SESSION 2: MYCOTOXIN BIOMARKER ANALYSIS

Determination of deoxynivalenol urinary biomarker(s)

Hans Mol, Hester van der Top
Outline

Mycotoxins in HBM4EU
Deoxynivalenol
Overview of method options
Example specific method & characteristics

Note: instruments, brand names, methods are for information, not HBM4EU endorsements
Mycotoxins in HBM4EU

**Mycotoxins:**
100's known
Currently 15 regulated in the EU in food commodities
Emerging issue

Included as substance group in
2\textsuperscript{nd} prioritisation round HBM4EU

**Prioritisation within the substance group:**

**Deoxynivalenol**
Fumonisin B1
Aflatoxin B1(?)

Others?

**Compound group leaders (CGLs):**
Paula Alvito, Susana Viegas, Maria João Silva
INSA / ESTeSL-IPL, Portugal

Currently in progress:
- Inventory of biomarker/matrices & methods
- Drafting of Scoping document
Exposure to mycotoxins

General population: food is the main route of exposure
⇒ EFSA is a main stakeholder

Regulated mycotoxins in food
EU Regulation 1881/2006
recommendation 2013/165/EU

Cereals/-products
Nuts, dried fruits,
various other products

In progress:
Extension other food items
DON derivatives
Ergot alkaloids
Alternaria toxins
......

<table>
<thead>
<tr>
<th>Mycotoxins</th>
<th>Max Limit range µg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>aflatoxin B1</td>
<td>2-12</td>
</tr>
<tr>
<td>aflatoxin B1,B2,G1,G2</td>
<td>4-15</td>
</tr>
<tr>
<td>aflatoxin M1</td>
<td>0.05</td>
</tr>
<tr>
<td>citrinin</td>
<td>2000 (RYR)</td>
</tr>
<tr>
<td>deoxynivalenol</td>
<td>200-1750</td>
</tr>
<tr>
<td>ergot sclerotia (kernels)</td>
<td>0.5 g/kg</td>
</tr>
<tr>
<td>fumonisin B1</td>
<td>200-4000</td>
</tr>
<tr>
<td>fumonisin B2</td>
<td></td>
</tr>
<tr>
<td>ochratoxin A</td>
<td>2-20</td>
</tr>
<tr>
<td>patulin</td>
<td>10-50</td>
</tr>
<tr>
<td>T-2 toxin</td>
<td>15-1000*</td>
</tr>
<tr>
<td>HT-2 toxin</td>
<td></td>
</tr>
<tr>
<td>zearalenone</td>
<td>20-400</td>
</tr>
</tbody>
</table>

*recommendation
HBM of mycotoxins

Relatively low number of studies
Usually NOT included in existing/on-going HBM monitoring programs
Recently increasing interest (modified forms, heterogeneity in food, ....)
Background information deoxynivalenol (DON)

Main source of exposure:
Cereals and cereal products
Grains, flour
Breakfast cereals
Bread/bakery products
Pasta
Beer

Health based guidance values:
group-TDI of 1 µg/kg bw per day for the sum of the four DON forms
group-ARfD of 8 µg/kg bw per eating occasion
Deoxynivalenol (DON)

**Main forms in food:**
- **DON-3-glucoside**: <20% (higher in beer)
- **15-acetyl-DON**: <7%
- **3-acetyl-DON**: <10%
- **DON**: ≥70%

**Main human biomarkers:**
- **DON-15-glucuronide**: ~58%
- **DON-3-glucuronide**: ~27%
- **DON-3-glucuronide**: ~14%
- **Free DON**: <7%

RIKILT, Wageningen, November 22nd 2018, part of 2nd HBM4EU Training School, Nijmegen, November 19th-23rd, 2018

Vidal et al, 2018
DON

Expected levels DON biomarkers in general population:

<table>
<thead>
<tr>
<th></th>
<th>Norway</th>
<th>UK</th>
<th>Italy</th>
</tr>
</thead>
<tbody>
<tr>
<td>detection rate%</td>
<td>99%</td>
<td>93%</td>
<td>76%</td>
</tr>
<tr>
<td>total DON</td>
<td>ng/ml</td>
<td>morning voids 2 days</td>
<td></td>
</tr>
<tr>
<td>median</td>
<td>3.6-7.3</td>
<td>10.7-12.6</td>
<td>4-13</td>
</tr>
<tr>
<td>min</td>
<td>&lt;0.015</td>
<td>0.9-5.1</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>max</td>
<td>19-43</td>
<td>29-59</td>
<td>14-51</td>
</tr>
</tbody>
</table>

