Modern Analytical Tools to Tackle an Old Problem: Mycotoxins in Food

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Mycotoxins are...

Aspergillus spp.
Penicillium spp.
Fusarium spp.
Alternaria spp.
Claviceps spp.
etc.

... toxic secondary fungal metabolites
Mycotoxins

History

- Epidemics of ergotism have been reported as early as **857 A.D.** resulting from the ingestion of cereals infected with ergot

- **1960:** Turkey X Disease
  Peanuts infected with *Aspergillus flavus*

- Isolation of the highly toxic fungal metabolite *aflatoxin* and **intensive research** on other fungi and toxins produced by them

- Effects: acute toxic, nephrotoxic, **carcinogenic**, immunosuppressive, estrogenic, hepatotoxic,…

- Annual losses of **several hundred million tons of food and feed** worldwide

- 100+ countries have **regulations** for the control of mycotoxins in food and feed
Sampling is major source of error
• regulation EC 401/2006: Methods of sampling and analysis for the official control of the levels of mycotoxins in foods

Careful choice of extraction solvents
• typically mixtures of water with organic solvents (MeOH, ACN, acetone)

SPE, IAC, MycoSep®, QuEChERS, dilute & shoot

Rapid methods (mostly antibody-based) or

Chromatographic methods
50 years of Mycotoxin Analysis
A Transition of Methods

Transition of methods over the last 50 years used in mycotoxin analysis based on the Web of Science®

Keywords: mycotox* & TLC & ELISA & GC & HPLC & LC-MS

Franz Berthiller
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LC-MS Multi-Mycotoxin Methods

Reasons

- Unified methods save time and costs

- For **accurate quantification** of regulated mycotoxins
  - high degree of accuracy
  - easy to handle, cost effective
Accurate Mycotoxin Quantification

Reasons

- European Commission Regulation (EC) No 1881/2006 maximum levels in maize for
  - aflatoxins (AFB₁, AFB₂, AFG₁, AFG₂)
  - fumonisins (FB₁, FB₂)
  - deoxynivalenol
  - ochratoxin A
  - zearalenone
  - (HT-2 toxin and T-2 toxin)

**BUT:**

- Electrospray ionisation (ESI)
  - matrix effects hamper accurate mass spectrometric quantification
Accurate Mycotoxin Quantification

**Approaches**

- **Dilution of the sample**
  - loss of sensitivity

- **Matrix matched calibration**
  - needed for each individual matrix

- **Standard addition to each sample**
  - multiple injections needed

- **Internal calibration**
  - ideally: internal standard (IS) behaves exactly the same as the analyte, but still distinctive
  - structurally related or similar compounds: zearalanone (ZAN) for zearalenone (ZEN)
  - \([^2\text{H}]-\) or \([^{13}\text{C}]-\)labelled compounds
Stable Isotope Dilution Assay (SIDA)  
Multiple Mycotoxins - Sample Preparation

1. Extraction
   - Centrifugation

2. Extraction
   - Centrifugation

   +IS

Transfer

Analysis

- Milling
  - grind and homogenise sample + weight-in
    - acetonitrile:water:formic acid (80:19.9:0.1, v:v:v)
      - 60 min at room temperature on a rotary shaker

- Centrifugation

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

Transfer 80 µL aliquot to HPLC vial with microinsert and add 20 µL ISTD-mix

UHPLC-MS/MS
Stable Isotope Dilution Assay
UHPLC-MS/MS Setup

- **1290 Infinity UHPLC (Agilent Technologies)**
  - C18 column
  - methanol-water gradient

- **6490 QQQ MS/MS (Agilent Technologies)**
  - single run, fast polarity switching, dynamic MRM

![Graph showing counts (x 10^4) vs. acquisition time (min) for various mycotoxins: DON, AFG₁, AFB₁, AFG₂, AFB₂, FB₁, T-2, HT-2, FB₂, OTA, ZEN.](image)
## SIDA Method
### Validation Results – Maize

