

Testing for Food Allergens: Avoiding Potential Pitfalls

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- Overview of Current Approach
 - Sampling (e.g., Collection, Analytical portion)
 - Extraction
 - Analysis
 - Interpretation
- Concerns & Solutions
 - Reference Materials & Calibrants
 - Matrix Effects
 - Specificity & Cross-reactivity
 - Food Processing
 - Heterogeneous Solid Mixtures
- The Future is NOW
- Illustrative Cases

Focus: Food

- Primary focus: Consumer's Perspective
 - Also applied to Ingredients, Facilities, etc.
- Detection of the Food
 - not just allergens, may detect non-allergenic marker 'unique' to the food
 - Ideally, should reflect (potential) allergenicity
 - Problem, how to gauge allergenicity

- Immunodiagnosics
- DNA-based Technology
- Mass Spectrometry

ALL 3 HAVE A ROLE

- Currently Using ELISAs

- **Detectability**
- **Antigenicity**
- **Bioavailability**
- **Allergenicity**

SAMPLING

- Representative of Lot
 - Size of Lot often not known
 - Relation between multiple ‘containers’ not known

IOM (Inspector’s Operational Manual) table based on a model designed for low molecular weight analytes that distribute somewhat homogeneously

IOM TABLE

Sample Schedule 13: Allergen Sample Schedule

SAMPLE SIZES WITH APPLICATION TO FOOD PRODUCTS FOR ALLERGENS*

(Listed below is the sample size needed for lab analysis. Collect all samples in duplicate, with the duplicate serving as the 702 (b) reserve sample)

| Product | Package type | Number of sample units | Unit size | Total sample |
|--|--------------|------------------------|-----------|--------------|
| Non-liquefied foods, i.e., cereals, cookies | Consumer | 20 | 1 lb | 20 lbs |
| Pre-liquefied foods, i.e., ice cream, chocolates | Consumer | 10 | 1 lb | 10 lbs |
| Paste or slurry type | Consumer | 24 | 8 oz | 12 lbs |
| | | 12 | 1 lb | 12 lbs |
| Fluid, i.e., beverages | Consumer | 10 | 16 fl. oz | 160 fl. oz |

IMPORTANT! WHEN TO SAMPLE: At the time of submission of this table to the IOM, only "for cause" allergen samples for peanut contamination should be collected. Test methods for additional allergens are under development and the field will be notified when they are available for regulatory purposes. The allergen compliance program, when issued, will provide additional sampling guidance. "For cause" sampling should be limited to instances where a consumer, medically determined to have a food allergy, experiences an adverse event believed due to the allergenic food, and the labeling of the suspect product does not indicate the presence of the allergen.

* See Laboratory Information Bulletin (LIB) # 4341, Application of Validated, Multiple Laboratory *Performance Test Methods*SM for the Detection of Peanuts in Food, Vol 21(2) 2005 for details regarding the analysis and quantitation of analytical samples.

Note: To be collected from random sites. May combine subs or maintain sub integrity depending on purpose of sampling

Note: Prepare composite following proper grinding and mixing procedures. Separate four 1-lb portions from composite.

Adapted from U.S. Food and Drug Administration, Office of Regulatory Affairs, Investigations Operations Manual, Chapter 4, Sample Schedule 6, Mycotoxin Sample Sizes, http://www.fda.gov/ora/inspect_ref/iom/ChapterText/sschedule6.html

<http://www.fda.gov/ICECI/Inspections/IOM/ucm127711.htm>

Antibody-based Assays

Advantage: Selectivity, detect w/o fractionation or imposition of associated selective pressures

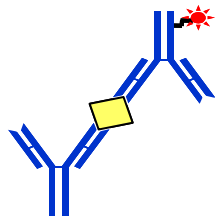
Commonly Used Approach:


Dilute into a matrix that 'overwhelms' original matrix.
Combine extraction & preparation into a single step
simplifies but may be counterproductive

remove particulates (especially for competitive assays)

ELISA

Sandwich ELISA



Immobilized antibody binds antigen (protein, ) unique to the allergenic food.

Second antibody with a bound marker (e.g., enzyme) binds to a second site on the antigen.

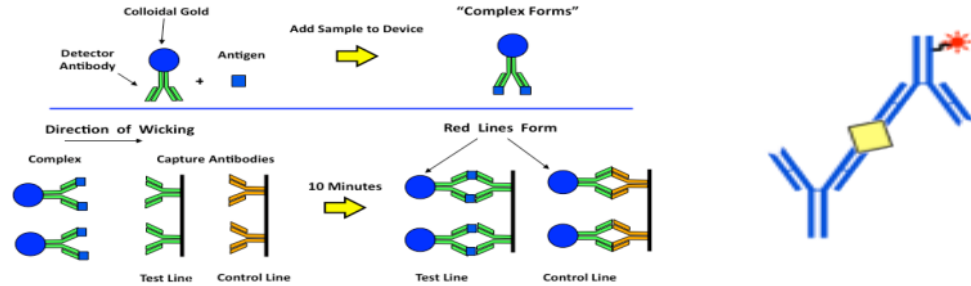
The presence of the marker indicates the likelihood of the presence of the allergenic food.

