

Research Note

Application of Multiantigen Profiling To Detect Pecan

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ABSTRACT

A problem often encountered in the detection and identification of undeclared tree nut food allergens is the lack of analytical methods. This problem is accentuated by the current trend, whereby the primary methods used to detect food allergens are antibody-based enzyme-linked immunosorbent assays (ELISAs) and the development of analyte-specific antibodies takes months. The recently developed xMAP food allergen detection assay (xMAP FADA) has the ability to generate multiantigen profiles with tree nuts, thereby providing a potential solution to this problem. The xMAP FADA includes 22 antibodies targeting peanut, soy, and nine tree nuts. The high number of antibodies to a diverse group of tree nuts and legumes and the propensity of tree nuts to cross-react have enabled the development of multiantigen profiling, whereby an analyte reacts with the various antibodies to generate a profile. Recently, a question arose regarding the possible presence of pecan dust at a manufacturer of pecan products that also stored fresh produce. The lack of suitable pecan ELISAs created an analytical challenge that was resolved using multiantigen profiling with the xMAP FADA. Pecan was detected on swab samples by using multiantigen profiling and confirmed by DNA analysis. The use of multiantigen profiling provided an analytical capability beyond what was possible with an analyte-specific analytical method.

Key words: Antigenic profiling; Food allergens; Multiplex assay; Pecan; xMAP food allergen detection assay

Food allergies affect more than 15 million people in the United States, approximately 4% of adults and 8% of children (11, 13). Each year, about 29,000 anaphylactic events occur, of which 150 result in death (1). There are more than 170 foods known to cause allergies, with diet patterns affecting allergic incidences (7, 9). There is no known cure for food allergies, thereby necessitating a strict avoidance diet as the only method to prevent adverse reactions. As such, consumers suffering from food allergies must rely on the accuracy of labels to declare the presence of any allergenic foods and the accuracy of manufacturers to prevent accidental inclusion of any allergenic foods. To assist manufacturers and consumers, the Food Allergen Labeling and Consumer Protection Act of 2004 (5) requires labeling of the eight major foods that cause 90% of all allergies in the United States.

Unfortunately, inadvertent cross-contact, accidental errors in manufacturing and labeling, and insufficiently designed food handling practices have resulted in the presence of undeclared food allergens in foods. This has resulted in the need for analytical methods by food producers, contract analytical laboratories, and regulatory laboratories to detect the presence of undeclared food allergens and help safeguard allergic consumers. The most commonly used analytical methods for the detection of food

allergens are enzyme-linked immunosorbent assays (ELISAs). The high specificity of the antibodies used in ELISAs makes possible the detection of low concentrations of food allergens in complex food matrices with high reliability.

Although ELISAs are highly specific, the antibodies can cross-react with homologous proteins. Distinguishing between these false positives and the targeted analyte requires independent confirmatory analyses. To reduce the likelihood of false positives, the U.S. Food and Drug Administration (FDA) requires concurrence between two ELISAs that require different extraction protocols and that are based on different antibodies. As a result, testing for multiple allergens can be time-consuming and expensive. A solution to this problem is the xMAP food allergen detection assay (xMAP FADA; Radix BioSolutions, Ltd., Georgetown, TX) (2). The xMAP FADA entails the use of 30 antibodies and two extraction protocols to detect 14 food allergens plus gluten. Most of the food allergens are detected using two antibodies, providing built-in confirmation. In addition, the presence of 22 antibodies for peanut, soy, and nine tree nuts makes it possible to observe cross-reactivity patterns between the legumes and tree nuts and to use these profiles as a secondary level of confirmation (3, 10). Additional research established that the profiles generated by legumes and tree nuts not specifically targeted by the antibodies could be used to detect these legumes and tree nuts,

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provided the profiles (multiantigen profiling) generated were unique (3).

