and 15 products.

However, strong IgE binding to 20 kDa proteins was not observed and minor binding was only observed in a few cases. Soy KTI does not appear to be a major ingestion allergen in this study population. Additionally, soy KTI has only been found as an ingested allergen in one case report, a woman that was previously sensitized to soy KTI through work in a laboratory, and is not believed to be a major ingestion allergen in the overall soy population (Moroz and Yang, 1980).

Gly m Bd 30K, also known as soybean vacuolar protein P34, is a 32 kDa oil body protein with IgE binding characteristics (Helm et al., 1998; Ogawa et al., 1993). While resistant to a number of denaturation treatments, Gly m Bd 30k may be coded by a single gene and represents 2% to 3% of the total protein content (Wilson et al., 2005). As expected by prediction of percent of total protein content, Gly m Bd 30k was found in all 16 products during relative quantitation at levels that were comparable but slightly less than the levels of KTI. Gly m Bd 30K is a monomeric glycoprotein and has been shown to react with 65% of soy-sensitive patients with atopic dermatitis (Helm et al., 1998; Ogawa et al., 1993; Wilson et al., 2005). In the current study, 16 of 31 (52%) of sera had IgE to proteins near 32 kDa but further identification work would need to confirm if binding was to Gly m Bd 30K or another protein.

Gly m Bd 28 K is a 26 kDa Asn-linked glycoprotein that has been shown to bind IgE from 25% of soy-allergic subjects (Hiemori et al., 2000; Ogawa et al., 2000; Tsuji et al., 1997). Additionally, a 23 kDa C terminal fragment of Gly m Bd 28K has been shown to have the same IgE reactivity as the 26 kDa form (Hiemori et al., 2000; Hiemori et al., 2004). In the current study, 11 of 31 (35%) of sera bound to proteins near 23 and 26 kDa with the strongest binding in soy flours but also present in soy milk to a lesser extent. Previous studies detected high levels of Gly m Bd 28K in tofu, SPI and soy milk (Tsuji et al., 1997) but our current study only identified it in 6 products: 2 soy flours, 1 SPC, 1 SPI, Almased® (SPI), and the soybean powder. No soy milks studied had identifiable levels of Gly m Bd 28K.

4. Conclusion

The results from this study show that LC-MS/MS is a viable method to identify the majority of allergenic soy proteins. Additional method refinements or new techniques such as antibody detection would be necessary to detect low abundance proteins such as Gly m 3 and Gly m 4. IgE binding using sera from soy-allergic subjects did not allow the identification of patterns that could be associated with the clinical histories and symptoms of these patients. In fact, patients with similar histories could exhibit intense binding or no binding at all. However, when IgE binding was present, the relative amount of an allergen in a sample correlated positively to the intensity of IgE binding to proteins at the expected molecular weight. Small differences in IgE binding were observed based on the form of soy (soy flour vs soy milk) known to elicit an allergic reaction. However, studies with more subjects would be necessary to confirm these initial findings. Finally, quantification by LC-MS/MS identified small variations in

the amounts of soy allergens per product type, but the results were not as drastic as one might expect after the extensive treatments applied to some of these products.

References

- Amnuaycheewa, P., Gonzalez de Mejia, E., 2010. Purification, characterisation, and quantification of the soy allergen profilin (Gly m 3) in soy products. *Food Chem* 119, 1671-1680.
- Barać, M.B., Stanojević, S.P., Jovanović, S.T., Pešić, M.B., 2004. Soy protein modification: A review. *Acta Periodica Technologica* 35, 3-16.
- Baur, X., Pau, M., Czuppon, A., Fruhmans, G., 1996. Characterization of soybean allergens causing sensitization of occupationally exposed bakers. *Allergy* 51, 326-330.
- Beardslee, T.A., Zeece, M.G., Sarath, G., Markwell, J.P., 2000. Soybean glycinin G1 acidic chain shares IgE epitopes with peanut allergen Ara h 3. *Int Arch Allergy Immunol* 123, 299-307.
- Branum, A.M., Lukacs, S.L., 2009. Food allergy among children in the United States. *Pediatrics* 124, 1-7.
- Crowell, D.N., John, M.E., Russell, D., Amasino, R.M., 1992. Characterization of a stress-induced, developmentally regulated gene family from soybean. *Plant Mol Biol* 18, 459-466.
- Egbert, W.R., 2004. Isolated Soy Protein, in: K. Liu (Ed.), *Soybeans as Functional Foods and Ingredients*. AOCS Publishing, Urbana, IL, pp. 134-162.
- Friedman, M., Brandon, D.L., 2001. Nutritional and Health Benefits of Soy Proteins. *J Agric Food Chem* 49, 1069-1086.
- Hefle, S.L., Furlong, T.J., Niemann, L., Lemon-Mule, H., Sicherer, S., Taylor, S.L., 2007. Consumer attitudes and risks associated with packaged foods having advisory labeling regarding the presence of peanuts. *J Allergy Clin Immunol* 120, 171-176.
- Helm, R.M., Cockrell, G., Connaughton, C., Sampson, H.A., Bannon, G.A., Beilinson, V., Nielsen, N.C., Burks, A.W., 2000. A soybean G2 glycinin allergen 2. Epitope mapping and three-dimensional modeling. *Int Arch Allergy Immunol* 123, 213-219.
- Helm, R.M., Cockrell, G., Herman, E., Burks, A.W., Sampson, H.A., Bannon, G.A., 1998. Cellular and molecular characterization of a major soybean allergen. *Int Arch Allergy Immunol* 117, 29-37.
- Hiemori, M., Bando, N., Ogawa, T., Shimada, H., Tsuji, H., Yamanishi, R., Terao, J., 2000. Occurrence of IgE antibody-recognizing N-linked glycan moiety of a soybean allergen, Gly m Bd 28K. *Int Arch Allergy Immunol* 122, 238-245.
- Hiemori, M., Ito, H., Kimoto, M., Yamashita, H., Nishizawa, K., Maruyama, N., Utsumi, S., Tsuji, H., 2004. Identification of the 23-kDa peptide derived from the precursor of Gly