Most ELISAs detect 1 $\mu\text{g/g}$ (ppm) of the allergenic food in the original sample. Since, a 10- fold (or greater) dilution of the sample is often included in the analysis and the protein being detected may be only 1% of the allergenic food, the amount of analyte in the 100 μL analytical sample may be < 0.1 ng.

Competitive ELISA Competition by analyte in sample results in decrease in response generated.

ANTIGEN CAPTURE CHROMATOGRAPHIC ASSAY

aka Dipstick or Lateral Flow Device (LFD)



Similar to ELISA: Ab-Ag-Ab-marker
Objective quantifiable response using reader
Concern: quality control effecting reliability
Advantages: cost, field-deployable, simple?



REFERENCE MATERIALS

Egg (RM 8445)

Milk (SRM 1549, NFD, MoniQA)

Peanut (SRM 2387)

Crustacean Black Tiger Prawn (equivalent to Japanese std)

Wheat Gluten (Sigma G5004, Equivalent to IRMM & Japanese std)

Peanut, Soy, Almond, Hazelnut, Walnut – in-house reference material from ground, dried, organic, de-shelled, unsalted beans*

**Mimic typical analyte, purified proteins or proprietary extracts not representative of target analytes.*

Analytical Approach

- Commercial ELISAs
- Multi-lab validated - extensively evaluated
- Analysis of replicates
- Extended dynamic range
 - serial dilution (ULOD 30,000 ppm)
- Controls - Method Extension
 - Rule out false positives
 - Rule out false negatives
 - Demonstrate a Dynamic Response
 - same food, if not possible mimic physical-chemical properties*
- Standardized Data Processing Workbooks

- Concurrence between two ELISAs
 - Different methods of extraction
 - Different target epitopes & antigens
 - Cannot target all allergenic elements
 - Not all allergenic elements known
 - Law specifies the food
 - Quantify relative to standards
 - VARIANCE
 - » ELISAs
 - » Processing
 - » Cultivars, growth conditions, etc.

Analytical Sensitivity (LOD)

- FALCPA does not specify enforcement levels
 - *Goal: LOD fit-for-purpose, DO NOT PURSUE ZERO*
 - *Clinical thresholds not established*
 - *Advantages / Disadvantages*
 - Adverse events support target levels > 5 ppm
 - Oral portion
 - Processing
 - Target
 - Current LODs 0.3 – 5 ppm
 - Application on a case-by-case basis
 - Consumer complaint
 - “Level characteristic of”
 - Target consumer (sensitive sub-population)

Gluten-free Regulation specifies 20 ppm

- Recognizes multiple sources
 - “gluten” what is it
 - content varies with source
- Enforcement not at 20.000 ppm
- **Focus on exposure from oral portion***
not average content across lot, but dosage

GOOD NEWS: 2015-16 Domestic survey of 720+ products from 3 commodity groups found only 4 violations.

ELISAs

- ELISAs for seven* of the Big Eight routinely used
- Analyte often not identical to calibrant ('processing')
 - target different epitopes & employ different antibodies
 - likely to generate different quantitative results

NEITHER IS WRONG

*Analytical methods for fish not adopted.

Commercial ELISA test kit target

multiplex DNA method for 55 species of fish

Hildebrandt 2010 (Anal Bioanal Chem. 2010 Jul;397(5):1787-96)

Does not cover all fish nor designed for speciation

Allergen Test Kits

| | | | | | | |
|---------------------|-----------|-------------|-------------|-------------|-------------|-------------|
| Peanut | R | A, A | V, V | P, P | G, - | S, S |
| Egg | R | | V, V | -, P | G, G | S, S |
| Milk | R | | V, V | -, P | G, - | S, S |
| Gluten/Wheat | R* | -, A | V, V | P, P | G, G | S, S |
| Soy | r | | | P, P | | S, S |
| Shellfish | R* | | V, - | P, - | G, - | S, S |
| Tree Nuts | | | | | | |
| Hazelnut | r | | V, V | P, P | G, - | S, S |
| Almond | r | | V, - | P, P | G, - | S, S |
| Walnut | r | | | P, - | | S, S |
| Fish | | | | | | |

R-NIST reference material available (R-non-NIST); r-standardization material, A-AOAC validated at suitable level ; V-multilaboratory validated; P-published; G-official governmental method (non-US); S- single lab validated by CFSAN (level 2 or higher).*

