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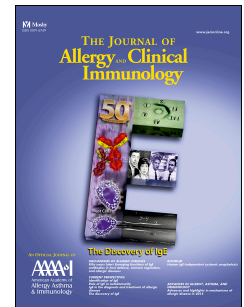
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**Specific allergen profiles of peanut foods and of diagnostic or therapeutic
allergenic products.**

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ABSTRACT

Rationale: 'Generic' immunoassays for peanut cannot discriminate between allergen levels in peanut derived food products or therapeutics. Clinical trials of oral immunotherapy are strengthened by using standardized peanut preparations, with defined doses of major allergens.

Objective: This paper describes measurement of Ara h 1, Ara h 2 and Ara h 6 in peanut foods and in peanut flour extracts used for allergy diagnosis and oral immunotherapy.

Methods: Monoclonal antibody-based enzyme immunoassays for Ara h 1, Ara h 2 and Ara h 6 were used to compare allergen levels in peanut (n=19) and tree nut (n=16) butter, peanut flour (n=11); oils (n=8); extracts used for diagnosis and oral immunotherapy (n=5); and the National Institute for Standards and Technology Peanut Butter Standard Reference Material® 2387.

Results: Roasted peanut butters contained 991-21,406µg/g Ara h 1 and exceeded Ara h 2 and Ara h 6 levels by 2-4 fold. Similarly, NIST SRM 2387 contained 11,275µg/g Ara h 1; 2,522µg/g Ara h 2 and 2,036µg/g Ara h 6. In contrast, peanut flours contained 787-14,631µg/g Ara h 2 and exceeded Ara h 1 levels by 2-20 fold. Flour extracts used for oral immunotherapy contained 394-505µg/ml Ara h 1, 1,187-5,270µg/ml Ara h 2 and 1,104-8,092µg/ml Ara h 6. In most cases, specific peanut allergens were not detected in tree nut butters or peanut oils.

Conclusions: The results show marked differences in specific peanut allergen profiles in peanut butter, flour and peanut preparations for clinical use. Roasting may increase Ara h 1 levels in peanut butter. Variability in allergen levels could affect the outcome of clinical trials of peanut oral immunotherapy, especially with respect to Ara h 1. Specific allergen measurements will improve standardization and provide accurate dosing of peanut preparations that are being used for oral immunotherapy.

Clinical Implications

Quantitative measurements of specific peanut allergens will improve the formulation and standardization of peanut allergen vehicles that are used for diagnostic purposes and for clinical trials of oral immunotherapy.

Capsule Summary

The specific (major) allergen content of peanuts foods and of peanut preparations used for allergy diagnosis and oral immunotherapy is highly variable. Allergen measurements provide essential tools for dosing of clinical products used in trials of oral immunotherapy.

Key Words

Peanut, peanut foods, peanut butter, peanut flour, peanut allergens, oral immunotherapy, allergen dosing, risk assessment, allergen standardization

Abbreviations

AGE	Advanced glycation end products
ELISA	Enzyme-linked immunosorbent assay
NIST SRM 2387	National Institute for Standards and Technology Peanut Butter Standard Reference Material® 2387
OIT	Oral immunotherapy
PBS	Phosphate buffered saline, pH 7.4
UNC	University of North Carolina at Chapel Hill
UVA	University of Virginia Health Sciences Center

Introduction

Allergen testing in the food industry relies on the use of 'generic' immunoassays for allergens and for other antigens or residues within the food to provide a measure of 'total' food allergen content (usually expressed in parts per million). This approach serves the purpose of having a test that can measure allergenic foods (e.g. peanut, milk, egg, soy, shellfish) that might contaminate the food supply and put allergic patients at risk of having symptoms. From an allergy perspective, generic ELISA tests for foods have several limitations. The specific food allergens/antigens that are measured in these assays are not defined and assays from different manufacturers cannot be directly compared. The inability to measure specific allergens means that the tests cannot discriminate between specific allergen levels in food products or in food preparations that are increasingly being used for oral immunotherapy (OIT) or for prophylactic approaches to food allergy prevention.⁽¹⁻⁴⁾ The allergenic potency of these preparations needs to be assessed relative to patients' sensitivities to specific allergens (e.g. peanut allergens Ara h 1, Ara h 2 and Ara h 6), which is not possible using generic ELISA.

A quantitative, monoclonal antibody-based ELISA for Ara h 1 was previously used to monitor allergen levels in peanut containing foods, including chocolate.^(5,6) Subsequently, this assay was used to investigate the effects of roasting on Ara h 1 levels in peanuts and expression of Ara h 1 in different peanut strains.⁽⁷⁾ Measurements of Ara h 1 were also used as a surrogate marker in clinical studies to assess the distribution of peanut allergens in schools and to investigate the transmission of peanut through saliva via kissing or sharing utensils.^(8,9) While Ara h 1 appeared to be a good marker for peanut, the assay sensitivity was somewhat low (~30ng/ml) which made it unsuitable for certain applications e.g. environmental exposure assessments. It also became clear that Ara h 2 was the most important peanut allergen in terms of biological potency and diagnostic efficacy.^(10,11) Ara h 6 is structurally related to Ara h 2 (50% amino acid identity) and has similar allergenic importance.⁽¹²⁾ Both Ara h 2 and Ara h 6 are

major allergens, eliciting IgE responses in 80-90% of peanut allergic patients and showing potent activity in basophil histamine release assays.^(10;11) The three dimensional structures of Ara h 1, Ara h 2 and Ara h 6 have been determined and these three allergens are well defined molecular markers of peanut.^(13;14)

Panels of monoclonal antibodies (mAb) to Ara h 2 and to Ara h 6 were used to develop sensitive ELISA that were specific for each allergen.⁽¹¹⁾ The aim of the present study was to compare Ara h 1, Ara h 2 and Ara h 6 profiles in peanut foods (peanut butter, flour, and oil) as well as in allergenic products that are used for diagnostic purposes and in preparations that are used for OIT. The results show wide variation in the profiles of specific peanut allergens across these sources. They also suggest that specific allergen measurements can improve allergen standardization of peanut preparations used for diagnostic and therapeutic purposes.

