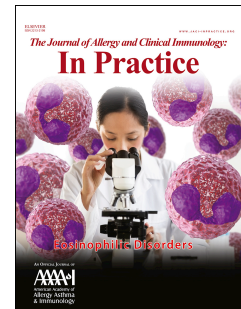


Journal Pre-proof

Irradiated Tree Nut Flours for Use in Oral Immunotherapy

Anusha Penumarti, PhD, Nicole Szczepanski, BS, Janelle Kesselring, BS, Elizabeth Gabel, BS, Rohini Sheth, BS, Jelena Berglund, PhD, Edwin H. Kim, MD MS, A. Wesley Burks, MD, Michael D. Kulis, PhD



PII: S2213-2198(20)31227-7

DOI: <https://doi.org/10.1016/j.jaip.2020.11.008>

Reference: JAIP 3250

To appear in: *The Journal of Allergy and Clinical Immunology: In Practice*

Received Date: 15 July 2020

Revised Date: 2 November 2020

Accepted Date: 3 November 2020

Please cite this article as: Penumarti A, Szczepanski N, Kesselring J, Gabel E, Sheth R, Berglund J, Kim EH, Burks AW, Kulis MD, Irradiated Tree Nut Flours for Use in Oral Immunotherapy, *The Journal of Allergy and Clinical Immunology: In Practice* (2020), doi: <https://doi.org/10.1016/j.jaip.2020.11.008>.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2020 Published by Elsevier Inc. on behalf of the American Academy of Allergy, Asthma & Immunology

Title: Irradiated Tree Nut Flours for Use in Oral Immunotherapy

Authors: Anusha Penumarti, PhD,^{1,2} Nicole Szczepanski, BS,^{1,2} Janelle Kesselring, BS,^{1,2} Elizabeth Gabel, BS,^{1,2} Rohini Sheth, BS,^{1,2} Jelena Berglund, PhD,³ Edwin H Kim, MD MS,^{2,4} A. Wesley Burks, MD,^{1,2} and Michael D. Kulis PhD^{1,2}

Affiliations:

¹ Department of Pediatrics, Division of Allergy, Immunology, and Rheumatology, University of North Carolina at Chapel Hill, Chapel Hill, NC

² University of North Carolina at Chapel Hill Food Allergy Initiative, Chapel Hill, NC

³ Duke Translational Medicine Institute, Duke University Medical Center, Durham, NC

⁴ Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC

Corresponding Author: Anusha Penumarti, PhD, Research Instructor, UNC

Chapel Hill, 116 Manning Dr, Mary Ellen Jones Building Room 3310B, Chapel Hill, NC 27599, Phone: 919-962-5482, anusha23@email.unc.edu

Funding: This research received no external funding.

Conflict of Interest Disclosure Statement:

Michael Kulis receives grant support to his institution from the National Institutes of Allergy and Infectious Diseases (NIH/NIAID) and the Department of Defense (DoD).

Edwin Kim reports clinical medical advisory board membership with DBV Technologies; consultancy with Aimmune Therapeutics, DBV Technologies, AllerGenis, Allakos, Ukko, and Vibrant America; and receives grant support to his institution from the National Institute of Allergy and Infectious Diseases (NIH/NIAID),

National Center for Complementary and Integrative Health (NIH/NCCIH), FARE and the Wallace Research Foundation.

Wesley Burks receives grant support to his institution from the National Institutes of Health and Food Allergy Research & Education; royalties from UpToDate; consulting honorariums from Astella Pharma Global Development, DBV Technologies, kaléo, N-Fold, LLC, and UKKO, Inc. as well as Aimmune Therapeutics, Consortia TX, Inc., and Prota Therapeutics for his service on their respective Scientific Advisory Boards. Dr. Burks owns stock in Allertein and Mastcell Pharmaceuticals.

All other authors have nothing to disclose.

