Detection and quantitation of various food allergens by LC-MS/MS

Volker Kruft¹, André Schreiber², Detlev Schleuder¹
¹SCIEX, Darmstadt (Germany), ²Concord, ON (Canada)

volker.kruft@sciex.com

Agenda

- Brief introduction
- LCMSMS workflow for protein allergen detection
- Quantitation of allergens by LCMSMS
- Conclusions
- Emerging LC-MS/MS applications food testing.
Prevalence of food allergies in the United States: 6% for children and 3.7% for adults → 15 million people.

Number of food allergies is rising.

Allergens: Complex mix of different chemicals but include proteins and chemicals such as sulphites.

Eight potential sources for allergens should always be declared on pre-packaged foods: Peanuts, tree nuts, eggs, milk, cereals containing gluten, shellfish, fish, and sulphites.
Allergens

- More likely to be murdered than to die from a severe reaction
- Chance to die from:
  - murder 11 in a million in EU
  - accident 324 in a million in EU
  - murder 61 in a million in US
  - accident 399 in a million in US
  - anaphylaxis 1.81 in a million
  - anaphylaxis 3.25 in a million (age under 19)
- EU label law: peanuts, tree nuts, soybeans, mustard, eggs, lupin, fish, gluten, sesame, celery, SO₂, molluscs, crustaceans
Allergens

Food allergy death less murder

By Caroline Scott-Thomas & al
27-Nov-2013

Food allergen labelling and information requirements under the EU Food Information for Consumers Regulation No. 1169/2011: Technical Guidance

Food allergies are unlikely to kill, but could lead to severe reactions.

Related tags: Food allergy, Allergens
Related topics: Science & Nutrition, Cereals and bakery preparations, Food safety and labelling, Fruit, vegetable, nut ingredients, Proteins, non-dairy

Food allergy sufferers are more likely to be murdered than to die from a severe reaction – but allergen labelling is about much more than fatality risk, says an allergen assessor.

According to a review published in Clinical and Experimental Allergy, the chance of being murdered is 11 in a million in any one year in Europe, and the chance of dying of any severe reaction is 1 in a million. The chance of being murdered is therefore 11 times higher than the chance of dying from a severe reaction.

EU label law: peanuts, tree nuts, soybeans, mustard, eggs, lupin, fish, gluten, sesame, celery, SO₂, molluscs, crustaceans
Why allergen detection is important

Food allergies are a significant and global health risk

- global
- no cure
- recognized as a problem
- LC-MS can:
  - multiplex
  - detect allergens directly
Allergens: Structural Database of Allergenic Proteins (SDAP) - https://fermi.utmb.edu/SDAP/index.html
Allergens:

Cashew: vicilin-like protein
High quality results:

- Less influence by food processing
- Highly specific
- Very sensitive
- Multiple allergens tested in single analysis
- Accurate quantitation possible

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LC-MS/MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allergen detection approach</td>
<td>Peptides of allergenic proteins are detected by their mass</td>
</tr>
<tr>
<td>LOD</td>
<td>Low part-per-million (ppm*), depending on the allergen</td>
</tr>
<tr>
<td>Number of allergens per analysis</td>
<td>&gt; 2 (multiple peptides per protein)</td>
</tr>
<tr>
<td>Effects of food processing</td>
<td>Not significantly impacted by food processing</td>
</tr>
<tr>
<td>Specificity</td>
<td>Highly specific</td>
</tr>
<tr>
<td>Sample preparation</td>
<td>Slightly laborious</td>
</tr>
<tr>
<td>Matrix influence</td>
<td>Potential matrix suppression causing reduced detectability and sensitivity (false negative results possible without proper control samples)</td>
</tr>
<tr>
<td>Analysis time</td>
<td>4+ hours</td>
</tr>
</tbody>
</table>
| Disadvantages                 | • Long analysis time and inability to analyze ingredients quickly during processing  
                                  • Larger investment in equipment and personnel training | |
| Advantages                    | • Direct detection of multiple peptides by distinct mass gives high specificity and accuracy  
                                  • Can identify multiple allergens in a single analysis  
                                  • Not susceptible to cross-reactivity  
                                  • Potential to accurately quantify the allergen of interest |
Detecting allergens = Detecting proteins

