science and policy
for a healthy future

HBM4EU project

Determination of aflatoxin biomarkers for acute and chronic exposure

Arnau Vidal, Sarah De Saeger & Marthe De Boevre

2nd HBM4EU Training School 2018

1. Overview

2. Aflatoxin biomarkers

3. Determination aflatoxin biomarkers

4. Quality Control and determination of ¨Unknowns¨

- International Agency for Research on Cancer (IARC)
- Classification according to evidence of carcinogenicity to humans

Group	Classification	Mycotoxins
1	Carcinogenic to humans	**aflatoxins**
2A	Probably carcinogenic to humans	/
2B	Possibly carcinogenic to humans	**ochratoxin A, sterigmatocystin and fumonisins**
3	Not classifiable as to its carcinogenicity to humans	**deoxynivalenol, nivalenol, T-2 toxin, diacetoxyscirpenol, zearalenone, citrinin and fusarenon-X**
4	Probably not carcinogenic to humans	/

- As DON, AFB1 has validated biomarkers

- Acute exposure: AFB-N7-guanine

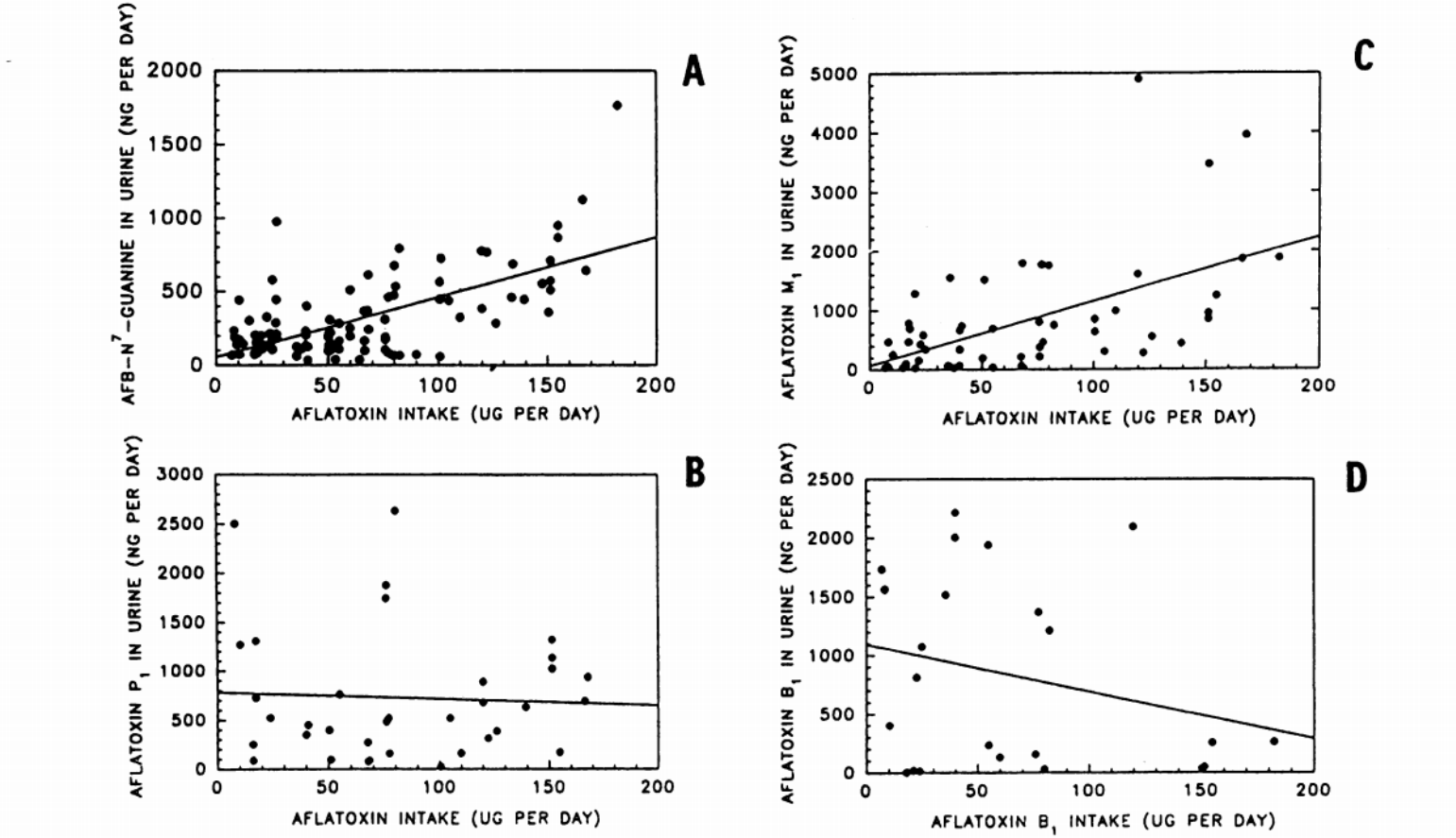


FIGURE 2. Linear regression analysis of daily (*A*) aflatoxin (AFB) N7-guanine, (*B*) AFM_1, (*C*) AFP_1, and (*D*) AFB_1 in urine compared with dietary aflatoxin intake from the previous day (*5*).

Groopman et al., 1992

- Acute exposure: AFB-N7-guanine

- Chronic exposure: AFB1-lysine

  - ✓ AFB1-lysine biomarker validated ELISA (Wild et al., 1992).
  - ✓ AFB1-lysine biomarker validated by LC-MS/MS (McMillan et al, 2018).

2.6 times more specific than ELISA technique

- Chronic exposure: AFB1-lysine
  - ✓ AF-lysine biomarker validated ELISA (Wild et al., 1992).