⇒ Target LOQ ≤0.5 ng/ml
Deoxynivalenol: target biomarkers

Options for determination of DON biomarkers:

1) Individual three main biomarkers
   - Availability of standards:
     - DON and $^{13}$C$_{15}$-DON readily available
     - DON-15-glucuronide
     - DON-3-glucuronide

   Only custom made $$$$$$

2) Total DON
   free DON + DON-glucuronides after deconjugation glucuronides

Pro: only 1 biomarker, ILIS available
Con: deconjugation step needed
### Methods from literature

**Without deconjugation:**
only free DON or DON-conjugates with custom made standards


<table>
<thead>
<tr>
<th><strong>With deconjugation (total DON, often also including DOM-1)</strong></th>
</tr>
</thead>
</table>
Methods from literature

Deconjugation

Extraction/cleanup

Instrumental analysis
Various options

**Chemical:** hydrolysis/cleavage under acid or alkaline conditions
Parameters: type/conc. acid/base, temperature, time
Example: addition of 37% HCl/70-100°C, 1-3h; 0.1-1 M NaOH
Remark: degradation of biomarker, degradation of matrix (dirty extracts)

**Biological:** enzymes, various options differing in activity specificity
source: molluscs (*Helix Pomatia*); bacterial (*E. Coli*)
most have β-glucuronidase activity, variation in activity/specificity
Parameters: enzyme, pH, units added, temperature, time,
biomarker-conjugate (isomers!)

*Helix Pomatia*: optimum pH
- β-glucuronidase  pH 4-5
- Arylsulfatase  pH ~6.5
- Other...(esterase..)

*E. Coli*: optimum pH
- β-glucuronidase  pH ~6-7
### Deconjugation different enzymes/conditions

<table>
<thead>
<tr>
<th>Ref</th>
<th>ml</th>
<th>Deconjugation enzyme</th>
<th>pH</th>
<th>time</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>1.0</td>
<td>β-gluc./arylsulf. (Helix P.) (Sigma)</td>
<td>5</td>
<td>18h</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>β-gluc (E. coli IX-A)</td>
<td>6.8</td>
<td>18h</td>
</tr>
<tr>
<td>8</td>
<td>1.0</td>
<td>β-gluc (E. coli IX-A)</td>
<td>6.8</td>
<td>18h</td>
</tr>
<tr>
<td>9</td>
<td>3.0</td>
<td>β-gluc./arylsulf. (Helix P.) (Roche)</td>
<td>5</td>
<td>18h</td>
</tr>
<tr>
<td>10</td>
<td>6.0</td>
<td>β-gluc./arylsulf. (Helix P.) (-)</td>
<td>-</td>
<td>overnight</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>β-gluc (E. coli IX-A)</td>
<td>7.4</td>
<td>overnight</td>
</tr>
<tr>
<td>12</td>
<td>0.5</td>
<td>β-gluc (E. coli IX-A)</td>
<td>7.4</td>
<td>16h</td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>β-gluc (E. coli IX-A)</td>
<td>6.8</td>
<td>18h</td>
</tr>
</tbody>
</table>

Does it matter?
[6] Effect of type of enzyme / units (18h, 37°C)
1 mL urine sample (spiked/pos?)

Fig. 2. Average of peak area (n = 3) using different types of β-glucuronidase enzymes at 5000 or 10000 U. HP (H. pomatia), EC (E. coli) and PV (P. vulgat).

HP = β-Glucuronidase Type 1 from Helix pomatia (Sigma); pH 5
EC = β-Glucuronidase Type IX from Escherichia coli (Sigma); pH 6.8
PV = β-Glucuronidase Type L-II from Patella vulgat (Sigma); pH 5
[13] Effect of enzyme concentration and time

1 mL positive urine sample + 1.5 ml phosphate buffer (75 mM, pH 6.8) containing X units of β-glucuronidase (Type IX from E. coli)


Figure S1. Effects of enzyme concentration and incubation time on the extent of deconjugation of DON-glucuronide.
[RIKILT] Effect of enzyme, pH, time

1 mL positive urine sample, experiments in triplicate (\(^{13}\)C\(^{15}\)-label after deconjugation)

