

Research Paper

Detection and Antigenic Profiling of Undeclared Peanut in Imported Garlic Using an xMAP Multiplex Immunoassay for Food Allergens

RONNIE O. PEDERSEN,^{1,2} TIM PETERS,³ RAKHI PANDA,² PAUL WEHLING,³ AND ERIC A. E. GARBER^{2*}

¹Office of Scientific and Professional Development, Office of the Chief Scientist, Office of the Commissioner, U.S. Food and Drug Administration, 10903 New Hampshire Avenue, Silver Spring, Maryland 20993; ²Division of Bioanalytical Chemistry, Office of Regulatory Science, Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, 5001 Campus Drive, College Park, Maryland 20740; and ³Medallion Labs, 9000 Plymouth Avenue North, Minneapolis, Minnesota 55427, USA

MS 16-485: Received 7 November 2016/Accepted 22 February 2017/Published Online 20 June 2017

ABSTRACT

A shipment of imported garlic powder was suspected of containing peanut. Samples (subs) collected from the shipment displayed considerable variability in peanut antigenicity when analyzed by enzyme-linked immunosorbent assay (ELISA). This raised questions regarding whether peanut was actually present, the amount present, and the basis for the variability in antigenic content. Analyses that used an xMAP multiplex assay for the detection of peanut and additional food allergens generated responses that were characteristic of peanut. Specifically, the relative intensities of two different peanut-specific antibodies coupled to beads (peanut-37 and -38) and the antigen profiles were identical to garlic controls spiked with peanut. In addition, the xMAP data did not indicate the presence of other allergens. Quantitative analyses indicated an approximately fivefold variation in peanut concentration among different subs. In contrast, within a sub, the apparent peanut concentration appeared constant. Particle size analyses of the garlic powder subs indicated a single distribution profile, with a peak at 380 μm . ELISA analysis of sieve-fractionated garlic powder from one of the subs indicated that slightly less than half of the detectable peanut was smaller than 212 μm , with the remainder almost evenly split between 212 and 300 μm and $>300 \mu\text{m}$. Modeling to predict possible oral exposure levels of peanut other than those directly measured requires additional research on the physicochemical properties of peanut and garlic, along with information on the production of the garlic powder.

Key words: Food allergen; Garlic; Multiplex; Peanut

Approximately 19 million adults and 26 million children in the United States suffer from food allergies (8, 20). Although recent advances in oral immunotherapy have shown promise in alleviating food allergies, a strict avoidance diet is currently the only reliable method to avoid an allergic reaction (1, 6, 15). As such, consumers with food allergies must rely on food labels to identify the presence of food allergens to avoid a potentially life-threatening reaction. To help safeguard the allergic consumer, Congress passed the Food Allergen Labeling and Consumer Protection Act of 2004 (Public Law 108-282, Title II), which requires that ingredients that include or are derived from the eight major food allergens (crustacean, egg, fish, milk, peanut, soy, tree nuts, and wheat) be declared on the label.

In early 2016, a shipment of garlic powder imported from a manufacturer in the People's Republic of China was suspected of containing undeclared peanut. Several methodologies exist for detecting peanut, including mass spectrometry, PCR, and enzyme-linked immunosorbent assay (ELISA) (2, 4, 9, 10, 12, 17, 21). Mass spectrometric

methods often rely on the detection of peptides generated by enzymatic digestion and display a high degree of specificity, but quantification can be problematic (11, 13, 17). PCR, likewise, displays excellent specificity but quantifies the DNA and not the allergenic protein (16, 21). ELISA technology is hampered by the inability to multiplex but, based on the specificity of the antibodies used, is able to detect analytes at low concentration in complex samples, with sample preparation and extraction entailing minimal effort.

A limitation of antibody-based technologies (e.g., ELISA) is the possibility of cross-reactivity with other antigens. Because of this, the U.S. Food and Drug Administration (FDA) relies on confirmatory results from two different ELISAs when using analytical results to support enforcement actions related to food allergen labeling requirements. However, in addition to being time-consuming and expensive, the reliance on two ELISAs does not guarantee that all cross-reactivities will be identified. To better address these and other concerns, an xMAP Food Allergen Detection Assay was developed that simultaneously detects 14 different allergens plus gluten (3). Twelve of the allergens are detected by at least two different antibodies,

* Author for correspondence. Tel: 240-402-2224; Fax: 301-436-2332; E-mail: Eric.Garber@fda.hhs.gov.

providing built-in confirmation. Multiple antibody detection in the xMAP Food Allergen Detection Assay also provides the opportunity to examine the ratio among the responses generated by the multiple antibodies, an approach referred to as antigenic profiling. Antigenic profiling not only increases confidence in the detection of an allergen but also can help detect and characterize the presence of other, unexpected antigens and inconsistencies that are easily missed using an ELISA assay. Antigenic profiling also decreases the likelihood of a cross-reactive analyte being mistaken for an allergen and helps distinguish antigenic features and the effects of food processing as it may relate to epitopic elements.

Multiple subsamples (subs) from the same lot of garlic powder suspected of containing peanut were analyzed for the presence of peanut and for peanut distribution. ELISA analyses, using the official regulatory protocol that requires the use of two ELISA test kits, indicated the presence of peanut, but at significantly different concentrations in the subs. This raised questions regarding whether peanut was the antigenicity detected, the basis for the heterogeneity, and how much peanut was potentially present in oral portions. The presence of peanut was confirmed using the xMAP Food Allergen Detection Assay. The intensity of the responses (median fluorescent intensity [MFI] values) generated by the bead sets for the detection of peanut were indicative of its presence. In addition, the antigenic profile was similar to that of a control (peanut-free) garlic powder spiked with the National Institute of Standards and Technology (NIST) peanut butter standard reference material (SRM) 2387. This result was in accordance with the results generated by commercial ELISA test kits for the detection of peanut.

In evaluating the risk to allergic consumers, the concern is the dose consumed in an oral portion, not the average concentration across a container, bag, batch, lot, or production run. The FDA has recently undertaken special efforts in its surveillance studies and in analyses of regulatory samples to specifically measure the dose in oral portions. The use of these data to predict the maximum oral dose a consumer may eat becomes complicated if the allergen is not uniformly distributed, as in the heterogeneous mixture model. This is especially a concern with allergens such as peanuts that are known to fragment and remain insoluble. If the fragmentation pattern is highly heterogeneous, then a single large particle might be found in a single portion or bite but not in multiple analyses across a production run. Thus, assessing the potential of a heterogeneous mixture to pose a health risk requires knowing the allergen's distribution in the product. These principles were applied to analyses for the presence of peanut in garlic powder. The distribution of peanut within multiple subs from the same lot was examined, as was the partitioning of peanut with size-fractionated garlic powder.

MATERIALS AND METHODS

Reagents. Phosphate-buffered saline (PBS; catalog no. P5368), Tween 20 (catalog no. P9416), and gluten (catalog no. G5400) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). BD Difco skim milk powder was purchased from Fisher Scientific

(Waltham, MA). NIST SRM 2387 peanut butter (NIST, Gaithersburg, MD), routinely used by the FDA as the SRM in the analysis of regulatory samples, was used as the peanut standard in these studies. All other reagents were of the highest technical grade available.