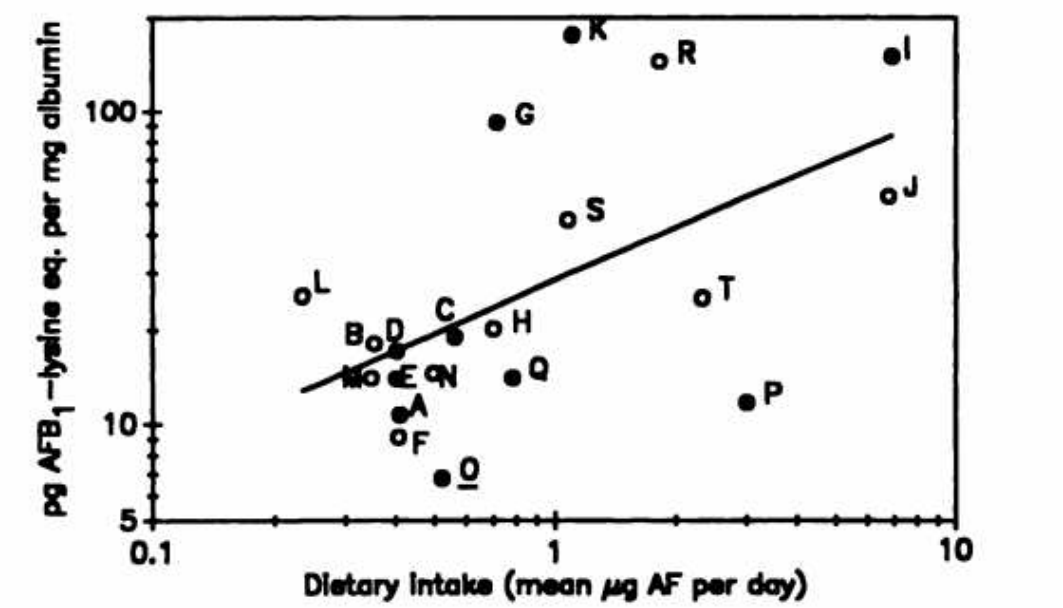
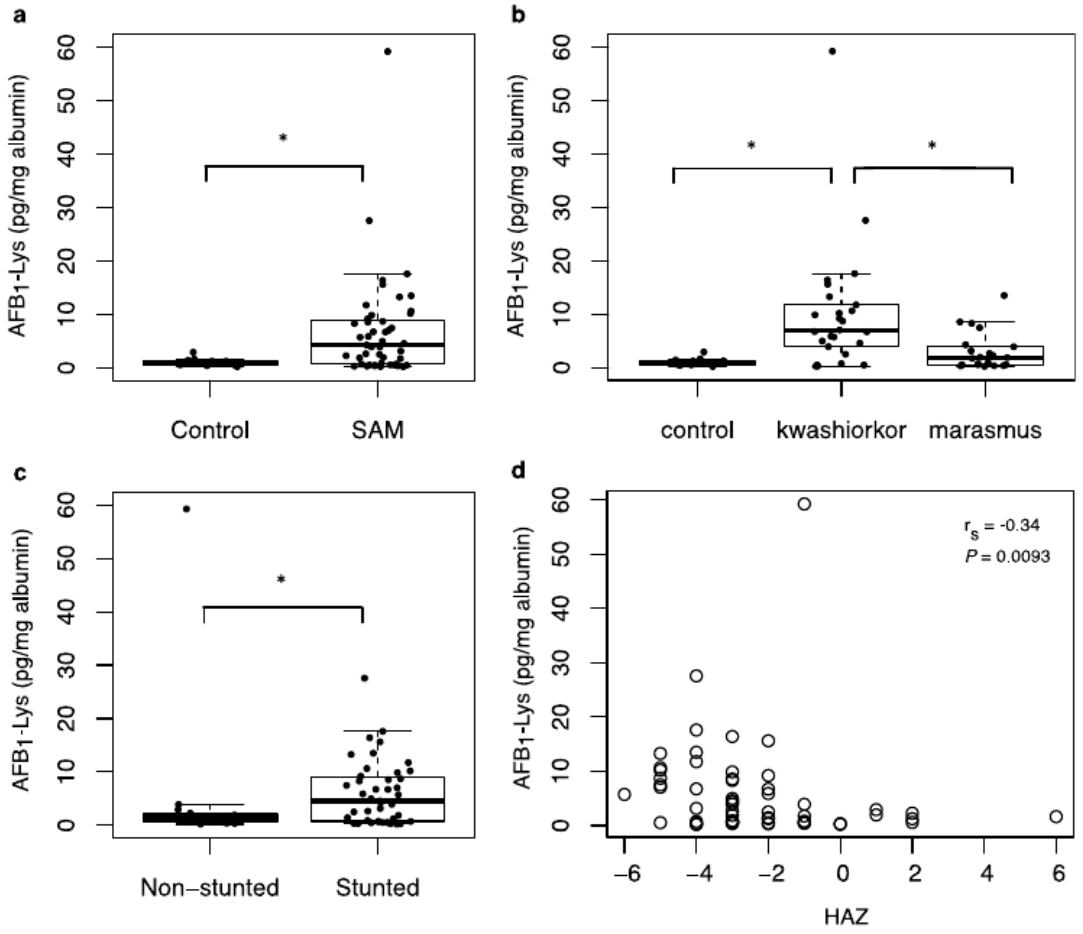


Fig. 4. Mean daily aflatoxin food intake over the 7-day period, plotted against the level of aflatoxin-albumin adduct on day 8 of the study. *Each point* represents one individual. ●, HB_sAg carriers; O, noncarriers. The *letters next to the points* represent the individuals in Table 1. Linear regression is plotted (correlation coefficient, $r = 0.55$; $P < 0.05$ on log-transformed values).