Protein digestion and peptide mapping analysis

ProteinPilot™ Database search

Literature search

Unique/signature peptide selection

**MRM Method**

<table>
<thead>
<tr>
<th>Q1</th>
<th>Q3</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>615.4</td>
<td>631.4</td>
<td>GDVLTVANVK</td>
</tr>
<tr>
<td>763.9</td>
<td>814.5</td>
<td>LLYPETGLRVR</td>
</tr>
<tr>
<td>743.4</td>
<td>813.4</td>
<td>SIDEVYVENLK</td>
</tr>
<tr>
<td>679.8</td>
<td>754.4</td>
<td>GEFGDVMLGDRYR</td>
</tr>
<tr>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

**MS/MS – Q3 m/z**

**Peptide Q1 m/z**
Allergen Analysis by LC-MS/MS

**Sample Preparation**

- Allergen protein
- Digest

**Peptides**

**Identification**

- Peptide - Q1 m/z
  - 785.8
  - 786.2
  - 786.8
  - 787.3

- Peptide - MS/MS m/z
  - Peaks marked with *

**Quantitation**

- LC
- Ionization
- MS/MS (MRM mode)
Selection of allergen proteins and peptides for detection

• Each peptide should be unique for the allergen marker protein.
• Each peptide should not be modified during food processing.
  - No post translation modification.
  - Can be detected in raw and baked allergens, and raw and baked food matrices.
• MRM transition selections with consideration on signal-to-noise ratios and reproducibility in matrix samples.
• During method development, evaluated at least 2 proteins for each allergen, 3 peptides each protein, and 3 MRM transitions each peptide.
  - Final method: 2 proteins for each allergen, 2 peptides each protein, and 2 MRM transitions each peptide (except 1 protein for egg and pine nut).
Sample analysis workflow

**Protein Extraction**

- Food sample
- Homogenize
- Lipid removal

**Protein Digestion**

- Reduction of protein disulfide bonds
  - TCEP & incubate at 60°C for 1 h
- Block cysteines
  - MMTS & incubate at RT for 15 min
- Protein digestion
  - Add trypsin & incubate at 37°C for 3 h

**LC/MS/MS analysis**

(12 allergens in one injection)

QTRAP 4500
Qualitative and quantitative methods for screening allergens

<table>
<thead>
<tr>
<th>Method 1 (Qualitative/Screening) Egg, Milk, Legumes &amp; Tree nuts</th>
<th>Method 2 (Quantitative) Gluten Quantitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Egg</td>
<td>1) Gluten</td>
</tr>
<tr>
<td>2) Milk</td>
<td>2) Barley</td>
</tr>
<tr>
<td>3) Almond</td>
<td>3) Oats</td>
</tr>
<tr>
<td>4) Brazil Nut</td>
<td>4) Rye</td>
</tr>
<tr>
<td>5) Cashew</td>
<td></td>
</tr>
<tr>
<td>6) Hazelnut</td>
<td></td>
</tr>
<tr>
<td>7) Pine Nut</td>
<td></td>
</tr>
<tr>
<td>8) Pistachio</td>
<td></td>
</tr>
<tr>
<td>9) Pecan</td>
<td></td>
</tr>
<tr>
<td>10) Walnut</td>
<td></td>
</tr>
<tr>
<td>11) Peanut</td>
<td></td>
</tr>
<tr>
<td>12) Soy</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Method</th>
<th>Screening Method</th>
<th>Gluten Quantitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrices</td>
<td>Baked products and cereals</td>
<td>Bakery products, dehydrated formula and fermented beverages</td>
</tr>
<tr>
<td>Detection limit:</td>
<td>LOD ≤ 10 ppm (allergen commodity concentration(^1))</td>
<td>LOQ ≤ 5 ppm (gluten protein concentration(^2))</td>
</tr>
<tr>
<td>Instrument</td>
<td>QTRAP 4500 system</td>
<td></td>
</tr>
<tr>
<td>Protocol</td>
<td>Sample preparation protocol included</td>
<td></td>
</tr>
<tr>
<td>Reagent kit</td>
<td>Future development</td>
<td></td>
</tr>
</tbody>
</table>

Definition:

1 Concentration of the physical substance/allergen, e.g. whole peanut.
2 Concentration of the gluten protein, e.g. glutenin
For each allergen detection of:
- 2 proteins
- 2 unique peptides per protein
- 2 selective MRM transitions per peptide