Methods

Peanut food products

Samples of commercial peanut butter (n=16), peanut flour (n=11), peanut powder (n=3) and refined or unrefined peanut oils (n=8) were purchased either from local supermarkets or online. Non-peanut butter (n=16) and non-peanut flour (n=9), including almond, hazelnut, cashew, walnut, pecan, soy, coconut, and garbanzo bean, were used as controls. Samples were selected to include representative store brands as well as organic and artisanal products. A complete list of all the products tested is provided in Table E.1 in the online repository.

The National Institute for Standards and Technology Peanut Butter Standard Reference Material® 2387 (NIST SRM 2387) was purchased from NIST (Gaithersburg, MD). This standard was established for validating methods for determining fatty acids, calories, vitamins, elements, amino acids and acrylamide and other components. The NIST SRM 2387 was prepared from raw Florunner peanuts from several suppliers which were roasted and then ground into creamy peanut butter. For further details, see the NIST SRM 2387 Certificate of Analysis at:

<https://www-s.nist.gov/srmors/certificates/2387.pdf>

Peanut allergenic products

Peanut skin test reagents were obtained from two U.S. allergen manufacturers: Greer Laboratories (Lenoir, NC, now Stallergenes-Greer) and ALK-ABELLO (Roundrock, TX).

Peanut flour extracts (n=3) were prepared for use in clinical trials of OIT at the Division of Pediatric Allergy, University of Virginia Health Sciences Center (UVA) and at the Department of Pediatric Allergy, University of North Carolina at Chapel Hill (UNC). The UVA peanut OIT Study involved twenty peanut allergic children ages 4–18 years who were enrolled in a pilot study of OIT (IRB #15098; ClinicalTrials.gov ID: NCT02350660). The peanut flour used in the

study was obtained from Greer Laboratories source materials department (product code RMF171P). A peanut protein extract solution was prepared according to Jones et al.⁽¹⁵⁾ Briefly, peanut proteins were extracted from the Greer sourced peanut flour in PBS, clarified by centrifugation (30,000g for 30 minutes) and sterilized by filtration. Protein concentration was determined and a stock solution was stored at -20°C. The UNC peanut OIT study enrolled 50 young children aged 9-36 months to determine the efficacy of OIT in terms of desensitization and sustained unresponsiveness (IRB #11-2307 and ClinicalTrials.gov ID: NCT00932828).⁽¹⁶⁾ The peanut flour used in the study was a 12% fat, light roasted peanut flour and was obtained from Golden Peanut Company (Alpharetta, GA). The extract was prepared using the same protocol as the extract produced at UVA.⁽¹⁵⁾

Immunoassays for peanut allergens

Measurements of Ara h 1, Ara h 2 and Ara h 6 were made by ELISA as described previously.^(5;11) The Ara h 1 ELISA used mAb 2C12 for allergen capture and biotinylated mAb 2F7 for detection. The Ara h 2 ELISA used mAb 1C4 for allergen capture and polyclonal rabbit anti-Ara h 2 for detection. The Ara h 6 ELISA used mAb 3B8 for allergen capture and biotinylated mAb 3E12 for detection. Purified in-house natural Ara h 1 (Lot 35106), Ara h 2 (Lot 35139) and Ara h 6 (Lot 35006) were used as allergen standards for each assay. Protein content of the standards was determined either by amino acid analysis or by absorption at 280nm. Samples were typically diluted to 1/100 and assayed at doubling dilutions across the ELISA plate.

Peanut butter, flour and powder samples and control samples were prepared by extracting 1g aliquots in 10ml PBS, pH 7.4, containing 0.05% Tween 20 and 1M NaCl. Samples were extracted for two hours at room temperature and centrifuged at 150xg for 20mins. The supernatants were stored at -20°C prior to analysis. The allergen content of peanut butter, flour

152 and powder samples was expressed as μg per gram of the actual food product extracted.

153 Peanut oils were diluted 1/100 in PBS, pH 7.4, containing 0.05% Tween 20 prior to assay.

154 Selected peanut butter and peanut flour extracts were assayed for total peanut antigen

155 by ELISA using a commercial kit (Veratox, Neogen, Lansing, MI).

156

RESULTS

Allergen profiles of peanut food products

The availability of ELISA for three major peanut allergens allowed quantitative comparisons of the allergen composition of peanut foodstuffs. Control curves for these assays were reproducible when different peanut flour extracts were compared, albeit with different levels of sensitivity: Ara h 1, Lower limit of detection (LLOD) 31ng/ml; Ara h 2, LLOD 2ng/ml; and Ara h 6, LLOD 0.8ng/ml (Figure 1). A peanut butter standard produced by the U.S. National Institute of Standards and Technology, SRM 2387, has been used by the food industry since 2003 for validating measurements of amino acids and other biochemical components of peanut butter. To our knowledge, NIST SRM 2387 is the only national standard for peanut butter (or any other peanut foodstuff). Control curves for the purified allergen standards, NIST SRM 2387 and selected peanut butter and peanut flour extracts were parallel (Figure E.1, online repository). These results provided validation that the ELISA could be used to make quantitative measurements of specific peanut allergens in different peanut matrices. Assay performance was also evaluated by spike recovery experiments using NIST SRM 2387 and peanut oil. Recovery rates using both matrices ranged from 80-99% (Table E.1, online repository). Reproducibility of the assays and the extraction procedures was also demonstrated using NIST SRM 2387, further validating the robustness of the ELISA (Table E.2, online repository).

Analysis of NIST SRM 2387 showed that it contained substantial amounts of Ara h 1, Ara h 2 and Ara h 6 (11,275µg/g, 2,522µg/g and 2,036µg/g, respectively) with high ratios of Ara h 1 to Ara h 2 (4.5) and to Ara h 6 (5.3). The allergen profiles of 16 peanut butter samples showed significant variability in Ara h 1 levels (991-21,406µg/g) and that Ara h 1 typically exceeded Ara h 2 and Ara h 6 levels by 2-4 fold (Table 1A). High ratios of Ara h 1:Ara h 2 were found in roasted peanut butter. Two samples of raw peanut butter (Essential Living Foods and

Vivapura), contained much lower levels of Ara h 1 than Ara h 2 (ratio 0.24), suggesting that roasting selectively increased measurable Ara h 1 in peanut butter. Overall, the mean ratio of Ara h 1: Ara h 2 and Ara h 1: Ara h 6 was 2.0 ± 0.6 and 2.7 ± 0.7 , respectively. Levels of Ara h 6 were similar to Ara h 2 in the peanut butter samples and in other peanut foodstuffs analyzed in this study (Table 1).