Wild et al., 1992

- Assess mycotoxin exposure with correct mycotoxin biomarker:



McMillan et al., 2018

- However, AF biomarkers in urine:
  - 4 metabolic pathways:
    - ✓ O-dealkylation: AFP1
    - ✓ Keto-reduction: AFL
    - ✓ Epoxidation: AFB1-8,9-epoxide
    - ✓ Hydroxylation: AFM1, AFP1, AFQ1 or AFB2
  - **AFQ1>AFM1** (Mykkanen et al., 2005)
  - **AFP1>AFB1-N7-guanine** (Groopman et al., 1992).
  - Lack of commercial standards

- *In vitro* study

Liver microsomes + mycotoxin + Tris-HCl buffer

Phase I

Nicotinamide adenine dinucleotide phosphate (NADPH)

Phase II

Uridine Diphosphate Glucuronic Acid (UDPGA)

Identification with High Resolution

- AFQ1
- AFP1

- *In vivo* study

Analysis of:

- AFB1 (standard)
- AFB2 (standard)
- AFG1 (standard)
- AFG2 (standard)
- AFM1 (standard)
- AFB1-lys (synthesised)
- AFB1-N7-guanine (synthesised)
- AFQ1 (no standard)
- AFP1 (no standard)

- Isotolabelled C13 AFB1 (Internal standard)

- *In vivo* study
  - Extraction method:
    - IAC columns:
      - ✓ NOT highly checked for AF conjugates.
      - ✓ Expensive.
      - ✓ Long time.
    - ELISA:
      - ✓ Higher LOD.
      - ✓ Less specific.
    - Dilute and shoot:
      - ✓ Small volume.
      - ✓ Fast.
    - Liquid/Liquid extraction:
      - ✓ You can concentrate.
      - ✓ Check recovery.

- *In vivo* study

Coutnry (matrix)	Mycotoxins	Extraction method	Limit of detection (ng/mL)	Average (ng/mL)	Reference
Belgium (Urine)	AFB1 AFB2 AFG1 AFG2 AFM1	Liquid/Liquid	0.001	Not detected	Heyndrickx et al., 2015
Belgium (Urine)	AFM1 AF-guanine	Liquid/Liquid with SPE column	0.01 0.85	Not detected	Njumbe Ediage et al., 2012
Italy (Urine)	AFB1 AFB2 AFG1 AFG2 AFM1	IAC Column	0.010 0.006 0.006 0.004 0.002	0.010 (0.8 %) 0.007 (0.8%) 0.058 (0.8%) 0.057 (11.1%) 0.042 (73.7%)	Ferri et al., 2017
Italy (Plasma)	AFB1 AFB2 AFG1 AFG2 AFM1	IAC Column	0.025 0.025 0.006 0.006 0.025	Not detected	Ferri et al., 2017

- *In vivo* study

Coutnry (matrix)	Mycotoxins	Extraction method	Limit of detection (ng/mL)	Average (ng/mL)	Reference
Ethiopia (Urine)	AFB1 AFB2 AFG1 AFG2 AFM1	Dilute and shoot	0.025	- 0.047 (4.5%) 0.061 (2.5%) 0.068 (3%) 0.064 (7%)	Ayelign et al., 2017
Cameroon (Urine)	AFM1 AFN7guanine	Liquid/liquid	0.01 0.83	0.33 (max = 4.7) (14 %)	Njumbe Ediage et al., 2013
Nigeria (Urine)	AFM1	ELISA	0.06	0.27 (98.8 %)	Chibundu et al., 2018
Malawi (Plasma)	AFB1lys	Liquid/Liquid	0.002	0.023 (73%)	Seetha et al., 2018
Nigeria (Plasma)	AFB1lys	Liquid/Liquid	0.022	0.0026	McMillan et al., 2018

QA-CONTROL in BIOMARKER ANALYSIS using LC-MS/MS

- First line control
- Second line control
- Third line control

- Identification of ‘unknowns’

- First line control
  - ✓ Assurance of a good performance of the analytical device and the correctness of the acquired results.
  - ✓ Analysis according to a quality control (QC)-scheme of a serie of unknown samples

- First line control
  1. A mix of calibrants in pure solvent = standard mix.
  2. Sample with pure injection solvent = mobile phase.
  3. Blank sample (urine/plasma/…).
  4. Spiked samples for the calibration curve (min. 5 points).
  5. Sample with pure injection solvent = mobile phase.
  6. Ten unknown samples.
  7. Control spike.
  8. Ten unknown samples.
  9. Control spike.

