HBM4EU science and policy for a healthy future

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ICI report 2nd round substances

Mycotoxins/round_02/2020

Deoxynivalenol biomarkers in urine

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1 Summary

Within the framework of the HBM4EU project, an interlaboratory comparison was organised for the determination of a biomarker for the mycotoxin deoxynivalenol (DON) in urine.

The study was performed in February/March 2020 and was conducted to assess the comparability and reliability of analytical methods across the participating expert laboratories.

The HBM4EU QAU had selected six expert laboratories for mycotoxin biomarkers in urine. The expert laboratories were from six different countries in Europe.

Each participant received two control materials of human urine to be analysed for total DON (total of free and conjugated). The laboratories were requested to perform a single analysis and to submit the results to the organiser within 3 weeks. Due to COVID-19 one laboratory could not analyse the sample before the deadline. An extension of the deadline was granted but after reopening of this laboratory in May, it decided to withdraw from the ICI. Hence, results were obtained from five laboratories.

A first assessment of comparability of results was done by calculation of the mean, the RSD, and the relative uncertainty of the mean. Results were compared against the mean through a Z-score when the relative uncertainty of the mean was within 17.5%. In this study, the uncertainty of the mean of the five laboratories exceeded this value meaning that the overall results were not comparable. It was noticed that, as in the first round, the lowest concentrations were obtained when using β-glucuronidase(/sulfatase) from *Helix Pomatia*. Therefore also in this round the consensus values were based on the results from laboratories using β-glucuronidase from *E. coli*.

All five laboratories reported quantitative results. Results were comparable for four laboratories.

The outcome of this second interlaboratory comparison for mycotoxin biomarkers in urine are summarised in **Table 1**.

Recommendations were made to improve comparability of results in the next round.

Table 1. Comparability of results for the biomarker of the mycotoxin deoxynivalenol (DON) in urine obtained in interlaboratory comparison/round 2.

Biomarker	Test material	Consensus (ng/ml)	Comparable results for X out of Y labs
DON (total)	R2A	32.7 [a]	4/5
	R2B	2.75[a]	4/5

[a] consensus value derived from laboratories using β-glucuronidase from E. coli.

2 Introduction

Mycotoxins have been included in HBM4EU as substances in the 2nd prioritisation round. The selection of the target mycotoxins and their most relevant biomarkers was previously done in WP9, and has been described in Deliverable report 9.5 v2.0 [1]. Based on this, and further considerations by the QAU and experts in the field, it was decided to include only the biomarker of deoxynivalenol (DON) as total DON (free and conjugates) in the anticipated analyses of samples from aligned studies in HBM4EU.

For the 2nd round substances, it was decided by WP9 to select a limited number of expert laboratories for analysis of HBM4EU samples. Laboratories were selected by the QAU according to criteria described in HBM4EU-SOP-QA-005 [2]. The selection criteria included:

1. Experience in analysis of all selected parameters in (the selected) human matrices at levels expected in the general population (proven experience, papers, reports, etc.)
2. Capacity for analysis (number of samples/time for analysis)
3. Limit of quantification of the method, i.e. sufficiently low for HBM4EU samples
4. Historical data of the successful participation in interlaboratory comparison exercises for the target substance (selected parameters)

This interlaboratory comparison is intended to assess the comparability and reliability of the analytical methods that laboratories will use for determination of the DON biomarker in samples analysed in the frame of HBM4EU. It forms an integral part of quality control, in addition to initial and ongoing in-house method validation.

This study has been organised by Wageningen Food Safety Research (WFSR) in the Netherlands, as part of the Quality Assurance program for biomonitoring analyses within the frame of HBM4EU. Participation in this exercise is mandatory for laboratories that will analyse HBM4EU samples.

This report describes the outcome of the 2nd round of interlaboratory comparisons for the DON biomarker in urine.

2.1 Confidentiality

In this report, the identity of the participants is treated as confidential. However, lab codes of the participants will be disclosed to the HBM4EU-QAU for performance assessment.

3 Control material

3.1 Preparation of control material

For this study, two control materials were prepared (R2A = material A, and R2B = material E). Burdened urine samples were used that were known to contain DON biomarkers at two different concentrations in the range expected for the general population.

