Accurate Quantitation of Regulated Mycotoxins by UHPLC/MS/MS

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EMEA Market Development Team
Agenda

- Mycotoxins
- EU Regulations
- Stable isotope dilution assay (SIDA) for the mycotoxins regulated in the European Union
  - Reasons
  - Approaches
  - Sample Preparation and Method
  - Results
- Summary
Mycotoxins

Background

- low molecular weight, toxic, secondary metabolites of fungi
- produced by e.g.:
  - *Fusarium* sp., *Aspergillus* sp., *Penicillium* sp.
- toxicity:
  - acute toxic, carcinogenic, mutagenic, teratogenic, estrogenic and immunotoxic effects

\[ \text{myces (Greek)} = \text{fungus} \]
\[ \text{toxicum (Latin)} = \text{toxic} \]

\[ = \text{Mycotoxin} \]
Mycotoxins
How many mycotoxins are there?

• Hundreds of compounds
• 2 Main classes:

**Major Mycotoxins**
- Aflatoxins
- Ochratoxins (OTA)
- Trichothecenes
- Zearalenone
- Fumonisins
- Patulin

**Minor Mycotoxins**
- Ergot alkaloids
- Citrinin
- Cyclopiazonic acid
- Sterigmatocystin
- Monoliformin
- Gliotoxin
- Citreoviridin
- Tremorgenic mycotoxins
- Penicillic acid
- Roquefortine
- 3-Nitropropionic acid
- Fusaproliferin
Mycotoxins
Chemical diversity – a challenge for the sample prep

Enniatins
beauvericin, enniatin A, A₁, B, B₁
APOLAR

Fumonisins
fumonisin B₁, FB₂, FB₃, hydrolyzed FB₁
POLAR, ACIDIC

Ergot alkaloids
ergotamin, ergocornin, ergovalin, dihydroergosin
POLAR, BASIC

beauvericin

FB₁

ergovalin
Mycotoxins
Why are they an issue?

- 100+ countries have regulations for the control of mycotoxins in food and feed

- >25% of all agricultural commodities are contaminated with mycotoxins

- annual losses of several hundred million tons of food worldwide

- annual economical losses: 1 billion USD (US only)
Mycotoxins
Infected food products

- Found in cereals, dried fruits, spices, grape, coffee, cocoa, fruit juices
- Secondary contamination in milk, eggs, meat

BIOMIN's Mycotoxin Survey Program 2012
Percentage of positive and negative samples worldwide
BIOMIN Newsletter, Vol. 11, No. 130
Mycotoxins
Relevance for food control

- Notifications concerning mycotoxins
  - (RASFF-Annual reports 2002-2008)
## Regulations for Mycotoxins

### European Commission Regulation 1881/2006 (EC)

<table>
<thead>
<tr>
<th>Analytes</th>
<th>MLs µg/kg (EC Reg. No 1881/2006)</th>
<th>Commodities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin B&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.1</td>
<td>processed cereal-based baby food</td>
</tr>
<tr>
<td></td>
<td>2.0-12</td>
<td>nuts and cereals</td>
</tr>
<tr>
<td></td>
<td>sum of aflatoxins:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.0-15.0</td>
<td></td>
</tr>
<tr>
<td>Deoxynivalenol</td>
<td>200</td>
<td>processed cereal-based baby food</td>
</tr>
<tr>
<td></td>
<td>500 - 1750</td>
<td>processed / unprocessed cereals, bread, pasta, breakfast cereals</td>
</tr>
<tr>
<td>Fumonisin B&lt;sub&gt;1&lt;/sub&gt;</td>
<td>200</td>
<td>processed maize-based baby food</td>
</tr>
<tr>
<td>Fumonisin B&lt;sub&gt;2&lt;/sub&gt;</td>
<td>800</td>
<td>maize-based breakfast cereals</td>
</tr>
<tr>
<td></td>
<td>1000 / 4000</td>
<td>maize / unprocessed maize</td>
</tr>
<tr>
<td>Patulin</td>
<td>10-50</td>
<td>fruit juices, apple products, baby food other than processed cereal-based foods</td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td>0.5</td>
<td>processed cereal-based baby food</td>
</tr>
<tr>
<td></td>
<td>3.0 / 5.0</td>
<td>processed / unprocessed cereals</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>dried vine fruit</td>
</tr>
<tr>
<td></td>
<td>15 / 20 / 80</td>
<td>spices / liquorice root / extract</td>
</tr>
<tr>
<td>Zearalenone</td>
<td>20</td>
<td>processed cereal-based baby food</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>bread, biscuits, breakfast cereals</td>
</tr>
<tr>
<td></td>
<td>75-350</td>
<td>processed / unprocessed cereals</td>
</tr>
</tbody>
</table>
Regulations for Mycotoxins
FDA Regulatory guidelines