- First line control

1. A mix of calibrants in pure solvent = standard mix.
2. Sample with pure injection solvent = mobile phase.
3. Blank sample (urine/plasma/…).
4. Spiked samples for the calibration curve (min. 5 points).
5. Sample with pure injection solvent = mobile phase.
6. Ten unknown samples.
7. Control spike.
8. Ten unknown samples.
9. Control spike.

<table>
<tr><th>What</th><th>Why</th><th>QC criteria</th><th>Consequences if QC-criteria are not fulfilled</th></tr>
<tr>
<td>Control spike</td>
<td>Check quantification during injection-sequence</td>
<td>
• The recovery of each compound needs to range between (concentration dependent):
<table>
<tr><td>Concentratie</td><td>Interval</td></tr>
<tr><td>≤ 1 µg/kg</td><td>50% – 120%</td></tr>
<tr><td>> 1 µg/kg – 10 µg/kg</td><td>70% - 110%</td></tr>
<tr><td>>10 µg/kg</td><td>80% - 110%</td></tr>
</table>
Or as determined as in the method validation (compound specific)

• Set-up a trend analysis!
</td>
<td>
• When recovery is NOT OK: quantification of all samples between control spike and previous control spike are not reliable. Re-analysis!

• The recovery of the control spike needs to be followed-up over a longer period. Visible trends need to be investigated when they falls out of an interval.
</td>
</tr>
</table>

- Second line control
  - ✓ Periodical evaluation (*eg* 2/year).
  - ✓ To check method with the acquired method validation (accuracy, LOD/LOQ, …).
  - ✓ New analyst.
  - ✓ …

  - ✓ Analysis of certified reference material.
  - ✓ Analysis of spiked sample by a third person.
  - ✓ Analysis of a blind, duplicated sample.

- Third line control
  - ✓ Quality control organised by an independent external organisation.
  - ✓ Interlaboratory test.
  - ✓ To compare and evaluate the performance of your developed method with other methods.
  - ✓ At least 1 x 3 years.

• Identification of unknowns

•Fulfilment of **4 identification criteria:**

1. **Minimum of 3** to more identification points because 2 **MRM-transitions** are present.

2. **S/N** every MRM-transition > 3.

3. **Relative peak-area** of selected ions has to correspond with those ions of the spike with a comparable concentration in an acceptable deviation.

4. **Relative retention time** of each MRM-transition should be within a range of 2.5% of the relative retention time of the spiked sample with a comparable concentration.

• Identification of unknowns

•Fulfilment of **4 identification criteria:**

1. **Minimum of 3** to more identification points because 2 **MRM-transitions** are present.

The relationship between a range of classes of mass fragment and identification points earned