Ten 1.5-lb (0.7-kg) subs from a lot of ground garlic imported from China that was suspected of peanut content were kindly supplied by the Office of Regulatory Affairs, FDA. The subs were collected from separate 50-lb (22.7-kg) bags distributed over four pallets, totaling 10,000 lb (4,536 kg). Garlic control samples were purchased at a local grocery store. Additional grinding of the garlic samples was done using a Tube Mill control (IKA Works, Inc., Wilmington, NC).

Immunochemical methods. The xMAP Food Allergen Detection Assay (Radix BioSolutions, Georgetown, TX) was used as recommended. One-gram samples were extracted with 40 mL of UD buffer (105 mM sodium phosphate, 75 mM NaCl, 2.5% Difco skim milk powder, 0.05% Tween 20) for 2 h while being rocked at room temperature (5, 7). Inasmuch as milk was included in the extraction buffer, the bead sets for the detection of milk were not included in the analyses. All samples were extracted in triplicate, with each extraction measured in one well, unless otherwise noted, and were read on a Bioplex 200 (Bio-Rad Laboratories, Inc., Hercules, CA). Quantitation was based on the calibration standards supplied by the manufacturer of the xMAP assay as a mixture of the 15 analytes. The eight-point calibration curves were fit to either a four- or five-parameter logistic plot and were used to calculate the apparent concentrations in the sample extracts. When plotting the calibration standards, calculating antigen concentrations, or determining response ratios among different bead sets (e.g., antigen profiling), the MFI values were used after subtracting the background responses, as determined using assay buffer. Because the antibodies have different binding affinities, when generating antigenic profiles or comparing the ratios among bead sets, it was important to compare the profiles or ratios generated by comparable amounts of the standard and analyte. To achieve this, the profiles or ratios generated by the samples were compared with those generated by a standard whose response was comparable to that of the sample with one of the bead sets. If an identical standard was not available, the two standards whose responses bracketed the sample's response were used. Although often the bead set with the greatest response was used as the basis for comparison (referred to as the anchor), bead sets with less intense responses were also appropriate as the anchor, provided that the signal or noise was suitable for distinguishing meaningful responses (3).

Veratox for Peanut Allergen (Neogen Corp., Lansing, MI), RIDASCREEN Fast Peanut (R-Biopharm, Inc., Washington, MO), and Peanut, Casein, and Gluten ELISA test kits from Morinaga Institute of Biological Science, Inc. (MioBS; U.S. distributor Crystal Chem, Downers Grove, IL) were used as recommended, except for a single modification. In an analysis of the size-fractionated peanut sample suspected of containing peanut using the Veratox for Peanut Allergen ELISA, two of the three analytical samples were only 2.5 g, with the amount of additive and PBS appropriately adjusted. The responses generated by the two 2.5-g samples were indistinguishable from the response generated by the 5-g sample. Otherwise, the RIDASCREEN Fast analysis was done according to the AOAC International performance tested methods program certified protocol, and the MioBS ELISA was done according to the multilaboratory validated overnight extraction protocol (12). Also, unless stated otherwise, all ELISA analyses were performed in triplicate, meaning that three separate analytical portions were extracted from scratch and the results were averaged.