COMMERCIAL ELISA TEST KITS

| FOOD | PRODUCT | SOURCE | TEL | CAT # | DESIGN | RANGE |
|---|---|------------|--------------|--------------------|------------|-------------------------------|
| Egg | Veratox [®] | Neogen | 800-234-5333 | 8450 | 4x12 | 2.5 – 25 ppm egg |
| Egg | Morinaga | Crystl Chm | 630-889-9003 | M2101 | plate | 0.65 – 41.6 µg egg/g |
| Gluten/Wheat | RIDASCREEN [®] <small>AOAC-RI method</small> | R-Biopharm | 877-789-3033 | R7001 | 12x8 | 5 – 80 ppm gluten |
| Gluten/Wheat | Morinaga | Crystl Chm | 630-889-9003 | M2103 | plate | 0.78 - 50 ng prtn/mL |
| Milk | Veratox [®] | Neogen | 800-234-5333 | 8470 | 4x12 | 2.5 – 25 ppm milk |
| Milk <small>baked or processed</small> | Morinaga | Crystl Chm | 630-889-9003 | M2102 | plate | 0.92 – 58.8 ppm milk |
| Milk <small>(opt for not processed)</small> | ELISA Systems | Pi Biosci | 206-714-5275 | EC52310 EB52110 | 6x8 6x8 | 1–10 ppm NFM 0.1-1 ppm BLG |
| Peanut | Veratox [®] | Neogen | 800-234-5333 | 8430 | 4x12 | 2.5 – 25 ppm peanut |
| Peanut | RIDASCREEN [®] Fast | R-Biopharm | 877-789-3033 | R6202 | 6x8 | 2.5 – 20 ppm peanut |
| Hazelnut | Veratox [®] | Neogen | 800-234-5333 | 8420 | 4x12 | 2.5 – 25 ppm hazelnut |
| Hazelnut | RIDASCREEN [®] Fast | R-Biopharm | 877-789-3033 | R6802 | 6x8 | 2.5 – 20 ppm hazelnut |
| Almond | Veratox | Neogen | 800-234-5333 | 8440 | 4x12 | 2.5 – 25 ppm almond |
| Almond | RIDASCREEN [®] Fast | R-Biopharm | 877-789-3033 | 6901 | 6x8 | 2.5 – 20 ppm almond |
| Walnut | BioKits | Neogen | 800-234-5333 | 902085J | 6x8 | 2.4 – 120 ppm walnut prtn |
| Walnut | AgraQuant [®] | Romer | 636-583-8600 | COKAL0948 | 6x8 | 2 – 60 ppm walnut 'prtn' |
| Crustacean | Maruha Nichiro kit 2 | Wako | 804-271-7677 | 385-05301 | plate | 0.78 - 50 ng prtn/mL |
| Crustacean | ELISA Systems | Pi Biosci | 206-714-5275 | EC52410 | 6x8 | 0.5 – 5 ppm tropomyosin |
| Soy | Veratox [®] | Neogen | 800-234-5333 | 8410 | 4x12 | 2.5 – 25 ppm SPI/4 |
| Soy | ELISA Systems | Pi Biosci | 206-714-5275 | ES53210 | 6x8 | 2.5 – 25 ppm SFP |

SPECIFICITY

- Cross-reactivity typically assessed at $\geq 0.1\%$
- IS THIS SUFFICIENT?

RECOVERY & EXTRACTION

- AOAC validation 80 – 120% (Schedule M)
- REALITY
 - Incurred versus spiked into food
 - Focus reproducibility, not 100% recovery
 - Recovery varies with extraction conditions
 - Reduced-denatured better than buffered-detergent
 - Detection of reduced-denatured better than 'native'
 - 'Better safe than sorry?'
- Report observed response 'characteristic of'
 - Not 'recovery-corrected'
 - Alongside measure the recovery of spiked analyte
 - FDA validations use incurred foods

2006 Milk in Dark Chocolate

NRL forced ELISA manufacturer to admit 30% recovery

Read validation report details

Too often, not realistic (not suitable for purpose)



Chocolate Soy & Dairy Protein Shake 03-23-2012

Consumer Complaint # 125400

'15 y/o boy with peanut & tree nut allergies (not soy) began to enter anaphylactic shock after sipping chocolate soy & dairy shake' 03-6-12

'investigator reports no peanuts /peanut products in the plant' 03-28-12

Contract lab: peanut < LOQ

NRL: peanut and almond < LOD (*importance of replicates & controls*)

Recalled

"company says people who have an allergy or severe sensitivity to peanuts and/or tree nuts may run the risk of a serious or life-threatening reaction if they consume the drink." – NY Daily News 4-6-12

Label: "although no peanut or tree nut containing ingredients were used to manufacture this product, allergic reactions to this product have been reported by peanut and tree nut sensitive people"

Legume Cross-sensitivity?

PROBLEM with ELISAs

- Detects single analytes
- Multiple targets requires multiple ELISAs
- Johnny ate a cookie and had a severe allergic reaction. Johnny is allergic to multiple food allergens*. Cost? Labor?

** Incidence rate for multiple food allergies estimated at 30% of the allergic population*

Cannot distinguish between cross-reactive proteins and target analyte

Concurrence between two ELISAs

- Costly, labor intensive, time consuming
- Still high probability of error

Is it ideal not to detect cross-reactive foods?

SOLUTION

MULTIPLEX ANALYSIS

EXTRACTION, PREPARATION, and ASSAY

DOES ONE SIZE FIT ALL?

IS OPTIMIZATION OF **ALL** REACTIONS NECESSARY?

LOD VALUES APPROPRIATE TO THE NEEDS?

