Analytical Roadmap, Reporting, Interpretation
Michael Walker
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2015 – Cumin and Paprika under suspicion

Contamination with almond – a risk for those with allergies
Lab tests compromised by ‘mahaleb’ - cross reacted to mimic almond

1. Why did this happen?
2. How were the analytical difficulties resolved?
3. Is there a roadmap around how to tackle this?
4. What is the law around this?
5. What should you offer as a testing lab & can your customers contribute?
6. And what do the results you get mean in terms of risk?
1a. Why did this happen?

Proteins

ELISA


1b. Why did this happen?
2a. How were the difficulties resolved?

ELISA

qPCR assay for Mahaleb

PCR

PCR screening assay

LC-MS/MS for Prunus Species-specific peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Processor km/mol</th>
<th>Almond</th>
<th>Mahaleb</th>
</tr>
</thead>
<tbody>
<tr>
<td>FYSSMLR</td>
<td>2+</td>
<td>428.2250</td>
<td></td>
</tr>
<tr>
<td>SGQELPF</td>
<td>2+</td>
<td>479.7824</td>
<td></td>
</tr>
<tr>
<td>DPQSSPF</td>
<td>2+</td>
<td>477.7310</td>
<td></td>
</tr>
<tr>
<td>DRQMVGILPLR</td>
<td>3+</td>
<td>485.6270</td>
<td></td>
</tr>
<tr>
<td>VPQQPPPPVPPPPFR</td>
<td>2+</td>
<td>694.8561</td>
<td></td>
</tr>
<tr>
<td>ALPCKLANKVSGDGR</td>
<td>3+</td>
<td>566.7825</td>
<td></td>
</tr>
<tr>
<td>ALPCKLANKVSGDGR</td>
<td>2+</td>
<td>537.6549</td>
<td></td>
</tr>
<tr>
<td>VGGMLFYQSFPS</td>
<td>2+</td>
<td>745.5852</td>
<td></td>
</tr>
<tr>
<td>TSWKNNALSQG</td>
<td>2+</td>
<td>716.3024</td>
<td></td>
</tr>
<tr>
<td>STLSHMLFLR</td>
<td>3+</td>
<td>483.2877</td>
<td></td>
</tr>
<tr>
<td>IDLYPSFPR</td>
<td>2+</td>
<td>571.8015</td>
<td></td>
</tr>
<tr>
<td>SYLKVYFQVFETDQSIDDQGR</td>
<td>3+</td>
<td>889.7442</td>
<td></td>
</tr>
</tbody>
</table>

2b. Summary of findings

<table>
<thead>
<tr>
<th>Cumin sample</th>
<th>Paprika sample</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ELISA</strong></td>
<td></td>
</tr>
<tr>
<td>Prunus protein +ve</td>
<td>Prunus protein +ve</td>
</tr>
<tr>
<td>~ quantification ✓</td>
<td>~ quantification ✓</td>
</tr>
<tr>
<td>Species specific ✗</td>
<td>Species specific ✗</td>
</tr>
<tr>
<td><strong>PCR</strong></td>
<td></td>
</tr>
<tr>
<td>Mahaleb-specific PCR +ve</td>
<td>Mahaleb-specific PCR –ve</td>
</tr>
<tr>
<td>Almond assay ✗</td>
<td>Almond assay ✗</td>
</tr>
<tr>
<td>PCR and melt curve ✓</td>
<td></td>
</tr>
<tr>
<td><strong>LC-MS/MS</strong></td>
<td></td>
</tr>
<tr>
<td>No peptides uniquely characteristic of almond were detected</td>
<td>2 peptides uniquely characteristic of almond were detected</td>
</tr>
<tr>
<td>Of 3 peptides known to be present in mahaleb 1 was detected</td>
<td>No peptides uniquely characteristic of mahaleb were detected</td>
</tr>
</tbody>
</table>
2c. Where can I find out more

1. Walker, Michael John, Duncan Thorburn Burns, Chris Elliott, M. Hazel Gowland, and E N Clare Mills, 2016, Flawed food allergen analysis–health and supply chain risks and a proposed framework to address urgent analytical needs, Analyst, 141, 24 - 35