Recently, the FDA inspected a facility that manufactured pecan products near where produce was stored. A high level of powder, potentially from the pecan deshelling and handling, was observed. This powder presence created concerns that the produce might be contaminated with undeclared pecan. Unfortunately, suitable analytical methods for pecan did not exist. Pecans and walnuts both belong to the same family (Juglandaceae); as such, pecans cross-react to the antibodies that are specific for walnut in the xMAP FADA (bead sets-47 and -48) but display a different multiantigenic profile. Thus, it should be possible to determine whether pecan was in the powder. Swab samples were collected at different locations of the facility and analyzed using the xMAP FADA (3). Pecan was detected in several of the swabs, and the presence of pecan was confirmed by DNA analysis.

MATERIALS AND METHODS

Reagents. Phosphate-buffered saline with Tween 20 (PBST) was obtained from Sigma (St. Louis, MO). Other reagents for UD buffer (105 mM sodium phosphate, 75 mM NaCl, 2.5% Difco skim milk powder, 0.05% Tween 20) were prepared as described previously (6). Raw whole walnuts and pecans were obtained from Nuts.com (Cranford, NJ), and upon visual inspection they were found to be free of contaminants.

Swab samples. Ten swab samples and two negative controls were collected at different locations within the facility (Fig. 1) by using sterile Falcon swabs (BD, Franklin Lakes, NJ). The pecan deshelling operation took place to the south side of the cold storage unit (cooler) where fresh produce was stored. Swab samples 1 and 2 were collected from the surface south of the shelling machine and from the table next to the shelling machine, respectively. Swab samples 5, 7, and 10 were collected from inside the cold storage unit. Swab samples 4 and 8 were collected from the outer wall surface of the cold storage unit, and swab sample 3 was collected from the floor in front of the cold storage unit, by the west side of the entrance. Swab samples 6 and 9, were from the area left of the forklift in front of the office and from the top of the front wheel frame of the forklift, respectively. Negative controls A and B were a blank swab and a wipe of the clean gloves used to collect swab samples, respectively. The analytical material collected by the swabs was transferred into PBST to generate the swab samples analyzed using the xMAP FADA and DNA analytical protocols.

Immunochemical analysis. The xMAP FADA buffered detergent extraction protocol using UD buffer was performed as described previously to generate multiantigen profiles (3). UD buffer was used to provide increased stringency and eliminate nonspecific binding typically observed with plant products, presumably due to lectins. All samples were analyzed in triplicate, and the averages are presented.

DNA analysis. DNA was extracted from aliquots of the swab samples using the DNeasy Plant Mini Kit (Qiagen, Germantown, MD) following the manufacturer's instructions. Pecan and peanut isolates were used as positive controls. DNA concentrations were quantified using a NanoDrop Lite spectrophotometer (Thermo-Fisher Scientific, Wilmington, DE). PCR amplification and Sanger sequencing were performed following the Plant DNA Barcode

Project, Smithsonian National Museum of Natural History, protocols (<http://botany.si.edu/projects/dnabarcode/details.htm>). Primers used to amplify the *trnL* region were as described previously (12). The resulting DNA sequences were assembled and aligned using Geneious 10.0.9 (Biomatters Ltd., Auckland, New Zealand). Additional sequences from other species belonging to the pecan and peanut families (Juglandaceae and Fabaceae, respectively) were mined from GenBank and included in the analysis. Data analysis was carried out as described previously (4, 8).

Real-time PCR was executed with a CFX96 Touch real-time PCR system with a C1000 thermal cycler (Bio-Rad, Hercules, CA) to assess the presence of peanuts in the swab samples. Each PCR reaction contained the following in a 12.5- μ L reaction volume: 1 $\times$ PCR buffer, 5 mM Mg, 1.2 mM deoxynucleoside triphosphates, 200 nM of each primer and probe for the internal amplification control, a constant copy number of 5×10^5 of the internal control template, and 0.625 U of Platinum *Taq* polymerase (Invitrogen/Life Technologies, Grand Island, NY). Primers and probes specific to peanut were designed to target the three chloroplast regions *trnL*, *rpl16*, and *matK* (unpublished data) and were used at 500 and 200 nM, respectively. DNA amplification was performed under the following conditions: 95°C for 3 min followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 65°C for 30 s, and extension at 65°C for 2 min. The resulting data were analyzed using the CFX Manager version 3.1 software (Bio-Rad).