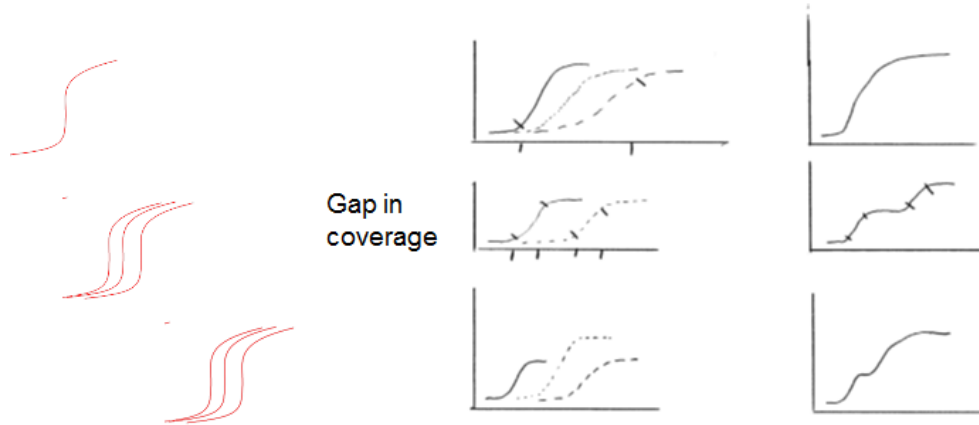
Focus: Target Level

COMPATIBILITY?

compromise?

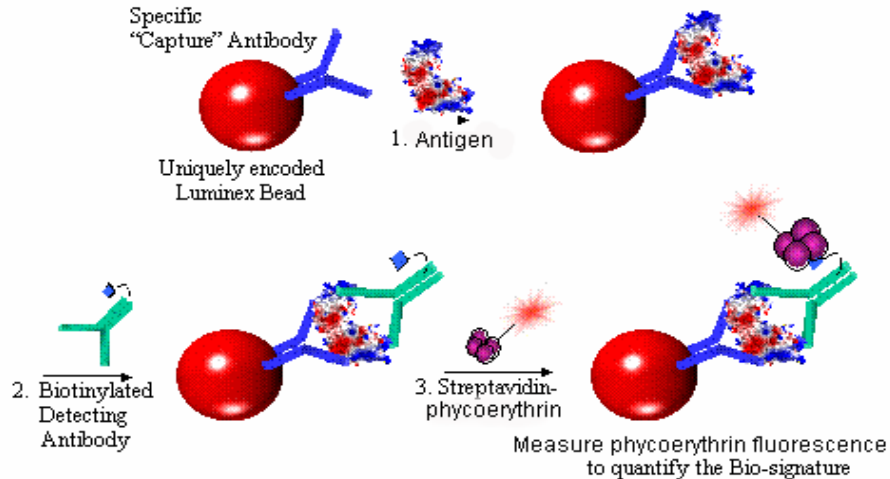
GETTING BEYOND HIGH AFFINITY & SHARP TITRATIONS

- Antibodies of varying affinity
 - Multi-antigen profiling
 - Extended dynamic range



Dynamic range determined by differences in affinities BUT must include in algorithm stipulations regarding differences

xMAP TECHNOLOGY



Adapted from Figure Courtesy of the Center for Innovations in Medicine, Biodesign Institute, Arizona State University

capture Ab conjugated to color coded beads,

xMAP FADA

- **Multiplex**
 - Horizontal – within an allergenic food
 - Vertical – multiple allergenic foods
- **Built-in Confirmation**
 - Redundancy
 - Characterization (antigenic profiling)
 - Distinguish between cross-reactive proteins
 - Kd-based MUST use comparable concentrations
 - Empirical approach
- **Adaptability**
 - Modular plug-and-play
 - Mix and match based on need

xMAP FADA

- Seven food allergens plus gluten
 - Egg
 - Milk
 - Soy
 - Tree Nuts (9: almond, Brazil nut, cashew, coconut, hazelnut, macadamia, pine nut, pistachio, walnut)
 - Peanut
 - Crustacean
 - Gluten / Wheat
- Two Extraction Protocols
 - Buffered-detergent (25 bead sets targeting food allergens)
 - Ideal for antigen profiling (22 bead sets target legumes & tree nuts)
 - Reduced-denatured (5 bead sets targeting food allergens)
 - Ideal for highly specific detection (egg, milk, peanut, and gluten)
- AssayChex Process Control Panel® (4 bead sets)

CASE 1

Gum Arabic

Spring 2011, contract lab reported peanut in gum arabic.

Lots varied from 0 to 100 ppm peanut

Data lacked controls, replicates, used single ELISA kit

Samples sent to NRL for Analysis

Differences between *Aciacia seyal* and *Acacia senegal*

Official sample was *A. senegal*

Veratox[®]: Elevated response equiv. to 40 ppm varied with gum

RIDASCREEN[®]: Elevated response but no dynamic response

Adding NFDM had minimal effect on ELISA performance

Morinaga ELISA: Elevated response but no dynamic response

GUM ARABIC

While FDA gathered RIDASCREEN[®] data, samples analyzed by an EU Contract Lab using the RIDASCREEN[®] ELISA reported initially no peanuts detected. Further examination of showed negative performance of the ELISA

Importance of controls

GUM ARABIC

Controls questioned reliability of ELISA with gum arabic
Unable to ascertain definitive proof of peanut presence.