The control materials were mixed and then aliquoted (5 ml) into coded polypropylene tubes with screwcap. The tubes were stored in the freezer (<-18°C). Part of the tubes were stored at -80°C as reference for future stability testing.

3.2 Homogeneity of control material

Homogeneity testing was done as described in HBM4EU-SOP-QA-002 [3]. Five tubes were randomly selected from the freezer and analysed in duplicate. The analysis results were processed according to the SOP using an Excel macro ("HBM4EU macro homogeneity test v1.xlsm"). The mean concentrations and relative standard deviations (RSD) as obtained during homogeneity testing, are included in Appendix 1. It was concluded that homogeneity was adequate for both control materials for the purpose of this interlaboratory comparison.

3.3 Stability of control material

For assessment of storage stability the procedures have been described in HBM4EU-SOP-QA-002 [3]. Previous assessments done by the organiser had shown that DON biomarkers do not significantly degrade when stored at -18°C during the period of the conduct of the interlaboratory comparison. Experimental verification will be done to confirm this at a later stage (during/after the 3rd round).

4 Organisational details

4.1 Participants

For the organisation of this 2nd interlaboratory comparison, WFSR contacted the six selected expert laboratories (HBM4EU laboratories from six different countries in Europe) and sent them an announcement letter by e-mail on December 17, 2019. The biomarker to be determined and the required LOQ were mentioned. It was indicated that the laboratories would receive in two test samples, to be analysed for total DON. Participation was free of charge. For this second round, a further announcement on planning was sent by mail on 7th February. Test results had to be submitted within the stipulated deadline (16th March, 2020).

Results were received within the deadline from five laboratories. One laboratory had to close down due to COVID-19 before it analysed the samples. An extension of the deadline was granted but after reopening of this laboratory in May, it withdraw from the ICI. Hence, results were obtained from five laboratories.

4.2 Dispatch and instructions

The test materials for determination of DON (5 ml each) were dispatched to the participants on 24th February. The samples were packed in an insulation box with ice packs and sent by courier. Instructions were sent by e-mail at the day of shipment (see **Appendix 2**). Participants were asked to check the content of the box upon receipt, to store the samples in the freezer, and to carry out a single analysis of the samples according to their routine method. The deadline for submission of results was 16th March 2020, which was extended for one laboratory due to COVID-19 (see 4.1).

For reporting of results an excel sheet was provided. In this excel sheet the participants were asked to report the biomarker concentration in ng/ml. In addition, the participants were asked to provide their method details (i.e. LOQ, deconjugation, cleanup, analysis technique, internal standards used, precision data). Based on observations from the first round, more details was asked for the enzyme and conditions used for deconjugation.

4.3 Deviations from SOPs

For the interlaboratory comparison, the HBM4EU-QA-SOPs [2,3] were followed. There were no deviations from the relevant SOPs, with the exception of the use of 5 replicate analysis (instead of 10) for homogeneity testing. The reason was the limited amount of control material available. This deviation was considered not to have an effect on the study outcome.

5 Data evaluation

Evaluation of comparability of the data was done according to HBM4EU-SOP-QA-005 [2]. This involves establishing a consensus value and assessing the deviation of the individual results from the consensus value by calculation of Z-scores.

5.1 Consensus value

The mean concentration derived from the expert laboratories is considered an acceptable consensus value in the interlaboratory comparison study when the relative uncertainty of the mean is ≤17.5%.

The relative uncertainty of the mean, is given by:

u = RSD / sqrt(N)

with u = relative uncertainty of the mean concentration from the expert labs

RSD = relative standard deviation of the mean concentration

N = the number of expert labs (after exclusion of outliers if applicable)

In case the uncertainty of the mean exceeds 17.5%, the results are checked for outliers using a Grubbs' test. If an individual value is identified as an outlier, it is rejected from the data set and the relative uncertainty calculated again when N is still ≥3. If u is still >17.5%, then no meaningful consensus expert value can be derived, and no objective reliable quantitative comparability assessment can be done.

It is recognised that with the small number of participants it could be less likely that outliers can be identified through statistical tests.

5.2 Target standard deviation (σ_T)

For calculation of the Z-scores, a fit-for-purpose relative target standard deviation (FFP-RSD_R) of 25% of the consensus value was used as target standard deviation.