Abstract

Background: Tree nut allergies affect an estimated 1% of the United States population and is lifelong in 90% of allergic individuals. Oral immunotherapy (OIT) for food allergies is an effective method to induce desensitization in a majority of participants in trials of peanut, egg, and milk OIT. Limited trials using tree nut OIT have been reported, possibly due to the lack of standardized drug products.

Objective: Food products used in OIT are considered drugs by the FDA since they are intended to modulate the individuals' immune responses to the food allergens. As such, OIT drug products must meet FDA standards for acceptable levels of microbes and undergo testing for allergenic proteins. We aimed to determine the suitability of walnut, cashew, hazelnut, and almond flours for use in OIT trials.

Methods: We employed gamma irradiation on commercially available walnut, cashew, hazelnut, and almond flours and tested their levels of microbial contamination, total protein and allergen content, along with stability of these parameters over time.

Results: Our results demonstrate that irradiation of tree nut flours greatly diminishes the levels of total aerobic bacteria, mold, yeast, *E. coli*, and salmonella, while there are no substantial changes in total protein or allergen content. Importantly, the microbial levels, protein, and allergen content remained stable over a 24 month period.

Conclusion: Irradiation of tree nut flours is a safe and effective method of processing to allow tree nut products to meet the FDA standards for OIT drug products.

Highlights Box

What is already known about this topic?

Food products used in oral immunotherapy (OIT) trials need to conform to FDA guidelines for orally delivered drugs. Limited information exists on the suitability of commercially available tree nut flours for use in OIT protocols.

What does this article add to our knowledge?

Tree nut flours are often contaminated with high levels of microbes and do not meet the FDA guidelines on OIT drug products. We demonstrated that gamma irradiation of walnut, cashew, hazelnut, and almond flours decreased bioburden without altering the allergenic protein content.

How does this study impact current management guidelines?

Irradiation of tree nut flours represents an effective method to generate drug products for use in OIT trials. These drug products remain stable for at least 24 months when refrigerated.

82 **Keywords:** Food allergy; tree nut allergy; oral immunotherapy; walnut; cashew;
83 hazelnut; almond; allergens

84

85 **Abbreviations:**

86 APC – Aerobic Plate Count

87 CoA – Certificate of Analysis

88 FDA – Food and Drug Administration

89 IND – Investigational New Drug

90 OIT – Oral Immunotherapy

91 SOP – Standard Operating Procedure

92 USDA – United States Department of Agriculture

1. Introduction

Food allergies are a public health concern affecting up to 10% of the US population (1). The severity of food allergies, particularly peanut and tree nut allergies is a significant concern as accidental exposures can result in life-threatening, or even fatal reactions (2, 3). The anxiety that accompanies food allergies can be disruptive, leading to a decreased quality of life for patients and their families (4). Additionally, the annual economic cost of food allergies in the United States is estimated to be \$25 billion (5).

Over the past decade, clinical trials have investigated the safety and efficacy of oral immunotherapy (OIT) for a variety of food allergies (6). OIT is administered in specific amounts of food proteins, usually in a powder form with the intent of modulating the immune response to the food inducing a state of allergic desensitization (7). In this sense, food-based OIT products are considered a drug. In January 2020, the US Food and Drug Administration (FDA) approved the first ever treatment for food allergies with Aimmune's Palforzia OIT product for peanut allergy following a successful Phase 3 trial (8). While this is a major achievement in the field of food allergy, there are no approved products for other severe allergies, such as those to tree nuts. Tree nuts consist of walnut, pecan, cashew, pistachio, almond, hazelnut, Brazil nut, pine nuts, and macadamia nuts. Importantly, tree nuts are commonly used in bakeries and a variety of confectionaries making them a source of food allergens that can lead to accidental exposures and allergic reactions (9). OIT trials for walnut (10) and hazelnut allergies (11) have recently been reported demonstrating desensitization in a substantial number of subjects. These trial results indicate that tree nut OIT can be efficacious and patients are likely to benefit from this form of therapy.