PCR inconclusive

Worse case, 100 ppm peanut in gum arabic

Western analysis detected 'peanut' bands at approx. 8 ppm

What is use of gum arabic in foods?

Maximum 43% in Martini mix

HHE

CASE 2



HAZELNUTS IN BEANS

Nov 30, 2012

CFIA found 5.3 ppm hazelnut in dried bean soup mix

Part of a monitoring survey

No consumer complaints, No reported allergic reactions.

Single ELISA test, No further data

Health 2 risk assigned by CFIA, Recall to the retail level

CONTRACT LAB ANALYSIS

Testing found

Black beans 4.6 ppm

Red Kidney Beans 5.4 ppm

Navy beans 6.4 ppm

Pinks 5.3 ppm

Pinto Beans 3.5 pm

Same ELISA as CFIA

No further information

2nd CONTRACT LAB WEIGHS IN

Data with Beans

Blacks 3 ppm, previous lab analysis was 4.6 ppm

Light reds 3 ppm, previous lab analysis was 5.4 ppm

Navy 4 ppm, previous lab analysis was 6.4 ppm

Pinks 3 ppm, previous lab analysis was 5.3 ppm

Pinto beans 0 ppm, previous lab analysis was 3.5 ppm

Still, No Controls

No indication of replicates

Same single test kit as previous

*“All beans that tested positive came from **Canada**”*

ANALYTIC KERFUFFLE

Company refuses to do RFR

Need for HHE?

Ask CFSAN

- no controls

- no replicates

- only one type of ELISA used

Shipped in RR cars not used for nuts

Company does not use nuts

No coatings, single use bags

Farmer's question

- Hazelnuts not grown within 1,000 miles.

BLAME IT ON SOY

Soy grown in northern plains

“Could *carry-over* soy cause false positives with Hazelnut ELISA”?

BUT...

WHY BLAME SOY
or CLAIM ANY VIOLATION...

- Cross-reactivity equiv. to < 10 ppm
- 4X dilution indistinguishable from bkgd.
- Cross-reactivity $< 0.001\%$
- False positive if $> 0.0003\%$

Conclusion: FDA: no action taken

IMPORTANCE of CONTROLS

CAUTIONS DERIVED

Variance within bean type

Different cultivars may have different content

Growth conditions affect protein content

Processing (cooking) affects antigenicity

What about allergenicity?

**Testing for allergens
in pure foods
is not uncommon**

- Peanut in Garlic 2016
- Peanut in Georgian Wheat 2016
- Peanut in Blueberries 2016

03/08/2017 can we TEST for PEANUT in ALMOND?



YES with xMAP

Try to mimic principle using ELISAs?
costly, labor intensive

Table Distinguishing between Almond and Roasted Peanut by Antigen Profiling with UD Buffer ^a

| Analyte | µg/mL | Almond | | Cashew | | Crust | HazelNut | | Macadamia | | Peanut | | Soy | |
|----------------|--------|--------|--------|--------|------|--------|----------|------|-----------|------|--------|--------|--------|--------|
| | | 12 | 13 | 18 | 19 | 22 | 29 | 30 | 33 | 34 | 37 | 38 | 45 | 46 |
| Almond | 25,000 | 1.00 | 0.58 | 0.21 | 0.13 | 0.0020 | 0.32 | 0.13 | 0.42 | 0.00 | 0.0259 | 0.0281 | 0.0048 | 0.0190 |
| | 500 | 1.00 | 0.65 | 0.17 | 0.07 | 0.0004 | 0.18 | 0.13 | 0.23 | 0.00 | 0.0037 | 0.0103 | 0.0006 | 0.0154 |
| | 12.5 | 1.00 | 0.65 | 0.10 | 0.03 | 0.0000 | 0.07 | 0.08 | 0.10 | 0.00 | 0.0009 | 0.0030 | 0.0000 | 0.0072 |
| Roasted Peanut | 25,000 | 0.0274 | 0.0031 | 0.06 | 0.04 | 0.0010 | 0.05 | 0.02 | 0.02 | 0.00 | 1.00 | 0.98 | 0.06 | 0.40 |
| | 500 | 0.0223 | 0.0004 | 0.02 | 0.02 | 0.0002 | 0.01 | 0.01 | 0.00 | 0.00 | 1.00 | 0.93 | 0.02 | 0.30 |
| | 12.5 | 0.0025 | < | 0.00 | 0.00 | < | 0.00 | 0.00 | 0.00 | < | 1.00 | 0.78 | < | 0.15 |

CASE 3

NOT Regulatory (FADA not formally adopted)

- SWABS taken and analyzed
 - Confirmed Pecan presence
 - Antigenic profiling enabled detection
 - One day, performed the equivalent of several weeks

analyst: C.Y. CHO

- Questions raised
 - What does analysis mean?
 - Validated environmental sampling – per ASTM, others?
 - If establish thresholds, how would data be interpreted?
 - If heat cleaned, does method still detect (inactive) analyte?