In contrast, peanut flour samples from five commercial sources contained higher levels of Ara h 2 (and Ara h 6) than Ara h 1 (9/11, 82%). Moreover, the Ara h 1 levels in peanut flour showed great variability (34-13,473 $\mu\text{g/g}$) (Table 1B). The lowest levels of Ara h 1 were found in "Dark Roast" peanut flour (34-122 $\mu\text{g/g}$) from two different suppliers. These samples also contained the lowest levels of Ara h 2, suggesting that dark roast processing significantly reduces overall allergen content. The mean ratio of Ara h 2: Ara h 1 in peanut flour samples was 7.3 ± 5.6 , with three samples containing 15 to 23fold more Ara h 2 than Ara h 1 (Table 1B, Figure 2). Ara h 2 levels in "Light Roast" peanut flour showed greater consistency, especially 12% fat, light roast flour in which the Ara h 1, Ara h 2 and Ara h 6 levels were of the same order of magnitude. Control curves for the light roast flours were similar (Figure E.1), indicating that antigenic reactivity was retained after the roasting process.

Peanut content of flour samples was also compared using one of the total peanut assays that are widely used in the food industry. The results did not correlate with the cumulative measurements of Ara h 1, Ara h 2 and Ara h 6 in the flour samples. For example, the light roast and dark roast Golden flour samples varied by 14-fold in cumulative allergen content, yet had very similar measures of total peanut in ppm (Table 1B). These results suggested that the total peanut ELISA was measuring peanut protein(s) other than the specific allergens measured in this study.

Control tree nut, soy nut or sunflower seed butters were below detection limit for all three allergen assays or contained trace amounts of Ara h 2 (<0.8ug/g). Significant Ara h 1 reactivity was detected in garbanzo bean/chickpea flour (14-16ug/g) (Table E1, online repository). Preliminary affinity purification data (not shown) suggests that garbanzo beans may produce an Ara h 1 homologue which would explain this apparent cross-reactivity.

Several peanut oils were tested, including unrefined, refined and expeller pressed oils, labeled as such by the manufacturers. No detectable Ara h 1, Ara h 2 or Ara h 6 was found in 7/8 oils. All three allergens were detected at 6-10µg/ml in one sample of roasted, unfiltered oil, which contained a sediment of peanut solids (see Table E.1, online repository, for the complete dataset of allergen profiles of peanut foodstuffs that were tested in the study).

Diagnostic reagents and OIT formulations

Specific peanut allergens were measured in diagnostic skin test products from two U.S. allergen manufacturers. The results showed that both manufacturers' test solutions contained high levels of Ara h 2 relative to Ara h 1, suggesting that they were prepared from peanut flour. The Ara h 2 levels of Manufacturer A skin test reagents were >2,000µg/ml, with ~50µg/ml Ara h 1. The Ara h 2 levels of Manufacturer B skin test reagents were 200-700µg/ml, with Ara h 1 ranging from 24-114µg/ml (Table 2). The Ara h 2 levels for both manufacturers are very high for a diagnostic skin test solution and exceed, for example, the allergen levels found in standardized dust mite and cat allergenic products that are licensed by the FDA.⁽¹⁷⁾ It should be noted that the levels of Ara h 1 are also broadly comparable to those found in standardized skin test products.

Oral immunotherapy is being developed for treatment and prevention of peanut allergy. Almost all peanut OIT studies use peanut flour to formulate food vehicles for treatment purposes.⁽¹⁵⁾ The dose of peanut used is reported as mg peanut flour, with a typical

228 maintenance dose being 300mg. Comparison of specific peanut allergen levels in flours used in
229 two OIT trials showed 3-10 fold more Ara h 2 and Ara h 6 than Ara h 1 (Table 3). The two
230 samples from UNC showed good reproducibility. The levels of Ara h 2 and Ara h 6 in the UVA
231 peanut vehicle were ~3-6 fold higher than those used by UNC, indicating that different
232 cumulative doses of specific allergens were being used in these OIT trials.

233

DISCUSSION

Allergen variance within and between peanut products

Ara h 1, Ara h 2 and Ara h 6 are immunodominant allergens among peanut sensitive individuals. Here, we show that the composition and concentrations of these three major allergens vary greatly in peanut butter, peanut 'powder' and, especially, in peanut flour, where specific allergen concentrations varied by as much as 20-fold. A striking feature of the results was that the levels of Ara h 1 in peanut butter were 2-4 fold higher than Ara h 2. This effect was most notable for the NIST SRM 2387 peanut butter standard that contained 4-5 fold more Ara h 1 than the other allergens. Conversely, Ara h 2 levels in peanut flour were up to 20 fold higher than Ara h 1. Ara h 6 levels closely paralleled those of Ara h 2 (for the purposes of this Discussion, any comments related to Ara h 2, also apply to Ara h 6.)

Roasting has previously been shown to increase the apparent antigenicity of Ara h 1 in peanuts and to increase IgE binding to peanut extracts.^(7;18;19) Thus high levels of Ara h 1 in peanut butter appear to be explained by the fact that the majority of the samples were prepared from roasted peanuts. The two peanut butter brands that used raw peanuts both contained 4-fold more Ara h 2 than Ara h 1 and had similar allergen profiles to peanut flour samples. For both peanut butter and peanut flour samples, there was reasonable consistency of allergen levels among multiple samples from the same food manufacturer. As expected, non-peanut butter samples were essentially free of peanut allergens, thus confirming their utility as a substitute food for peanut allergic patients. The molecular mechanisms of roasting, and of the effects of food processing in general, on peanut allergens are poorly understood. Recent mass spectrometry studies have identified advanced glycation end products (AGE) which modify lysine residues on Ara h 1 (and Ara h 3), but not Ara h 2.⁽²⁰⁾ IgE antibodies recognize both AGE-

modified and unmodified Ara h 1, which suggests that other mechanism(s) contribute to food processing effects on IgE and mAb binding.