Our group has developed a process for manufacturing investigational drug products for several food allergies used in a variety of OIT clinical trials. A previous publication has detailed the process and FDA guidelines required to produce the OIT drug products (12). Over the past few years, we have expanded our manufacturing process to include walnut, cashew, hazelnut, and almond. The difficulty with these particular foods is that many vendors supply these at USDA grade but they do not meet the FDA guidelines in terms of bioburden (aerobic plate count, yeast, mold, *E. coli*, and *Salmonella*) for orally delivered drug products. Thermal processing methods like roasting, toasting, and microwaving failed to decrease the bioburden in pilot studies. To circumvent this problem, we tested irradiation as a method to reduce bacterial contamination. Gamma irradiation is a type of cold sterilization that is approved by the FDA for the use on certain foods to effectively eliminate organisms that cause foodborne illness, such as *Salmonella* and *E. coli*, extend the shelf life of foods, destroy insects in or on tropical fruits, inhibit sprouting (e.g. potatoes) and delay ripening of fruit to increase longevity and sterilize foods, which can then be stored for years without refrigeration. The FDA has approved a variety of foods for irradiation in the United States including: beef and pork, crustaceans (e.g. lobster, shrimp, and crab), fresh fruits and vegetables, lettuce and spinach, poultry, seeds for sprouting (e.g. alfalfa sprouts), shell eggs, molluscan shellfish (e.g. oysters, clams, mussels, and scallops), spices and seasonings.

The aim of this study was to determine the effects of gamma irradiation on walnut, cashew, hazelnut, and almond flours for use in oral immunotherapy (OIT) trials. Specifically, we aimed to quantify relative amounts of the major allergens in these flours and bioburden levels present in irradiated flours and assess whether these parameters change over a period of twenty-four months.

2. Methods

2.1. Investigational New Drug Application

The work presented here reflects studies conducted under an IND with the FDA (IND# 77,468). The details described are contained within the CMC section of the IND.

2.2. Sources of flours for OIT manufacturing

All bulk flours are accepted after visual inspection and review of the accompanying Certificate of Analysis (CoA) from the vendors. Walnut and hazelnut flours were purchased from Holmquist (Lynden, Washington). Both flours were roasted and defatted by the manufacturer. Partially defatted cashew flour (whole roasted, blanched) was purchased from nuts.com. Almond flour was purchased from Honeyville (Brigham City, Utah) and was blanched by the manufacturer. Flours were purchased in 50 lb. quantities and stored at 2-8°C.

2.3. Irradiation

The flours to be processed were packed in 18x20x24 cartons weighing 50 lb and shipped at ambient temperature for irradiation. Irradiation was performed by Sterigenics (Haw River, NC). Flours were exposed to Minimum/Maximum Dose of kGy, on a scale of 5.0 kGy – 30.0 kGy. This is the maximum FDA permitted dose for microbial disinfection of dry preparations as per 21 CFR 179.26(b). The irradiated flours were then accepted based on the Certificate of Processing by Sterigenics. The Certificate of Processing includes identity of the product processed, maximum and minimum dose exposure, and pass/fail result.

2.4. Bioburden, toxin, and total protein content testing

Bacterial tests, aflatoxin, and total protein content were performed by Deibel Laboratories (Lincolnwood, IL). 100 g per sample type of irradiated walnut, cashew, hazelnut, and almond flours were weighed for bioburden testing. The weighed flours were shipped at ambient temperature to Diebel Laboratories. The following tests were conducted for each flour at each time point: Total Aerobic Plate Count (APC), Mold, and Yeast (SOP 4560, Modified US); Presence of *E. coli* and *Salmonella* (SOP2004, Modified USP), Aflatoxin (Neogen Veratox assay, AOAC RI-05091); and Protein percentage (Titration, Factor 5.30, AOAC 991.20).