<table>
<thead>
<tr>
<th>Analyte</th>
<th>External calibration Rₐ [%] ± RSD [%] (1))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin B₁</td>
<td>35 ± 4</td>
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<tr>
<td>Aflatoxin B₂</td>
<td>42 ± 4</td>
</tr>
<tr>
<td>Aflatoxin G₁</td>
<td>46 ± 5</td>
</tr>
<tr>
<td>Aflatoxin G₂</td>
<td>40 ± 6</td>
</tr>
<tr>
<td>Deoxynivalenol</td>
<td>41 ± 10</td>
</tr>
<tr>
<td>Fumonisin B₁</td>
<td>330 ± 6</td>
</tr>
<tr>
<td>Fumonisin B₂</td>
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<td>Zearalenone</td>
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\(1\) Apparent recovery ± relative standard deviation
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<tr>
<td></td>
<td>$R_A\ [%] \pm RSD\ [%]$ (^1)</td>
<td>$R_A\ [%] \pm RSD\ [%]$ (^1)</td>
</tr>
<tr>
<td>Aflatoxin $B_1$</td>
<td>35 ± 4</td>
<td>105 ± 6</td>
</tr>
<tr>
<td>Aflatoxin $B_2$</td>
<td>42 ± 4</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Aflatoxin $G_1$</td>
<td>46 ± 5</td>
<td>101 ± 5</td>
</tr>
<tr>
<td>Aflatoxin $G_2$</td>
<td>40 ± 6</td>
<td>101 ± 8</td>
</tr>
<tr>
<td>Deoxynivalenol</td>
<td>41 ± 10</td>
<td>99 ± 9</td>
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<tr>
<td>Fumonisin $B_1$</td>
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Validation Results – Maize

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<th>Internal calibration $R_A$ [%] ± RSD [%] $^1$</th>
<th>$R_E$ [%] $^2$</th>
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<tbody>
<tr>
<td>Aflatoxin $B_1$</td>
<td>35 ± 4</td>
<td>105 ± 6</td>
<td>108</td>
</tr>
<tr>
<td>Aflatoxin $B_2$</td>
<td>42 ± 4</td>
<td>100 ± 4</td>
<td>107</td>
</tr>
<tr>
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<td>46 ± 5</td>
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<td>109</td>
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</tbody>
</table>

$^1$ Apparent recovery ± relative standard deviation

$^2$ Extraction recovery
## SIDA Method
### Limits of Quantification

<table>
<thead>
<tr>
<th>Analyte</th>
<th>LOQ (µg/kg)</th>
<th>MLs (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin B&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.1</td>
<td>2.0 – 5.0 (0.1)</td>
</tr>
<tr>
<td>Aflatoxin B&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Aflatoxin G&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Aflatoxin G&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>Deoxynivalenol</td>
<td>12</td>
<td>500 – 1750 (200)</td>
</tr>
<tr>
<td>Fumonisin B&lt;sub&gt;1&lt;/sub&gt;</td>
<td>4.5</td>
<td>sum fumonisins 800 – 4000 (200)</td>
</tr>
<tr>
<td>Fumonisin B&lt;sub&gt;2&lt;/sub&gt;</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>HT-2 toxin</td>
<td>3</td>
<td>indicative values 3) sum 15-1000</td>
</tr>
<tr>
<td>T-2 toxin</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td>0.4</td>
<td>3.0 – 5.0 (0.5)</td>
</tr>
<tr>
<td>Zearalenone</td>
<td>3</td>
<td>50 (20)</td>
</tr>
</tbody>
</table>

1) Limit of quantification (S/N = 10) for maize
2) Maximum levels for various commodities according to European Union Commission Regulation 1881/2006 and its amendments (numbers in brackets: baby food)
3) Indicative values in various cereals according to European Union Recommendation 165/2013

**OTA:** 0.45 µg/kg
Stable Isotope Dilution Assay

Summary

Validation of a Stable Isotope Dilution Assay for the Accurate Quantitation of Mycotoxins in Maize Using UHPLC/MS/MS

Application Note 5991-2808

DOI 10.1007/s00216-012-5757-5

Stable isotope dilution assay for the accurate determination of mycotoxins in maize by UHPLC-MS/MS

Elisabeth Varga · Thomas Glauner · Robert Köppen · Katharina Mayer · Michael Sulyok · Rainer Schuhmacher · Rudolf Krksa · Franz Berthiller

