2011

A Sensitive, Specific, and Robust Enzyme-linked Immunosorbent Assy (elisa) for Macadamia Nut Seed Protein Detection

Julia Katz

ABSTRACT

This research project focused on macadamia nut detection. Two specific aims were proposed: (i) develop a competitive inhibition ELISA with a sensitivity of 10 ppm (ii) assess the assay specificity and robustness. The assay specificity of the rabbit anti-macadamia pAb-based ELISA was evaluated by determining assay interference by several select food matrices. The assay robustness was assessed by determining its ability to detect the presence of proteins in variously processed nut seeds. The optimized ELISA has a detection range of 3.2 to 400 ng of macadamia nut protein/mL of BSB. The protein G-purified rabbit anti-macadamia nut protein polyclonal antibodies did not exhibit detectable cross-reactivity with tested protein extracts prepared from several seed and food matrices. The assay was able to detect the seed proteins extracted from macadamia nut seeds subjected to select thermal processing methods.

KEY WORDS: ELISA, Macadamia Nut Seed, Protein, Immunoreactivity

THE FLORIDA STATE UNIVERSITY

COLLEGE OF HUMAN SCIENCES

A SENSITIVE, SPECIFIC, AND ROBUST ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR MACADAMIA NUT SEED PROTEIN DETECTION

By

JULIA KATZ

A Thesis submitted to the
Department of Nutrition, Food and Exercise Sciences
in partial fulfillment of the requirements for graduation with
Honors in the Major

Degree Awarded:
Summer, 2011

The members of the Defense Committee approve the thesis of Julia Katz defended on April 11, 2011.

_____

Dr. Shridhar K. Sathe
Thesis Director

_____

Dr. Jodee Dorsey
Committee Member

_____

Dr. Patricia Spears
Committee Member

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
BSB	Borate saline buffer
ELISA	Enzyme-linked immunosorbent assay
g	Gram
h	Hour
HDL	High density lipoprotein
IC_{50}	Inhibition concentration at 50%
LDL	Low density lipoprotein
LSD	Least significant difference
M	Molar
Min	Minute
NFDM	Nonfat dried milk
pAbs	Polyclonal antibodies
PCR	Polymerase chain reaction
PNPP	*p*-nitrophenyl phosphate
Ppm	Parts per million
RT	Room temperature
SEM	Standard error of mean
TBS-T	Tris buffered saline-Tween 20
v/v	Volume/volume
W	Watt
w/v	Weight/volume

INTRODUCTION

The U.S. is one of the top producers of macadamia nut seeds, a common tree nut, with Hawaii leading the production (1, 2). Native to Australia, macadamia nut seeds belong to a genus that contains seven species, two of which, *Macadamia integrifolia* and *Macadamia tetraphylla,* are of commercial importance (3). Due to their mild nutty flavor and crunchy texture, macadamia nut seeds are widely used by confectioners and bakers in numerous food products. The nut seeds contain ~66% fat of which 77.43% is comprised of monounsaturated fatty acids (MUFAs), making it one of the highest MUFA containing tree nuts. Oleic acid, palmitoleic acid, and erucic acid comprise 58.51%, 18.69%, and 0.23% of total MUFAs (4). Studies have shown that MUFAs may provide health benefits (5, 6, 7), such as a reduction in LDL and an increase in HDL (6).

While beneficial, nut seed consumption is of concern for sensitive individuals suffering from food allergies as tree nuts are one of the eight food groups (milk, eggs, soybeans, crustaceans, fish, wheat, peanuts and tree nuts) that account for 90% of food induced allergic reactions (8). Type I food allergies are defined as an adverse immune response to food proteins. Type I food allergies are characterized by cross-linking of two IgE molecules by the antigen (a food protein) on the surface of a mast cell/basophil. The portion of the protein molecule that binds with the IgE molecule is known as the epitope. Although the exact mechanism underlying food allergies remains unknown, such IgE cross-linking by the antigen on the surface of the affected mast cells/basophils seems to be the critical step in triggering an allergic response in sensitive individuals (9).

The recent rise in food allergies in Western countries is of concern as currently there is no known cure for food allergies. It has been reported that there is a 3% to 6% prevalence of adverse immune responses to foods (10). A 2008 report from the Centers for Disease Control and Prevention indicated an 18% increase in childhood food allergy from 1997 to 2007 (11). More than 1% of the U.S. population continues to report allergies specifically for peanuts and/or tree nuts, with the prevalence in children increasing by 0.9% from 2002 to 2008 (12). A recent study of 278 tree nut allergic patients found that among the 101 patients reactive to the tree nut challenge, 13% exhibited moderate and 4% severe allergic reactions particularly to macadamia nuts (13).