2.5. Stability testing

100 g of each of the irradiated flours were weighed into six separate containers for protein and microbial testing. All containers were kept at 2-8°C. The 100 g samples were removed from the refrigerator at approximately 3, 6, 9, 12, 18, and 24 months and shipped overnight to Deibel Laboratories for microbial testing. SDS-PAGE analysis was also done on these irradiated flours to determine the percent variance of proteins from unprocessed/non-irradiated flours. All test samples were compared to the 0 month unprocessed flours, which were stored frozen at -20°C, and run on the same gel as the test samples.

2.6. SDS-PAGE and densitometric scanning to determine allergen content

Irradiated walnut, cashew, hazelnut, and almond flour samples were subjected to SDS-PAGE to visualize known allergens from each food source (Table 1) (13). Proteins were first reduced with 50 mM DTT. These allergens were also used to track protein stability over time. 20 µL of samples were loaded in duplicate and run on a 4%-12% gradient polyacrylamide gel. An image of the gel was captured and densitometric scanning was performed on the image for assessing allergens using Image J software (NIH) (12). Duplicate samples were averaged and percent

difference in the proteins for each current lot from the previous lot was calculated with the following equation: $((\text{Current lot} - \text{Previous lot}) / \text{Previous lot}) \times 100\%$.

3. Results

3.1. Contaminated lots

Upon initial purchase of flours to manufacture OIT doses, we procured flours from a variety of vendors and checked their levels of contamination. Table 2 shows FDA standards for a non-aqueous drug product being delivered by the oral route. Table 3 shows results for each of the flours from sources that ultimately failed our quality control, as the bioburden levels were too high. For example, unprocessed walnut flour had high APC, mold, was presumptive for Salmonella, and tested positive for E.coli. Levels of APC, yeast, and mold were high in non-irradiated cashew flour, which also tested presumptive for Salmonella. While unprocessed hazelnut flour tested positive for Salmonella, unprocessed almond flour had very high APC levels.

3.2. Analysis of bioburden in irradiated flours

Following irradiation, bioburden tests were done in triplicate (Samples 1-3) on three different days and microbial levels were again examined. Table 4 shows the results post-irradiation. Importantly, we can see that bioburden is substantially reduced in irradiated flours and these now meet the FDA standards for orally-delivered drug products in flour form.

3.3. Analysis of proteins in irradiated flours

Each lot of flour was subjected to total protein analysis and then SDS-PAGE analysis to look at individual major allergens. Table 5 shows total protein content in each lot. Total protein content is consistent between irradiated and non-irradiated flours. SDS-PAGE analysis was used to determine the presence of major allergens known to exist in walnut, cashew, hazelnut, and almond. Figure 1 shows the

SDS-PAGE results and highlights the allergens of interest. The allergens evaluated in each of the flours is shown in Table 1. Table 6 shows protein variance of each allergen from unprocessed flour. Allergen levels do not change significantly (<30% as defined in our IND).

3.4. Stability of irradiated flours

Stability of the flours in terms of bioburden and proteins was examined over a 24 month period. Following irradiation, all flours were stored refrigerated at 2-8°C. At approximately 3, 6, 9, 12, 18, and 24 months microbial testing and SDS-PAGE analysis were performed. Table 7 and 8 show the results from long-term storage. After 24 months of regular testing, irradiated flours have maintained bioburden levels that are within the FDA standards for orally administered drugs (Table 6A). The percent variance in allergen levels for each irradiated flour has remained stable with less than a 30% variance in abundance between each test (Table 6B).

4. Discussion

OIT for food allergies has been investigated over the past decade and has shown promise, as it has a generally acceptable safety profile and efficacy in a majority of subjects, including alterations in the antigen-specific immune response (14, 15). Foods in a powdered form are used as OIT drug products for peanut, egg, and milk allergies (16-18). Importantly, OIT is likely to become increasingly practiced with the FDA-approval of Palforzia for peanut allergy. It is of utmost importance to thoroughly characterize food products for allergen content, biological contamination, and stability prior to use. Typically, products are purchased and used without further processing. However, we recently discovered an issue with tree nuts suggesting that many suppliers' products are not ready for OIT use due to bacterial, mold, and yeast contamination that fall outside of the FDA's established guidelines for

orally-administered drugs. We added the additional step of gamma irradiation to bring the biological contamination to meet the FDA's standards.