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Limit µg/kg</th>
<th>Commodities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxins, sum</td>
<td>20</td>
<td>All foods except milk</td>
</tr>
<tr>
<td>Patulin</td>
<td>50</td>
<td>Apple juice, apple juice concentrate, apple components in processed food</td>
</tr>
<tr>
<td>Deoxynivalenol</td>
<td>1000</td>
<td>Finished wheat products</td>
</tr>
<tr>
<td>Fumonisins sum of $B_1$, $B_2$, $B_3$</td>
<td>2000</td>
<td>Degermed dry milled corn products (e.g. flaking grits, corn grits, corn meal, corn flour)</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>Cleaned corn intended for popcorn</td>
</tr>
<tr>
<td></td>
<td>4000</td>
<td>Whole of partially degemermed dry milled corn products (e.g. flaking grits, corn grits, corn meal, corn flour)</td>
</tr>
<tr>
<td>Aflatoxin $M_1$</td>
<td>0.5</td>
<td>Milk</td>
</tr>
</tbody>
</table>
Accurate quantitation of mycotoxins

Reasons

  - set maximum limits (ML) for mycotoxins
- Single target versus multi-target methods

**BUT:**

- Electrospray ionisation (ESI)
  - matrix effects hamper accurate mass spectrometric quantification
- Quantification of regulated mycotoxins at a very high degree of accuracy is required
Matrix effects in ESI-MS and quantitation

Approaches

- Dilution of the sample
  - method less sensitive

- Matrix matched calibration
  - tedious
  - differences within one commodity not compensated

- Standard addition to each sample
  - more runs
  - more costs (time and standards)

- Internal calibration
  - similar compounds (ZAN for ZEN)
  - deuterium or $^{13}$C-labelled compounds
  - In the past: only single analyte or group analyte IS-addition
  - usually associated with rather high costs
Stable Isotope Dilution Assay (SIDA) Aims

- Development of a method fulfilling:
  - covering all regulated mycotoxins in solid food matrices
  - providing best possible accuracy
  - easy to handle
  - cost effective

- Stable isotope dilution assay (SIDA) for LC-MS/MS
  - 11 mycotoxins
  - $^{13}$C-labelled compounds as internal standards
  - validation of the method for maize
Multiple Reaction Monitoring
Principles

Quad Mass Filter (MS1)

Collision Cell

Quad Mass Filter (MS2)

Spectrum with background ions (from ESI)
Q1 lets only target ion 210 pass through
Collision cell breaks ion 210 apart
Q3 monitors only characteristic fragments 158 from ion 210 for quant

Chromatogram

High background
Low background
iFunnel Technology Revolutionizes Ion Sampling
Proven Sensitivity Breakthrough

Agilent Jet Stream
Hexabore Capillary
Dual Stage Ion Funnel

10X Higher Gas Sampling
Sample preparation
Universal extraction procedure

1. Sampling
   - grind and homogenize sample + weight-in

2. Extraction
   - acetonitrile:water:formic acid (80:19.9:0.1, v:v:v)
     60 min at room temperature on a rotary shaker

3. Extraction
   - acetonitrile:water:formic acid (20:79.9:0.1, v:v:v)
     30 min at room temperature on a rotary shaker

4. Centrifugation

5. Centrifugation

6. Dry Down

7. Up-take

Agilent 6490 QqQ
Stable Isotope Dilution Assay (SIDA)

HPLC method

Agilent 1290 Infinity LC system consisting of:
- binary pump
- wellplate sampler
- column compartment

HPLC method

Separation column: ZORBAX Eclipse Plus C-18 RRHD column, 100 x 2.1 mm, 1.8 µm @ 30°C