5. M Walker et al., 2017, Almond or Mahaleb? Resolution of allergen ELISA findings in cumin and paprika by molecular biology and protein mass spectrometry, JAOAC Int http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac/pre-prints/content-jaoac_170405

3a. Roadmap

- ELISA
  - -ve for almond – stop and report
  - Positive for almond – PCR or LC-MS/MS

- PCR
  - Real-Time PCR for Specific Detection of Prunus mahaleb
  - PCR and melt curve for common Prunus species

- MS
  - Tryptic digestion and LC-MS/MS for peptides specific to almond, mahaleb or other Prunus species

- Cross contamination
- Bioinformatics
- Reference Materials
- Reference methods
1. Select ELISA for allergen of interest
2. Check cross reactivity stated in ELISA specification, discuss with kit manufacturer, including kit validation.
3. Plan your analyses according to allergen of interest and known cross reactivity of the kit including conducting the necessary quality assurance
4. If ELISA is Prunus negative report all common Prunus species not detected with LoDs for each of the common species from your validation study.
5. If ELISA is Prunus positive, report ‘Prunus species detected’ with LoDs for each of the common species from your validation study
6. If it is necessary to know which Prunus is present move on to PCR

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Strongly suspect *P. mahaleb*?
Apply real-time PCR assay for *P. mahaleb*

Course objective: know how to apply this method and estimate order of magnitude concentration for *P. mahaleb* as ELISA ‘almond equivalents’ and DNA copy number % (i.e. ‘between 0.01 % and 0.1 %’ or ‘between 0.001 % and 0.01 %’).
Course objective: know how to apply this method and be able to specify to their client which Prunus species is present and a probable concentration range (see above).

In some circumstances it may be imperative to be sure that a Prunus protein is present

Course objective: know how to apply (or outsource) the LC-MS/MS method.
4a. What is the law around this?

Responsibility for safe and properly labelled food rests with those who make and sell it … Recital 30, & Art. 3.3, Regulation (EC) No 178/2002 laying down the general principles and requirements of food law

4b. What is the law around this?

**General Food Law prohibits adulteration & sale of unsafe food**

Regulation (EC) No 178/2002:

- Article 8 prohibits adulteration of food and fraudulent, deceptive or any other practices which mislead consumers
- Article 14 prohibits the sale of unsafe food such as food injurious to health, including the particular health sensitivities of any specific category of consumers [e.g. but not exclusively people with food allergy] where the food is intended for that category of consumers

**Labelling addresses allergen avoidance risks**

*Codex Alimentarius* General Standard for the Labelling of Prepackaged Foods harmonises globally mandatory disclosure of the presence of allergens (list of 8)

Regulation (EU) No 1169/2011, Annex II, inclusion in prepacked food of any of 14 major allergens triggers, with limited exemptions, specific labelling requirements extended in 2014 to non-prepacked food, including catering establishments
4c. What about cross contamination?

HACCP – Article 5 Regulation (EC) No 852/2004 on the hygiene of foodstuffs – the principles
(a) identify hazards e.g. allergens
(b) identify critical control points
(c) establish critical limits
(d) establish & implement effective monitoring procedures
(e) establish corrective actions
(f) verify that the ↑ measures are working effectively
(g) documents and records
(h) review the procedure and make any necessary changes

4d. Avoiding cross contamination in practice

BRC7 imposes allergen RA and RM including:
Risk assessments of all raw materials, and an inventory and labelling of all allergic materials handled on site
Risk assessed documentation of potential contamination routes
Zoned (segregated areas and dedicated equipment) for storing and handling allergenic ingredients
Production scheduling to minimise cross contamination risk
Validation of production processes to support any “free-from” claim
Validation of the effectiveness of factory cleaning procedures to remove allergens
Harvesting, storage, transport, processing incl. milling, & cleaning of equipment
4e. UK and other law around this?

**Food Safety Act 1990** - enabling powers for all food regulations, including labelling.

The main criminal offences:
- rendering food injurious to health (Section 7),
- selling, to the purchaser’s prejudice, food which is not of the nature or substance or quality demanded (Section 14) and
- falsely or misleadingly describing or presenting food (Section 15).