HP = β-Glucuronidase/aryl sulfatase from *Helix Pomatia* (Merck 104114); 1 ml urine + 2 ml 200mM NaAc buffer + 10 µl enzyme

E.Coli = β-Glucuronidase from *Escherichia coli* Type IX-A (Sigma G7396); 1 ml urine + 2 ml 75 mM phosphate buffer + 3000 U enzyme
Extraction/cleanup

**Dilute & shoot**
+ very straightforward
+ ability to simultaneously detect other mycotoxin biomarkers (and more)
  - ion suppression (depending on dilution)
  - interferences
  - Insufficient sensitivity (depending on instrument)
⇒ LOQs reported for DON 0.5-13 ng/ml

**Generic cleanup**
± additional step in sample prep
± ability to simultaneously detect multiple other mycotoxins biomarkers
± ion suppression (depending on urine equiv./ml extract)
± Interferences (depending on chemistries of cleanup)
±/+ adequate sensitivity (depending on instrument)

  e.g. SPE C18, OASIS (PRiME) HLB, .....
Specific cleanup
± additional step in sample prep
- only one or few mycotoxin biomarkers in one method
+ minor matrix effects/ion suppression (depending on urine equiv./ml extract)
+ little interferences (depending on chemistries of cleanup)
+ low LOQs (depending on instrument)

Immunoaffinity cleanup columns (IAC)
DONPrep (R-Biopharm Rhone Ltd)
DonStar™ IAC (Romer labs)
DONTest WB (Vicam)
Immunoclean CF DON from Aokin
Neocolumn for DON (Neogen Europe Ltd)
and more.....

Multi IAC cleanup?
[aflatoxins, ochratoxin A, fumonisins, deoxynivalenol, zearalenone, nivalenol, T-2 and HT-2 toxins]
e.g. Myco6in1 [10]
## Methods from literature

### Deconjugation different enzymes/conditions

**Extraction/cleanup: SPE or IAC**

**Instrumental analysis: LC-MS/MS, GC-MS(/MS)**

<table>
<thead>
<tr>
<th>Ref</th>
<th>ml</th>
<th>Deconjugation enzyme</th>
<th>pH</th>
<th>time</th>
<th>Extr./cleanup</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>1.0</td>
<td>β-gluc./arylsulf. (Helix P.) (Sigma)</td>
<td>5</td>
<td>18h</td>
<td>SPE C18</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>β-gluc (E. coli IX-A)</td>
<td>6.8</td>
<td>18h</td>
<td>IAC</td>
</tr>
<tr>
<td>8</td>
<td>1.0</td>
<td>β-gluc (E. coli IX-A)</td>
<td>6.8</td>
<td>18h</td>
<td>IAC</td>
</tr>
<tr>
<td>9</td>
<td>3.0</td>
<td>β-gluc./arylsulf. (Helix P.) (Roche)</td>
<td>5</td>
<td>18h</td>
<td>IAC</td>
</tr>
<tr>
<td>10</td>
<td>6.0</td>
<td>β-gluc./arylsulf. (Helix P.) (-)</td>
<td>-</td>
<td>overnight</td>
<td>multi-IAC+SPE (HLB)</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>β-gluc (E. coli IX-A)</td>
<td>7.4</td>
<td>overnight</td>
<td>IAC</td>
</tr>
<tr>
<td>12</td>
<td>0.5</td>
<td>β-gluc (E. coli IX-A)</td>
<td>7.4</td>
<td>16h</td>
<td>SPE (HLB)</td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>β-gluc (E. coli IX-A)</td>
<td>6.8</td>
<td>18h</td>
<td>μSPE HLB (96 well)</td>
</tr>
</tbody>
</table>
**Instrumental Analysis**