Mobile phase:
A: 5 mM HCOONH₄ + 0.1% formic acid
B: methanol + 5 mM HCOONH₄ + 0.1% formic acid

Flow: 0.35 ml/min

Gradient:
0.00 min 30 % B
0.50 min 30 % B
8.00 min 100 % B
9.50 min 100 % B
9.60 min 30 % B

Inj.Vol.: 3 µl
Stable Isotope Dilution Assay (SIDA) MS method

Spray chamber conditions:
Gas temp.: 140°C  
Dry gas: 16 l/min  
Nebulizer: 25 psi  
Sheath gas temp: 350°C  
Sheath gas flow: 11 l/min

<table>
<thead>
<tr>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>CapVoltage:</td>
<td>4000 V</td>
</tr>
<tr>
<td>Nozzle voltage</td>
<td>0 V</td>
</tr>
</tbody>
</table>

Automatic setup of MRM tables based on selected cycle time, retention times and retention time windows for the individual compounds

- Cycle time: 400 ms
- Interscan delay: 3.5 ms
- Total No. of MRMs: 33
- Maximum No. Of concurrent MRMs: 12
- Minimum Dwell time: 39.8 ms
- Maximum Dwell time: 196.5 ms
Stable Isotope Dilution Assay (SIDA) Chromatogram

- due to same MRM transitions baseline separation required for:
  - aflatoxin G1 and $^{13}$C-aflatoxin B1
  - aflatoxin G2 and $^{13}$C-aflatoxin B2

Spiked maize sample, 6490 QqQ
Stable Isotope Dilution Assay (SIDA) Chromatogram

- due to same MRM transitions baseline separation required for:
  - fumonisin B2 and B3

![Graph showing Cpd 18: Fumonisin B2 + B3: +ESI MRM (706.4000 -> 336.4000)](image)
Internal calibration in solvent
Aflatoxin B1

- Challenging compound due to low MLs
  - 0.1 µg/kg in processed cereal based baby food
  - 2 to 12 µg/kg in nuts and cereals

Overlay of 4 individual calibrations acquired within 45 hour worklist.
Internal calibration in solvent
Ochratoxin A

- Challenging compound due to low MRLs
  - 0.5 µg/kg in processed cereal based baby food
  - 3.0 / 5.0 µg/kg in processed / unprocessed cereals
  - 10.0 µg/kg in dried vine fruit

Overlay of 4 individual calibrations acquired within 45 hour worklist.
Validation of SIDA method
Experimental setup and results

- Linear range (external calibration in solvent)
  - 4 orders of magnitude for all toxins, 5 orders for Aflatoxins, T-2, and ZEN
- Costs
  - Additional price per IS per sample is between US$0.03 to 0.66
  - Price for all 11 IS per sample < US$5.23
- Full validation for maize
  - Maize:  - matrix for which most mycotoxins are regulated
    - known for matrix effects and matrix interferences
  - Spiking with native mycotoxins before extraction
  - Six concentration levels with 3 replicates
  - Spiking with $^{13}$C-labelled mycotoxins before analysis to compensate matrix effects in ESI
  - No sample clean-up
Validation of SIDA method in maize
Extraction of spiked blank maize and reference materials

- Blank maize sample spiked with native mycotoxins before extraction
  - includes 10-fold dilution of matrix in the final extract due to extraction procedure
Validation of SIDA method
Results – Sample preparation

- **Extraction efficiency**
  - Determined by spiking of blank samples before extraction
  - First extraction: 80% acetonitrile content (60 min)
    - recovery between 80 and 110% except for FB1 and FB2
  - Second extraction: 20% acetonitrile content (30 min)
    - improved extraction recovery for FB1 and FB2 to approx. 90%

- **Matrix effects**
  - Signal suppression
    - 50 to 60% aflatoxins
    - 50% DON
  - Signal enhancement
    - Fumonisins, HT-2, T2, OTA
  - Effectively compensated by ISTD
Validation of SIDA method
Results for maize