Irradiation is often practiced on foods consumed by the public (19). For example, beef, pork, crustaceans (e.g. lobster, shrimp, and crab), fresh fruits and vegetables, poultry, mollusks (e.g. oysters, clams, mussels, and scallops), spices, and seasonings are irradiated prior to human consumption. Our experiments demonstrate that irradiation of tree nut flours led to a decrease of bioburden levels without significantly altering the protein content. This is important because high levels of contamination could cause illness after regular ingestion, especially in young children who are often the subjects undergoing OIT in clinical trials.

We also examined the stability of the flours when stored refrigerated, as OIT trials are often conducted for several years and subjects require new doses approximately every two weeks during the build-up phase and every three months during the maintenance phase (20). After 24 months of regular testing, irradiated tree nut flours have maintained bioburden levels that are within the FDA standards for orally administered drugs. This finding allows us to utilize the same irradiated drug products for many months, helping to keep costs lower and drug product on-hand.

The allergen quantity in a given food may be variable from batch-to-batch and certainly between vendors (21). A sudden increase in allergen quantity could prove problematic for a research subject taking OIT as a higher amount of allergen could cause allergic reactions. To track protein quantities, we utilized a total protein assay and an allergen content approach. Total protein levels were not affected by irradiation and were very stable over a 24 month period. Additionally, allergens in the tree nut flours remained stable, with less than a 30% variance in concentration between each test over 24 months. These data allow us to conclude that irradiation

does not have a significant impact on protein or allergen content in these tree nut products. While it remains to be studied whether irradiation causes structural alterations of the tree nut allergens, a study using the purified peanut allergen Ara h 6 demonstrated that the alpha-helical nature of the protein was modified, leading to decreased binding to polyclonal IgG (22). However, it's important to note that irradiated wheat flour was used successfully in an OIT protocol for wheat allergy (23), indicating that irradiated food products are likely still effective for OIT.

In conclusion, gamma irradiation of tree nut flours, including walnut, cashew, hazelnut, and almond is sufficient to reduce bioburden levels to below the thresholds set by the FDA for orally-administered drug products. Under our IND, these tree nut flours have been administered to research subjects within clinical trials. As OIT use expands, we encourage investigators and allergists in practice to consider the importance of irradiation of tree nut flours prior to use in OIT protocols.