Statistical analysis. Ward's minimum clustering analysis of the median fluorescent intensity (MFI) data presented in Figure 2 was performed using JMP 12.0.1 (SAS Institute Inc., Cary, NC), and other analyses and calculations were performed using Microsoft Excel 14.0, packaged as part of Office Professional Plus 2010 (Microsoft Corp., Redmond, WA), as described previously (3).

Although the variances in the xMAP FADA data generated by replicates analyzed using a single xMAP FADA kit were typically <2%, the MFI data were compared based on two significant figures and the processed data (e.g., bead set ratios) were compared based on one significant figure. Further limiting data interpretation is that the collection of surface samples by using swabs and that the recovery of analyte from the swabs is neither standardized nor validated. Thus, anything dependent on quantification (e.g., potential levels of exposure due to cross-contact into a food product) cannot be assessed reliably. Fortunately, regulatory thresholds for food allergens and quantification of potential exposure levels due to cross-contact into a food product are not required. As the technology is refined and extensively validated, the use of more significant figures to distinguish between fine differences and estimation of potential cross-contact levels from food allergens on a surface should be possible.

RESULTS AND DISCUSSION

xMAP FADA analysis. The xMAP FADA multiantigen analyses of the 10 swab samples and the two negative controls are presented in Figure 2. Figure 2 lists the MFI responses of the samples with walnut bead set-47 and the associated profiles generated upon normalizing the responses to that of bead set-47. If a sample contained pecan as the prominent food allergen it would display a profile similar to pecan at the same concentration as evidenced by a similar MFI response with walnut bead set-47. This analysis was often a four-step process that involved the swab samples and standards (i.e., pecan and walnut) analyzed at 0.063, 0.125, 1.25, 12.5, 250, and