MAP of SWAB SAMPLES

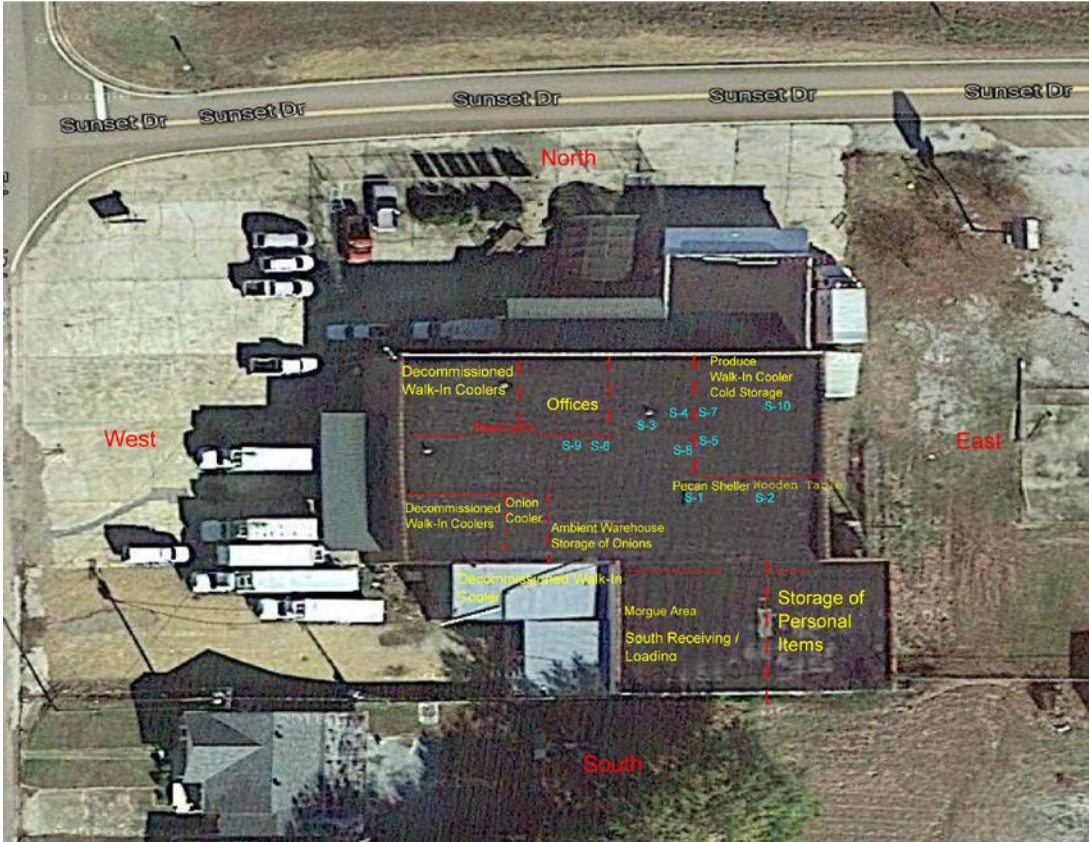


Table 1. Antigenic Profile of Pecan and Walnut Standards^a

| Sample | µg/mL | Almond | | Brazil nut | | Cashew | | Coconut | | Cru ^b | | Gluten | | Hazelnut | | Mac ^b | | Peanut | | Pine nut | | Pistachio | | Soy | | Walnut | | |
|--------------------|-------|--------|-----|------------|-----|--------|-----|---------|-----|------------------|----------------|--------|------|----------|------|------------------|------|--------|------|----------|------|-----------|------|-----|------|--------|-----------------------|------|
| | | -12 | -15 | -34 | -15 | -15 | -19 | -20 | -21 | -22 | -27 | -25 | -29 | -30 | -33 | -34 | -37 | -35 | -39 | -42 | -43 | -44 | -45 | -46 | -47 | -48 | -47 (MP) ^c | |
| Pecan | 25000 | 0.0 | 0.0 | 0.1 | 0.0 | 0.2 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.4 | 0.2 | 0.3 | 0.0 | 0.1 | 0.0 | 0.1 | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 | 1.0 | 0.5 | 5144 |
| | 250 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | < ^d | 0.0 | 0.0 | 0.4 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.5 | 3874 |
| | 12.5 | < | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | < | < | < | < | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | < | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.3 | 2209 |
| | 1.25 | < | < | < | 0.0 | 0.0 | 0.0 | < | 0.0 | < | < | < | < | 0.1 | 0.0 | 0.0 | < | < | < | < | < | < | 0.0 | < | < | < | 1.0 | 0.2 |
| Walnut | 25000 | 0.1 | 0.0 | 0.7 | 0.1 | 0.4 | 0.6 | 0.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.7 | 0.3 | 0.8 | 0.0 | 0.7 | 0.3 | 0.1 | 0.0 | 0.3 | 0.1 | 0.0 | 0.2 | 1.0 | 0.5 | 8474 |
| | 250 | 0.0 | 0.0 | 0.3 | 0.0 | 0.3 | 0.3 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.4 | 0.2 | 0.2 | 0.0 | 0.3 | 0.1 | 0.0 | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 | 1.0 | 0.5 | 7564 |
| | 12.5 | 0.0 | 0.0 | 0.1 | 0.0 | 0.1 | 0.1 | 0.0 | 0.0 | < | < | < | < | 0.2 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 | 0.0 | < | 0.0 | 1.0 | 0.4 | 4959 |
| | 1.25 | < | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | < | < | < | < | < | 0.1 | 0.0 | 0.0 | < | 0.0 | 0.0 | 0.0 | < | 0.0 | 0.0 | < | 0.0 | 1.0 | 0.2 | 3001 |
| 0.125 | < | < | 0.0 | < | 0.0 | 0.0 | 0.0 | < | < | < | < | < | 0.0 | 0.0 | 0.0 | < | 0.0 | < | < | < | 0.0 | < | < | < | < | 1.0 | 0.1 | 544 |
| Skgs ^e | | 4.2 | 4.0 | 9.2 | 5.7 | 14.0 | 4.0 | 11.3 | 4.0 | 6.0 | 7.7 | 39.7 | 15.0 | 7.0 | 17.7 | 4.0 | 11.0 | 13.2 | 16.3 | 6.3 | 11.7 | 3.0 | 10.7 | 6.3 | 9.52 | 10.7 | 85 | |
| Stdev ^e | | 0.3 | 0.0 | 0.6 | 0.6 | 1.0 | 0.0 | 0.6 | 0.0 | 0.0 | 0.6 | 1.2 | 1.0 | 0.0 | 0.6 | 0.0 | 0.3 | 0.6 | 0.6 | 0.6 | 0.6 | 0.0 | 0.6 | 0.6 | 2.5 | 0.6 | 2 | |