**Scheduled MRM™ algorithm**
(Detection around expected RT)

Screening for eggs, milk, peanuts, soy beans, and tree nuts (including almonds, Brazil nuts, cashew nuts, hazelnuts, pecans, pine nuts, pistachios, and walnuts)

Separate method for the quantitation of gluten (adjusted sample prep)
Method Verification using Spiked Food (Raw and Backed)

Bread spiked with 100 ppm

Cookie spiked with 100 ppm

Method fully verified for dough, bread, cookies, wine and cereal.
Method partly tested on spices (peanuts and almonds) and chocolate (more extensive cleanup needed and high end mass spec recommended)
Allergen Detection in Commercial Samples

- Egg-, milk-, and nut-free cookie
- Peanut cookie
- Hazelnut cookie
- Hazelnut bread
Quantitation of Allergens

Bread spiked with hazelnuts and peanuts to evaluate linearity for quantitation.
Quantitation of Allergens

Bread spiked with hazelnuts and peanuts to evaluate linearity for quantitation.

(0 to 500 ppm)

Largest challenge for development of quantitation methods is availability of standards, internal standards and reference materials.

Also guidance for method development would be useful specifying what to quantify: amount of allergen or amount of protein in food.
LC-MS/MS to ELISA correlations in bread and cookie

**Hazelnut Protein 1**
- BREAD
  - Peptide 1: $y = 3.23x + 16.76$, $R^2 = 0.98$
  - Peptide 2: $y = 3.30x + 12.14$, $R^2 = 0.99$

**Peanut Protein 1**
- BREAD
  - Peptide 1: $y = 1.60x - 10.91$, $R^2 = 1.00$
  - Peptide 2: $y = 1.53x - 3.34$, $R^2 = 1.00$

**Hazelnut Protein 1**
- COOKIE
  - Peptide 1: $y = 2.97x - 8.89$, $R^2 = 1.00$
  - Peptide 2: $y = 2.90x - 3.43$, $R^2 = 1.00$

**Peanut Protein 1**
- COOKIE
  - Peptide 1: $y = 1.38x + 18.73$, $R^2 = 0.99$
  - Peptide 2: $y = 1.42x + 15.16$, $R^2 = 1.00$
Detection of Peanuts and Almonds in Spices

10 mg peanut in 1 g cumin

10 mg almond in 1 g paprika

Multi-allergen method was evaluated for identification and quantitation of peanuts and almonds in spices following the series of food recalls in 2015.
Quantitation of Wheat Gluten (5 to 1000 ppm with IS)

- Cookies
- Beer
- Infant formula
## Precision and Accuracy of Wheat Gluten Measurements

<table>
<thead>
<tr>
<th>Food Matrix</th>
<th>QC Sample</th>
<th>Concentration (ppm)</th>
<th>Mean (ppm)</th>
<th>Accuracy (%)</th>
<th>%CV (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cookies</td>
<td>LQC</td>
<td>10.0</td>
<td>11.2</td>
<td>111.7</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>MQC</td>
<td>400</td>
<td>391.4</td>
<td>97.8</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>800</td>
<td>735.9</td>
<td>92.0</td>
<td>3.2</td>
</tr>
<tr>
<td>Beer</td>
<td>LQC</td>
<td>10.0</td>
<td>9.4</td>
<td>93.7</td>
<td>17.4</td>
</tr>
<tr>
<td></td>
<td>MQC</td>
<td>400</td>
<td>355.6</td>
<td>88.9</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>800</td>
<td>924.0</td>
<td>115.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Baby formula</td>
<td>LQC</td>
<td>10.0</td>
<td>9.9</td>
<td>98.8</td>
<td>18.8</td>
</tr>
<tr>
<td></td>
<td>MQC</td>
<td>400</td>
<td>447.3</td>
<td>111.8</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>800</td>
<td>767.4</td>
<td>95.9</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Accuracies between 88 and 116%
%CV less than 20%
## Wheat Gluten Concentration in Different Food Samples