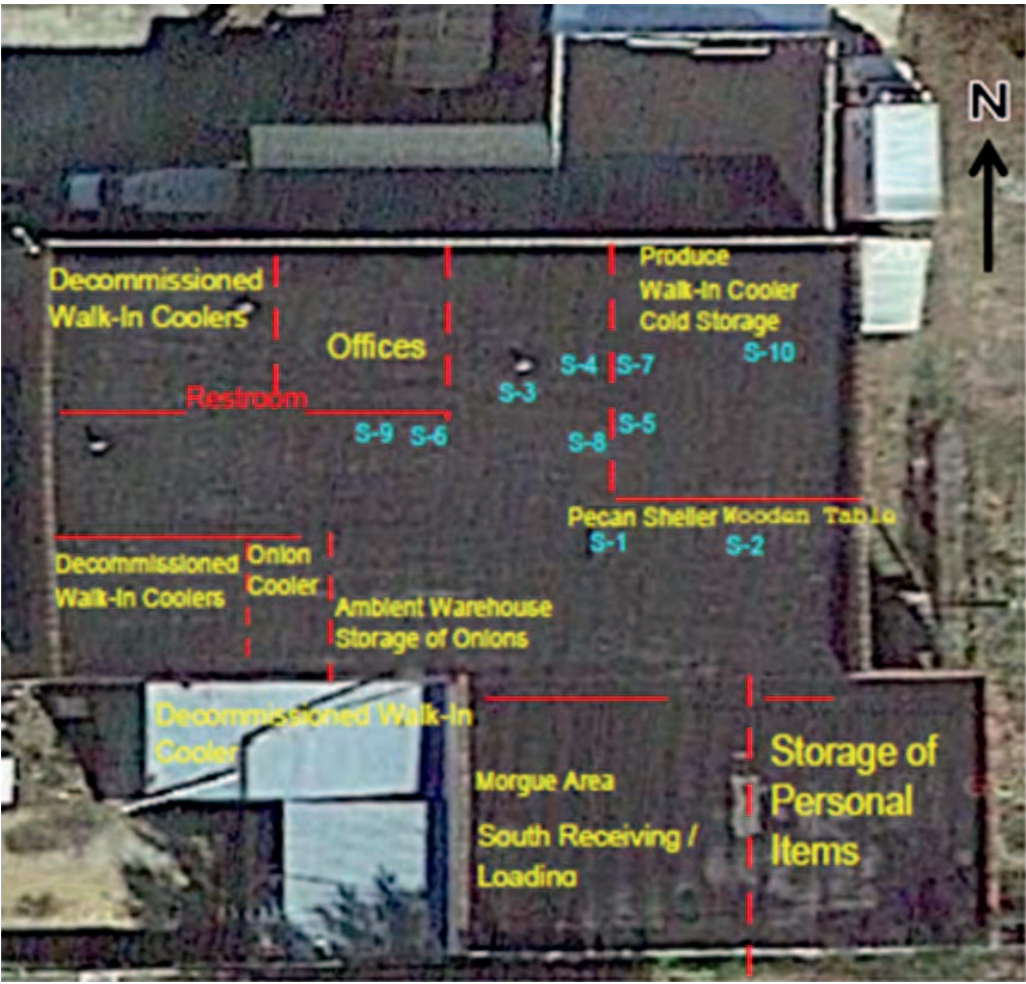


FIGURE 1. Firm diagram with swab collection sites. Swabs were collected from the following sites: 1, pecan sheller; 2, bottom left corner of wooden table; 3, floor in front of the primary cooler; 4, bottom of the door of the primary cooler; 5, two tomatoes in the box exposed to air; 6, area left of the forklift; 7, crack in the floor inside the cooler, directly behind the cooler entry door; 8, metal conduit on the outside wall of the cooler; 9, top of the left front wheel frame of the forklift; 10, area on a wooden frame of a pallet, holding cases of cabbage. Swab samples are designated by an “S” followed by the number of the sample site (e.g., S-1).