The variability of Ara h 2 levels in peanut flour was largely attributable to dark roast flour containing either 12% or 28% fat. These samples had the lowest Ara h 2 levels, but also had extremely low levels of Ara h 1, suggesting that dark roast processing degrades both allergens, especially Ara h 1. Although 12% fat light roast or dry roasted peanut flours also had higher levels of Ara h 2, the differences in Ara h 2: Ara h 1 ratios were not as marked and some samples contained similar levels for all three allergens (e.g. Old Virginia Byrd Mill and Golden Peanut Company).

Comparison of testing modalities

Peanut flour is widely used in the food manufacturing and processing industry and is incorporated into baked goods, confectionary and desserts. Trace contamination of other foods with peanut has traditionally been measured using total peanut antigen ELISA. The results show a weak correlation between total peanut protein and specific allergen content of peanut flour. Recent studies by Jayasena et al compared the specificity and sensitivity of six commercial peanut ELISA kits for measuring Ara h 1, Ara h 2, Ara h 3 and Ara h 6. The data convincingly showed that 5/6 peanut ELISA kits measured predominantly Ara h 3, with weak reactivity to other allergens, while one kit predominantly measured Ara h 2 and Ara h 6.⁽²¹⁾ The total peanut kit that was used in the present study was an 'Ara h 3' kit, which explains why the results did not correlate with specific allergen data reported here. Almost all of the peanut flour samples were measured to contain >1,000,000 ppm total peanut in the commercial kit (Table 1B). The most likely explanation for these results is that the flour samples contained higher concentrations of Ara h 3 than the assay standard for total peanut used in the kit.

Clinical significance of variability in peanut allergens levels

(i) Peanut oils

The present analysis of allergen profiles in peanut foods was not designed to investigate whether the consumption of a particular peanut food might affect IgE mediated sensitization to a specific allergen(s). However, the results have some bearing on whether peanut oil contributes towards allergenicity. Early studies indicated that peanut oil did not contain protein allergen(s).^(22;23) Subsequent studies reported that concentrated protein from peanut oil gave positive skin tests, leucocyte histamine release and IgE inhibition activity in highly sensitive peanut allergic patients.⁽²⁴⁾ Epidemiologic studies showed a significant correlation between positive peanut challenge tests in children and use of peanut creams or emollients that contained peanut oils. This led to the hypothesis that allergic sensitization to peanut occurred because of exposure to allergen via peanut oils present in creams and emollients that were applied to inflamed skin.⁽²⁵⁾

In the present study, 7/8 peanut oils were below the limit of detection in the Ara h 1, Ara h 2 and Ara h 6 ELISA. That is <8ng/ml for the most sensitive assay (Ara h 6). All three allergens were detected at 6-10µg/ml in unfiltered peanut oil, suggesting that the absence of allergen in refined peanut oils was not explained by the extraction methods used. The data are consistent with clinical studies which showed that highly refined peanut oils did not cause allergic reactions in double blind crossover food challenge studies.⁽²⁶⁾ While the possibility that peanut oil contains allergens other than those measured in this study cannot be excluded, this appears to be unlikely. Over the ~15 years since the earlier publications that reported allergens in peanut oil, manufacturers may have improved the refining process to remove all protein. Certainly, given the current results, creams and emollients could be tested for specific allergens to ascertain whether these products pose a significant exposure risk.

(ii) Diagnostic and therapeutic products

Peanut allergenic products for clinical use showed variability in allergen content. The diagnostic products had much lower levels of Ara h 1 than Ara h 2, raising questions about the source materials from which these extracts were prepared. The relative deficiency of Ara h 1 in skin test solutions is cause for concern, although the absolute concentrations of Ara h 1 in the extracts (24-114µg/ml) are in line with those present in other FDA standardized allergenic products. Concerns about specific allergen content may also apply to use of peanut products for diagnostic peanut challenges. Double blind placebo controlled food challenges typically use peanut flour, whereas peanut butter is often used in clinical practice or for open challenges in research. The stark differences in allergen content between these foods suggests a need for improved standardization and, possibly, manufacture of peanut food standards that can be used for challenge testing. Similarly, the data on allergen profiles of peanut vehicles used in OIT suggests a need for improved standardization of formulations used in clinical trials so that dosing regimens can be more directly compared.

Variability in the composition of peanut flour is clearly demonstrated in our studies. The results indicate that there could be differences in peanut allergen dosing in clinical trials depending on the manufacturer or supplier of the flour. Weight of flour *per se* in mg does not adequately reflect allergen content. By assessing the specific allergen profiles of OIT study materials, it will be possible to make more accurate and precise estimates of dosing and to determine effective allergen levels that are associated with tolerance to peanut. In a companion paper, we have estimated the doses of Ara h 1, Ara h 2, and Ara h 6 present in a peanut snack (Bamba®) that were associated with the prevention of peanut allergy.⁽³⁾ The ability to compare specific antibody responses, cellular responses and inflammatory markers with specific allergen concentrations in peanut vaccines will facilitate mechanistic studies of food allergy.

One of the limitations of the present study was the limited number of diagnostic and OIT trial materials that were available for analysis. Given the increasing success of peanut OIT trials, more OIT products will become available for allergen testing. In addition, allergen measurements are now being used for assessing the dose of epicutaneous immunotherapy products. It can be envisioned that specific allergen profiles will increasingly be used for standardization purposes to improve the quality of peanut vehicles used for immunotherapy. Recent proteomic profiling of peanut allergens by mass spectrometry further supports this approach.⁽²⁷⁾

In conclusion, there are significant quantitative and qualitative differences in the specific allergen profiles of peanut containing foods and in the ratios of Ara h 1 to Ara h 2 and Ara h 6. Allergen profiles are affected by roasting and by the fat composition (in peanut flour). Differences in allergen profiles are also apparent in diagnostic and therapeutic peanut allergen formulations. The data suggests that specific allergen measurements will improve standardization and provide valuable information on allergen dosing for optimal results in OIT as part of future trials. We have recently developed specific ELISA for Ara h 3 and Ara h 8 which will expand the peanut allergen profile. These assays are also being developed on multiplex array platforms, so that all major peanut allergens can be profiled in a single assay. Peanut allergen profiling will expand the scope of peanut OIT studies so that immunologic and other responses to treatment can be directly correlated to the doses of specific allergens used for therapy or for peanut allergy prevention.