Since there is no cure for food allergies as yet, the best option for sensitive individuals is to avoid allergen containing foods. Unfortunately, accidental ingestion of the offending foods is common (9, 13). Although food labeling and good manufacturing practices minimize such accidental exposure of sensitive individuals to trace amounts (ppm) of macadamia nut that may be present in food, they do not eliminate it. Therefore, in order to protect consumers, sensitive, practical, and economical methods (with a detection limit of at least 10 ppm) for macadamia nut protein detection must be developed. Among several possible methods that may be used for such purpose (e.g. PCR, DNA, spectrophotometric, mass spectrophotometric, and others), ELISA is currently the most practical choice.

The current lack of adequate practical methods for the detection of macadamia nut proteins prompted this research. The specific aims were i) to develop a sensitive, specific and robust ELISA that can detect at least 10 ppm of macadamia nut seed proteins in foods and ii) to determine whether such a method is suitable for the purpose of detecting trace amounts of macadamia nut proteins in commercially produced foods.

MATERIALS AND METHODS

Materials: Protein G-purified anti-macadamia nut seed protein rabbit pAbs, and various flours from defatted food matrices were prepared and stored at -20 °C. Macadamia nut seeds were purchased from Trader Joe's (Needham Heights, MA) and New Leaf Market (Tallahassee, FL), defatted, and stored at -20°C. All required chemicals and reagents were from Fisher Scientific Co. (Pittsburgh, PA) and were of ACS grade. Alkaline phosphatase labeled goat anti-rabbit IgG, BSA (Fraction V grade, 98% purity), and PNPP substrate were from Sigma Chemical Co. (St. Louis, MO). Ninety-sex well polyvinyl microtiter ELISA plates were from Costar (Cambridge, MA).

Methods:

Rabbit Polyclonal Antibody Preparation and Purification. The anti-macadamia nut pAbs were produced in the same manner as previously stated in Sharma et al. (17).

Preparation of Protein Extract. Whole macadamia nuts were ground in an Osterizer blender (speed setting "grind"; Galaxy model 869-18R, Jaden Consumer Solutions, Boca Raton, FL) to homogeneous flour and defatted for 8 hours using a Soxhlet apparatus with petroleum ether (boiling point range of 38.2 - 54.3 °C) as the extraction solvent. The defatted samples were dried for 24 hours under a fume hood at RT (~25 °C) and then passed through a 40 mesh sieve. The powdered samples were stored in air-tight plastic containers and stored at -20 °C. Protein extracts from defatted macadamia nut flours and several other defatted flours were prepared using 0.1 M BSB (0.1 M H_3BO_3, 0.025 M $Na_2B_4O_7$, 0.075 M NaCl, pH 8.45) (flour-to-solvent ratio of 1:10 w/v) by continuous vortexing for 1 h at RT followed by centrifugation (Centrifuge 5415 D, Brinkmann Instruments Inc.,Westburg, NY) at 16,000*g* for 20 min at RT. Aliquots of

the supernatants were analyzed within 24 hours of preparation, and the remainder was stored at -20 °C until further use.

Protein Estimation. The soluble protein content of the macadamia nut supernatants was determined using the method of Lowry et al. (14). The protein content of food matrices with supernatants dark in color was assessed using the Bradford assay (15). BSA was the standard protein for both methods, with concentrations of 0-600 µg/mL for the Bradford assay and 0-200 µg/mL for the Lowry assay.

ELISA. Competitive inhibition ELISA was used with protein G-purified rabbit anti-macadamia nut pAbs (0.6 mg/mL). Ninety-six well polyvinyl microtiter plates were coated with 50 µL of 10 µg/mL of macadamia protein extract per well (500 ng of macadamia protein/well) prepared in the coating buffer (48.5 mM citric acid, 103 mM Na_2HPO_4, pH 5.0) and incubated for 1 h at 37 °C. Plates were washed three times with TBS-T (10 mM Tris, 0.9% w/v NaCl, 0.05% v/v Tween 20, pH 7.6) and then blocked with 200 µL/well of 5% w/v NFDM in TBS-T for 1 h at 37 °C, and again washed three times with TBS-T. Concurrently, 90 µL of pAb, diluted 1:30,000 v/v in TBS-T containing 1% NFDM, was added to the top row of wells of a second uncoated plate. The remaining wells of the uncoated plate contained 80 µL of pAb, diluted 1:30,000 v/v in TBS-T containing 1% NFDM. Ten microliters of 0.1 mg/mL standard macadamia protein extract (inhibitor) was added in the first row of wells, and a 5 times serial dilution was carried out in the subsequent wells. The plates were incubated at 37 °C for 1 h. Fifty microliters of the content from each well of the uncoated plate were transferred to the respective well of the coated plate, incubated for 1 h at 37 °C, and washed three times with TBS-T. The coated plates were then incubated with a 1:5,000 dilution of alkaline phosphatase-labeled goat anti-rabbit IgG in TBS-T for 1 h at 37 °C. The plates were washed three times with TBS-T. Proceeding, 50 µL of PNPP