<table>
<thead>
<tr>
<th>Analyte</th>
<th>LOQ in µg/kg</th>
<th>( R_A^{**} ) in %</th>
<th>RSD in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin B₁</td>
<td>0.04</td>
<td>105</td>
<td>6</td>
</tr>
<tr>
<td>Aflatoxin B₂</td>
<td>0.04</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>Aflatoxin G₁</td>
<td>0.05</td>
<td>101</td>
<td>5</td>
</tr>
<tr>
<td>Aflatoxin G₂</td>
<td>0.24</td>
<td>101</td>
<td>8</td>
</tr>
<tr>
<td>Deoxynivalenol</td>
<td>2.5</td>
<td>99</td>
<td>9</td>
</tr>
<tr>
<td>HT-2 toxin</td>
<td>2.0</td>
<td>98</td>
<td>7</td>
</tr>
<tr>
<td>T-2 toxin</td>
<td>0.17</td>
<td>99</td>
<td>6</td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td>0.23</td>
<td>93</td>
<td>7</td>
</tr>
<tr>
<td>Zearalenone</td>
<td>0.97</td>
<td>103</td>
<td>11</td>
</tr>
<tr>
<td>Fumonisin B₁</td>
<td>2.5</td>
<td>101</td>
<td>10</td>
</tr>
<tr>
<td>Fumonisin B₂</td>
<td>0.64</td>
<td>88</td>
<td>7</td>
</tr>
</tbody>
</table>

** average for triplicate samples and 6 spiking levels
Validation of SIDA method
Results for official test materials

<table>
<thead>
<tr>
<th>No</th>
<th>Analyte</th>
<th>assigned value $^a$ ± STDEV [µg kg$^{-1}$]</th>
<th>measured value $^b$ ± STDEV [µg kg$^{-1}$]</th>
<th>Status $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TM_01</td>
<td>ZEN</td>
<td>83 ± 4.5</td>
<td>86 ± 10</td>
<td>ok</td>
</tr>
<tr>
<td>TM_02</td>
<td>Sum AFs</td>
<td>3.79 ± 1.67</td>
<td>4.6 ± 0.2</td>
<td>ok</td>
</tr>
<tr>
<td></td>
<td>AFB$_1$</td>
<td>1.87 ± 0.83</td>
<td>2.3 ± 0.1</td>
<td>ok</td>
</tr>
<tr>
<td></td>
<td>AFB$_2$</td>
<td>0.51 ± 0.23</td>
<td>0.6 ± 0.03</td>
<td>ok</td>
</tr>
<tr>
<td></td>
<td>AFG$_1$</td>
<td>0.96 ± 0.43</td>
<td>1.0 ± 0.1</td>
<td>ok</td>
</tr>
<tr>
<td></td>
<td>AFG$_2$</td>
<td>0.52 ± 0.23</td>
<td>0.7 ± 0.1</td>
<td>ok</td>
</tr>
<tr>
<td>TM_03</td>
<td>FB$_1$</td>
<td>1650 ± 53</td>
<td>1960 ± 198</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>FB$_2$</td>
<td>461 ± 16</td>
<td>496 ± 32</td>
<td>ok</td>
</tr>
<tr>
<td>TM_04</td>
<td>DON</td>
<td>1714 ± 64</td>
<td>1660 ± 145</td>
<td>ok</td>
</tr>
<tr>
<td>TM_05</td>
<td>DON</td>
<td>901 ± 55</td>
<td>908 ± 79</td>
<td>ok</td>
</tr>
<tr>
<td></td>
<td>ZEN</td>
<td>79 ± 13</td>
<td>84 ± 10</td>
<td>ok</td>
</tr>
<tr>
<td>TM_06</td>
<td>FB$_1$</td>
<td>2630 ± 370</td>
<td>2300 ± 233</td>
<td>ok</td>
</tr>
<tr>
<td></td>
<td>FB$_2$</td>
<td>690 ± 170</td>
<td>578 ± 38</td>
<td>ok</td>
</tr>
<tr>
<td>TM_07</td>
<td>FB$_1$</td>
<td>270 ± 55</td>
<td>223 ± 23</td>
<td>ok</td>
</tr>
<tr>
<td></td>
<td>FB$_2$</td>
<td>&lt; 80</td>
<td>55 ± 4</td>
<td>ok</td>
</tr>
</tbody>
</table>
Validation of SIDA method

Published in Anal. Bioanal. Chem, 402 (9) 2675-2686
Implementation of SIDA method
Romer LC/MS Mycotoxin kit – developed to fit the application