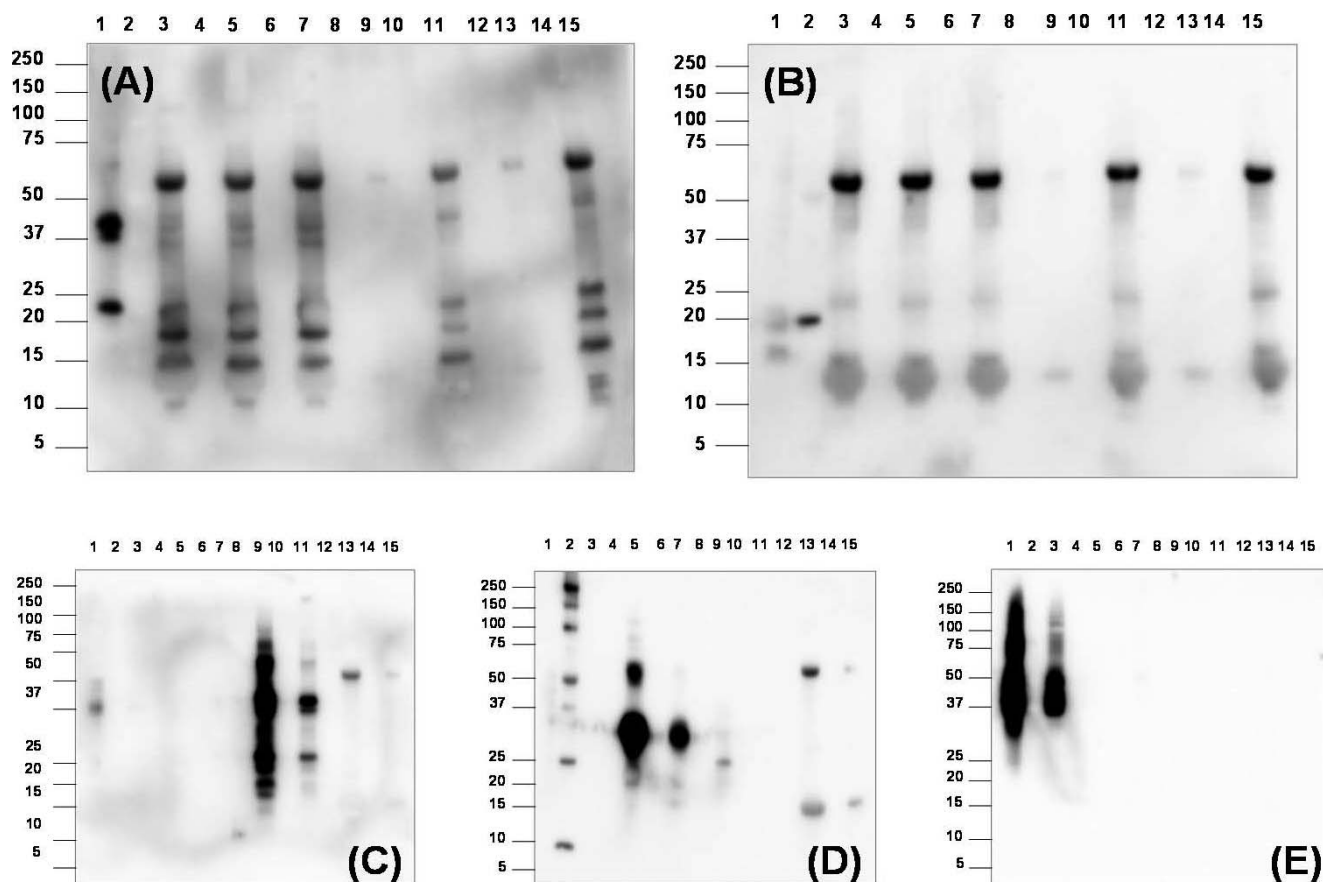


FIGURE 1. Western blot analysis using the detector antibodies from the (A) Veratox Peanut Allergen ELISA (diluted 10 $\times$), (B) MIOBS Peanut ELISA (diluted 10 $\times$), (C) Veratox Peanut Allergen ELISA (diluted 20 $\times$), (D) MIOBS Casein ELISA (diluted 10 $\times$), (E) MIOBS Gluten ELISA (diluted 10 $\times$). Immunoblots A and B consisted of the following: lane 1, 1,000 ppm of NIST SRM 2387; lane 2, molecular weight markers; lane 3, 100,000 ppm of garlic control (GC); lane 4, empty; lane 5, GC supplemented with 20 ppm of SRM 2387; lane 6, empty; lane 7, GC supplemented with 2.5 ppm of SRM 2387; lane 8, empty; lane 9, sub F diluted 10-fold; lane 10, empty; lane 11, sub F; lane 12, empty; lane 13, sub G diluted 10-fold; lane 14, empty; lane 15, sub G. Immunoblots C, D, and E consisted of the following: lane 1, 1,000 ppm of gluten; lane 2, molecular weight markers; lane 3, 100 ppm of gluten; lane 4, empty; lane 5, 10,000 ppm of nonfat dry milk; lane 6, empty; lane 7, 1,000 ppm of nonfat dry milk; lane 8, empty; lane 9, 10,000 ppm of SRM 2387; lane 10, empty; lane 11, 1,000 ppm of SRM 2387; lane 12, empty; lane 13, 100,000 ppm of GC; lane 14, empty; lane 15, 10,000 ppm of GC. The prestained molecular weight markers were photographed prior to visualizing the chemiluminescence (data not shown). The molecular weight markers may also generate chemiluminescent bands depending on the use of (strept)avidin-biotin in the design of the antibody-horseradish peroxidase conjugates.

NIST SRM 2387 peanut butter was used to prepare peanut standard solutions and spiked samples using the procedure described for spiking on a per mass basis (19). The calibration standards supplied with the xMAP assay, consisting of a mixture of the NIST SRM 2387 and the other calibration standards, were used for low-level spiking from 0.2 to 1 $\mu\text{g/g}$.

Western blot. Samples for Western immunoblot analysis were diluted with PBS with 0.1% Tween 20, extracted for 2 h at room temperature, and centrifuged at 10,000 $\times g$ for 15 min. The proteins in the supernatant were fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and were immunoblotted as previously described (14). Immunoblot analysis entailed using the detector antibodies of the Veratox for Peanut Allergen (diluted 1/10 and 1/20, v/v), MIOBS Peanut (diluted 1/10, v/v), MIOBS Casein (diluted 1/10, v/v), and MIOBS Gluten (diluted 1/10, v/v) ELISA test kits. The use of polyclonal antibodies increases the probability of detecting antigenic epitopes. In addition, because the MIOBS antibodies were generated to detect reduced-SDS denatured proteins, they were compatible with the electrophoretic conditions and routinely performed well in Western

blot analyses. To help identify and characterize the protein bands visualized in the Western blots, Precision Plus Protein Dual Xtra Prestained Protein Standards (catalog no. 1610377, Bio-Rad) were run alongside the various samples. The molecular weight markers were readily observed without the addition of chemiluminescent reagents and were photographed for use in estimating the size of the protein bands visualized by Western blot. Depending on the use of (strept)avidin-biotin in the construction of the antibody-horseradish peroxidase conjugates used in the Western blots, some or all of the markers were also sometimes visualized in the chemiluminescent analysis of the blots (e.g., Fig. 1B and 1D). A Syngene G:Box Chemi XX9 imager (Syngene, Frederick, MD) was used to measure chemiluminescence.

Particle size analysis and size fractionation by sieving.

Size distribution analysis was conducted using a Microtrac S3500 (Microtrac, Inc., Largo, FL) with isopropyl alcohol (refractive index 1.38) as the carrier fluid. The particles were designated as absorbing with an irregular shape. A 15-mL portion of isopropyl alcohol (enough to fully suspend the sample) was added to the powdered sample in a 50-mL tube and was mixed by inversion to

minimize aggregation or any partitioning that might affect achieving a homogenous distribution of the particles in the slurry. A 50% flow was used to transport and disperse the sample prior to and during analysis. Dispersion in the instrument was assisted by 30 s of 40-W ultrasonics. The test consisted of three 10-s run times that were averaged for the final result.

Size fractionation was done using 3-in. U.S. Standard sieves no. 50 and 70 (W.S. Tyler, Mentor, OH) with mesh sizes of 300 and 212 μm , respectively. Nine-gram sample portions were placed in the center of the top sieve of a stack consisting of the two sieves and a bottom pan and were shaken with periodic tapping using a Gilson Performer III 3-in. Sieve Shaker (Gilson Company, Inc., Lewis Center, OH) with a vibration setting of 6. Fractionation was repeated three times for both 1 and 6 h. The fractionated material was subsequently ground to ensure uniformity among the analytical samples and to maximize extraction efficiency. A mass-weighted sum was calculated as the sum of each sieve fraction signal weighed by that sum's mass fraction of the 9 g, according to the following equation:

$$\sum_i \frac{m_i}{m_T} \text{OD}_i$$

Where m_i is the mass of fraction i (>300 , 300 to 212 , <212), m_T is the total mass recovered after fractionation, and optical density (OD) $_i$ is the OD measurement for the i fraction. Reported value is the average of three replicates.

The questions of whether 1 or 6 h of agitation and tapping were sufficient to fractionate the garlic powder and whether the processing fragmented the powder particles were examined. Average mass fractions for 1-h sieving were 0.38 ± 0.05 , 0.37 ± 0.01 , and 0.25 ± 0.04 for the >300 -, 300 - to 212 -, and <212 - μm fractions, respectively. For 6-h sieving, the mass fractions were 0.34 ± 0.02 , 0.36 ± 0.01 , and 0.30 ± 0.01 . A slight increase in material was observed to accumulate in the smaller size fraction with the extended sieving. A common rule defines the end point of sieving as when no more than 1% by mass per min passes through the sieve (18). Using this rule, it was concluded that sieving was completed after 1 h. It cannot be ruled out that a small percentage of material could have broken down during 6 h of fractionation, but the differences between 1 and 6 h of fractionation were negligible.

Distribution of peanut in the fractionated garlic powder was determined using MIOBS Peanut ELISA test kits. The sensitivity of the MIOBS Peanut ELISA, low background absorbance (0.020 ± 0.001 , $n = 3$, typically <0.05), an upper, maximum absorbance consistent with providing good sensitivity and resolving power (0.98 ± 0.01 , $n = 3$, typically ≥ 1), low coefficient of variability (typically $<10\%$), and the need for small analytical portions were compatible with the analytical needs. The distribution of the results was expressed as the measured absorbance values and not as the calculated amounts of peanut, so that the reliability of the data relative to the dynamic range could be appreciated. The data plotted as the calculated micrograms of peanut per milliliter were comparable to the data plotted based on the absorbance (not shown), probably because the measured responses were within the central linear range of the calibration curve.