Open access
LC-MS Multi-Mycotoxin Methods

Reasons

- Unified methods save time and costs

- For **accurate quantification** of regulated mycotoxins
  - high degree of accuracy
  - easy to handle, cost effective

- For **(semi-)quantification of 200+ mycotoxins**
  - analysis of food and feed mixtures of several commodities
  - monitoring of changes in regional fungal spread (climate change)
Multi-Mycotoxin Quantification
The Need to Determine More Toxins in Food

Berthiller et al. (2005) J. Chrom. A
- 9 mycotoxins, APCI, polarity switching
- Mycosep clean-up
- LOD deoxynivalenol: 65 pg on column

- 39 mycotoxins, ESI, 2 runs (pos. and neg.)
- no clean-up
- LOD deoxynivalenol: 10 pg on column

extended to:
- 87 toxins (Sulyok et al., 2007)
- 186 metabolites (Vishwanath et al., 2009)

Malachová et al. (2014) J. Chrom. A
- 295 fungal and bacterial metabolites
- LOD deoxynivalenol: 0.3 pg on column

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LC-Tandem-Mass Spectrometry
Multiple Reaction Monitoring (MRM)

Collision gas $N_2$

MS1 | CID | MS2

Focusing | Precursor Ion Selection | Fragmentation | Product Ion Selection | Detector

static (classical) MRM
dynamic (scheduled) MRM

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Multi-Mycotoxin Quantification Method Workflow

1. **Sampling**
2. **Milling**
3. **Extraction**
   - **Osterizer Blender (household mill)**
   - acetonitrile:water:acetic acid (79:20:1, v:v:v)
   - 90 min at RT on rotary shaker
4. **Filtration**
5. **Clean-up**
6. **Analysis**
7. **Evaluation**

Multi-Mycotoxin Quantification Method Workflow

Sampling → Milling → Extraction

representative sample

Osterizer Blender (household mill)

acetonitrile:water:acetic acid (79:20:1, v:v:v)
90 min at RT on rotary shaker

“dilute and shoot” approach
acetonitrile:water:acetic acid (20:79:1, v:v:v)

Multi-Mycotoxin Quantification
Method Workflow

Sampling → Milling → Extraction → Analysis → Evaluation

- LC-MS/MS (1290 series UHPLC + QQQ 6460) eluents (acidified methanol/water mixtures)
  - pos. and neg. ionisation mode

- Mass Hunter (manual correction);
  - linear, 1/x weighted calibration curves
Multi-Mycotoxin Quantification
Chromatogram for a total of 191 compounds

- 65 analytes validated for almonds, hazelnuts, peanuts and pistachios.
- Semi-quantitative method for 126 analytes.

ESI + SRM of a spiked hazelnut sample (medium level)

- Count vs. acquisition time (min)
- Multi-Mycotoxin Quantification Chromatogram for a total of 191 compounds
- ESI + SRM of a spiked hazelnut sample (medium level)

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Multi-Mycotoxin Quantification
Market Survey - Nuts

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Number of contaminated samples (n = 53)

% cont of total nuts

- Aflatoxin B₁: 34
- Sterigmatocystin: 49
- Ochratoxin A: 15
- Ochratoxin B: 11
- Alternariol: 45
- Alternariolmethylether: 51
- Macrosporin: 57
- Tentoxin: 42
- Beauvericin: 79
- Enniatin B: 62
- HT-2 toxin: 28
- T-2 toxin: 28
- Emodin: 53
- 3-Nitropropionic acid: 47
- Mycophenolic acid: 55

Almonds
Hazelnuts
Peanuts
Pistachios

Aspergillus metabolites
Fusarium metabolites
Alternaria metabolites
Penicillium met.
Development and validation of a (semi-)quantitative UHPLC-MS/MS method for the determination of 191 mycotoxins and other fungal metabolites in almonds, hazelnuts, peanuts and pistachios

Elisabeth Varga · Thomas Glauner · Franz Berthiller · Rudolf Krška · Rainer Schuhmacher · Michael Sulyok

Screening and Quantitation of 191 Mycotoxins and Other Fungal Metabolites in Almonds, Hazelnuts, Peanuts, and Pistachios Using UHPLC/MS/MS