Native mycotoxins:
- Mix 3 (fumonisins)
- Mix 9 (aflatoxins)
- Mix 8 (fusarium toxins)
- Ochratoxin

13C labelled mycotoxins:
- Mix 12 (13C fumonisins)
- Mix 11 (13C aflatoxins)
- Mix 10 (13C fusarium toxins)
- [13C20]-Ochratoxin
Typical performance during method implementation

Typical results of the SIDA method for mycotoxins

Aflatoxin B1 Chromatograms

Reproducibility:

Table 1 Area and peak height of selected mycotoxins (area of ISTD = 100%)

<table>
<thead>
<tr>
<th>Level</th>
<th>Area (n = 3)</th>
<th>Peak Height (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90.2 ± 3.2</td>
<td>90 ± 3</td>
</tr>
<tr>
<td>2</td>
<td>87.1 ± 2.8</td>
<td>87 ± 2.5</td>
</tr>
<tr>
<td>3</td>
<td>85.4 ± 2.3</td>
<td>85 ± 2.1</td>
</tr>
<tr>
<td>4</td>
<td>83.7 ± 2.2</td>
<td>83 ± 2.0</td>
</tr>
<tr>
<td>5</td>
<td>82.0 ± 2.1</td>
<td>82 ± 1.9</td>
</tr>
<tr>
<td>6</td>
<td>80.3 ± 2.0</td>
<td>80 ± 1.8</td>
</tr>
<tr>
<td>7</td>
<td>78.6 ± 1.9</td>
<td>78 ± 1.7</td>
</tr>
<tr>
<td>8</td>
<td>76.9 ± 1.8</td>
<td>76 ± 1.6</td>
</tr>
<tr>
<td>9</td>
<td>75.2 ± 1.6</td>
<td>75 ± 1.5</td>
</tr>
<tr>
<td>ISTD</td>
<td>100 ± 0</td>
<td>100 ± 0</td>
</tr>
</tbody>
</table>

Potential compounds for method extension (selection)

Compounds might be added by copying transitions and conditions to the method. It has to be mentioned that additional internal standards might be required to compensate for matrix effects. Though the instrument is capable to run many more compounds it should be noted that adding more compounds to the method might result in a different characteristic in terms of sensitivity, matrix effects and robustness.

Table 12 MRM transitions and conditions of compounds which might be added for method extension.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor</th>
<th>Product</th>
<th>Collision Energy</th>
<th>Cell Accelerator</th>
<th>Polarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin M1</td>
<td>329.1</td>
<td>273.1</td>
<td>20</td>
<td>5</td>
<td>positive</td>
</tr>
<tr>
<td>Aflatoxin M2</td>
<td>331.1</td>
<td>273.1</td>
<td>24</td>
<td>5</td>
<td>positive</td>
</tr>
<tr>
<td>Neosolanol</td>
<td>400.2</td>
<td>305.1</td>
<td>4</td>
<td>5</td>
<td>Positive</td>
</tr>
<tr>
<td>Fusarenone X</td>
<td>399.1</td>
<td>263.0</td>
<td>10</td>
<td>3</td>
<td>Negative</td>
</tr>
<tr>
<td>Nivalenol</td>
<td>357.1</td>
<td>281.0</td>
<td>12</td>
<td>3</td>
<td>Negative</td>
</tr>
</tbody>
</table>
Analysis of regulated mycotoxins in infant formula
Poster presented at the NACRW 2013
Increased confidence by using triggered MRM
Aflatoxin M1 in Infant Formula, fortified at 0.02 µg/kg
Summary

- **UHPLC-MS/MS method**
  - Improved chromatographic resolution
- **Multiple extraction steps**
  - Enhancement of extraction efficiency especially for fumonisins
- **Dynamic MRM with fast polarity switching**
  - Most abundant ionization mode and maximized dwell times within a single run
- **Addition of internal standards after extraction**
  - Compensation for matrix effects
  - Minimized costs
- **Apparent recoveries of 88 to 105% for all mycotoxins**
  - Evaluated by extraction of spiked maize samples
  - Validated by correct quantitation of 12 official test materials covering most toxins
- **Sensitivity suitable for MRLs**
  - Improved sensitivity of G6490 allows to omit sample concentration resulting in easier handling and improved robustness
Thanks!

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