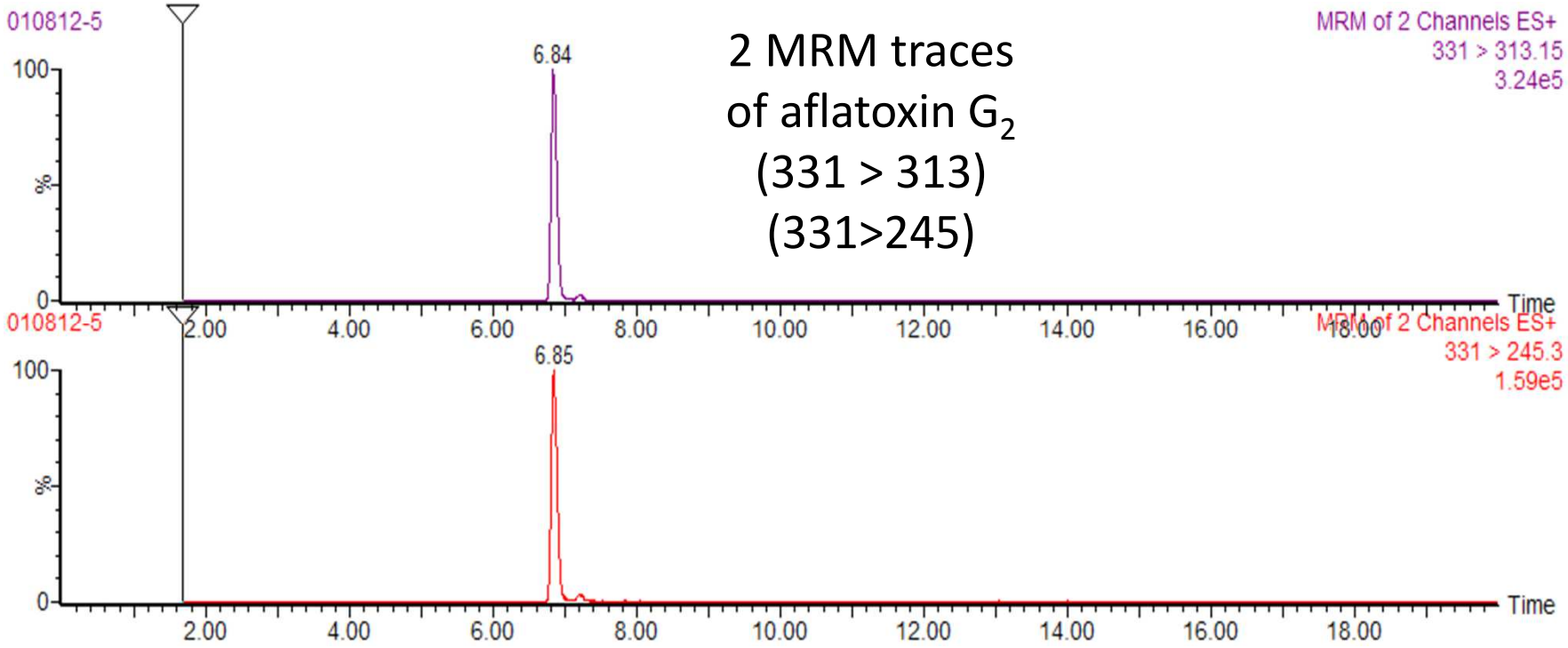
MS technique	Identification points earned per ion
Low resolution mass spectrometry (LR)	1,0
LR-MS^n precursor ion	1,0
LR-MS^n transition products	1,5
HRMS	2,0
HR- MS^n precursor ion	2,0
HR-MS^n transition products	2,5

X 2 (MRM transitions) = 3 identification points

• Identification of unknowns

•Fulfilment of **4 identification criteria:**

2. S/N every MRM-transition > 3.



2 MRM traces of aflatoxin G_2 (331 > 313) (331>245)

• Identification of unknowns

•Fulfilment of **4 identification criteria:**

3. Relative peak-area of selected ions has to correspond with those ions of the spike with a comparable concentration in an acceptable deviation.

Relative intensity (% of mean peak)	***Accepted limits LC-MS/MS***
> 50%	± 20%
> 20% – 50%	± 25%
> 10% - 20%	± 30%
≤ 10%	± 50%