substrate dissolved in alkaline phosphatase substrate buffer was added to each well to detect the alkaline phosphatase and incubated at 37 °C in the dark for ~20 min. The reaction was stopped with 3M NaOH. The developed color, as a result of PNPP hydrolysis, was measured at 405 nm using an ELISA reader.

Cross-reactivity of pAb. The protein G-purified rabbit anti-macadamia pAbs were tested for cross- reactivity against 61 different food ingredients (**Table 1**). The matrices selected are common food items that could be prepared with macadamia nuts. Extracts of the food matrices were prepared using BSB (flour-to-solvent ratio of 1:10 w/v) by continuous vortexing for 1 h at RT followed by centrifugation at 16,000*g* for 20 min at RT. Aliquots of the supernatants were analyzed for their protein content using either the Bradford or Lowry method. In the inhibition ELISAs, the protein extracted from the food matrix was used as the inhibitor. A standardized protein concentration of 0.1 mg/mL was used, followed by a 5 times serial dilution in the subsequent wells. On each microtiter plate, a macadamia nut standard curve was prepared. For food ingredients with negligible protein content (0-0.1 mg/mL, for example, spices, salt, sugar), 90 µL of the BSB extract was used. Immunoreactivity was measured by using the four-parametric equation of the macadamia nut standard curve (**Table 2, 3**). The reactive protein in the food ingredient sample (C1) was determined using the sample absorbance closest to the IC_{50} of the macadamia nut standard curve. The percent immunoreactivity of the food ingredient was calculated as follows:

% immunoreactivity of the sample = [C1/total protein in the well corresponding to the C1] x 100

Table 1. Cross-reactivity testing matrices

Category	Food Matrix
Tree nuts	Almond, brazil nut, cashew nut, coconut, hazelnut, Inca peanut, pecan, pine nut, pistachio, Spanish peanut, Virginia peanut, walnut
Seeds	Sesame, sunflower
Cereals	All purpose wheat flour, barley, corn, millet, oat bran, rice flour, rye, sorghum, whole wheat flour
Dairy	NFDM, vanilla ice cream
Legumes	Black bean, black gram, chick pea, green peas, lentil, lima bean, navy bean, soybean
Fruits	Banana, cherry, pineapple, raisin
Vegetables	Asparagus, broccoli, carrot, green pepper, mushroom, red potato, spinach
Spices	Black pepper, cardamom, cinnamon, garlic, mustard seed, nutmeg
Confectionery	Baking powder, Baker's sweet chocolate, Baker's unsweetened chocolate, brown sugar, cocoa, dark chocolate, egg white, egg yolk, salt, sugar, tapioca

Thermal Processing. Whole macadamia nuts from Trader Joe's and New Leaf Market were subjected to different thermal treatments. Three nuts, ~7 to 10 g, in duplicates were used for each treatment, and all processes were repeated three times. Treatments included pressure cooking in an autoclave (dry cycle) at 121 °C and 15 psi for 15 and 30 min, blanching in boiling water for 5 and 10 min, and microwave heating at 500 W for 1 and 3 min as described previously by Su et al. (16). Processed and unprocessed macadamia nuts were ground in a blender, crushed with a mortar and pestle, defatted and passed through a 40 mesh sieve. The flours were stored at -20 °C until further use. The soluble proteins from the processed and unprocessed macadamia nuts were extracted in BSB and analyzed for their protein content using the Lowry assay. Stock solutions (0.1 mg/mL) were prepared from the protein extracts. The IC_{50} ratios of the processed to unprocessed macadamia nut seed proteins were compared by ELISA.

Statistics. All experiments were carried out in duplicates or triplicates, and data are reported as mean ± SEM. Data were analyzed for statistical significance by one-way ANOVA using SPSS statistical software (version 18.0; Chicago, IL) and Fisher's LSD test ($p = 0.05$).