RESULTS AND DISCUSSION

Ten subs (designated A to J) from a single lot of powdered garlic imported from China were collected and analyzed using the Veratox for Peanut Allergen and RIDASCREEN Fast Peanut ELISAs for the presence of peanut by the Office of Regulatory Affairs of the FDA. The

Veratox results indicated the presence of 10 to 90 ppm of peanut in the subs (i.e., 22, 17, 30, 22, 14, 90, 30, 10, 10, 15 ppm), whereas the RIDASCREEN Fast ELISA indicated 9 to 21 ppm (i.e., 21, 15, 21, 18, 13, 18, 12, 9, 20, 21 ppm) of peanut. The amount of peanut detected in a specific sub displayed little variance among the replicates; the average coefficient of variability of the Veratox data was 17%, and values ranged from 5 to 33%. Although the two ELISAs agreed about the presence of peanut, the specific amounts detected in the 10 subs varied between the two ELISAs, with the Veratox ELISA indicating higher amounts of peanut in sub F and sub G. No regulatory action was taken against the product because the estimated oral exposure from these two high subs did not indicate a health risk associated with excessive, atypical garlic consumption. Controls indicated no false positives with ground garlic from an alternative manufacturer; there were no false negatives when the peanut-free garlic was spiked with the peanut standard; and peanut-free garlic samples spiked with 5, 25, and 50 $\mu\text{g}/\text{mL}$ peanut displayed an average recovery of $51\% \pm 3\%$ across the three sets of spiked samples, each analyzed in duplicate on four ELISA plates ($n_{\text{TOT}} = 24$; average recovery of the RIDASCREEN Fast ELISAs, $52\% \pm 3\%$). In spite of the agreement between the two ELISAs about the presence of peanut, and despite the proper performance of the ELISAs with the various control samples, the results left questions unanswered: (i) could the quantitative differences between the results generated by the Veratox and RIDASCREEN Fast ELISAs indicate a cross-reactive protein source? and (ii) what were the implications of the apparent heterogeneous distribution of the analyte and the consistent recovery of 50% with the spiked garlic control samples? To address these questions, five of the subs (designated A, D, F, G, and I) were subjected to additional analyses designed to confirm whether peanut was present and whether the distribution was heterogeneous, possibly due to particle size.

A 25-g portion of material from each sub was finely ground to ensure homogeneity of a sufficient amount of sample so that triplicate analyses could be performed (from scratch) and multiple methods compared. Without replication, a single positive response is considered inconclusive. Further, the application of multiple methods might provide insight into the antigenic content. To avoid loss of information due to excessive dilution, the size of the analytical portion was limited to 25 g, the equivalent of five oral portions. As such, the degree of maximum dilution (fivefold if four of the portions were analyte free) would not be able to mask an aberrant amount of antigenicity in a single oral portion using the xMAP and MIOBS assays (limit of quantification < 1 ppm).

xMAP food allergen assay. The subs labeled A, D, F, G, and I were analyzed using the xMAP Food Allergen Detection Assay's buffered-detergent extraction protocol. This assay uses 22 bead sets with antibodies specific for legumes (peanut and soy) and nine tree nuts, in addition to five bead sets for the detection of crustacean, egg, and gluten, a repertoire sufficient for antigen profiling (Table 1). Prior comparisons of results generated by different plates

TABLE 1. *xMAP analysis of sub D and garlic controls^a*

Sample	Almond		Brazil nut		Cashew		Coconut		Crustacean	
	12	13	14	15	18	19	20	21	22	
Plate 1 ^b										
Sub D	492 ± 37	139 ± 9	73 ± 8	25 ± 1	106 ± 8	114 ± 7	255 ± 26	18.7 ± 0.6	24.2 ± 0.8	
GC	21 ± 2	21 ± 1	30 ± 2	19.7 ± 0.6	41.5 ± 0.5	30 ± 7	119 ± 9	16 ± 1	19.7 ± 0.6	
S1	804 ± 19	345 ± 11	447 ± 11	107 ± 3	591 ± 18	126 ± 4	1,417 ± 28	43 ± 2	331 ± 10	
Background	9.7 ± 0.6	16.3 ± 0.6	27.3 ± 0.6	14 ± 1	44.7 ± 0.6	16.2 ± 0.8	38.3 ± 0.6	13.2 ± 0.3	21.5 ± 0.9	
Plate 2										
GC+2387	45 ± 5	20 ± 3	174 ± 195	20 ± 1	214 ± 227	129 ± 85	170 ± 16	13.7 ± 0.6	21.3 ± 0.6	
S1	596 ± 19	241 ± 4	312 ± 6	53.3 ± 0.6	439 ± 10	99 ± 2	1,162 ± 30	33 ± 1	194 ± 4	
Background	8.0 ± 0.0	12.7 ± 0.6	28 ± 1	11.7 ± 0.6	46.3 ± 0.6	14.0 ± 0.0	39.7 ± 0.6	11.7 ± 0.6	21.7 ± 0.6	
Peanut										
Gluten										
Sample	25	26	27	28	29	30	33	34	37	38
Plate 1										
Sub D	1,946 ± 29	3,479 ± 21	150 ± 8	1,618 ± 9	97 ± 7	51 ± 4	311 ± 13	35 ± 4	9,206 ± 600	6,992 ± 390
GC	1,867 ± 16	3,380 ± 30	40 ± 3	426 ± 45	48 ± 1	16.3 ± 0.6	112 ± 3	22.3 ± 0.6	30.3 ± 0.6	42 ± 2
S1	2,679 ± 55	4,862 ± 32	79 ± 2	541 ± 7	390 ± 12	96 ± 4	4,138 ± 137	171 ± 3	782 ± 79	508 ± 43
Background	2,042 ± 14	3,739 ± 36	16.0 ± 0.0	141 ± 1	32 ± 1	15.0 ± 0.0	42.7 ± 0.6	15.7 ± 0.6	22.0 ± 0.0	20.7 ± 0.6
Plate 2										
GC+2387	2,401 ± 23	4,341 ± 75	51 ± 4	721 ± 42	88 ± 63	101 ± 93	109 ± 9	18.0 ± 0.0	8,718 ± 442	6,215 ± 319
S1	3,063 ± 37	5,656 ± 40	63 ± 2	432 ± 9	302 ± 4	70 ± 1	3,004 ± 65	115 ± 2	510 ± 9	335 ± 13
Background	2,503 ± 4	4,505 ± 74	16.0 ± 0.0	155 ± 3	32 ± 1	13.0 ± 0.5	39.7 ± 0.6	13.0 ± 0.0	21 ± 1	23 ± 1
Walnut										
Soy										
Sample	39	42	43	44	45	46	47	48		
Plate 1										
Sub D	574 ± 68	36.0 ± 0.0	38 ± 3	68 ± 4	303 ± 94	2,646 ± 537	237 ± 9	73 ± 3		
GC	510 ± 35	30.7 ± 0.6	23 ± 1	36 ± 3	57 ± 26	239 ± 146	196 ± 2	34.3 ± 0.6		
S1	483 ± 8	55.3 ± 0.6	1,498 ± 62	66 ± 4	427 ± 5	1,798 ± 63	514 ± 21	124.3 ± 0.6		
Background	107.0 ± 0.0	16 ± 1	23.7 ± 0.6	15.7 ± 0.6	19.7 ± 0.6	15 ± 1	52 ± 1	19.3 ± 0.6		
Plate 2										
GC+2387	372 ± 14	22.8 ± 0.3	99 ± 110	47 ± 19	120 ± 93	2,359 ± 1,160	157 ± 4	63 ± 50		
S1	336 ± 2	39.2 ± 0.8	1,072 ± 34	50.0 ± 0.0	262 ± 4	1,214 ± 29	337 ± 11	83 ± 2		
Background	79 ± 3	13 ± 1	22.2 ± 0.3	12.3 ± 0.6	18.7 ± 0.6	13.3 ± 0.6	50 ± 2	15.7 ± 0.6		

^a GC, garlic control; GC+2387, GC with SRM2387; S1, first nonzero calibration standard. Values are average responses of triplicate analyses of sub D, GC, GC+2387, S1, and background (buffer) samples expressed as the median fluorescence intensity ± standard deviation.