**DON**

**GC-MS(/MS):** requires derivatisation

[6] dry extract: 100 µL of BSA + TMCS + TMSI (3:2:3); 20 min 80°C

**LC-MS/MS:**

<table>
<thead>
<tr>
<th>Mycotoxin</th>
<th>ESI</th>
<th>precursor ion (m/z)</th>
<th>product ion 1 a)</th>
<th>product ion 2 a)</th>
<th>product ion 3 a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DON</td>
<td>pos</td>
<td>M+H</td>
<td>297</td>
<td>249</td>
<td>231</td>
</tr>
<tr>
<td></td>
<td>neg</td>
<td>M+acetate b)</td>
<td>355</td>
<td>265</td>
<td>295</td>
</tr>
<tr>
<td>13C15-DON</td>
<td>pos</td>
<td>M+H</td>
<td>312</td>
<td>263</td>
<td>245</td>
</tr>
<tr>
<td></td>
<td>neg</td>
<td>M+acetate b)</td>
<td>370</td>
<td>279</td>
<td>310</td>
</tr>
</tbody>
</table>

a) the relative abundance or optimum S/N for the transitions depend on the instrument and matrix, and needs to be experimentally optimized/verified.

b) when formate and/or formic acid is used in the mobile phase, formate adducts instead of acetate adducts can be formed.

c) measurement of acetate as product ion can be rather non-specific and is therefore not recommended.

https://www.wur.nl/en/Research-Results/Research-Institutes/rikilt/Reference-laboratory/European-Union-Reference-Laboratory-1/Library-EURL-MP.htm#eurl_mp_methods_from_2018
Methods from literature

DON

Deconjugation different enzymes/conditions

Extraction/cleanup: SPE or IAC

Instrumental analysis: LC-MS/MS, GC-MS(/MS)

<table>
<thead>
<tr>
<th>Ref</th>
<th>ml</th>
<th>Deconjugation enzyme</th>
<th>pH</th>
<th>time</th>
<th>Extr./cleanup</th>
<th>analysis</th>
<th>scope</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>1.0</td>
<td>β-gluc./arylsulf. (Helix P.) (Sigma)</td>
<td>5</td>
<td>18h</td>
<td>SPE C18</td>
<td>GC-MS*</td>
<td>DONs</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>β-gluc (E. coli IX-A)</td>
<td>6.8</td>
<td>18h</td>
<td>IAC</td>
<td>LC-MS</td>
<td>DONs</td>
</tr>
<tr>
<td>8</td>
<td>1.0</td>
<td>β-gluc (E. coli IX-A)</td>
<td>6.8</td>
<td>18h</td>
<td>IAC</td>
<td>LC-MS &amp;HRMS</td>
<td>DONs</td>
</tr>
<tr>
<td>9</td>
<td>3.0</td>
<td>β-gluc./arylsulf. (Helix P.) (Roche)</td>
<td>5</td>
<td>18h</td>
<td>IAC</td>
<td>LC-MS/MS</td>
<td>DONs</td>
</tr>
<tr>
<td>10</td>
<td>6.0</td>
<td>β-gluc./arylsulf. (Helix P.) (−)</td>
<td>-</td>
<td>overnight</td>
<td>multi-IAC+SPE (HLB)</td>
<td>LC-MS/MS</td>
<td>DONs +[a]</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>β-gluc (E. coli IX-A)</td>
<td>7.4</td>
<td>overnight</td>
<td>IAC</td>
<td>LC-MS/MS</td>
<td>DONs</td>
</tr>
<tr>
<td>12</td>
<td>0.5</td>
<td>β-gluc (E. coli IX-A)</td>
<td>7.4</td>
<td>16h</td>
<td>SPE (HLB)</td>
<td>LC-MS/MS</td>
<td>DONs +[b]</td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>β-gluc (E. coli IX-A)</td>
<td>6.8</td>
<td>18h</td>
<td>μSPE HLB (96 well)</td>
<td>LC-MS/MS</td>
<td>DONs</td>
</tr>
</tbody>
</table>

*After derivatisation: 100 µL of BSA + TMCS + TMSI (3:2:3); 20 min 80°C

DONs include DOM-1 (acetyl-DONs)

[a] DON, DOM-1, aflatoxin M1, ochratoxin A, fumonisin B1, α-zearalenol, β-zearalenol

[b] DON, DOM-1, aflatoxin M1, alternariol, citrinine/dihydrocitrinine, fumonisin B1, ochratoxin A, α-zearalenol, β-zearalenol, zearalenone
**Example specific method**

DON

**Thaw urine, vortex, take 1.0 ml aliquot**

**Deconjugation**
Add 2 ml 75 mM phosphate buffer pH 6.8
Add 250 µl enzyme solution (~3000 U β-Glucuronidase *E. coli* type IX-A)
37°C overnight (at least 16h)
Cool down, add 2 ml milliQ water
Add $^{13}$C$_{15}$-DON internal standard