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428 Legends to Figures

429 Figure 1

430 Immunoassay control curves. Serial dilutions of the allergen standard for each assay (●) were
431 ran in parallel with three peanut flour extracts, (●), (●), (●).

432 Figure 2

433 Comparison of specific peanut allergen concentrations in peanut butter (red symbols) and
434 peanut flour (blue). Geometric mean values are indicated by the horizontal lines.

TABLE 1

A: Peanut Butter

Sample	Description	Ara h 1 (µg/g)	Ara h 2 (µg/g)	Ara h 6 (µg/g)	Ratio Ara h 1: Ara h 2
NIST SRM 2387	Reference Material	11,275	2,522	2,036	4.47
Earth Balance	Creamy	12,659	4,866	4,107	2.59
Jif	Creamy	11,491	3,549	2,677	3.24
Kroger	Natural creamy	9,970	4,467	3,245	2.23
Smucker's	Natural	13,906	4,951	3,935	2.81
Walmart	Natural crunchy	10,323	4,568	3,439	2.26
Whole Foods	365, crunchy	21,406	7,859	5,786	2.72
Essential Living Foods	<u>Raw</u> jungle peanut	3,415	14,485	18,394	0.24
Vivapura	<u>Raw</u> jungle peanut	5,665	24,311	14,439	0.23
Wonder Nat Foods	Better'n peanut butter	991	1,805	685	0.55

B: Peanut Flour

Flour ¹	Description ²	Ara h 1 (µg/g)	Ara h 2 (µg/g)	Ara h 6 (µg/g)	Total Peanut (ppm)

MK	Prebiotic Blend	8,762	7,372	5,784	1,579,836
PP	Natural 12% Fat Light Roast	26,947	15,007	9,181	6,093,056
OVB	12% Fat Light Roast	10,942	12,787	10,528	1,107,289
OVB	12% Fat Dark Roast	158	3,239	2,155	>1000
OVB	28% Fat Light Roast	1,147	7,816	6,816	336,180
Golden	12% Fat Light Roast	12,559	16,322	12,769	3,626,987
Golden	28% Fat Dark Roast	68	1,574	1,249	2,762,537
Nuts.com	Unspecified roast	10,525	29,262	29,128	>10,000,000

Footnote:

- 1 Sources of peanut flour: Montebello Kitchens, Gordonsville, VA (MK); Protein Plus, Fitzgerald, GA (PP); Old Virginia Byrd Mill, Ashland, VA (OVB); Golden Peanut Company, Alpharetta, GA (Golden); Nuts.com (Cranford, NJ).
- 2 Light Roast flour is derived from peanuts that have been roasted at 250°F for 11min, then 315°F-330°F for additional 14 min. Dark Roast flour is from peanuts that have been roasted at 250°F for 11min, then 350°F for additional 14 min.

TABLE 2

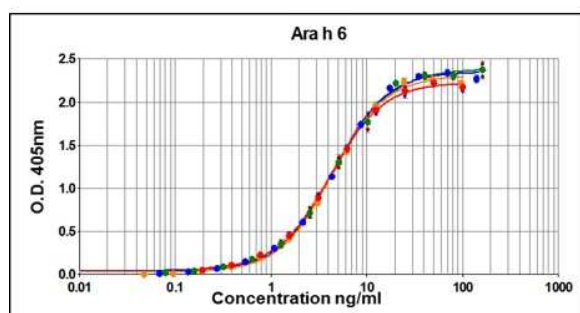
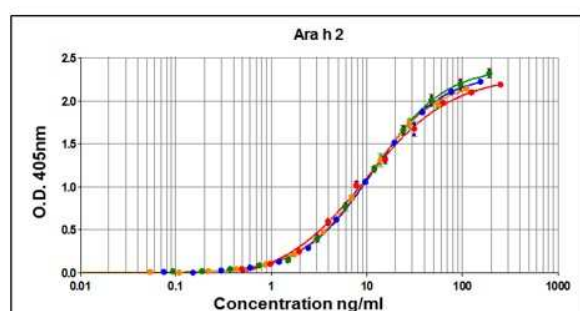
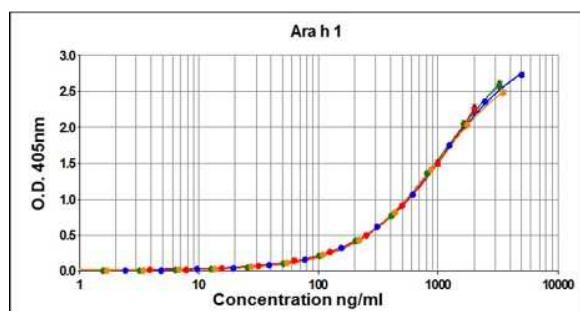
Ara h 1, Ara h 2 and Ara h 6 levels in diagnostic skin test extracts (US).

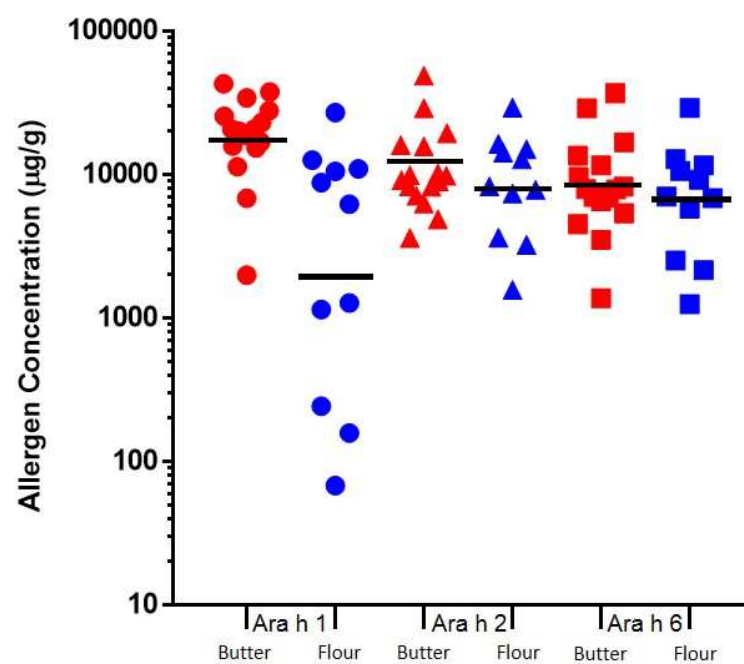
Sample	Ara h 1 ($\mu\text{g/ml}$)	Ara h 2 ($\mu\text{g/ml}$)	Ara h 6 ($\mu\text{g/ml}$)
Manufacturer A-1	55	2,038	1,783
Manufacturer A-2	41	2,311	1,860
Manufacturer B-1	89	670	1,529
Manufacturer B-2	24	234	312
Manufacturer B-3	65	215	390
Manufacturer B-4	114	232	342