5.3 Z-scores

The Z-score (Z) was calculated as follows:

$$Z = \frac{x - C}{\sigma_T}$$

with x = result submitted by the laboratory;

C = consensus value;

σ_T = target standard deviation, here 0.25*C

When the Z-score is within -2 and +2 (-2 ≤ Z≤ 2), the results are considered sufficiently comparable.

6 Results and discussion

6.1 Results submitted by participants

In total, six laboratories from six European countries participated in this study. Quantitative results were reported by five laboratories. One laboratory withdraw from the ICI, due to COVID-19 related issues. The individual results of the laboratories are included in **Appendix 3**.

6.2 Analysis methods

The method details provided by the laboratories are included in **Appendix 4 and 5**.

For the determination of total DON, all laboratories used a method involving enzymatic deconjugation and determination of the total free DON by LC-MS (various MS techniques). The volume of urine used for the analysis varied from 0.5-3.0 ml. For deconjugation three laboratories used ß-Glucuronidase from *E. coli*, and two laboratories used ß-Glucuronidase or ß-Glucuronidase/ Arylsulfatase from *Helix Pomatia*, after adjustment of the pH to a certain value. Deconjugation was performed at 37°C for 15 (overnight) to 24 hours (full details on enzyme/conditions used are included in **Appendix 5**). In all cases a cleanup step was performed (involving a concentration at the same time in most cases). Three laboratories used a dedicated immuno-affinity column (IAC) cleanup. Two laboratories used a more generic SPE cleanup procedure. The extracts were analysed by LC triple quadrupole LC-MS/MS (3x), LC-MS/HRMS (Q-Orbitrap, 1x), and LC-HRMS (Orbitrap, 1x). With one exception, all laboratories used isotopically labelled DON as internal standard, added to the urine sample before deconjugation (3x), or to final extract before LC-MS/MS analysis (1x). MS measurement was done as positive ion (2x) or negative ion (with acetate adduct as precursor, 3x).

6.3 Consensus values

For all biomarkers the mean, (R)SD and the relative uncertainty of the mean were determined. The results are included in **Appendix 3**. As in the first round, laboratories using enzymes from *Helix Pomatia* reported the lower concentrations for total DON compared to the laboratories using enzymes from *E.coli*. The means of the two sub-sets were significantly different (t-test, 95% confidence). The lower total DON concentration in case of *Helix Pomatia* had also been observed by the organiser in a previous study. To gain more insight in the effect of the enzyme/conditions on total DON for the materials used in current ICIs, additional experiments were performed by the organiser. As a first step, two enzymes/conditions were compared: ß-Glucuronidase from *E. coli* (pH 6.8) and ß-Glucuronidase/ Arylsulfatase from *Helix Pomatia* (pH 4.5), both overnight, 37°C. With the *Helix Pomatia* based enzymes the total DON concentration was 1-37% lower, with an average of 20%. Two of the participating expert laboratories also compared *Helix Pomatia* and *E.coli* based enzymes, in one case using samples from round 1, in the other case from round 2. One laboratory also found a substantial difference (*E.coli* giving higher concentrations for total DON), the other found only minor differences. It is clear that the enzyme/conditions used may affect the concentration of total DON obtained, and that the effect appears not to be the same for all urine samples. A more detailed follow up experiment will be done by the organiser at a later stage. For now, the intermediate conclusion is that enzymes from *E.coli* result in the same or higher concentrations of total DON compared to enzymes from *Helix Pomatia*. For this reason, as was done in the first round, it was decided to calculate the consensus value based on the results from the three laboratories using enzymes from *E.coli*. The uncertainty of the mean of these three was <17.5%. The assigned values for the R2A (material A) and the R2B (material E) were 32.7 and 2.75 ng/ml, respectively.

6.4 Assessment of laboratory performance

The performance of the individual laboratories for each of the biomarkers was assessed by the determination of Z-scores based on the assigned value (consensus value of three labs in this case) and a fit-for-purpose relative standard deviation of 25% (see 5.3). The results are considered sufficiently comparable when Z-scores are within -2 and +2 ($-2 \leq Z \leq 2$). For information, as additional indication for comparability, the deviation of the individual results relative to the consensus value is also included in **Appendix 3**.