RESULTS AND DISCUSSION

Optimization of ELISA Standard Curve

A mean standard curve and standard error of mean were derived from 24 experimental trials. With an inhibitor concentration of 0.1 mg/mL, a 1:30,000 pAb dilution and a 5 times serial dilution, the average IC_{50} value, or the amount of antigen needed to inhibit 50% of the optical density signal, was found to be 73.7±5.4 ng/mL, equal to 0.074 ppm of macadamia nut soluble protein from defatted flours. The protein G-purified rabbit anti-macadamia pAbs can sensitively detect macadamia nut seed protein (range 3.2 to 400 ng/mL) from macadamia nut seed protein extracts prepared using the BSB buffer as the extraction solvent.

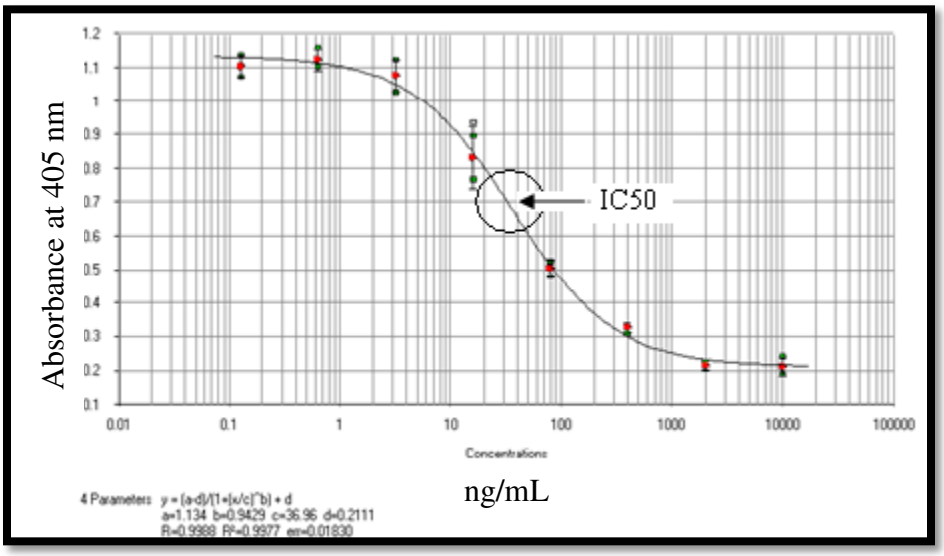

Figure 1. Sample Inhibition ELISA for BSB soluble macadamia nut seed proteins (n=61)

Specificity of the rabbit anti-macadamia nut pAbs

The specificity of the protein G-purified rabbit anti-macadamia nut pAbs was determined by checking the pAbs against various food matrices. When trying to detect macadamia nut proteins in food items, it is important only to recognize the offending proteins. Various food ingredients may cross-react with the anti-macadamia nut rabbit pAbs, leading to a non-specific assay. For example, if the food matrix is cross-reactive with the pAbs one may observe false positives. Alternatively, when the food matrix inhibits the detection of the target protein even when it is present in the food, one obtains false negative results. In either case, the assay utility diminishes. The matrices that were selected are common food items that may be prepared with macadamia nuts. For example, macadamia nuts may be added to bakery items that are prepared with common confectionary ingredients, such as chocolate, sugar or tapioca. Macadamia nuts also could be added to a trail mix that may contain other tree nuts, peanuts, seeds and dried fruits. Other food ingredients may include legumes, vegetables, cereals, and spices. All of these food matrices could contain proteins that interact with the pAbs (i.e. cross-reactive) and were therefore assessed.

Table 2. Cross-reactivity of rabbit anti-macadamia nut pAbs with various food matrices (n=3)