m Bd 28K, a major soybean allergen, as a new allergen. *Biochim Biophys Acta* 1675, 174-183.

Holzhauser, T., Wackermann, O., Ballmer-Weber, B.K., Bindslev-Jensen, C., Scibilia, J., Perono-Garoffo, L., Utsumi, S., Poulsen, L.K., Vieths, S., 2009. Soybean (*Glycine max*) allergy in Europe: Gly m 5 (β -conglycinin) and Gly m 6 (glycinin) are potential diagnostic markers for severe allergic reactions to soy. *J Allergy Clin Immunol* 123, 452-458.

Houston, N.L., Lee, D.-G., Stevenson, S.E., Ladics, G.S., Bannon, G.A., McClain, S., Privalle, L., Stagg, N., Herouet-Guicheney, C., MacIntosh, S.C., Thelen, J.J., 2011. Quantitation of Soybean Allergens Using Tandem Mass Spectrometry. *J Proteome Res* 10, 763-773.

Ito, K., Sjölander, S., Sato, S., Movérare, R., Tanaka, A., Söderström, L., Borres, M., Poorafshar, M., Ebisawa, M., 2011. IgE to Gly m 5 and Gly m 6 is associated with severe allergic reactions to soybean in Japanese children. *J Allergy Clin Immunol* 128, 673-675.

Julka, S., Kuppanan, K., Karnoup, A., Dielman, D., Schafer, B., Young, S.A., 2012. Quantification of Gly m 4 Protein, A Major Soybean Allergen, By Two-Dimensional Liquid Chromatography with Ultraviolet and Mass Spectrometry Detection. *Anal Chem* 84, 10019-10030.

Kim, S.H., Hara, S., Hase, S., Ikenaka, T., Toda, H., Kitamura, K., Kaizuma, N., 1985. Comparative study on amino acid sequences of Kunitz-type soybean trypsin inhibitors, Tia, Tib, and Tic. *J Biochem (Tokyo)* 98, 435-448.

Kleine-Tebbe, J., Wangorsch, A., Vogel, L., Crowell, D.N., Hausteiner, U.-F., Vieths, S., 2002. Severe oral allergy syndrome and anaphylactic reactions caused by a Bet v 1-related PR-10 protein in soybean, SAM22. *J Allergy Clin Immunol* 110, 797-804.

Kunitz, M., 1947. Crystalline soybean trypsin inhibitor II. General properties. *J Gen Physiol* 30, 291-310.

Liu, K., 2004. Soybeans as a Powerhouse of Nutrients and Phytochemicals, in: K. Liu (Ed.), *Soybeans as Functional Foods and Ingredients*. AOCS Publishing, Urbana, IL, pp. 1-22.

Maruyama, N., Katsube, T., Wada, Y., Oh, M.H., Barba De La Rosa, A.P., Okuda, E., Nakagawa, S., Utsumi, S., 1998. The roles of the N-linked glycans and extension regions of soybean beta-conglycinin in folding, assembly and structural features. *Eur J Biochem* 258, 854-862.

Mikić, A., Perić, V., Đorđević, V., Srebrić, M., Mihailović, V., 2009. Anti-nutritional factors in some grain legumes. *Biotech Anim Husbandry* 25, 1181-1188.

Mittag, D., Vieths, S., Vogel, L., Becker, W.-M., Rihs, H.-P., Helbling, A., Wüthrich, B., Ballmer-Weber, B.K., 2004. Soybean allergy in patients allergic to birch pollen: clinical

investigation and molecular characterization of allergens. *J Allergy Clin Immunol* 113, 148-154.

Moroz, L.A., Yang, W.H., 1980. Kunitz soybean trypsin inhibitor. A specific allergen in food anaphylaxis. *N Engl J Med* 302, 1126-1128.

Nordlee, J.A., 1995. Screening of soybean (*Glycine max*) germplasm for binding to immunoglobulin E by immunoelectroblotting, Food Science and Technology. University of Nebraska-Lincoln, Lincoln, NE.