Comparable results were obtained for four laboratories. This included one laboratory using enzymes from *Helix Pomatia* which had a negative bias but still within the range considered acceptable. The other laboratory (MEL4), also using *Helix Pomatia*, had a stronger negative bias and Z-scores below -2. Of course, besides the deconjugation conditions, other factors may also play a role in the variability of analysis results. Not using labelled DON as internal standard (MEL4) could have been another factor.

6.5 Conclusions and recommendations

A second interlaboratory comparison was done for the biomarker of the mycotoxin deoxynivalenol (DON) in urine amongst six selected HBM4EU laboratories.

Conclusions:

- Comparable results were obtained for both samples by four out of five laboratories.
- As in the first round, a significant difference between use of two enzymic deconjugation procedures was observed.
- The use of *Helix Pomatia* ß-Glucuronidase(/Arylsulfatase) for deconjugation appears to give lower concentrations of total DON, although the effect may differ for different urine samples.
- Standardizing enzymatic deconjugation may improve comparability of results for total DON.

Recommendations

- Laboratories should be aware that deconjugation of DON-glucuronides may be affected by the enzyme/conditions and are encouraged to verify this themselves.
- One laboratory did not use the isotopically labelled internal standard. The use is a general recommendation, especially in LC-MS-based urine analysis.

7 References

[1] Deliverable Report D 9.5 Prioritised list of biomarkers, matrices and analytical methods for the 2nd prioritisation round of substances, v2.0. https://www.hbm4eu.eu/deliverables/

[2] HBM4EU-SOP-QA-005 “Organisation of the Quality Assurance and Quality Control Program for the 2nd prioritized substances”

[3] HBM4EU-SOP-QA-002 “Preparation of test materials for ICI / EQUAS”

Appendix 1 Homogeneity data

	Control material A			Control material E	
	total DON			total DON	
	replicate-1	replicate-2		replicate-1	replicate-2
1	33.75	31.79		2.23	2.00
2	33.20	33.23		2.13	2.26
3	33.60	32.86		2.32	2.00
4	32.39	34.29		2.18	2.10
5	32.47	33.94		2.24	2.13
6					
7					
8					
9					
10					
grand mean	33.151			2.159	
Stdev	0.779			0.106	
VC%	2%			5%	
Cochran's test					
C	0.377			0.538	
Ccrit	0.841			0.841	
C < Ccrit →	No outliers detected			No outliers detected	
target σ_{FFP}	8.288			0.540	
s_x =	0.221			0.0321	
s_w =	1.008			0.1360	
s_s =	0.000			0.0000	
critical=0.3σ_{FFP}	2.486			0.1619	
s_s < critical?	Homogeneity adequate			Homogeneity adequate	
s_w< 0.5*σ_{FFP}?	Method suited			Method suited	

Appendix 2 Copy of letter of instructions sent together with test samples

WAGENINGEN
UNIVERSITY & RESEARCH

HBM4EU science and policy for a healthy future

Postbus 230 | 6700 AE WAGENINGEN The Netherlands

WFSR

DATE
February 24th, 2020

SUBJECT
Instruction letter for ICI study mycotoxin biomarkers in urine

OUR REFERENCE
HBM4EU ICI-DON R2

POSTAL ADDRESS
Akkermaalsbos 2
6708 WB, Wageningen
The Netherlands

INTERNET
www.wur.nl

CoC NUMBER
09098104

HANDLED BY
Hans Mol

TELEPHONE
+31 317 480318

EMAIL
hans.mol@@wur.nl

Dear participant,

Thank you for participation in the interlaboratory comparison study **HBM4EU ICI-Mycotoxins/round_02** for the determination of total deoxynivalenol (DON) in human urine.

You will receive a parcel containing 2 urine samples. Each sample contains 5 ml of urine.

Instructions:

- Upon receipt, store the samples in the freezer until analysis.
- Confirm the receipt by email to hans.mol@wur.nl
  You should receive the sample in frozen condition. If not or in case of damage, please indicate that in your mail.
- Before analysis, thaw and re-homogenize the samples according to your laboratory's procedure.
- Please carry out a **single analysis** for each sample.
- Results are to be reported as total DON, in ng/ml urine.
- For reporting, please use the excel file provided by us through email: "ICI-study Mycotoxins-DON-round_02_results and method information v1.xlsx'. Also provide your method details through this excel sheet.
- The deadline for submission of results is strict and is **16th March 2020.**

Please contact us if you have any questions or need any assistance.