TABLE 3

Comparison of Allergen Levels in Peanut Vehicles Used for Oral Immunotherapy

	Sample	Ara h 1 ($\mu\text{g/ml}$)	Ara h 2 ($\mu\text{g/ml}$)	Ara h 6 ($\mu\text{g/ml}$)	Ratio Ara h 2: Ara h 1
UNC	1	394	1,187	1,104	3.0
	2	505	1,431	1,448	2.8
UVA	1	487	5,270	8,093	10.8





Online Repository Materials

**Specific allergen profiles of peanut foods and of diagnostic or therapeutic
allergenic products.**

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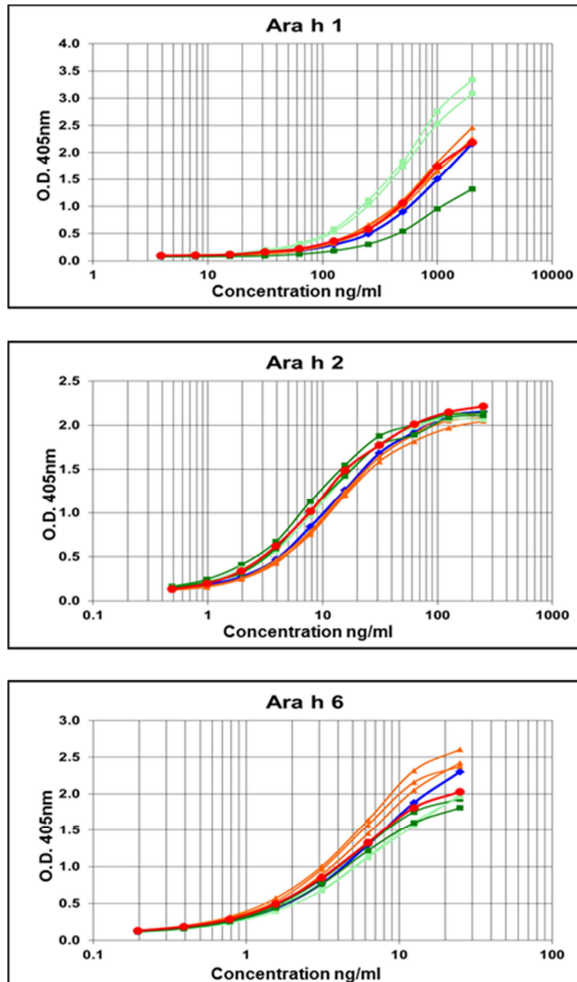
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19 FIGURE E.1

20



26

27 Comparison of control curves for allergen standards and peanut food extracts. Serial

28 dilutions of allergen Standards (●), NIST SRM 2387 (◆), peanut butter extracts (▲) and

29 extracts of light roast (■) or dark roast peanut flour (■) were compared in the Ara h 1, Ara h

30 2 and Ara h 6 ELISA. These curves illustrate the greater sensitivity of the Ara h 2 and Ara h

31 6 ELISA compared with Ara h 1. There was a high degree of parallelism between the assays

32 and in most cases the control curves were superimposable, except for dark roast peanut

33 flour extract (Ara h 1).

TABLE E.1

Recovery of Peanut Allergen in Spiked Samples

Allergen	Spiked Sample	Measured Conc. (ng/ml)	Theoretical Conc. (ng/ml)	% Spike Recovery
Ara h 1	NIST SRM 2387 peanut butter	2,417	2,770	87
	Peanut oil	382	479	80
Ara h 2	NIST SRM 2387 peanut butter	791	801	99
	Peanut oil	60	67	89
Ara h 6	NIST SRM 2387 peanut butter	807	827	98
	Peanut oil	42	47	90

Extracts of NIST SRM 2387 peanut butter reference material and peanut oil were spiked with Ara h 1, Ara h 2, and Ara h 6, and tested by ELISA to determine peanut allergen concentration. Spike recovery was calculated by dividing the measured allergen concentration in spiked samples by the total theoretical concentration, multiplied by 100. Allergen recovery from spiked samples ranged from 80 to 99%.

Assay Procedure

Extracts of NIST SRM 2387 peanut butter reference material and peanut oil (roasted, unrefined) were tested by ELISA to determine Ara h 1, Ara h 2, and Ara h 6 concentration. The peanut butter extract was diluted 100-fold in ELISA assay buffer (0.05% PBS-Tween 20, 1% Bovine Serum Albumin, pH 7.4) to achieve a concentration in the quantifiable range of each ELISA. The peanut oil was diluted 10-fold in assay buffer to provide a miscible sample. Control spikes were prepared in assay buffer using purified, natural Ara h 1, Ara h 2, and Ara h 6 (Indoor Biotechnologies, Inc.). Spike amounts for each allergen were: Ara h 1, 850ng/ml; Ara h 2, 125ng/ml; Ara h 6, 85ng/ml. Spiked samples were prepared by mixing equal volumes of control spike and diluted peanut butter extract or peanut oil. Unspiked samples, spiked samples, and control spikes were tested at multiple dilutions by ELISA.

56 Plates were developed using ABTS and read at 405nm on a microplate reader (BioTek
57 Instruments). Allergen concentration was quantified using Ara h 1, Ara h 2, and Ara h 6
58 allergen standards (see Methods).
59

TABLE E.2

Reproducibility of extraction using NIST SRM 2387 peanut butter
standard reference material

Measurement	Ara h 1 ($\mu\text{g/g}$)	Ara h 2 ($\mu\text{g/g}$)	Ara h 6 ($\mu\text{g/g}$)
1	11,373	2,681	1,887
2	10,628	2,505	2,157
3	11,823	2,380	2,063
Mean	11,275	2,522	2,036

On three separate days, one gram of NIST peanut butter was weighed then extracted in 10 mL of phosphate buffered saline with 0.05% Tween 20 for 2 hours at room temperature. The extracts were measured for Ara h 1, Ara h 2 and Ara h 6 using ELISA.