References

1. Gupta RS, Warren CM, Smith BM, Jiang J, Blumenstock JA, Davis MM, et al. Prevalence and Severity of Food Allergies Among US Adults. *JAMA Netw Open*. 2019;2(1):e185630.
2. Bock SA, Munoz-Furlong A, Sampson HA. Fatalities due to anaphylactic reactions to foods. *J Allergy Clin Immunol*. 2001;107(1):191-3.
3. Clark AT, Anagnostou K, Ewan PW. Cashew nut causes more severe reactions than peanut: case-matched comparison in 141 children. *Allergy*. 2007;62(8):913-6.
4. Greenhawt M. Food allergy quality of life and living with food allergy. *Curr Opin Allergy Clin Immunol*. 2016;16(3):284-90.
5. Gupta R, Holdford D, Bilaver L, Dyer A, Holl JL, Meltzer D. The economic impact of childhood food allergy in the United States. *JAMA Pediatr*. 2013;167(11):1026-31.
6. Wood RA. Food allergen immunotherapy: Current status and prospects for the future. *J Allergy Clin Immunol*. 2016;137(4):973-82.
7. Kulis M, Wright BL, Jones SM, Burks AW. Diagnosis, management, and investigational therapies for food allergies. *Gastroenterology*. 2015;148(6):1132-42.
8. Investigators PGoC, Vickery BP, Vereda A, Casale TB, Beyer K, du Toit G, et al. AR101 Oral Immunotherapy for Peanut Allergy. *N Engl J Med*. 2018;379(21):1991-2001.
9. Eigenmann PA, Lack G, Mazon A, Nieto A, Haddad D, Brough HA, et al. Managing Nut Allergy: A Remaining Clinical Challenge. *J Allergy Clin Immunol Pract*. 2017;5(2):296-300.
10. Elizur A, Appel MY, Nachshon L, Levy MB, Epstein-Rigbi N, Pontoppidan B, et al. Walnut oral immunotherapy for desensitisation of walnut and additional tree nut allergies (Nut CRACKER): a single-centre, prospective cohort study. *Lancet Child Adolesc Health*. 2019;3(5):312-21.
11. Moraly T, Pelletier de Chambure D, Verdun S, Preda C, Seynave M, Vilain AC, et al. Oral Immunotherapy for Hazelnut Allergy: A Single-Center Retrospective Study on 100 Patients. *J Allergy Clin Immunol Pract*. 2020;8(2):704-9 e4.
12. Berglund JP, Szczepanski N, Penumarti A, Beavers A, Kesselring J, Orgel K, et al. Preparation and Analysis of Peanut Flour Used in Oral Immunotherapy Clinical Trials. *J Allergy Clin Immunol Pract*. 2017;5(4):1098-104.
13. Smeeckens JM, Bagley K, Kulis M. Tree nut allergies: Allergen homology, cross-reactivity, and implications for therapy. *Clin Exp Allergy*. 2018;48(7):762-72.
14. Gernez Y, Nowak-Wegrzyn A. Immunotherapy for Food Allergy: Are We There Yet? *J Allergy Clin Immunol Pract*. 2017;5(2):250-72.
15. Smeeckens JM, Kulis MD. Evolution of Immune Responses in Food Immunotherapy. *Immunol Allergy Clin North Am*. 2020;40(1):87-95.
16. Varshney P, Jones SM, Scurlock AM, Perry TT, Kemper A, Steele P, et al. A randomized controlled study of peanut oral immunotherapy: clinical desensitization and modulation of the allergic response. *J Allergy Clin Immunol*. 2011;127(3):654-60.
17. Burks AW, Jones SM, Wood RA, Fleischer DM, Sicherer SH, Lindblad RW, et al. Oral immunotherapy for treatment of egg allergy in children. *N Engl J Med*. 2012;367(3):233-43.
18. 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. *J Allergy Clin Immunol*. 2008;122(6):1154-60.

19. Maherani B, Hossain F, Criado P, Ben-Fadhel Y, Salmieri S, Lacroix M. World Market Development and Consumer Acceptance of Irradiation Technology. *Foods*. 2016;5(4).
20. Vickery BP, Scurlock AM, Kulis M, Steele PH, Kamilaris J, Berglund JP, et al. Sustained unresponsiveness to peanut in subjects who have completed peanut oral immunotherapy. *J Allergy Clin Immunol*. 2014;133(2):468-75.
21. Filep S, Block DS, Smith BRE, King EM, Commins S, Kulis M, et al. Specific allergen profiles of peanut foods and diagnostic or therapeutic allergenic products. *J Allergy Clin Immunol*. 2018;141(2):626-31 e7.
22. Luo C, Hu C, Gao J, Li X, Wu Z, Yang A, et al. A potential practical approach to reduce Ara h 6 allergenicity by gamma irradiation. *Food Chem*. 2013;136(3-4):1141-7.
23. Nowak-Wegrzyn A, Wood RA, Nadeau KC, Pongratic JA, Henning AK, Lindblad RW, et al. Multicenter, randomized, double-blind, placebo-controlled clinical trial of vital wheat gluten oral immunotherapy. *J Allergy Clin Immunol*. 2019;143(2):651-61 e9.