With kind regards,

Hans Mol hans.mol@wur.nl
Hester van den Top hester.vandentop@wur.nl
Ingrid Elbers pt.wfsr@wur.nl

100years 1918 — 2018

Appendix 3: Overview results DON biomarker Round-2.

Biomarker	total DON					
Control material	R2A (material A)			R2B (material E)		
Conc. hom. test (ng/ml)	33.2			2.16		
Assigned value (ng/ml)	32.7			2.75		
Rel. uncertainty	13%			13%		
Lab code	ng/ml	%DA	Z	ng/ml	%DA	Z
MEL1 (a)	40.5	24%	0.9	3.41	24%	1.0
MEL2	did not analyse			did not analyse		
MEL3 (a)	31.8	-3%	-0.1	2.71	-1%	-0.1
MEL4 (b)	10.8	-67%	-2.7	1.09	-60%	-2.4
MEL5 (b)	19.9	-39%	-1.6	1.730	-37%	-1.5
MEL6 (a)	25.9	-21%	-0.8	2.13	-23%	-0.9
N	5			5		
mean	25.8			2.2		
SD	11.31			0.89		
RSD	44%			40%		
Rel. uncertainty	20%			18%		
N (a)	3			3		
mean (a)	32.7			2.750		
SD (a)	7.34			0.641		
RSD (a)	22%			23%		
Rel. uncertainty (a)	13%			13%		
N (b)	2			2		
mean (b)	15.35			1.410		
SD (b)	6.435			0.453		
RSD (b)	42%			32%		
Rel. uncertainty (b)	30%			23%		

%DA = percent deviation from consensus value

Z = Z-score

(a) deconjugation using E. Coli ß-Glucuronidase

(b) deconjugation using Helix Pomatia ß-Glucuronidase(/Arylsulfatase)

The means of (a) and (b) differed significantly, consensus is based on (a).

Appendix 4: Method details for determination of total DON in urine, provided by the laboratories.

		PRETREATMENT					
Lab	LOQ (ng/ml)	Pretreatment	urine aliquot used (ml)	pH adjustment (provide buffer and pH)	Deconjugation	time(hrs) / temp (°C)	post deconjugation adjustment of sample (pH, dilution, ...)
MEL1	0.5	centrifugation	0.5	pH 7.4, PBS	E. coli B-glucuronidase	16h / 37 °C	none
MEL2	samples not analysed						
MEL3	0.5	none	1.0	pH 6.8, phosphate buffer (75 mM)	E. coli B-glucuronidase	over night / 37 °C	none
MEL4	0.1	none	2.0	pH 4.7, acetate buffer (0.1M)	Helix Pomatia (B-glucuronidase)	24 / 37	-
MEL5	0.3	centrifugation	2.5	pH 5, acetate buffer	Helix Pomatia (B-gluc/sulfatase)	20h, 37°C	PBS buffer, pH = 7.4
MEL6	0.05	none	3.0		E. coli B-glucuronidase	15h / 37°C	

	EXTRACTION & CLEANUP		INSTRUMENTAL ANALYSIS					
Lab	Technique	specify IAC or SPE column or LLE solvent	Separation technique	injection volume (µl)	Column	Detection technique	for MS(/MS): ionisation	Quantifier transition/ion (m/z x>y)
MEL1	SPE (off-line)	Oasis HLB Prime 30cc	(U)HPLC	10	Acquity HSS T3 1.8µm (100x2.1mm)	MS/MS (triple/Qtrap)	ESI neg	354.9 -> 265.0
MEL2								
MEL3	IAC	DONTEST (VICAM)	(U)HPLC	10	Restek, Ultra Aqueous C18 3 µm 100x2,1 mm	MS/MS (triple/Qtrap)	ESI-pos	297 > 249
MEL4	IAC	DONPREP (R-Biopharm)	(U)HPLC	10	Acquity UPLC HSS T3 (100x2.1 mm, 1.8 µm	Orbitrap	ESI neg	m/z 265.1081
MEL5	IAC	DON-Star	(U)HPLC	20	Biphenyl column	MS/MS (triple/Qtrap)	ESI neg	355 > 59
MEL6	SPE (off-line)	Oasis HLB	(U)HPLC	20	C18	Orbitrap	ESI pos	

Appendix 4 continued. Method details for determination of total DON in urine, provided by the laboratories.