^b Samples spread over two plates from the same lot. To facilitate analysis, the background and S1 responses are listed for both plates.

^c Elevated responses of the egg-25 and egg-26 characteristic of the bead sets. In all cases, the samples generated responses comparable to background and <S1.

TABLE 2. Determination of peanut concentration in garlic powder^a

Sample	Peanut (μg/g)		RSD (%)	
	37	38	37	38
xMAP multiplex assay ^b				
Garlic control spiked at 18 ppm ^c	8.6	8.6	1	0.5
Garlic control spiked at 4.5 ppm	2.4	2.4	1	1.3
Garlic control spiked at 1.0 ppm	1.2	1.4	2	3
Sub A	17	18	2	5
Sub D	9	10	9	8
Sub F	51	51	8	7
Sub G	28	29	11	10
Sub I	12	13	4	5
Veratox peanut allergen				
Sub A	51		9	
Sub D	26		29	
Sub F	134		13	
Sub G	81		13	
Sub I	30		20	

^a RSD, relative standard deviation. Concentrations interpolated using test kit standards. Samples analyzed in triplicate.

^b xMAP assay peanut concentrations determined using bead sets 37 and 38. Background responses subtracted from MFI values prior to interpolation.

^c NIST SRM 2387 used to spike garlic control at 4.5 and 18 ppm; xMAP calibration standard used to spike at 1 ppm.

from the same lot of xMAP assay test kits showed low plate-to-plate variability (3). In this study, absolute MFI responses of the calibration standards and some of the background responses were significantly different among plates, as indicated in Table 1. Therefore, to compare the MFI responses measured using different plates, whether from the same or different lots, the responses (minus background) were either normalized to the calibration standards or were processed to plate-independent parameters (e.g., ratios among responses by different bead sets, the basis of antigen profiling). These ensured that the results would be analyzed in a manner consistent with the foci of this study, namely, to establish the presence of peanut, confirm the presence of peanut using antigen profiling, determine peanut concentration relative to the calibration standards, determine peanut distribution, and gather information useful for modeling heterogeneous mixtures and food allergen detection.

Samples from all five subs had comparable MFI profiles, with varying intensities of peanut. Sub D displayed MFI responses with peanut bead sets 37 and 38 of $9,200 \pm 600$ and $6,990 \pm 390$, respectively, characteristic of 9.2 and 9.6 μg/g peanut in the garlic powder. Signals more than one standard deviation above the first nonzero standard (S1) were observed for bead sets gluten-27, gluten-28, pine nut-37, and soy-46. The same antigenic profile was observed for a garlic control spiked with 13 μg/g peanut using SRM 2387 (GC+2387) with comparable MFI intensities, except for gluten-27. A garlic control not spiked with peanut (GC) displayed no significant response with peanut-37 and peanut-38 (<S1) but did generate a response with pine nut-37 within one standard deviation above S1.

To better assess the presence of peanut, the ratios of various bead set responses (MFI minus background) were compared. Sub D generated strong responses with the

peanut-specific bead sets with a peanut-37/peanut-38 ratio of 1.3, which was comparable to the 1.4 observed for peanut in garlic control powder (GC+2387). The lesser responses generated by sub D with the bead sets specific for pine nut and soy were comparable to the results generated by the garlic control samples. Specifically, the pine nut-39/pine nut-42 ratio for the GC was 17, comparable to sub D and GC+2387. This was considerably different from the ratios of 8 and 9 for the pine nut standard in the S1 and S2 calibration standards, which had responses that bracketed the response generated by sub D (data not shown). This indicates that the pine nut-39 signal was most likely not due to the presence of pine nut but likely was due to a cross-reactivity from the garlic. For soy, the ratios of soy-46/soy-45 for sub D and GC+2387 were 9 ± 3 and 20 ± 18 , respectively, with sub D and GC+2387 generating comparable responses with soy-46 of $2,650 \pm 540$ and $2,359 \pm 1,160$, respectively. In contrast, the S1 and S2 calibration standards generated soy-46/soy-45 ratios of 4 (data not shown). This also indicates that the soy signals were not due to the presence of soy but rather to cross-reactivity of antibodies with the peanut in garlic. The responses observed with the gluten-specific bead sets were inconclusive. For gluten-28/gluten-27, the ratios observed for S1 on plate 1 and plate 2, for sub D, and for GC+2387 were 9, 7, 11, and 14; this variance is inconsistent with concluding definitively whether gluten or a cross-reactivity component was present in either sub D or GC+2387.

The average recovery for bead sets peanut-37 and peanut-38 with SRM 2387 peanut butter spiked into powdered garlic at 18.2 and 4.5 μg/g were 47 and 53%, respectively, with relative standard deviation <2% ($n = 3$, within plate). Using the calibration standard mixture supplied with the xMAP assay to spike garlic powder at 1 μg/g peanut resulted in a considerably higher average

recovery of 128% (Table 2). The higher recovery can be explained by multiple potential causes, including the higher variance (increased background and noise) typical for low concentrations of analyte that approach the lower limit of the dynamic range of the assay.

An indicator of the precision of the xMAP assay was obtained by comparing the results generated by a single extract of a peanut in garlic sample analyzed in triplicate, on two plates, on different dates. The sample, which generated a response (MFI) characteristic of 5 ppm, displayed a relative standard deviation of 7 and 6% with bead sets peanut-37 and peanut-38, respectively.

The five subs of imported garlic powder were reanalyzed using the Veratox ELISA and indicated peanut concentrations considerably different from those measured using the xMAP assay (Table 2). This difference was not unexpected because the two assays rely on different antibodies and different extraction protocols. However, interestingly, the relative concentrations of peanut in the different subs displayed similar peanut concentration patterns with both assays ($D < I < A < G < F$), and both definitively indicated heterogeneity across the subs. Because only five subs were analyzed in detail (plus another five less extensively), and given the heterogeneity of results in those subs, there is the possibility that another sub might exceed the 134 $\mu\text{g/g}$ that was detected using the Veratox ELISA (51 $\mu\text{g/g}$ by xMAP). However, without additional sampling and knowledge of the size of the production run or how the peanut was introduced (e.g., in transport, storage, or agricultural production), it is difficult to statistically estimate the maximum concentration that may be present. Triplicate measurements of the subs suggest relatively homogenous distribution of peanut in each sub, as evidenced by low coefficients of variance (Table 2).