342 **Figure legends:**

343 **Figure 1.** SDS-PAGE gels comparing proteins in unprocessed and irradiated
344 walnut, cashew, hazelnut and almond flours. All samples were run in triplicate.

Table 1. Allergens that were assessed for each flour.

Flour Source	Allergens [Molecular Weight]
Walnut	Jug r 2 [~44 kD] and Jug r 1 [~6 kD]
Cashew	Ana o 1 [~50 kD] and Ana o 3 [~5 kD]
Hazelnut	Cor a 11 [~47 kD] and Cor a 14 [~6 kD]
Almond	Pru du 6 [~42 and 44 kD]

Table 2. FDA standards for orally delivered drugs.

Total Aerobic Microbial Count (cfu/g)	<10 ³
Yeast (cfu/g)	<10 ²
Mold (cfu/g)	<10 ²
Salmonella (in 1 g)	Absent
E.coli (in 1 g)	Absent
Aflatoxin	<15 ppb

Table 3. Microbial levels in two lots of unprocessed walnut, cashew, hazelnut and almond flours.

	Walnut		Cashew		Hazelnut		Almond	
	Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2
APC (cfu/g)	>4000	840	>2500	>2500	90	490	>2500	>2500
Yeast (cfu/g)	<10	<10	40	310	70	50	<10	<10
Mold (cfu/g)	>2500	190	110	<10	<10	<10	<10	10
Salmonella	Negative	Presumptive	Presumptive	Presumptive	Presumptive	Positive	Negative	Negative
E.coli	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Aflatoxin (ppb)	<2	<2	<5	<5	<5	<5	5	<5

Table 4. Bioburden levels in Irradiated Walnut, Cashew, Hazelnut and Almond flours.

Walnut	Irradiated Sample 1	Irradiated Sample 2	Irradiated Sample 3
APC	<10 cfu/g	<10 cfu/g	<10 cfu/g
Mold	<10 cfu/g	<10 cfu/g	<10 cfu/g
Yeast	<10 cfu/g	<10 cfu/g	<10 cfu/g
E.coli	Negative	Negative	Negative
Salmonella	Negative	Negative	Negative
Aflatoxin	<5 ppb	<5 ppb	<5 ppb
Cashew	Irradiated Sample 1	Irradiated Sample 2	Irradiated Sample 3
APC	<10 cfu/g	<10 cfu/g	<10 cfu/g
Mold	<10 cfu/g	<10 cfu/g	<10 cfu/g
Yeast	<10 cfu/g	<10 cfu/g	<10 cfu/g
E.coli	Negative	Negative	Negative
Salmonella	Negative	Negative	Negative
Aflatoxin	<5 ppb	<5 ppb	<5 ppb
Hazelnut	Irradiated Sample 1	Irradiated Sample 2	Irradiated Sample 3
APC	<10 cfu/g	<10 cfu/g	<10 cfu/g
Mold	<10 cfu/g	<10 cfu/g	<10 cfu/g
Yeast	<10 cfu/g	<10 cfu/g	<10 cfu/g
E.coli	Negative	Negative	Negative
Salmonella	Negative	Negative	Negative
Aflatoxin	<5 ppb	<5 ppb	<5 ppb
Almond	Irradiated Sample 1	Irradiated Sample 2	Irradiated Sample 3
APC	<10 cfu/g	<10 cfu/g	<10 cfu/g
Mold	<10 cfu/g	<10 cfu/g	<10 cfu/g
Yeast	<10 cfu/g	<10 cfu/g	<10 cfu/g
E.coli	Negative	Negative	Negative
Salmonella	Negative	Negative	Negative
Aflatoxin	<5 ppb	<5 ppb	<5 ppb

Table 5. Total protein content in unprocessed and irradiated (run in triplicate) flours.