Ingredient	% Immunoreactivity (Mean ± SEM)	Ingredient	% Immunoreactivity (Mean ± SEM)
Tree nuts		Green pea	1.700±1.624
Almond	1.185±0.643	Lentil	0.351±0.107
Brazil nut	0.210±0.077	Navy bean	0.038±0.018
Cashew nut	1.984±0.361	**Fruits**	
Coconut	0.116±0.024	Banana	0.032±0.028
Hazelnut	0.049±0.013	Cherry	0.924±0.073
Pecan	0.888±0.856	Pineapple	0.419±0.211
Pine nut	0.055±0.019	Raisin	0.076±0.003
Pistachio	0.113±0.033	**Vegetables**	
Walnut	0.021±0.002	Asparagus	0.462±0.050
Oil Seeds		Carrot	1.002±0.119
Inca Peanut	0.068±0.007	Green pepper	1.121±0.073
Sesame	0.630±0.119	Mushroom	0.560±0.273
Soybean	0.040±0.009	Red potato	0.031±0.012
Spanish peanut	0.067±0.062	Spinach	0.027±0.007
Sunflower	0.505±0.141	**Spices**	
Virginia Peanut	0.008±0.006	Black pepper	0.674±0.128
Cereals		Cardamom	1.834±0.800
All purpose flour	0.363±0.282	Cinnamon	6.999±0.374
Barley	0.640±0.606	Garlic	0.000±0.000
Corn	0.224±0.094	Nutmeg	0.468±0.158
Millet	0.042±0.021	Salt	0.535±0.086
Oat bran	0.090±0.047	**Confectionery**	
Rice flour	0.000±0.000	Baking powder	0.340±0.025
Rye	0.101±0.051	Baker's sweet chocolate	0.020±0.006
Sorghum	0.459±0.230	Baker's unsweet chocolate	0.040±0.021
Whole wheat flour	0.089±0.012	Brown sugar	0.519±0.042
Dairy		Cocoa	0.003±0.003
Vanilla ice cream	5.276±3.03	Dark chocolate	0.934±0.148
Legumes		Sugar	0.140±0.013
Black bean	1.226±1.083	**Egg**	
Black gram	0.734±0.281	Egg White	0.575±0.332

Data are expressed as mean ± SEM. Fischer's LSD=1.53 (p=0.05, n=3). Differences between two means exceeding the LSD value are significant. The reactive protein in the food ingredient sample was determined using the sample absorbance closest to the IC_{50} of the macadamia nut standard curve.

Table 3. Cross-reactivity of select food matrices (n=2) as tested by standardized ELISA

Ingredient	% Immunoreactivity (Mean ± SEM)
Broccoli*	16.810±0.637
Chick pea*	27.773±2.014
Egg yolk	0.788±0.742
Lima	4.430±0.539
Mustard seed	2.452±2.446
NFDM	2.137±1.964
Tapioca	2.968±2.147

Data are expressed as mean ± SEM. Fischer's LSD=4.28 (p=0.05, n=2). Differences between two means exceeding the LSD value are significant. The reactive protein in the food ingredient sample was determined using the sample absorbance closest to the IC_{50} of the macadamia nut standard curve.
*Statistically significantly different.

None of the above matrices, except broccoli and chick pea, were found to be cross-reactive with the protein G-purified rabbit anti-macadamia nut pAbs. Broccoli and chick pea had immunnoreactivities greater than 10% with the anti-macadamia nut pAbs, and are therefore considered significant. These matrices must be further investigated to assure cross- reactivity. The % immunoreactivities of all other food ingredients were found to be significantly different from that of macadamia nut. This means that if macadamia nut is incorporated with these food ingredients, the assay would still be able to specifically detect macadamia nut protein.

Effects of Thermal Processing on Macadamia Nut Protein Immunoreactivity

During preparation, food matrices containing macadamia nuts may undergo various types of thermal processing, such as autoclaving, blanching, frying, microwave heating, and roasting. These processes could denature the proteins in macadamia nuts, causing modification or destruction of the epitope, causing the loss of detection of macadamia nut proteins by the anti-macadamia nut pAbs. It is necessary to determine whether the immunoreactivity of macadamia

nut proteins is compromised by various thermal treatments to determine whether the assay is robust towards thermal processing.

The IC_{50} ratios of processed to unprocessed macadamia nut seed protein extracts by ELISA were compared (**Table 4**). For macadamia nuts from Trader Joe, the data suggest that there was no reduction in immunoreactivity upon processing compared to the unprocessed macadamia standard. A comparison with the macadamia nuts purchased from New Leaf market suggests that there was a reduction in immunoreactivity when the nuts were microwaved for 3 min at 500 W and autoclaved at 121 °C and 15 psi for 30 min. The cause of the reduced immunoreactivity may be due to the difference in source since nuts from Trader Joe underwent the same processes. Given that the assay still detected macadamia nut proteins after thermal processing, it is robust towards thermal processing methods.