Western blot. Samples from sub F and sub G were analyzed by Western blot (Fig. 1) using the detection antibodies from the Veratox (Fig. 1A) and MIOBS (Fig. 1B) Peanut ELISA kits. Both detection antibodies are polyclonal; the Veratox antibodies display specificity for Ara h 3 and Ara h 1, whereas the MIOBS antibodies preferentially recognize Ara h 2 and Ara h 6, with weaker binding to Ara h 1 and Ara h 3 (9). The detector antibody from the Veratox Peanut Allergen ELISA test kit (Fig. 1A) generated intense bands at 24 and 37 kDa with SRM 2387 peanut butter (lane 1), consistent with its known specificity for Ara h 3. Sub F (lane 11) and sub G (lane 15) also showed faint bands at 24 and 37 kDa when blotted using the Veratox detector antibody, consistent with the presence of peanut in both subs. However, additional bands of high intensity were observed with both subs at 15, 18, and 60 kDa, which were also observed with GC (lane 3) and GC+2387 (lanes 5 and 7). Using the detector antibody from the MIOBS Peanut ELISA (Fig. 1B), bands at 15 and 60 kDa were observed with sub F (lane 11), sub G (lane 15), GC (lane 3), and GC+2387 (lanes 5 and 7). These bands were not observed with the SRM 2387 peanut butter (lane 1), which displayed bands at 16 and 20 kDa. An additional twofold dilution of

the Veratox detector antibody (net 20-fold dilution) resulted in only the 60-kDa garlic-derived protein being visualized and in loss of the bands at 15 and 18 kDa (Fig. 1C). Thus, the interactions between the Veratox antibodies and the 15- and 18-kDa garlic-derived proteins may be relatively weak. Additional immunoblots using the detector (polyclonal) antibodies from the MIOBS Gluten and Casein ELISA test kits (Fig. 1D and 1E) indicated a complex specificity by these garlic-derived proteins. The gluten detector antibody did not show any binding to garlic-derived proteins (Fig. 1E, lanes 13 and 15). In contrast, the casein detector antibody generated bands at approximately 14 and 60 kDa with both garlic samples (Fig. 1D, lanes 13 and 15) and a weak band at approximately 24 kDa with 10,000 ppm of SRM 2387 that was not observed at 1,000 ppm (Fig. 1D, lanes 9 and 11). As expected, the antibodies against casein and gluten showed strong binding to milk and gluten, respectively (Fig. 1D, lanes 5 and 7; Fig. 1E, lanes 1 and 3).

The question became whether the bands at 15, 18, and 60 kDa with the Veratox peanut antibodies (15 and 60 kDa with the MIOBS peanut antibody) represented specific antigenic interactions due to homology between the garlic-derived proteins and antigenic peanut proteins or whether the garlic-derived proteins recognized a feature of the antibodies that was not universally present on all antibodies. Whatever the mechanism, it must also explain why garlic did not generate false positives with the ELISAs. One possibility is that the capture antibody did not recognize the same cross-reactive epitope as the detector antibody used in the Western blot. Alternatively, lack of binding between the Veratox antibody and two of the garlic-derived proteins upon dilution would be consistent with weak binding, which, under the stringent conditions of the ELISA, might not occur. Further, if the number of binding sites were only one per protein, it would mean that the garlic-derived proteins could not mediate the binding of both the capture and detector antibodies, thus making it impossible for the garlic-derived proteins to generate false positives in a sandwich ELISA. Though this scenario might explain how garlic might appear inconsequential in ELISA analyses, the situation may be more complicated. If the garlic-derived proteins were competing for the antigen recognition site on the antibody, the garlic protein would act as a competitor and would distort the binding curve and antigen quantitation. Binding elsewhere on the antibody might also affect quantitation by affecting the thermodynamics of antibody-antigen interaction. In a worst-case scenario, it is possible that sufficient distortion of the thermodynamics of antigen-antibody interaction might generate false negatives, which could possibly explain the 50% recoveries measured with spiked samples using the Veratox ELISA. Thus, unless demonstrated otherwise, it might be advisable whenever antibody-based assays to detect analytes in garlic are used, that the calibration curves entail varying the concentration of analyte in garlic.

The observation of a protein band at approximately 24 kDa generated by the detector antibody from the MIOBS Casein ELISA with 10,000 ppm of SRM 2387 does not appear to affect the performance of the Casein ELISA. The

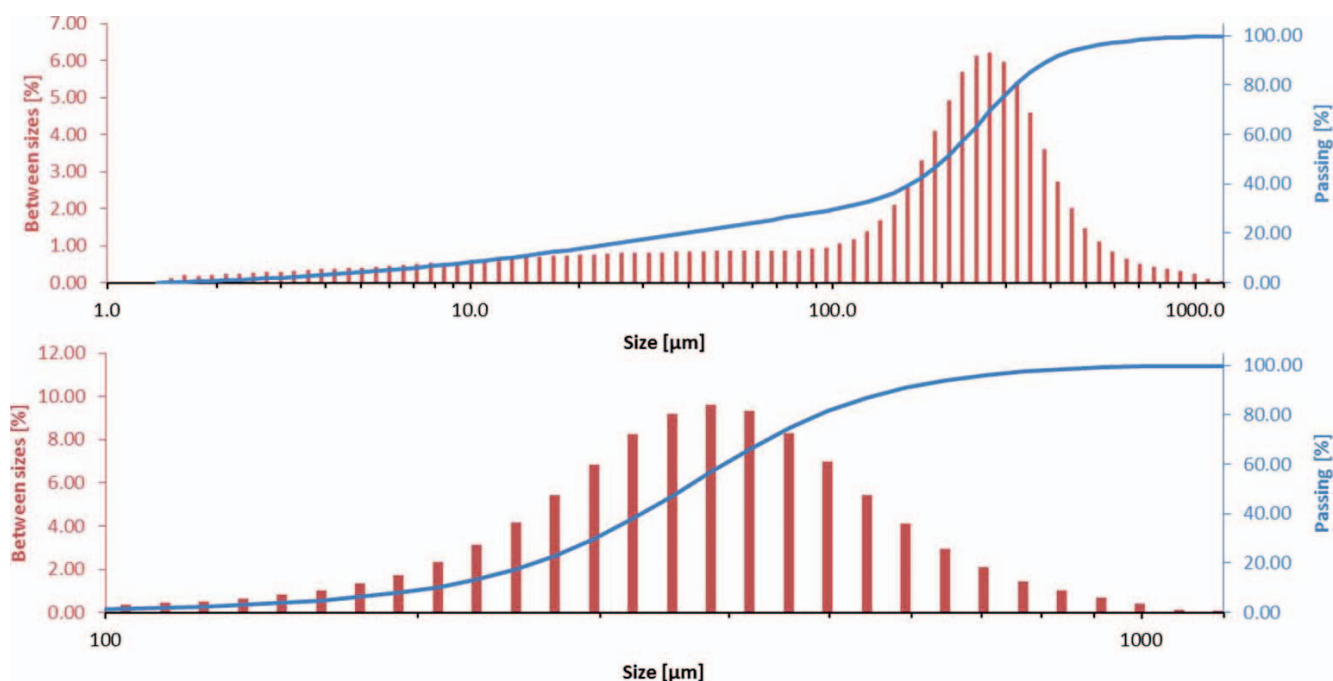


FIGURE 2. Particle size distribution of garlic powder suspected of undeclared peanut. Sub F (A) prior to and (B) after grinding. The major peak at 380 μm shifts to 290 μm with the appearance of a secondary peak at 40 μm after grinding.

Casein ELISA has no record of displaying cross-reactivity with peanut (12). SRM 2387 contains only roasted peanuts, sugar, partially hydrogenated vegetable oils (48% rapeseed, 40% cottonseed, and 12% soybean oil), and salt. It is possible that the interaction of the detector antibody with the peanut-derived protein is too weak, as evidenced by no band being generated with 1,000 ppm of SRM 2387; the maximum concentration of peanut possible in the analytical sample of the ELISA is 2,500 ppm, owing to the 400-fold dilution associated with sample extraction and preparation. Alternatively, the sandwich ELISA would not generate false positives if the band visualized in the immunoblot was due to a single epitope per protein. Further research is necessary to characterize this protein, although its importance to the analytical performance of the Casein ELISA is questionable.

