Accurate quantitation of regulated mycotoxins by UHPLC-MS/MS

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Outline

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

Background

- myces (Greek) = fungus
- toxicum (Latin) = toxic

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

\[ = \text{Mycotoxin} \]

- 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
Mycotoxins
Why are they an issue?

- >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)
- 100+ countries have regulations for the control of mycotoxins in food and feed
Mycotoxins
Relevance for food control

- Notifications concerning mycotoxins
  - (RASFF-Annual reports 2002-2008)
Food contaminants online
FERA FC24 database access

- Free access to the RASFF alerts and notifications via FC24 database
- Registration through Agilent website
Accurate quantitation of mycotoxins

Reasons

  - set maximum residue levels (MRL) 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
  - until this year: 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
Sample preparation
Universal extraction procedure

1. Extraction
   - Centrifugation

2. Extraction
   - Centrifugation
     + ISTD

Grind and homogenize sample + weight-in

Acetonitrile:water:formic acid (80:19.9:0.1, v:v:v)
60 min at room temperature on a rotary shaker

Acetonitrile:water:formic acid (20:79.9:0.1, v:v:v)
30 min at room temperature on a rotary shaker

Dry Down

Agilent 6490 QqQ
Stable Isotope Dilution Assay (SIDA)

HPLC method

Agilent 1290 Infinity LC system consisting of:
- binary pump
- wellplate sampler
- column compartment
- diode array detector (not used)

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></th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>CapVoltage</td>
<td>4000 V</td>
<td>3000 V</td>
</tr>
<tr>
<td>Nozzle voltage</td>
<td>0 V</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
Dynamic MRM functionality
Comparison of MRM and DMRM

- 2 x shorter cycle times supports narrow chromatographic peaks, more analytes or longer dwell per analyte.
Dynamic MRM functionality
DMRM simulation
Stable Isotope Dilution Assay (SIDA) Chromatogram

- due to same MRM transitions baseline separation required for:
  - fumonisin B2 and B3
  - aflatoxin G1 and $^{13}$C-aflatoxin B1
  - aflatoxin G2 and $^{13}$C-aflatoxin B2

Spiked maize sample, 6490 QqQ
**Internal calibration in solvent**

**Aflatoxin B1**

- Challenging compound due to low MRLs
  - 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.**

- 0.0075 ng/ml
- 0.0225 ng/ml
- 0.075 ng/ml
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.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.6</td>
<td>0.0077</td>
</tr>
<tr>
<td>13.8</td>
<td>0.0230</td>
</tr>
<tr>
<td>25.3</td>
<td>0.0765</td>
</tr>
</tbody>
</table>
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 0.01 to 1.40 €
  - Price for all 11 IS per sample < 2.00 €

- 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 13C-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 % ± RSD in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin $B_1$</td>
<td>0.04</td>
<td>105 ± 6</td>
</tr>
<tr>
<td>Aflatoxin $B_2$</td>
<td>0.04</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Aflatoxin $G_1$</td>
<td>0.05</td>
<td>101 ± 5</td>
</tr>
<tr>
<td>Aflatoxin $G_2$</td>
<td>0.24</td>
<td>101 ± 8</td>
</tr>
<tr>
<td>Deoxynivalenol</td>
<td>2.5</td>
<td>99 ± 9</td>
</tr>
<tr>
<td>HT-2 toxin</td>
<td>2.0</td>
<td>98 ± 7</td>
</tr>
<tr>
<td>T-2 toxin</td>
<td>0.17</td>
<td>99 ± 6</td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td>0.23</td>
<td>93 ± 7</td>
</tr>
<tr>
<td>Zearalenone</td>
<td>0.97</td>
<td>103 ± 11</td>
</tr>
<tr>
<td>Fumonisin $B_1$</td>
<td>2.5</td>
<td>101 ± 10</td>
</tr>
<tr>
<td>Fumonisin $B_2$</td>
<td>0.64</td>
<td>88 ± 7</td>
</tr>
</tbody>
</table>

** average for triplicate samples and 6 spiking levels
DMRM database for mycotoxins
Customize your mycotoxin method

- Multi-mycotoxin method for 242 mycotoxins and other fungal metabolites has been developed
  - Validated for different nuts
  - Transitions are shortly available as DMRM database

![Graph showing various mycotoxins](image-url)
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 18 reference materials covering all toxin groups

- Sensitivity suitable for MRLs
  - Improved sensitivity of G6490 allows to omit sample concentration resulting in easier handling and improved robustness
Questions
Validation of SIDA method
Method characteristics and requirements

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Linear range (ng/mL)</th>
<th>LOQs (maize) µg/kg</th>
<th>MRLs (EC Reg. No 1881/2006)</th>
<th>Commodities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin B₁</td>
<td>0.0075 - 74.6</td>
<td>0.04</td>
<td>0.1</td>
<td>processed cereal-based baby food</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.0-12</td>
<td>nuts and cereals</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>sum of aflatoxins: 4.0-15.0</td>
<td></td>
</tr>
<tr>
<td>Deoxynivalenol</td>
<td>0.23 - 225</td>
<td>2.5</td>
<td>200</td>
<td>processed cereal-based baby food</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>500 - 1750</td>
<td>processed / unprocessed cereals, bread, pasta, breakfast cereals</td>
</tr>
<tr>
<td>Fumonisin B₁</td>
<td>0.075 - 249</td>
<td>2.5</td>
<td>200</td>
<td>processed maize-based baby food</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>800</td>
<td>maize-based breakfast cereals</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1000 / 4000</td>
<td>maize / unprocessed maize</td>
</tr>
<tr>
<td>Fumonisin B₂</td>
<td>0.075 - 251</td>
<td>0.64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HT-2 toxin</td>
<td>0.2 - 202</td>
<td>2.0</td>
<td></td>
<td>implementation of MRLs is expected in the near future</td>
</tr>
<tr>
<td>T-2 toxin</td>
<td>0.023 - 75.4</td>
<td>0.17</td>
<td></td>
<td>unprocessed cereals and cereal products</td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td>0.023 – 23.0</td>
<td>0.23</td>
<td>0.5</td>
<td>processed cereal-based baby food</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.0 / 5.0</td>
<td>processed / unprocessed cereals</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10.0</td>
<td>dried vine fruit</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15 / 20 / 80</td>
<td>spices / liquorice root / extract</td>
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<tr>
<td>Zearalenone</td>
<td>0.076 - 252</td>
<td>1.0</td>
<td>20</td>
<td>processed cereal-based baby food</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td>processed / unprocessed cereals</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>75-350</td>
<td>bread, biscuits, breakfast cereals</td>
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</tbody>
</table>

Implementation of MRLs is expected in the near future.