Table 4. Effect of thermal processing on immunogenicity of macadamia nut seed proteins assessed by inhibition ELISA

Thermal Processing	**ELISA (Mean ± SEM)**	
	Trader Joe	New Leaf
Unprocessed control	1.00±0	1.00±0
Microwaving at 500 W for 1 min	1.18±0.26	1.01±0.11
Microwaving at 500W for 3 min	1.16±0.06	0.48±0.17
Blanching at 100°C for 5 min	1.38±0.04	0.95±0.2
Blanching at 100°C for 10 min	0.93±0.18	0.93±0.17
Autoclaving at 121°C & 15 psi for 15 min	0.56±0.06	0.87±0.17
Autoclaving at 121°C & 15 psi for 30 min	0.91±0.25	0.51±0.04
LSD	0.46	0.42

Data are expressed as percent immunoreactivity as compared to corresponding unprocessed control, mean ± SEM (p=0.05, n=3). Differences between two means within the same column exceeding the LSD value are significant.

CONCLUSIONS

In conclusion, a competitive inhibition ELISA for the detection of macadamia nut seed protein was developed with sensitivity lower than 10 ppm and a detection range of 3.2 to 400 ng/mL. The assay was robust and specific for macadamia nut seed protein.

Macadamia nuts are often incorporated into other food items for texture and flavor. Future research should include ELISA assessment of macadamia nut seed protein recovery from spiked and unspiked food matrices. Determining whether the ELISA can detect the presence of macadamia nut proteins in the presence of other food matrices is vital for consumer protection.

REFERENCES

1. USDA. Market and Trade Data, Foreign Agricultural Service. <www.fas.usda.gov>. 15 May 2010.
2. USDA Economics, Statistics and Market Information System. <http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID =1377>. 28 September 2010.
3. Steiger, D. L.; Moore, P. H.; Zee, F.; Liu, Z.; Ming, R. Genetic relationships of macadamia cultivars and species revealed by AFLP markers. *Euphytica*. **2003**, *132*, 269-277.
4. Venkatachalam, M.; Sathe, S. K. Chemical composition of selected nut seeds. *J. Agric. Food Chem*. **2006**, *54*, 4705-4714.
5. Curb, J. D.; Wergowske, G.; Dobbs, J. C.; Abbott, R. D.; Huang, B. Serum lipid effects of a high-monounsaturated fat diet based on macadamia nuts. *Arch. Intern. Med.* **2000**, *160*, 1154-1158.
6. Garg, M. L.; Blake, R. J.; Wills, R. B. H.; Clayton, E. H. Macadamia nut consumption modulates favourably risk factors for coronary artery disease in hypercholesterolemic subjects. *Lipids*. **2007**, *42*, 583-587.
7. Kris-Etherton, P.M.; Pearson, T. A.; Wan, Y.; Hargrove R. L.; Moriarty, V. F.; Etherton, T. D. High–monounsaturated fatty acid diets lower both plasma cholesterol and triacylglycerol concentrations. *Amer. J. Clin. Nutr*. **1999**, *70*, 1009-1015.
8. Tiwari, R. S.; Venkatachalam, M.; Sharma, G. M.; Su, M.; Roux, K. H.; Sathe, S. K. Effect of food matrix on amandin, almond (*Prunus dulcis* L.) major protein, immunorecognition and recovery. *LWT - Food Sci. Technol.* **2010**, *43*, 675-683.

9. Sathe, S. K.; Teuber, S. S.; Roux, K. H. Effects of food processing on the stability of food allergens. *Biotechnol. Adv*. **2005**, *23*, 423-429.

10. Sicherer, S. H.; Sampson, H. A. Epidemiology of food allergy. *J. Allergy Clin. Immunol*. **2011**, *127*, 594-602.

11. Sicherer, S. H.; Sampson, H. A. Food allergy. *J. Allergy Clin. Immunol*. **2010**, *125*, S116-S126.

12. Sicherer, S. H.; Munoz-Furlong, A.; Godbold, J. H.; Sampson, H. A. US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. *J. Allergy Clin. Immunol*. **2010**, *125*, 1322-1326.

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

14. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265-275.

15. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem*. **1976**, *72*, 248-254.

16. Su, M.; Venkatachalam, M.; Teuber S. S.; Roux K. H.; Sathe, S. K. Impact of γ- irradiation and thermal processing on the antigenicity of almond, cashew nut and walnut proteins. *J. Sci. Food Agric*. **2004**, *84*, 1119-1125.

17. Sharma G. M.; Roux, K. H.; Sathe, S. K. A sensitive and robust competitive enzyme-linked immunosorbent assay for Brazil nut (*Bertholletia excelsa* L.) detection. *J. Agric. Food Chem.* **2009**, *57*, 769-776.