**General Food Regulations 2004** (as amended) amend the Food Safety Act 1990 to enforce Regulation 178/2002 in GB, (similar legislation in NI)

European Framework Directive on **Safety and Health at Work** (Directive 89/391 EEC and daughter legislation) that covers liabilities in the workplace

**Compensation in civil law** for loss or damage caused by an allergic reaction to a food supplied is a foreseeable risk for food businesses


4. Summary - what is the law around this?

[Diagram showing food allergens, mainstream products ingredients, 'free from' products, pre-packed / non-prepacked labelling law, all products cross-contaminants, general food law, HACCP, PAL, thresholds]
5a. What should you offer as a testing lab?

Clear reporting of
- the method of analysis
- the units reported (allergen protein or allergenic food)
- any associated method uncertainty or method cross reactivity

$[X]$ mg/kg as $Y$
- $[X]$ = best estimate of concentration in the sample received after in-laboratory homogenisation, extraction and analysis by a validated method, and
- $Y$ is EITHER the allergen protein OR the name of the food

In my opinion if whole food is reported conversion factor from allergen protein to whole food must be given.

Allergen or (preferably) allergen protein should be specified every time a datum is given in a method or report.

Contextual awareness

THESE EXPECTATIONS DO NOT COME CHEAPLY

5b. What should your customer’s input be?

Awareness
- different tests measure different things (DNA vs protein, different proteins by different ELISAs …)
- effects of processing and cooking on the response of certain tests
- there are uncertainties in the risk assessment
- there are data gaps

Do not take as set in stone the concentration as-reported by the laboratory especially at levels approaching the LoD or LoQ

Appreciate the sensitivity of your risk assessment to sampling uncertainty

Appreciate the sensitivity of your risk assessment to analytical uncertainty

Ideally include a laboratory representative in the incident control team.
6a. What do the results mean in terms of risk?

**Allergen risks to consumers**

**Catering supply chain**
- Ingredients ‘forgotten about’ (e.g. marinades)*
- Cross-contamination or fraud

**Manufactured foods**
- Mis-packs – putting a product in the wrong packaging
- Inadequate labelling of an allergenic ingredient
- Cross contamination

* Added by Hazel Gowland during seminar

6b. What do the results mean in terms of risk?

**Thresholds**

Limits for allergen proteins in food below which most of the food allergic population will not react

Acknowledges impossibility of proving zero risk or absolute certainty

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*Crevel, et al., 2007, Food and Chem Toxicol, 45, 691-701*
6c. The severity of allergic reaction depends on...

- Genetics
  - atopy, may be $10^6$ difference between least & most sensitive
- Dose
- Matrix – e.g. fat, pH, binding….
- Food processing
- Exercise
- Medication (NSAIs)

- Alcohol
- Asthma
- Concurrent or recent infection
- Individual
  - Age, knowledge experience
- Situation
  - ...

6d. The Allergen Bureau VITAL grid – deterministic RA

Typical protein levels in allergenic foods and ACTION LEVELS with caveats

Milligram per kilogram allergen protein ‘action levels’ derived from
- the estimated eliciting dose extrapolated from dose-distribution curve
- the food serving size.

The eliciting dose is the predicted amount of allergenic food that may provoke an allergic reaction in a given percent of the population.
6e. Allergen Bureau Action Levels

Intentionally added allergens must be declared on the product label (e.g. in L/I).
Must review cross contact allergens for opportunities to reduce or eliminate
If cannot be eliminated, should be labelled as specified by the appropriate Action Level:

**Action Level 1** – precautionary cross contact statement is not required for the relevant allergen under evaluation

**Action Level 2** – precautionary cross contact labelling statement is required for the relevant allergen using the standard VITAL statement.

Precautionary labelling should only be used after a thorough assessment of the risk

**NEVER** as a substitute for good manufacturing practice (GMP) or as a generic disclaimer.

**The ONLY precautionary statement to be used in conjunction with VITAL is:** “May be present: [name of allergen]”

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6f. The calculation

MUST read the whole document!
AB recommends use their guide and excel spreadsheet (free…)

Calculation is:–

\[
\text{Action Level transition} = \frac{\text{Ref Dose (mg)} \times 1000}{\text{Ref Amount (g)}}
\]

**Reference Dose** the milligram protein level (total protein from an allergenic food) below which only the most sensitive individuals (between 1% and 5% depending on the quality of the data set available) in the allergic population are likely to experience an adverse reaction.