69 TABLE E.3 Ara h 1, Ara h 2 and Ara h 6 concentrations in peanut foods and related products: complete dataset

70

Bold values indicate the highest and lowest concentrations of Ara h 1, Ara h 2 and Ara h 6 in the peanut butter and flour samples tested.

Table E3 Ara h 1, Ara h 2 and Ara h 6 concentrations in peanut foods and related products: complete dataset

Peanut Butter				Ara h 1 µg/g	Ratio		Ara h 2 µg/g	Ratio		Ara h 6 µg/g	Ratio	
					1 to 2	2 to 1		2 to 6	6 to 2		1 to 6	6 to 1
1	Kroger	Peanut	<u>Natural</u> Creamy	9,970	2.23	0.45	4,467	1.38	0.73	3,245	3.07	0.33
2	Kroger	Peanut	Creamy	8,418	1.65	0.61	5,098	1.29	0.77	3,945	2.13	0.47
3	Wonder Natural Foods Corp. "Better'n Peanut Butter"	Peanut	Original	991	0.55	1.82	1,805	2.63	0.38	685	1.45	0.69
4	Essential Living Foods	<u>Raw</u> Peanut	Wild Jungle Peanut Butter	3,415	0.24	4.24	14,485	0.79	1.27	18,394	0.19	5.39
47	Vivapura	<u>Raw</u> Peanut	Stone Ground Wild Jungle Peanut	5,665	0.23	4.29	24,311	1.68	0.59	14,439	0.39	2.55
46	Fresh Market	Peanut	Fresh Ground	18,761	1.94	0.52	9,677	1.16	0.87	8,376	2.24	0.45
40	Whole Foods	Peanut	Fresh Ground	7,875	1.92	0.52	5,740	0.86	1.17	4,796	1.64	0.61
39	Whole Foods 365	Peanut	Crunchy	21,406	2.72	0.37	7,859	1.36	0.74	5,786	3.70	0.27
35	Smucker's	Peanut	Creamy	17,052	2.14	0.47	7,986	1.18	0.84	6,745	2.53	0.40
36	Smucker's Natural	Peanut		10,321	2.50	0.40	4,131	1.19	0.84	3,480	2.97	0.34
37	Smucker's Natural	Peanut	Creamy	13,906	2.81	0.36	4,951	1.26	0.79	3,935	3.53	0.28
44	Earth Balance	Coconut & Peanut Spread	Creamy	12,659	2.59	0.39	4,886	1.19	0.84	4,107	3.08	0.32
41	Crazy Richard's	Peanut	<u>Natural</u> Chunky	7,653	2.44	0.41	3,131	1.39	0.72	2,255	3.39	0.29
42	Walmart Great Value	Peanut	<u>Natural</u> Crunchy	10,323	2.26	0.44	4,568	1.33	0.75	3,439	3.00	0.33
43	Jif Whips	Peanut	Creamy	9,289	3.80	0.26	2,443	1.39	0.72	1,756	5.29	0.19
45	Jif	Peanut	Creamy	11,491	3.24	0.31	3,549	1.33	0.75	2,677	4.29	0.23

Table E3 Ara h 1, Ara h 2 and Ara h 6 concentrations in peanut foods and related products: complete dataset

NIST SRM 2387			Ara h 1 µg/g	Ratio		Ara h 2 µg/g	Ratio		Ara h 6 µg/g	Ratio	
				1 to 2	2 to 1		2 to 6	6 to 2		1 to 6	6 to 1
Sample 1	Peanut Butter	Oct-2013 Room Temp	11,373	4.24	0.24	2,681	1.42	0.70	1,887	6.03	0.17
Sample 2	Peanut Butter	May-2014	10,628	4.24	0.24	2,505	1.16	0.86	2,157	4.93	0.20
Sample 3	Peanut Butter	Jun-2014	11,823	4.97	0.20	2,380	1.15	0.87	2,063	5.73	0.17

Peanut Oil				Ara h 1 µg/ml	Ratio		Ara h 2 µg/ml	Ratio		Ara h 6 µg/ml	Ratio	
					1 to 2	2 to 1		2 to 6	6 to 2		1 to 6	6 to 1
20	Spectrum	Peanut	Unrefined Hi-Oleic	<DL <0.63			<DL <0.02			<DL <0.008		
21	Spectrum	Peanut	Refined, Organic, Hi-Oleic	<DL <0.63			<DL <0.02			<DL <0.008		
22	Bell Plantation	Peanut	Roasted, <u>Unfiltered</u> Extra Virgin	8.11	0.81	1.23	10.00	1.52	0.66	6.56	1.24	0.81
23	InterNational Foods "Loriva"	Peanut	Pure Roasted, Expeller Pressed	<DL <0.63			<DL <0.02			<DL <0.008		
24	Whole Foods "365 Everyday Value"	Peanut	Roasted, Unrefined	<DL <0.63			<DL <0.02			<DL <0.008		
50	Golden Peanut Company	Peanut	Premium	<DL <0.63			<DL <0.02			<DL <0.004		
51	Golden Peanut Company	Peanut	<u>Crude</u> Roasted	<DL <0.63			0.02 <0.02	2.00	0.50	0.01 <0.004		
52	Baar	Peanut	Expeller Cold Pressed Massage Oil	<DL <0.63			<DL <0.02			<DL <0.004		

Table E3 Ara h 1, Ara h 2 and Ara h 6 concentrations in peanut foods and related products: complete dataset