<table>
<thead>
<tr>
<th>Food Matrix</th>
<th>Sample ID</th>
<th>Gluten (ppm)</th>
<th>Sample ID</th>
<th>Gluten (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Labeled as Gluten-free</strong></td>
<td></td>
<td></td>
<td><strong>Gluten containing</strong></td>
<td></td>
</tr>
<tr>
<td>Cookies</td>
<td>Choco Chip</td>
<td>1.3</td>
<td>Cremica Crisp</td>
<td>7990</td>
</tr>
<tr>
<td></td>
<td>Lemon</td>
<td>1.1</td>
<td>McVities Digestive</td>
<td>557</td>
</tr>
<tr>
<td></td>
<td>Nutty Fibre Choco Chip</td>
<td>1.9</td>
<td>Sunfeast Marie Light</td>
<td>7970</td>
</tr>
<tr>
<td><strong>Baby formula</strong></td>
<td>Nestum rice</td>
<td>n.d.</td>
<td>Cerelac multi-grain</td>
<td>493</td>
</tr>
<tr>
<td></td>
<td>Cerelac rice</td>
<td>n.d.</td>
<td>Cerelac wheat apple</td>
<td>833</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cerelac wheat honey</td>
<td>538</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cerelac wheat mix fruit</td>
<td>504</td>
</tr>
</tbody>
</table>
The wheat gluten concentration was calculated based on the signature peptides of glutenin. Wheat gluten concentration was below 20 ppm. However, much higher concentrations of hordein characteristic for barley were detected.
Gluten-free as defined by the FDA

- FDA defines “gluten free” as < 20 ppm
- Our allergen method can be used for gluten detection
Why use LC-MS/MS for food allergen testing

**Increase throughput**
Detect 12 key allergens in different types of matrices at once

**Reduce false positive and false negative results**
Enhance selectivity through peptide mass detection, reducing false results due to cross reactivity or matrix effects

**Improve accuracy and reliability**
Detect allergens at levels as low as 10 ppm or as high as 500 ppm without worry
Top 10 Foods at Risk: Authenticity

- Olive oil
- Meat and fish
- Organic produce
- Milk
- Grains
- Honey and maple syrup
- Coffee and tea
- Spices (chili, saffron)
- Fruit juices
- Wine

EU highlights top ten foods at risk of fraud

By Caroline Scott-Thomas
21-Oct-2013

Olive oil, fish and organic foods are at the highest risk of food fraud in Europe, according to a new draft report from the European Union – but meat is not in the top ten, despite this year’s high-profile horse meat scandal.

Wine for cats launched in Japan

By Mark Astley
17-Oct-2013

The report underlines that risk of food fraud is greatest when potential economic gains are large and chances of getting caught are slim. Most of the at-risk foods have been exposed to fraud recently, including olive oil, fish and organic products.

Related tags: Food fraud, Adulteration
Related topics: Public Concerns, Testing

RELATED NEWS:
Proposed regulation overhaul could cost industry, FSA warns
Top 10 Foods at Risk: Authenticity (historical)

- Olive oil
- Meat and fish
- Organic produce
- Milk
- Grains
- Honey and maple syrup
- Coffee and tea
- Spices (chili, saffron)
- Fruit juices
- Wine
Why bother?

- Religious and ethical concerns
- Health risks
- Legal implications
Horse Meat Scandal in Europe

New LC-MS/MS based methods test for horse protein in beef meat and at the same time measure veterinary drugs residues.
Meat Scandal in early 20th century

In America, in the days when Chicago stockyards were first building a reputation as being fairly disgusting (early 20th century), people sang this:

Mary had a little lamb
when she saw it sicken
she sent it to Chicago
and it came back labeled "chicken."
Comparison of beef, horse and pig reference material
Comparison of beef and beef contaminated by 10% and 1% horse meat

Beef

1% Horse in Beef

10% Horse in Beef

Horse

Location of horse meat marker
The effect of moving to using MRM3 in the detection of pork at a concentration of 0.13 % pork spiked into beef using the Qtrap® 6500
**Pork Marker**

- XIC of +MRM (62 pairs): Exp 1, 376.100/322.200 Da ID: Myosin-4/-1 (N5)_SALAHAVQSSR_5 (Pig) from Sample 5 (Mix 2) of MRM Measurements, Max. 8800.0 cps.

- XIC of +MRM (62 pairs): Exp 3, 376.100/322.200 Da ID: Myosin-4/-1 (N5)_SALAHAVQSSR_5 (Pig) from Sample 5 (Mix 2) of MRM Measurements, Max. 8800.0 cps.

- EPI (376.10) Charge (+0) CE (35) CES (15) FT (4.38627): Exp 3, 2.476 min from Sample 5 (Mix 2) of MRM Measurements, Max. 1.7e6 cps.

10 % Pork in Beef

Pure Beef