Ogawa, T., Bando, N., Tsuji, H., Nishikawa, K., Kitamura, K., 1995. α -Subunit of β -conglycinin, an allergenic protein recognized by IgE antibodies of soybean sensitive patients with atopic dermatitis. *Biosci Biotechnol Biochem* 59, 831-833.

Ogawa, T., Bando, N., Tsuji, H., Okajima, H., Nishikawa, K., Sasaoka, K., 1991. Investigation of the IgE-binding proteins in soybeans by immunoblotting with the sera of the soybean-sensitive patients with atopic dermatitis. *J Nutr Sci Vitaminol (Tokyo)* 37, 555-565.

Ogawa, T., Samoto, M., Takahashi, K., 2000. Soybean allergens and hypoallergenic soybean products. *J Nutr Sci Vitaminol (Tokyo)* 46, 271-279.

Ogawa, T., Tsuji, H., Bando, N., Kitamura, K., Zhu, Y.-L., Hirano, H., Nishikawa, K., 1993. Identification of the soybean allergenic protein, Gly m Bd 30K, with the soybean seed 34-kDa oil-body-associated protein. *Biosci Biotechnol Biochem* 57, 1030-1033.

Osborne, N., Koplin, J.J., Martin, P.E., Gurrin, L.C., Lowe, A.J., Matheson, M.C., Ponsonby, A.-L., Wake, M., Tang, M.L.K., Dharmage, S.C., Allen, K.J., 2011. Prevalence of challenge-proven IgE-mediated food allergy using population-based sampling and predetermined challenge criteria in infants. *J Allergy Clin Immunol* 127, 668-676.

Panda, R., 2012. Soybean allergy: Effect of genetic modification (GM), heat and enzymatic treatment on overall allergenicity, Food Science and Technology. University of Nebraska, Lincoln, Nebraska.

Pieretti, M.M., Chung, D., Pacenza, R., Slotkin, T., Sicherer, S.H., 2009. Audit of manufactured products: use of allergen advisory labels and identification of labeling ambiguities. *J Allergy Clin Immunol* 124, 337-341.

Prak, K., Nakatani, K., Katsube-Tanaka, T., Adachi, M., Maruyama, N., Utsumi, S., 2005. Structure-function relationships of soybean proglycinins at subunit levels. *J Agric Food Chem* 53, 3650-3657.

Quirce, S., Fernández-Nieto, M., Escudero, C., Cuesta, J., de las Heras, M., Sastre, J., 2006. Bronchial responsiveness to bakery-derived allergens is strongly dependent on specific skin sensitivity. *Allergy* 61, 1202-1208.

- Rabjohn, P., Helm, E.M., Stanley, J.S., West, C.M., Sampson, H.A., Burks, A.W., Bannon, G.A., 1999. Molecular cloning and epitope analysis of the peanut allergen Ara h 3. *J Clin Invest* 103, 535-542.
- Rihs, H.-P., Chen, Z., Ruëff, F., Petersen, A., Rozynek, P., Heimann, H., Baur, X., 1999. IgE binding of the recombinant allergen soybean profilin (rGly m 3) is mediated by conformational epitopes. *J Allergy Clin Immunol* 104, 1293-1301.
- Rona, R.J., Keil, T., Summers, C., Gislason, D., Zuidmeer, L., Sodergren, E., Sigurdardottir, S.T., Lindner, T., Goldhahn, K., Dahlstrom, J., McBride, D., Madsen, C., 2007. The prevalence of food allergy: a meta-analysis. *J Allergy Clin Immunol* 120, 638-646.
- Sicherer, S.H., Sampson, H.A., 2010. Food allergy. *J Allergy Clin Immunol* 125, S116-S125.
- Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. *Anal Biochem* 150, 76-85.
- Stevenson, S.E., Woods, C.A., Hong, B., Kong, X., Thelen, J.J., Ladics, G.S., 2012. Environmental effects on allergen levels in commercially grown non-genetically modified soybeans: assessing variation across north america. *Front Plant Sci* 3, 196.
- Taylor, S.L., Bush, R.K., Busse, W.W., 1986. Avoidance diets - how selective should we be? *N Engl J Allergy Proc* 7, 527-532.
- Tsuji, H., Bando, N., Hiemori, M., Yamanishi, R., Kimoto, M., Nishikawa, K., Ogawa, T., 1997. Purification and characterization of soybean allergen Gly m Bd 28K. *Biosci Biotechnol Biochem* 61, 942-947.
- Wilson, S., Blaschek, K., Gonzalez de Mejia, E., 2005. Allergenic proteins in soybean: processing and reduction of P34 allergenicity. *Nutr Rev* 63, 47-58.
- Xiang, P., Beardslee, T.A., Zeece, M.G., Markwell, J., Sarath, G., 2002. Identification and analysis of a conserved immunoglobulin E-binding epitope in soybean G1a and G2a and peanut Ara h 3 glycinins. *Arch Biochem Biophys* 408, 51-57.
- Zeece, M.G., Beardslee, T.A., Markwell, J.P., Sarath, G., 1999. Identification of an IgE-binding region in soybean acidic glycinin G1. *Food Agric Immunol* 11, 83-90.