^a Samples extracted using UD buffer. Responses expressed normalized relative to the response of Walnut-47 (another bead set, colored green). Normalized responses >0.2 in red. All MM values used in calculations after subtraction of average background response from average of triplicate sample analysis.

^b Abbreviations for crustacean (Cru) and Macadamia (Mac)

^c MM response used to generate normalized responses.

^d < indicates signal less than or equal to below the background response.

^e Average MM background response (and standard deviation) measured for the bead set.

Analyst C.Y.Cho



Table 1B. Antigenic Profile Analysis of Swab Samples

| Sample | Almond | | Brazil nut | | Cashew | | Coconut | | Cru d | Gluten | | Hazelnut | | Mac d | | Peanut | | Pine nut | | Pistachio | | Soy | | Walnut | | | |
|--------|--------|-----|------------|-----|--------|-----|---------|-----|-------|--------|------|----------|-----|-------|-----|--------|------|----------|-----|-----------|-----|------|-----|--------|------|-----------|------|
| | -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 (MFI) | |
| Sub 1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.1 | 0.0 | 0.0 | < | 0.0 | 0.0 | 0.3 | 0.1 | 0.1 | 0.0 | 0.1 | 0.1 | 0.0 | 0.0 | 0.1 | 0.0 | < | 0.0 | 1.0 | 0.4 | 2491 | |
| Sub 2 | < | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | < | 0.0 | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 | < | 0.0 | 0.0 | < | 0.0 | 1.0 | 0.3 | 1920 | |
| Sub 3 | 0.0 | < | 0.0 | < | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.1 | 0.0 | 0.0 | < | 0.3 | 0.5 | 0.0 | < | 0.0 | 0.0 | < | 0.0 | 1.0 | 0.1 | 1146 | |
| Sub 4 | 0.0 | < | 0.0 | < | 0.0 | 0.0 | 0.0 | 0.0 | < | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 | < | 1.4 | 1.8 | 0.0 | < | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.1 | 1152 | |
| Sub 5 | < | < | < | < | < | < | < | < | < | 0.2 | 0.5 | 0.1 | < | < | < | 0.3 | 0.6 | < | < | < | < | < | < | 1.0 | 0.1 | 40 | |
| Sub 6 | 0.0 | 0.0 | 0.1 | 0.0 | 0.2 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.5 | 0.2 | 0.4 | 0.0 | 0.1 | 0.1 | 0.1 | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 | 1.0 | 0.6 | 5033 | |
| Sub 7 | 0.3 | 0.0 | 0.0 | 0.0 | 0.1 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.8 | 0.2 | 0.1 | 0.1 | 0.0 | 10.6 | 5.6 | 0.2 | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 | 0.4 | 1.0 | 0.3 | 1471 |
| Sub 8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.2 | 0.1 | 0.0 | 0.0 | 0.7 | 0.9 | 0.0 | < | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.3 | 2594 | |
| Sub 9 | < | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 | < | 0.0 | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 | 0.1 | 0.1 | 0.0 | < | 0.0 | 0.0 | < | 0.0 | 1.0 | 0.3 | 1720 | |
| Sub 10 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | < | 0.0 | 0.2 | 0.1 | 0.0 | 0.0 | 0.0 | 0.4 | 0.7 | 0.0 | < | 0.0 | 0.0 | < | 0.0 | 1.0 | 0.1 | 1128 | |
| Sub A | < | < | < | < | < | < | < | < | < | < | < | 0.1 | < | < | < | < | < | < | < | < | < | < | < | 1.0 | 0.2 | 70 | |
| Sub B | < | < | < | < | < | < | < | < | < | < | < | < | < | < | < | < | < | < | < | < | < | < | < | < | < | < | < |
| Bkgd | 4.5 | 4.0 | 9.2 | 6.2 | 15.3 | 4.3 | 12.3 | 4.3 | 6.7 | 7.3 | 39.0 | 15.3 | 7.3 | 17.5 | 4.0 | 11.3 | 12.5 | 26.3 | 7.0 | 12.0 | 3.3 | 11.3 | 6.5 | 86.7 | 11.3 | 87 | |
| Stdev | 0.5 | 0.0 | 0.8 | 0.3 | 1.2 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 1.7 | 0.6 | 0.6 | 0.9 | 0.0 | 0.6 | 0.9 | 1.2 | 0.0 | 1.0 | 0.6 | 0.6 | 0.5 | 3.4 | 0.6 | 3.4 | |