		Almond		Brazil nut		Cashew		Coconut		Cru		Gluten		Hazelnut		Mac		Peanut		Pine nut		Pistachio		Soy		Walnut		Walnut
Sample	µg/ml	-12	-13	-14	-15	-18	-19	-20	-21	-22	-27	-28	-29	-30	-33	-34	-37	-38	-39	-42	-43	-44	-45	-46	-47	-48	-47	
Pecan	25,000	-	-	0.2	-	0.3	0.1	0.1	-	-	-	-	0.6	0.3	0.3	-	0.3	0.1	0.1	-	0.1	-	-	0.1	1	0.7	15445	
Swab 6	UK*	-	-	0.1	-	0.2	0.1	0.1	-	-	-	-	0.6	0.2	0.3	-	0.2	0.1	0.1	-	0.1	-	-	-	1	0.7	14925	
Walnut	12.5	-	-	0.1	-	0.1	0.1	-	-	-	-	-	0.3	0.1	0.1	-	-	-	-	-	0.1	-	-	-	1	0.4	12280	
Walnut	250	-	-	0.3	-	0.3	0.3	0.2	-	-	-	-	0.5	0.3	0.3	-	0.3	0.1	-	-	0.2	-	-	-	1	0.6	17420	
Pecan	12.5	-	-	-	-	-	-	-	-	-	-	-	0.3	-	-	-	-	-	-	-	-	-	-	-	1	0.3	6674	
Swab 1	UK	-	-	-	-	0.1	-	-	-	-	-	-	0.3	0.1	0.1	-	0.1	0.1	-	-	-	-	-	-	1	0.4	7967	
Swab 2	UK	-	-	-	-	-	-	-	-	-	-	-	0.2	-	-	-	-	0.1	0.1	-	-	-	-	-	1	0.3	7450	
Swab 9	UK	-	-	-	-	-	-	-	-	-	-	-	0.2	-	-	-	-	0.1	0.2	-	-	-	-	-	1	0.3	6515	
Walnut	1.25	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	1	0.2	8260	
Walnut	12.5	-	-	-	0.1	-	0.1	0.1	-	-	-	-	0.3	0.1	0.1	-	-	-	-	-	0.1	-	-	-	1	0.4	12280	
Control Swab A	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	41	
Control Swab B	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	
Swab 5	UK	-	-	-	-	-	-	-	-	-	0.1	0.4	-	-	-	-	0.3	0.4	-	-	-	-	-	-	1	0.1	211	
Swab 3	UK	-	-	-	-	-	-	-	-	-	-	-	0.2	0.1	-	-	-	0.5	0.5	-	-	-	-	-	1	0.1	5066	
Swab 4	UK	-	-	-	-	-	-	-	-	-	-	-	0.5	0.1	-	-	-	1.7	1.4	-	-	-	-	-	1	0.1	4753	
Swab 7	UK	0.4	-	-	-	0.1	0.1	0.1	-	-	-	-	1.0	0.2	0.1	0.1	-	4.6	2.9	0.3	-	0.2	-	0.5	1	0.3	5329	
Swab 8	UK	-	-	-	-	0.1	-	-	-	-	-	-	0.1	0.3	0.1	-	-	0.8	0.6	-	-	-	-	-	1	0.4	9010	
Swab 10	UK	0.1	-	-	-	-	-	-	-	-	-	-	0.4	0.1	-	-	-	0.8	0.8	-	-	-	-	-	1	0.1	5266	
BKG		11	11	28	12	53	9	43	12	21	52	333	68	14	49	11	26	30	90	14	31	8	27	19	89	19		
SD		1	1	1	0	3	0	2	1	1	2	8	2	0	1	1	2	1	5	1	1	1	1	1	5	1		

FIGURE 2. Comparison of multiantigen profiles generated by swab samples. The background MFI for each bead set was subtracted from each of the responses and then normalized to the signal (response) observed with walnut bead set-47 (listed in green). Normalized responses of particular note are presented in red. Hyphen (-) is used to indicate normalized responses less than 0.05. UK indicates that the concentration of sample was not known. The background responses and associated standard deviations are listed in the rows labeled BKG and SD, respectively. Cru and Mac are abbreviations for crustacean and macadamia nuts, respectively. All samples were analyzed in triplicate, and the averages are presented.

25,000 µg/mL (ppm). The initial focus was on the most reliable data, which often entailed larger MFI responses, except when the MFI was characteristic of a saturation response. Using the results obtained with swab sample 6 as an example, the following steps were performed:

(i) The dominant MFI response was used to select the proper standards for comparison. Swab sample 6 generated an MFI response of 15,000 with bead set-47. Pecan at 25,000 µg/mL generated an MFI response of 15,000, whereas walnut at 12.5 and 250 µg/mL generated MFI responses of 12,000 and 17,000, respectively.

(ii) The ratio between the two bead sets of the dominant antigenic response (walnut) were compared. Swab sample 6 generated a ratio of 0.7, pecan at 25,000 µg/mL generated a ratio of 0.7, and walnut at 12.5 and 250 µg/mL generated a ratio of 0.4 and 0.6, respectively.