**Reference amount** = defined by manufacturer and is the maximum amount of a food eaten in a typical eating occasion. This may be the same as the “serving size”
6g. Example Soya

- Reference dose soya = 1 milligram soya protein
- Reference amount (serving size) = 40 g
- Action Level transition point = 1*(1000/40) = 25 mg kg\(^{-1}\)
  - Above 25 mg soya protein per kg product precautionary cross contact labelling statement is required for the relevant allergen using the standard VITAL statement.
  - Can convert soya protein to soya using the protein content of the soy ingredient you are using or the AB typical levels

- If serving size is 100 g transition is 1*(1000/100) = 10 mg kg\(^{-1}\)

6h. Reference doses

<table>
<thead>
<tr>
<th>Allergen</th>
<th>Reference dose (mg of protein)</th>
<th>Action Level mg/kg 50g portion</th>
<th>Action Level mg/kg 250g portion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peanut ED 1 %</td>
<td>0.2</td>
<td>4</td>
<td>0.8</td>
</tr>
<tr>
<td>Cow’s milk ED 1 %</td>
<td>0.1</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>Egg ED 1 %</td>
<td>0.03</td>
<td>0.6</td>
<td>0.12</td>
</tr>
<tr>
<td>Hazelnut ED 1 %</td>
<td>0.1</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>Soya ED 5 %</td>
<td>1.0</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>Wheat ED 5 %</td>
<td>1.0</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>Cashew ED 5 %</td>
<td>0.1</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>Mustard ED 5 %</td>
<td>0.05</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>Lupin ED 5 %</td>
<td>4.0</td>
<td>80</td>
<td>16</td>
</tr>
<tr>
<td>Sesame seed ED 5 %</td>
<td>0.2</td>
<td>4</td>
<td>0.8</td>
</tr>
<tr>
<td>Shrimp ED 5 %</td>
<td>10</td>
<td>200</td>
<td>40</td>
</tr>
</tbody>
</table>

6i. What do almond results mean in terms of risk?

- No almond reference dose – hazelnut ED 1% of 0.1 mg protein is a guide
- 50 gram portion of cumin, 0.1 mg almond protein
  \[= \frac{0.1 \times 1000}{50} = 2 \text{ mg/kg (ppm)}\]
- Almond ~ 20% protein thus 2 mg/kg almond protein is 10 mg/kg whole almond
- Cumin and other spices used at low levels (say around 1%) in most foods
- Cumin added to a food at 1% = 1000 mg/kg almond in the cumin would result in:
  - 10 ppm almond in the compound food.
  - This would not be expected to be harmful to most almond allergic consumers

6j. Why can’t I work to 1000 ppm of whole almond in my cumin?

Because there are uncertainties.

These include:
- varying concentrations of almond across the cumin batch,
- there might be poor analytical recovery,
- the measurement uncertainty of my measurements might be high (its usually higher than you might suppose)
- no reference dose for almond so people with almond allergies might be more sensitive than we assumed
- might be extra sensitivity - concurrent infection, exercise, lack of sleep, or other factors - need for a safety margin – say x100

Thus working to 10 mg/kg whole almond in cumin is a good starting point
6k. Probabilistic Risk Assessment

ED$_{01}$ = underlying risk that 1 in 100 allergic individuals may have a reaction.
Is this an acceptable balance of risk?
May be to a business selling 1000 units/week, but not for 100,000 units/week
Temptation to opt for the analytical LoD as a default action limit, which may not bear any relation to true risk
Probabilistic RA

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6l. Probabilistic Risk Assessment

[Diagram showing distribution of cross-contact allergen content in product “population” vs. amount of allergen]

Crevel, et al., 2007, Food and Chem Toxicol, 45, 691-701
6m. Probabilistic Risk Assessment

- Probabilistic assessment includes
  - concentration distribution of the allergen in the product,
  - the number of customers buying the product, or
  - typical population consumption patterns from diet and nutrition surveys, and
  - the prevalence of the allergy

- iFAAM Integrated Approaches to Food Allergen and Allergy Management
  - Tier 1
  - Tier 2

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