Analyst C.Y.Cho

CASE 4

CUMIN 2014

- CFIA detected peanut & almond in spice mix
- FARRP traced problem to cumin
- FDA confirmed ELISA results

BUT....



CUMIN

Table Responses Generated by Cumin and Almond Normalized to the Response of the Major Bead Set for the Analyte ^a

| Analyte | µg/mL | Almond | | Brazil nut | | Cashew | | Coconut | | Crust | Gluten | | Hazelnut | | Macadamia | | Peanut | | Pine nut | | Soy | | Walnut | | | |
|---------|-------|--------|------|------------|------|--------|------|---------|------|-------|--------|------|----------|------|-----------|------|--------|------|----------|------|------|------|--------|------|------|------|
| | | 12 | 13 | 14 | 15 | 18 | 19 | 20 | 21 | | 22 | 27 | 28 | 29 | 30 | 33 | 34 | 37 | 38 | 39 | 42 | 43 | 44 | 45 | 46 | 47 |
| Cumin A | | 0.58 | 0.26 | 0.30 | 0.09 | 0.40 | 0.22 | 0.20 | 0.09 | 0.06 | 0.05 | 0.31 | 0.28 | 0.09 | 0.20 | 0.11 | 0.76 | 1.00 | 0.06 | 0.08 | 0.25 | 0.11 | 0.14 | 0.15 | 0.02 | 0.07 |
| Cumin B | | 0.72 | 0.43 | 0.25 | 0.05 | 0.51 | 0.19 | 0.16 | 0.04 | 0.02 | 0.03 | 0.30 | 0.27 | 0.09 | 0.18 | 0.05 | 0.76 | 1.00 | 0.04 | 0.04 | 0.22 | 0.06 | 0.10 | 0.11 | 0.01 | 0.05 |
| Peanut | 0.10 | 0.10 | 0.02 | 0.07 | 0.01 | 0.27 | 0.08 | 0.10 | 0.01 | 0.02 | 0.02 | 0.02 | 0.05 | 0.03 | 0.07 | 0.01 | 0.73 | 1.00 | 0.04 | 0.01 | 0.08 | 0.03 | 0.11 | 0.10 | 0.07 | 0.03 |
| Almond | 0.10 | 1.00 | 0.52 | ≤.01 | ≤.01 | 0.47 | ≤.01 | ≤.01 | ≤.01 | ≤.01 | ≤.01 | 0.04 | 0.03 | 0.02 | 0.02 | ≤.01 | ≤.01 | ≤.01 | ≤.01 | ≤.01 | 0.02 | ≤.01 | ≤.01 | ≤.01 | ≤.01 | ≤.01 |

^a One gram of tree nut or roasted peanut was extracted with PBST and analyzed using the xMAP FADA . The average MFI values (minus background), of triplicate analyses, were normalized to the maximum response by the major bead set designed specifically to detect the analyte (green). Cross-reactivities (fractional responses) ≥0.1 are listed in red.

Responses with Brazil nut-14, -15, Cashew-19, Hazelnut-29, Macadamia-33, and Pistachio-43 (highlighted) inconsistent with either peanut or almond.

Raised questions, was peanut and almond actually present?

Mahleb did not explain either the complex antigenic profile or the strong response with a hazelnut ELISA.

PCR confirmed peanut, almond, & hazelnut; MS confirmed peanut

CUMIN

- Relying solely on an ELISA result akin to blinders.
- Use of orthogonal methods (MS and PCR) enabled definitive confirmation of peanut's presence and other analytes.
- Without orthogonal testing, there would have been reasonable doubt regarding the ELISA results and the presence of a cross-reactive protein source.

CASES 5 & 6

Peanut in Garlic Powder

- Extensive variation in detectable content
- Pattern not characteristic of variability associated with analytical method
 - Replicates displayed excellent reproducibility

*analysts R.O Pedersen & NRL
paper in-press JFP*

Peanut in Georgian Wheat & Various Products

- Extensive variation in detectable content
- Pattern not characteristic of variability associated with analytical method
 - Replicates displayed excellent reproducibility

HETEROGENEOUS SOLID MIXTURES

- Replicate analysis to determine degree of heterogeneity
 - Variability between / within subs
 - Goal: determine potential oral exposure
 - NOT the average content across a production run
scientifically may be of interest but focus is oral exposure
- Analytical modeling **MUST** be appropriate
 - Averaging as if homogeneous potentially dangerous



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