Peanut Flour				Ara h 1 µg/g	Ratio		Ara h 2 µg/g	Ratio		Ara h 6 µg/g	Ratio	
					1 to 2	2 to 1		2 to 6	6 to 2		1 to 6	6 to 1
29	Montebello Kitchens	Peanut Flour	Prebiotic Blend	4,381	1.19	0.84	3,686	1.27	0.78	2,892	1.51	0.66
30	Protein Plus	Peanut Flour	Roasted All <u>Natural</u> 12% Fat Light Roast	13,473	1.80	0.56	7,504	1.63	0.61	4,591	2.94	0.34
31	Old Virginia Byrd Mill	Peanut Flour	12% Fat - Light Roast Peanut Flour	3,096	0.44	2.29	7,091	1.23	0.82	5,782	0.54	1.87
61	Old Virginia Byrd Mill	Peanut Flour	12% Fat - Light Roast Peanut Flour	5,471	0.86	1.17	6,394	1.21	0.82	5,264	1.04	0.96
32	Old Virginia Byrd Mill	Peanut Flour	12% Fat - <u>Dark</u> Peanut Flour	79	0.05	20.50	1,620	1.50	0.67	1,077	0.07	13.64
62	Old Virginia Byrd Mill	Peanut Flour	12% Fat - <u>Dark</u> Peanut Flour	122	0.07	14.94	1,816	1.44	0.69	1,258	0.10	10.36
33	Old Virginia Byrd Mill	Peanut Flour	28% Fat - Light Peanut Flour	574	0.15	6.81	3,908	1.15	0.87	3,408	0.17	5.94
63	Old Virginia Byrd Mill	Peanut Flour	28% Fat - Light Peanut Flour	636	0.15	6.49	4,123	1.18	0.85	3,505	0.18	5.51
58	Golden Peanut Company	Peanut Flour	12% Fat - Light Roast Peanut Flour	6,280	0.77	1.30	8,161	1.28	0.78	6,385	0.98	1.02
59	Golden Peanut Company	Peanut Flour	28% Fat - <u>Dark</u> Roast Peanut Flour	34	0.04	23.30	787	1.26	0.79	624	0.05	18.49
34	Nuts.com	Peanut Flour	Dry Roasted	5,262	0.36	2.78	14,631	1.00	1.00	14,564	0.36	2.77

Table E3 Ara h 1, Ara h 2 and Ara h 6 concentrations in peanut foods and related products: complete dataset

Non-Peanut Butter				Ara h 1 µg/g	Ratio		Ara h 2 µg/g	Ratio		Ara h 6 µg/g	Ratio	
					1 to 2	2 to 1		2 to 6	6 to 2		1 to 6	6 to 1
5	The Hain Celestial Group "MaraNatha"	Almond May contain Peanut	All Natural Roasted Creamy	5.50	110.00	0.01	0.05 <0.10			<DL <0.04		
6	The Hain Celestial Group "MaraNatha"	Almond May contain Peanut	All Natural <u>Raw</u> Creamy	<DL <3.125			<DL <0.10			<DL <0.04		
7	Barney Butter	Almond Peanut Free	Smooth	<DL <3.125			<DL <0.10			<DL <0.04		
8	Barney Butter	Almond Peanut Free	Crunchy	<DL <3.125			<DL <0.10			<DL <0.04		
9	Barney Butter	Almond Peanut Free	<u>Bare</u> , Smooth	<DL <3.125			<DL <0.10			<DL <0.04		
10	Justin's	Almond Not Peanut Free Facility	Classic, All- <u>Natural</u>	<DL <3.125			0.09 <0.10			<DL <0.04		
11	Artisana	Almond Peanut Free	Organic, <u>Raw</u>	<DL <3.125			<DL <0.10			<DL <0.04		
38	Trader Joe's	Almond	Creamy	<DL <3.125			<DL <0.10			<DL <0.04		
12	Justin's	Hazelnut Not Peanut Free Facility	All <u>Natural</u> Chocolate Hazelnut Butter Blend	<DL <3.125			0.08 <0.10			<DL <0.04		
13	Artisana	Cashew Peanut Free	Organic <u>Raw</u>	<DL <3.125			<DL <0.10			<DL <0.04		
14	Artisana	Pecan w/Cashews Peanut Free	100% Organic <u>Raw</u> Pecan Butter with Cashews	<DL <3.125			0.09 <0.10			<DL <0.04		
15	Artisana	Walnut w/Cashew Peanut Free	100% Organic <u>Raw</u> Walnut Butter with Cashews	<DL <3.125			0.40 <0.10			<DL <0.04		
16	The Hain Celestial Group "MaraNatha"	Sunflower Seed May contain Peanut	All <u>Natural</u>	<DL <3.125			<DL <0.10			<DL <0.04		
17	The SoyNut Butter Co. "I.M. Healthy"	SoyNut Peanut Free	Creamy	<DL <3.125			<DL <0.10			<DL <0.04		
48	Fastachi	Pistachio	Roasted	<DL <3.125			0.06 <0.10	1.50	0.67	0.04 <0.04		
49	Wilderness Poets	Macadamia	Raw	<DL <3.125			<DL <0.10			<DL <0.04		

Table E3 Ara h 1, Ara h 2 and Ara h 6 concentrations in peanut foods and related products: complete dataset

Non-Peanut Flour				Ara h 1 µg/g	Ratio		Ara h 2 µg/g	Ratio		Ara h 6 µg/g	Ratio	
					1 to 2	2 to 1		2 to 6	6 to 2		1 to 6	6 to 1
25	Bob's Red Mill	Coconut	Organic	<DL <3.125			0.05 <0.10			<DL <0.04		
26	Bob's Red Mill	Garbanzo Bean	Stone Ground	14.16	283.10	0.004	0.05 <0.10			<DL <0.04		
57	Nuts.com	Chickpea		16.04	229.07	0.00	0.07 <0.10	0.64	1.57	0.11	145.77	0.01
27	Bob's Red Mill	Almond Meal/ Flour	Finely Ground	<DL <3.125			<DL <0.10			<DL <0.04		
28	Bob's Red Mill	Soy Flour	Organic. Whole Grain. 35% Protein	1.43 <3.125	28.50	0.04	0.05 <0.10			<DL <0.04		
53	Food to Live	Lupini Bean	(whole beans ground in lab)	<DL <3.125			0.08 <0.10	0.31	3.25	0.26		
54	Eat Seed	Sesame Seed	Roasted, Ground	0.78 <3.125			<DL <0.10			<DL <0.04		
55	Nuts.com	Green Pea		<DL <3.125			<DL <0.10			<DL <0.04		
56	Nuts.com	White Bean		2.70 <3.125	6.67	0.15	0.41	0.59	1.70	0.69	3.91	0.26

Bold values indicate the highest and lowest concentrations of Ara h 1, Ara h 2 and Ara h 6 in the peanut butter and flour samples tested.