	QUANTIFICATION			CRITERIA USED FOR IDENTIFICATION		
Lab	specify which internal standard you used for quantification	moment of addition of internal standard to sample?	Preparation of calibration standards	retention time tolerance used (minutes or % from ref. std)	for MS(/MS): number of ions/transitions used for identification	ion ratio tolerance used (% relative or absolute from ref. std)
MEL1	$^{13}C_{20}$-DON	to final extract	cal stds prepared in blank urine processed as samples	<2%	2	<20%
MEL2						
MEL3	13C20-DON	before deconjugation	cal stds prepared in solvent/eluent	0.1	2	±30%
MEL4	none	not applicable	cal stds prepared in solvent/eluent	0.05 min	HRMS - 3 ions	± 20 %
MEL5	fully 13C labeled DON	before deconjugation	cal stds prepared in blank urine processed as samples	±15 s	1	±20%
MEL6	DON (13C15)	before deconjugation	cal stds prepared in blank urine processed as samples	0.03 min		

Appendix 5. Details on enzyme/conditions used for deconjugation.

	MEL1	MEL2	MEL3	MEL4	MEL5	MEL6
Enzyme	β-Glucuronidase from Escherichia coli	samples not analysed	β-Glucuronidase from Escherichia coli	Beta-glucuronidase from Helix pomatia	Helix Pomatia (β-Glucuronidase/Arylsulfatase)	E. coli B-glucuronidase
Specification/description	Type IX-A, lyophilized powder, 1,000,000-5,000,000 units/g protein (30 min assay)		Type IX-A, lyophilized powder, 1,000,000-5,000,000 units/g protein (30 min assay)	Type HP-2, aqueous solution, ≥ 100 000 units/mL	The glycosides that β-D-glucuronic acid forms with a variety of compounds containing hydroxyl groups, hydrolyse readily in the presence of β-glucuronidase. Sulfate esters of many phenols are hydrolyzed in the presence of arylsulfatase.	type IX-A lyophilized powder 1,000,000-5,000,000 units/g protein (30 min assay)
Source of enzyme (not necessarily the same as the	Sigma-Aldrich		Sigma-Aldrich	Sigma Aldrich / Merck	Roche	Sigma-Aldrich
Amount	727 mg			25 mL	10 mL	250 KU
Supplier	Sigma-Aldrich		Sigma-Aldrich	Sigma Aldrich / Merck	Sigma Aldrich	Sigma-Aldrich
Article number	G7396 500KU		G7396 250KU	G7017	10127698001	G7396-250KU
Batch number	SLBQ5263V		028M4113V	SLCB5079 (exp. 01/2021)	38964024	028M4113V
Units	500 KU		250 KU	≥ 100 000 units/mL		250 KU
Solution/dilution prepared in lab (if applicable)	dissolved in PBS, pH 7.4; diluted with PBS to reach a final concentration of 3000U per 0.5mL		entire content dissolved in 20 ml 75 mM phosphate buffer, pH 6.8 resulting in 12,500 units/ml	preparation of a mixture of beta-glucuronidase (200 µL) and buffer (4.8 mL)	-	Solution 3000 Units/ml in 0,6M Ammonium acetate .
volume of enzyme solution added to urine	0.5mL containing 3000U in PBS; pH 7.4		240 µl (=3000 units)	equivalent of 80 µL, corresponding to 4000 units/mL of urine	40 µl	0,6 mL (=1800 Units)
volume urine	0.5 mL		1.0 ml	2 mL	2,5 ml	3 mL
Buffer / buffer solution added to urine (if applicable)	PBS, pH 7.4		2 ml phosphate buffer 75 mM pH 6.8	2 mL of acetate buffer with beta-glucuronidase	acetic acid/ acetate buffer, pH = 5	