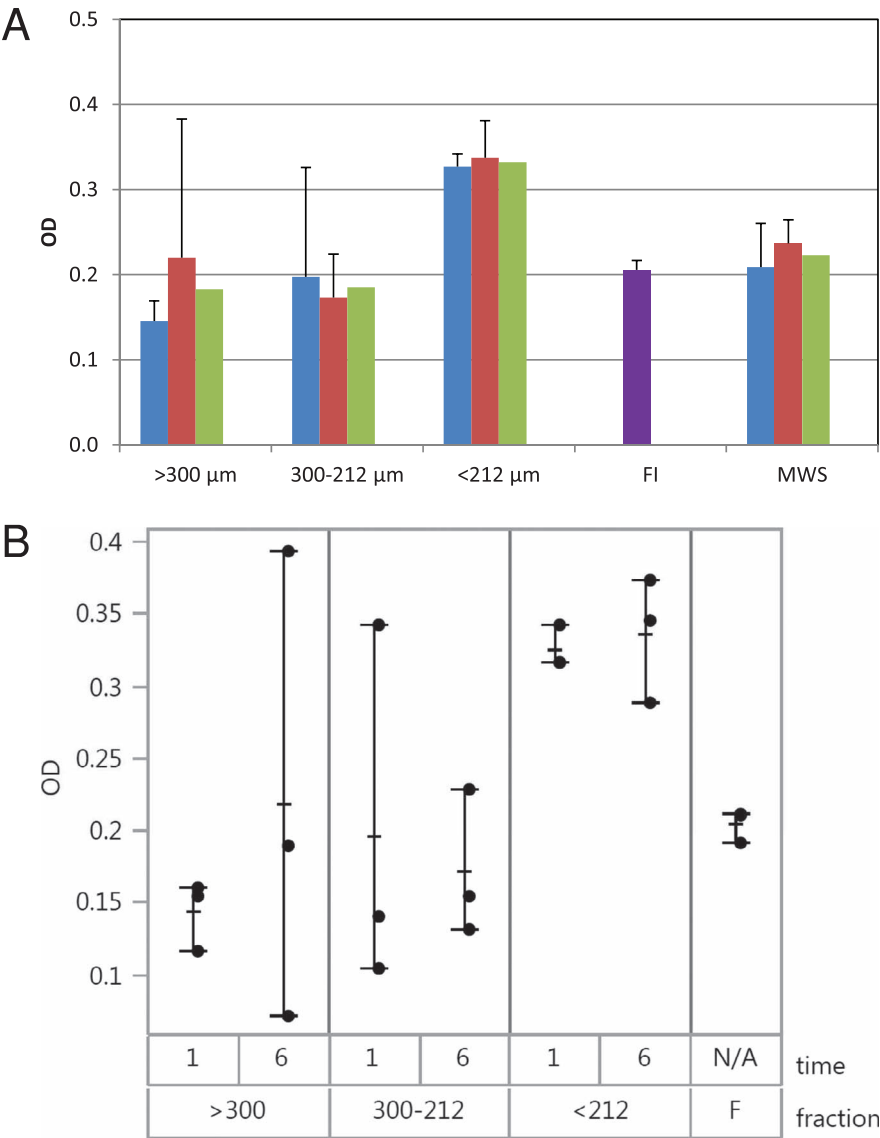
Particle size. The particle size distribution of each sub was measured before and after grinding (Fig. 2). Each sample showed a similar distribution. Prior to any grinding, a major peak was observed at 380 μm . Following grinding, this peak shifted to 290 μm , and a secondary peak at 40 μm appeared.

Fractionation of the garlic powder from sub F was performed using 300- and 212- μm sieves with the fractions (>300, 212 to 300, and <212) collected after 1 and 6 h of shaking. The fractions were analyzed for peanut using the MIOBS Peanut ELISA. The replicates of the >300- and 212- to 300- μm fractions displayed considerably more variation in the ELISA data (OD representative of peanut concentration) than the <212- μm fraction or the unfractionated garlic powder (Fig. 3). The higher variances generated by the larger fractions (>300 and 212 to 300 μm) may represent greater variability associated with the sampling of larger

peanut particles. Interestingly, the <212- μm fraction indicated a higher peanut content (OD values) than the other fractions. This was irrespective of whether the fractionation process was performed for 1 or 6 h, indicating that the distribution was likely not due to the peanut particles undergoing fragmentation during the separation process. The possibility that the increase in detectable peanut observed with the <212- μm fractions might reflect increased extraction efficiency in the preparation of the ELISA samples with the smaller particles was somewhat counteracted by including a grinding step as part of the sample preparation prior to sample extraction. Also, the observation that the mass-weighted sum was comparable to the peanut content detected in the unfractionated garlic powder confirms that the amount of peanut recovered from the three fractions was not distorted. Thus, the higher content of peanut detected in the <212- μm fraction was probably not an artifact of the fractionation process or associated analyses.

Analytical concerns. The complexity of foods, the variability in content, and the use of different forms of processing make the reliance on analytical methods that generate a “one-dimensional” response potentially unreliable. Methods that detect multiple properties of an analyte offer a potential solution to this problem. The more orthogonal the parameters measured, the more confirmatory the results. Thus, the use of two ELISAs to detect peanut provides one level of confirmation, as does the use of two bead sets for the detection of peanut in an xMAP assay. However, comparing the intensities (ratio) of the responses generated by the two ELISAs (or the ratio of the intensities generated by the two bead sets) with the ratio of intensities generated by a reference material, at the concentration

FIGURE 3. To determine peanut particle size distribution in sub F, sub F was fractionated using 300- and 212- μm sieves; the fractions were subsequently ground and analyzed using MIOBS Peanut ELISA. (A) Average absorbance ($\text{OD}_{450\text{ nm}}$) of triplicate analyses generated by the fractions produced from (blue bar) 1 and (red bar) 6 h of sieving; (green bar) average of the $\text{OD}_{450\text{ nm}}$ values generated by 1 and 6 h of fractionation; (purple bar) sub F not subjected to fractionation. Mass-weighted sum (MWS) is the sum of each size fraction mathematically weighed against the fraction of total mass sieved. Error bars represent one standard deviation. (B) The distribution of $\text{OD}_{450\text{ nm}}$ values (individual and averaged) for the various fractions and times of fractionation.



ascribed to the sample, offers the possibility of a higher level of confirmation. A potentially higher level of confirmation is achieved by not only examining strong interactions characteristic of the analyte (e.g., two bead sets specifically designed to recognize peanut) but by also looking at weak interactions that are more likely to be easily altered; this is the basis of antigen profiling. The high sensitivity of the xMAP assay makes it possible to follow these weak interactions. The imported garlic powder not only displayed the antigenicity characteristic of peanut, but also the relative intensities of the responses generated by the bead sets for peanut and the relative intensities of the cross-reactivity of peanut with antibodies for other analytes.