**Extraction/cleanup**
IAC (Vicam DONTEST)
drain, rinse 2 ml milliQ water
load entire deconjugated sample
rinse 2 ml milliQ water
elute 2 ml MeOH (soak & then elute)
Evaporate to dry 55°C/N2
Reconstitute in 200 µl 20% MeOH/water, vortex
Transfer into filter vial/press through

**LC-MS/MS analysis**
Inject 10-25 µl
Acquity UPLC 100 x 2.1 mm 1.8µm HSS T3; 35°C, 0.40 ml/min
5 mM NH$_4$Ac, 0.1% acetic acid, MeOH gradient
Sciex Qtrap 6500, ESI positive: m/z 297>249, 297>231; label: 312>263

Quantification: multi-level solvent standards with $^{13}$C$_{15}$-DON internal standard
Validation

Pre-screen urine analysis for blank samples
6 different urine samples

Validation set:
Procedural blank (water)
Urine
Urine spikes @ 0.5, 1, 5 ng/ml single analysis

Calibrants in solvent equivalent to 0.25, 0.5, 1.25, 2.5, 5, 7.5 ng/ml urine
Solvent injection <> carry-over
Validation results

Calibration (solvent standards with 13C-DON)

<table>
<thead>
<tr>
<th>ng/ml (urine equiv.)</th>
<th>rel. response</th>
<th>BCC ng/ml</th>
<th>deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.467</td>
<td>0.25</td>
<td>2%</td>
</tr>
<tr>
<td>0.50</td>
<td>0.901</td>
<td>0.50</td>
<td>0%</td>
</tr>
<tr>
<td>1.25</td>
<td>2.255</td>
<td>1.26</td>
<td>1%</td>
</tr>
<tr>
<td>2.50</td>
<td>4.320</td>
<td>2.42</td>
<td>-3%</td>
</tr>
<tr>
<td>5.00</td>
<td>9.132</td>
<td>5.13</td>
<td>3%</td>
</tr>
<tr>
<td>7.50</td>
<td>13.243</td>
<td>7.44</td>
<td>-1%</td>
</tr>
</tbody>
</table>

y = 1.778x + 0.0155

R² = 0.9994
Validation results

Matrix effects (data from analysis sequence)

Peak area of $^{13}$C-DON in solvent standards and urine extracts.

**Matrix effect**
- min: 67%
- max: 84%
- average: 77%

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## Validation results

### Recovery (accuracy) & precision

<table>
<thead>
<tr>
<th>Deoxynivalenol in urine (n=6)</th>
<th>recovery</th>
<th>RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 ng/ml</td>
<td>100%</td>
<td>7%</td>
</tr>
<tr>
<td>1 ng/ml</td>
<td>104%</td>
<td>2%</td>
</tr>
<tr>
<td>5 ng/ml</td>
<td>106%</td>
<td>3%</td>
</tr>
</tbody>
</table>

### Identification*: $t_r$ & ion ratio stability

$t_r$ within sequence within ±0.02 min

<table>
<thead>
<tr>
<th>Ion ratio</th>
<th>solvent</th>
<th>urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>average</td>
<td>51%</td>
<td>50%</td>
</tr>
<tr>
<td>min</td>
<td>47%</td>
<td>48%</td>
</tr>
<tr>
<td>max</td>
<td>52%</td>
<td>51%</td>
</tr>
<tr>
<td>tolerance lower</td>
<td>36%</td>
<td></td>
</tr>
<tr>
<td>tolerance upper</td>
<td>66%</td>
<td></td>
</tr>
</tbody>
</table>

*Guidance Identification criteria SANTE/12089/2016

Validation

LOQ: lowest validated level meeting quan/qual criteria: 0.5 ng/ml
LOD: not established, S/N of low levels spikes and blanks inspected

Chromatograms indicate lower LOQ is feasible
⇒ If needed: additional validation at lower level
Contacts

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RIKILT – Wageningen University & Research

Speaker’s information

Hans Mol is senior scientist at the department of Natural Toxins and Pesticides, RIKILT, Wageningen, The Netherlands. He is an analytical chemist with more than 20 years of experience in determination of pesticides, mycotoxins, and their metabolites in food, environmental and biological samples.

In HBM4EU he is involved in WP9 (9.4 Quality Assurance, organisation of ICI/EQUAS, 9.1 biomarker/method inventory)