Application Note 5991-4991
LC-MS Multi-mycotoxin Methods

Reasons

- Unified methods save time and costs

- For **accurate quantification** of regulated mycotoxins
  - high degree of accuracy
  - easy to handle, cost effective

- For **(semi-)quantification of 200+ mycotoxins**
  - analysis of food and feed mixtures of several commodities
  - monitoring of changes in regional fungal spread (climate change)

- For **screening** of a huge variety of mycotoxins
  - identification of mycotoxins in unlikely matrices
  - screening of co-occurring analytes to evaluate synergistic effects

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Contaminant Screening
Using HR-MS instruments

- **Advantages**
  - in MS mode -> **fast data acquisition**
  - post-acquisition data analysis (at least in MS mode)
  - potential screening for **unknown contaminants**
    - MS-mode: high mass accuracy -> sum formulas
    - MS/MS-mode: sum formulas of fragments
  - **unambiguous identification possible without standards**

- **Disadvantages**
  - higher limit of detections compared to QQQs

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6550 iFunnel QTOF
Mycotoxin Screening Database

- Contains compound information:
  - name
  - formula
  - exact mass
  - retention time
  - external IDs (e.g. CAS)
  - IUPAC name
  - structural information

mycotoxins and related metabolites: 455 entries
Acquiring target HR-MS/MS spectra from standards
- import using mass correction based on (fragment) structures
- only explainable fragments are imported → exact masses for precursors and fragments

302 compounds with spectra
LC-HR-MS/MS Screening

Classical Workflow

1) HR-MS full scan

total ion chromatogram of a naturally contaminated hazelnut sample

2) Find-by-Formula (FBF)

extracted ion chromatogram & extracted MS spectrum of mycophenolic acid

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3) MS/MS scan
specify precursor mass, retention time (RT), RT window and collision energy

4) Library confirmation
extracted MS/MS spectra at different collision energies

CE 10 eV

CE 20 eV

CE 40 eV

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Library hit confirming the presence of the immunosuppressive mycotoxin mycophenolic acid in a hazelnut sample.

Mycophenolic acid ($\text{C}_{17}\text{H}_{20}\text{O}_6$) produced by several *Penicillium* spp. has low acute toxicity and is immunosuppressive.
**LC-HR-MS/MS Screening**

**All Ions Approach**

1) **HR-All Ions MS/MS acquisition**

Fragmentation without precursor selection

Measurement with “low energy channel” (no collision energy, MS scan) and at least one “high energy channel” (collision energy e.g. 20 eV)

<table>
<thead>
<tr>
<th>Focusing</th>
<th>NO Precursor Ion Selection</th>
<th>NO Fragmentation</th>
<th>MS Scan</th>
<th>Detector</th>
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LC-HR-MS/MS Screening
All Ions Approach

2) Database search and library confirmation

I) Database search: applying “Find by Formula” algorithm on low channel
II) Automatic extraction of fragment ion chromatograms in high energy channel
III) Co-elution scoring of fragment and parent EICs for qualification and library confirmation
LC-HR-MS/MS Screening
All Ions Approach

Co-elution score
Major function to qualify compounds based on similarity of peak shapes

All Ions approach
- worse limits of detection (more noise)
- limited with collision energies

+ only one injection for each polarity
+ post-acquisition MS/MS information
LC-HR-MS/MS Screening

Summary

- **General unknown screening** using HR-MS instrumentation
- Identification by **HR-MS/MS library spectra**
- Standards not mandatory for unambiguous identification
- **Retrospective data-analysis** also in MS/MS mode possible
Conclusions

Summary

- **LC-MS/MS** is increasingly used for the **simultaneous quantification and identification** of mycotoxins and other food contaminants.

- **Stable Isotope Dilution Analysis** is used for quantification of mycotoxins in food and feed offering **highest possible accuracy** at affordable additional costs for internal standards.

- (Semi-)quantitative **LC-MS/MS methods allow** for screening and **quantification of 200+ mycotoxins**.

- **LC-HR-MS screening** with exact mass MS/MS libraries allow **unambiguous identification for a practically unlimited number of compounds** even without standards and **in retrospective data analysis**.
Thanks for the attention!

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