	Unprocessed Flour	Irradiated Flour		
	Sample 0	Sample 1	Sample 2	Sample 3
Walnut	35.18%	34.96%	34.30%	35.16%
Cashew	17.60%	17.22%	18.06%	17.31%
Hazelnut	26.87%	28.24%	28.01%	27.97%
Almond	23.62%	23.17%	23.28%	23.19%

Table 6. Percent variance in protein concentration of Irradiated flours (run in triplicate) compared to unprocessed flours.

		Irradiated Flour		
		Sample 1	Sample 2	Sample 3
Walnut	Jug r 2	12.62%	3.40%	2.69%
	Jug r 1	9.46%	2.51%	1.26%
Cashew	Ana o 1	3.83%	4.53%	4.71%
	Ana o 3	3.12%	8.21%	0.47%
Hazelnut	Cor a 11	5.43%	3.82%	3.00%
	Cor a 14	3.32%	3.92%	0.92%
Almond	Pru du 6	4.72%	0.46%	0.98%

Table 7. Bioburden and total protein results of irradiated flours at 0-24months.

Walnut	0 month	3 month	6 month	9 month	12 month	18 month	24 month
APC	<10 cfu/g	10 cfu/g	10 cfu/g	<10cfu/g	<10cfu/g	<10 cfu/g	<10 cfu/g
Yeast	<10 cfu/g	<10 cfu/g	<10 cfu/g	10 cfu/g	10 cfu/g	<10 cfu/g	<10 cfu/g
Mold	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
Salmonella	Negative	Negative	Negative	Negative	Negative	Negative	Negative
E.coli	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Aflatoxin	<5 ppb	<5 ppb	<5 ppb	<5 ppb	<5 ppb	<5 ppb	<5 ppb
Protein%	35.16%	-	34.65%	-	34.59%	-	35.24%
Cashew	0 month	3 month	6 month	9 month	12 month	18 month	24 month
APC	<10 cfu/g	<10 cfu/g	10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
Mold	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
Yeast	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
E.coli	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Salmonella	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Aflatoxin	<5 ppb	<5 ppb	<5 ppb	<5 ppb	<5 ppb	<5 ppb	<5 ppb
Protein%	17.31%	-	17.46%	-	17.34%	-	17.50%
Hazelnut	0 month	3 month	6 month	9 month	12 month	18 month	24 month
APC	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
Mold	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
Yeast	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
E.coli	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Salmonella	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Aflatoxin	<5 ppb	<5 ppb	<5 ppb	<5 ppb	<5 ppb	<5 ppb	<5 ppb
Protein%	27.97%	-	27.61%	-	27.53%	-	27.90%
Almond	0 month	3 month	6 month	9 month	12 month	18 month	24 month
APC	<10 cfu/g	10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
Yeast	<10 cfu/g	<10 cfu/g	<10 cfu/g	10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
Mold	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
Salmonella	Negative	Negative	Negative	Negative	Negative	Negative	Negative
E.coli	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Aflatoxin	<5 ppb	<5 ppb	<5 ppb	<5 ppb	<5 ppb	<5 ppb	<5 ppb
Protein%	23.19%	-	23.56%	23.89%	23.24%	-	23.71%

Table 8. Percent variance in allergen content of irradiated flours over 24 months.

		0 month	3 month	6 month	9 month	12 month	18 month	24 month
Walnut	Jug r 2	12.62%	6.60%	7.15%	3.29%	4.28%	16.90%	2.11%
	Jug r 1	9.46%	1.19%	19.50%	0.66%	4.49%	15.53%	5.58%
Cashew	Ana o 1	4.71%	8.79%	7.63%	14.50%	6.94%	12.58%	2.10%
	Ana o 3	0.47%	7.34%	9.09%	4.64%	10.95%	8.60%	3.81%
Hazelnut	Cor a 11	3.00%	3.10%	16.76%	2.02%	3.49%	11.26%	12.68%
	Cor a 14	0.92%	3.31%	6.81%	3.62%	12.39%	25.24%	1.74%
Almond	Pru du 6	0.98%	1.82%	0.60%	5.85%	1.74%	1.33%	3.46%