Another concern too often ignored is the probability of a heterogeneous distribution of an allergen. Too often, multiple analyses are averaged as if the target analyte was uniformly distributed, with the variances viewed as representing random error. Based on similar reasoning, massive amounts of a food containing multiple oral portions are often composited prior to analysis to obtain an average content. Both the overaveraging of data and the compositing of too many oral portions can generate misleading

conclusions regarding potential oral exposure. The assumption of homogeneity may be appropriate for milk or a finely ground material (e.g., grain or flour) thoroughly mixed with another solid of comparable particle size, density, and physicochemical properties. However, food allergens that form insoluble particulates (e.g., tree nuts and legumes), or that are introduced nonuniformly, pose a concern. In this study, the peanut in garlic powder was heterogeneously distributed between subs but, within a sub, was apparently homogeneously distributed. The majority of peanut particles in sub F were <212 μm , considerably less than the 380- μm size favored by the garlic powder. Unfortunately, extrapolation of the analytical data to predict probable exposure levels was not possible because of a lack of information regarding production (e.g., size of a run, relationship during production of the subs, production details) and the physicochemical properties of peanut and garlic (e.g., tensile strength, fragmentation patterns). Considering the size and complexity of the knowledge gap, an empirical approach to the problem may provide a route to creating a database that can be applied to developing a model that may approximate distribution and exposure probabilities.

ACKNOWLEDGMENTS

Thanks are expressed to Chung Y. Cho (FDA), William Nowatzke, Ph.D., and Kerry Oliver, Ph.D. (Radix BioSolutions), for their openness and guidance regarding the xMAP multiplex assay. Appreciation is also expressed to Thomas Kuntz, Jennifer Canale, Gary Yuen, James M. Farrow, Ph.D., and Donald H. Burr, Ph.D. (FDA), for providing background information regarding the imported garlic.

REFERENCES

- Burks, A. W., S. M. Jones, R. A. Wood, D. M. Fleischer, S. H. Sicherer, R. W. Lindblad, D. Stablein, A. K. Henning, B. P. Vickery, A. H. Liu, A. M. Scurlock, W. G. Shreffler, M. Plaut, H. A. Sampson, and Consortium of Food Allergy Research (CoFAR). 2012. Oral immunotherapy for treatment of egg allergy in children. *N. Engl. J. Med.* 367:233–243.
- Cheng, F., J. Wu, J. Zhang, A. Pan, S. Quan, D. Zhang, H. Kim, X. Li, S. Zhou, and L. Yang. 2016. Development and inter-laboratory transfer of a decaplex polymerase chain reaction assay combined with capillary electrophoresis for the simultaneous detection of ten food allergens. *Food Chem.* 199:799–808.
- Cho, C. Y., W. Nowatzke, K. Oliver, and E. A. E. Garber. 2015. Multiplex detection of food allergens and gluten. *Anal. Bioanal. Chem.* 407:4195–4206, Erratum 408:6509–6510.
- Cucu, T., L. Jacxsens, and B. De Meulenaer. 2013. Analysis to support allergen risk management: which way to go? *J. Agric. Food Chem.* 61:5624–5633.
- Dayan-Kenigsberg, J., A. Bertocchi, and E. A. E. Garber. 2008. Rapid detection of ricin in cosmetics and elimination of artifacts associated with wheat lectin. *J. Immunol. Methods* 336:251–254.
- Du Toit, G., G. Roberts, P. H. Sayre, H. T. Bahnson, S. Radulovic, A. F. Santos, H. A. Brough, D. Phippard, M. Basting, M. Feeney, V. Turcanu, M. L. Sever, M. Gomez Lorenzo, M. Plaut, G. Lack, and LEAP Study Team. 2015. Randomized trial of peanut consumption in infants at risk for peanut allergy. *N. Engl. J. Med.* 372:803–813, Erratum 375:398.
- Garber, E. A. E., K. V. Venkateswaran, and T. W. O'Brien. 2010. Simultaneous multiplex detection and confirmation of the proteinaceous toxins abrin, ricin, botulinum toxins, and *Staphylococcus* enterotoxins A, B, and C in food. *J. Agric. Food Chem.* 58:6600–6607.
- Gupta, R. S., E. E. Springston, M. R. Warrier, B. Smith, R. Kumar, J. Pongracic, and J. L. Holl. 2011. The prevalence, severity, and distribution of childhood food allergy in the United States. *Pediatrics* 128:E9–E17.
- Jayasena, S., M. Smits, D. Fiechter, A. de Jong, J. Nordlee, J. Baumert, S. L. Taylor, R. H. Pieters, and S. J. Koppelman. 2015. Comparison of six commercial ELISA kits for their specificity and sensitivity in detecting different major peanut allergens. *J. Agric. Food Chem.* 63:1849–1855.
- Koeberl, M., D. Clarke, and A. L. Lopata. 2014. Next generation of food allergen quantification using mass spectrometric systems. *J. Proteome Res.* 13:3499–3509.
- Korte, R., S. Lepski, and J. Brockmeyer. 2016. Comprehensive peptide marker identification for the detection of multiple nut allergens using a non-targeted LC-HRMS multi-method. *Anal. Bioanal. Chem.* 408:3059–3069.
- Matsuda, R., Y. Yoshioka, H. Akiyama, K. Aburatani, Y. Watanabe, T. Matsumoto, N. Morishita, H. Sato, T. Mishima, R. Gamou, Y. Kihira, and T. Maitani. 2006. Interlaboratory evaluation of two enzyme-linked immunosorbent assay kits for the detection of egg, milk, wheat, buckwheat, and peanut in foods. *J. AOAC Int.* 89:1600–1608.
- Newsome, G. A., and P. F. Scholl. 2013. Quantification of allergenic bovine milk alpha (S1)-casein in baked goods using an intact N-15-labeled protein internal standard. *J. Agric. Food Chem.* 61:5659–5668.
- Panda, R., H. F. Zoerb, C. Y. Cho, L. S. Jackson, and E. A. E. Garber. 2015. Detection and quantification of gluten during the brewing and fermentation of beer using antibody-based technologies. *J. Food Prot.* 78:1167–1177.
- Perkin, M. R., K. Logan, A. Tseng, B. Raji, S. Ayis, J. Peacock, H. Brough, T. Marrs, S. Radulovic, J. Craven, C. Flohr, G. Lack, and EAT Study Team. 2016. Randomized trial of introduction of allergenic foods in breast-fed infants. *N. Engl. J. Med.* 374:1733–1743.
- Scaravelli, E., M. Brohee, R. Marchelli, and A. J. van Hengel. 2008. Development of three real-time PCR assays to detect peanut allergen residue in processed food products. *Eur. Food Res. Technol.* 227:857–869.
- Sealey-Voyksner, J., J. Zweigenbaum, and R. Voyksner. 2016. Discovery of highly conserved unique peanut and tree nut peptides by LC-MS/MS for multi-allergen detection. *Food Chem.* 194:201–211.
- Smith, T. (ed.). 2014. Manual on test sieving methods: guidelines for establishing sieve analysis procedures, 5th ed. ASTM International, West Conshohocken, PA.
- Trucksess, M. W., V. A. Brewer, K. M. Williams, C. D. Westphal, and J. T. Heeres. 2004. Preparation of peanut butter suspension for determination of peanuts using enzyme-linked immunoassay kits. *J. AOAC Int.* 87:424–428.
- Verrill, L., R. Bruns, and S. Lucciolli. 2015. Prevalence of self-reported food allergy in US adults: 2001, 2006, and 2010. *Allergy Asthma Proc.* 36:458–467.
- Zhang, W. J., Q. Cai, X. Guan, and Q. Chen. 2015. Detection of peanut (*Arachis hypogaea*) allergen by real-time PCR method with internal amplification control. *Food Chem.* 174:547–552.

Reproduced with permission of copyright owner.
Further reproduction prohibited without permission.