• Identification of unknowns

•Fulfilment of **4 identification criteria:**

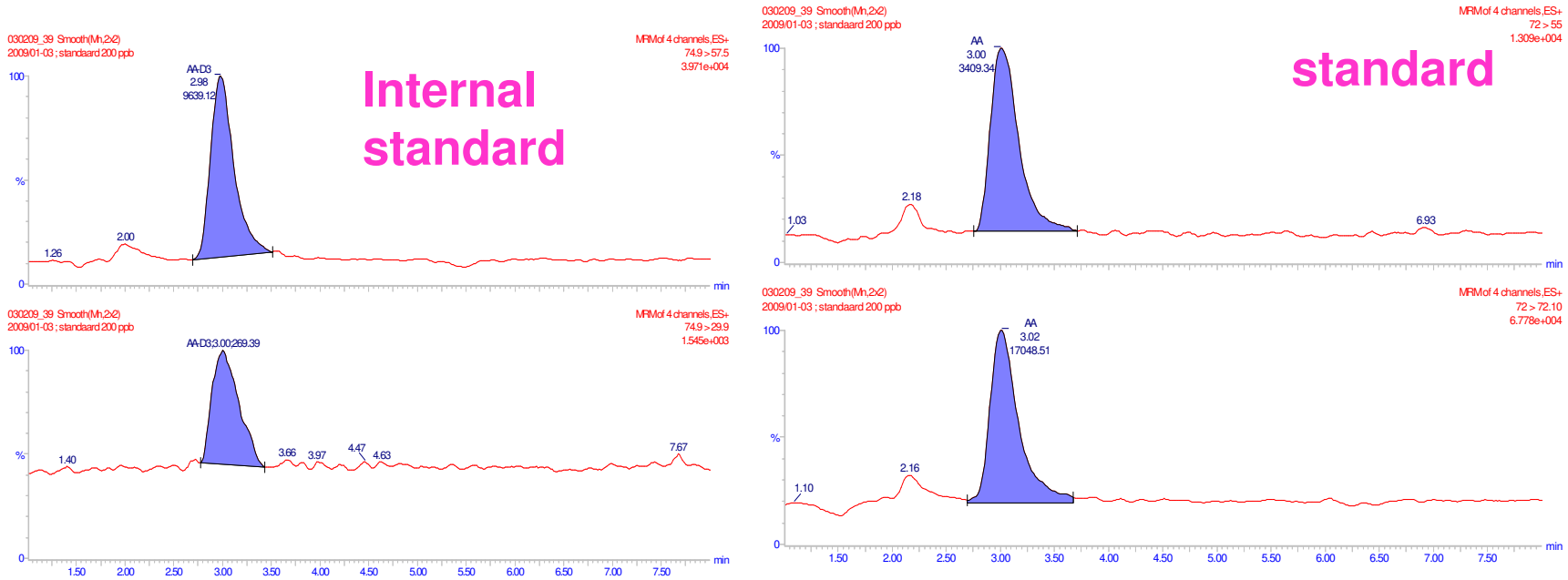
3. Relative peak-area of selected ions has to correspond with those ions of the spike with a comparable concentration in an acceptable deviation.

- Compare with spike with similar concentration.
- Relative peak area spike: (area ion 331>313)/(area ion 331>245) = x
- Range determined on spike: [x - limit; x + limit].
- Relative peak area unknown: (area ion 331>313)/(area ion 331>245) = z
- x - limit < z > x + limit.

- Identification of unknowns

•Fulfilment of **4 identification criteria:**

4. Relative retention time of each MRM-transition should be within a range of 2.5% of the relative retention time of the spiked sample with a comparable concentration.



• Identification of unknowns

•Fulfilment of **4 identification criteria:**

4. Relative retention time of each MRM-transition should be within a range of 2.5% of the relative retention time of the spiked sample with a comparable concentration.

- Compare with calibration standard (spike)
- Relative retention time spike: (RT MYCO)/(RT IS) = r
- Range calculated on spike: [r – 2.5%; r + 2.5%]
- Relative retention time unknown: (RT Unkown)/(RT IS) = t
- r – 2.5% < t > r + 2.5%

- Identification of unknowns

•Fulfilment of 4 identification criteria

1. minimum of 3 to more iden… ecause 2 MRM-transitions are prese…
2. S/N every MRM-tra…
3. relative peak-ar… to correspond with those … a comparable concentra… viation
4. rel… ach MRM-transition should be … of the relative retention time of th… with a comparable concentration.

Only when these 4 criteria are fulfilled, you can state that the mycotoxin biomarker is PRESENT in your unknown sample!

Contacts

Arnau Vidal

Arnau Vidal PhD works as a FWO PostDoc at the Centre of Excellence on Mycotoxicology and Public Health, Ghent University, Belgium.

Email:

Arnau.vidalcorominas@ugent.be

Marthe.deboevre@ugent.be

Sarah.desaeger@ugent.be

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 733032.