(iii) Using the data (Fig. 2), the ratios of the responses normalized to the dominant bead set (walnut-47) were compared with those of the standards. As previously stated, focus was first placed on the higher, more reliable ratios that are tabulated below for swab sample 6 and the appropriate pecan and walnut standards:

	Hazelnut-29	Macadamia-33	Hazelnut-30	Peanut-37
Swab sample 6	0.6	0.3	0.2	0.2
Pecan, 25,000 µg/mL	0.6	0.3	0.3	0.3
Walnut, 12.5 µg/mL	0.3	0.1	0.1	<0.1
Walnut, 250 µg/mL	0.5	0.3	0.3	0.3

(iv) The remaining ratios in the profiles were compared with ratios of 0.1 understood to be less reliable due to poor signal to noise.

Using the abovementioned criteria, swab samples 1, 2, and 9 were characterized as comparable to the 12.5 µg/mL pecan standard. Swab sample 1 generated a greater response than either swab sample 2 or 9, consistent with a greater concentration of pecan. Swab sample 1 also displayed slight antigenicity with cashew-18, hazelnut-30, and macadamia-33, an additional characteristic of a higher pecan concentration.

In contrast, control swab samples A and B and swab sample 5 generated MFI responses indicative of being pecan free, whereas the MFI responses generated by swab samples 3, 4, 7, 8, and 10 were inconclusive for the presence of pecan. As exemplified by swab sample 8, a strong MFI response with bead set-47 (9,000) and a bead set-48/bead set-47 ratio of 0.4, both consistent with 12.5 µg/mL pecan, were observed. However, the response with bead set-37, -38, -28, and -30 was inconsistent with pecan. It is possible that the MFI responses reflected the presence of multiple food antigens, but presently the database, precision, and accuracy of the xMAP FADA make it impossible to conclude beyond any reasonable doubt that pecan was present in swab samples 3, 4, 7, 8, and 10. Similarly, the xMAP FADA

could not conclusively establish that peanut was present in swab sample 7, despite strong MFI responses with peanut bead set-37 and -38. Interestingly, if swab sample 8 was analyzed using a walnut ELISA, it would have seemed to conclusively support the presence of pecan with no indication that the results were not straightforward.

Cluster analysis. Cluster analysis was performed using Ward's minimum clustering method on the MFI responses of pecan, walnut, and the swab samples. The patterns were consistent with the profile data. Specifically, the MFI response generated by swab sample 6 clustered with 25,000 µg/mL pecan and swab samples 1, 2, and 9 clustered with 12.5 µg/mL pecan. However, swab samples 3, 4, 7, 8, and 10 did not cluster with either pecan or walnut.

DNA analysis. Swab samples 1, 4, 6, 7, 8, and sub A (a negative control) were analyzed using DNA barcoding, targeting the chloroplast *trnL* region. Sequence data generated from swab samples 1 and 6 matched 100% to pecan (positive control) and were consistent with the xMAP FADA data. In contrast, pecan DNA was not detected in swab samples 4, 7, and 8 by using DNA barcoding, consistent with the xMAP FADA data being inconclusive for the presence of pecan in these samples.

Given the strong MFI responses generated by swab sample 7 with peanut bead set-37 and -38, real-time PCR using peanut-specific primers was performed on swab samples 1, 4, and 7. Peanut DNA was not detected in any of these swab samples. The apparent contradiction between the xMAP FADA data and the DNA analyses may reflect several possibilities. The possibility that peanut material does not contain the targeted DNA sequences of the primers is unlikely. Another possibility that the xMAP FADA detected material that contained epitopes homologous to peanut cannot be disqualified. Because these are regulatory samples, the inconsistencies and lack of a definitive explanation make it necessary to classify the presence of peanut in these samples as inconclusive.

In conclusion, positive responses for pecan were observed in the samples collected south of the pecan deshelling machine and from the forklift. However, the samples collected from inside and around the entrance of the cold storage unit used to store produce were all negative for pecan. Thus, although there may be a concern that the deshelling of pecan can generate pecan powder that could pose a cross-contamination threat, the cold storage unit seemed to provide a sufficient physical barrier for protecting the stored produce.

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