HBM4EU science and policy for a healthy future

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ICI report 2nd round substances

Mycotoxins/round_03/2020

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 June/July 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. Due to COVID-19 related capacity issues, one expert lab could no longer participate in the program.

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.

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%. This was the case for one sample (R3A). For that sample, the results reported by all five labs were comparable. For the other sample (R3B), the uncertainty of the mean of the five laboratories was too high. No reliable mean could be established and no z-scores assigned.

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

Table 1. Comparability of results for the biomarker of the mycotoxin deoxynivalenol (DON) in urine obtained in interlaboratory comparison/round 3.

Biomarker	Test material	Consensus (ng/ml)	Comparable results for X out of Y labs
DON (total)	R3A	3.08 [a]	5/5
	R3B	(1.00)[b]	-

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

[b] uncertainty of the mean too high, results of the labs were not comparable. The value between brackets is concentration as established during homogeneity study.

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 3rd 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 (R3A = material C, and R3B = material F). Human 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 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]. After preparation and characterisation of the six control materials used in the three ICI rounds (Dec 2019/Jan 2020), tubes were stored at -18°C. In addition, a set of tubes was stored at -80°C. The stability of total DON in urine when stored at -18°C was determined by analysis of six samples stored at -18°C against six samples stored at -80°C. Here is was assumed that no degradation occurs at -80°C. The two sets of six samples for each control material were analysed on 24.07.2020. No statistic instability was detected. In addition, results of the samples stored at -18°C and analysed in July 2020 were also compared to the initial analysis by WFSR in January 2020. Differences were below 10% (for material D 12%), further proving stability and consistency of results after storage/re-analysis. A summary of the results is provided in **Appendix 2**. Based on this, it can be concluded that total DON in urine is stable for at least 6 months.

4 Organisational details

4.1 Participants

For the organisation of this 3rd 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 two test samples, to be analysed for total DON. Participation was free of charge. For this third round, further announcements on planning were sent by mail on 18th May and 18th June, to find out and ensure all laboratories were operational again following COVID-19 lockdowns, and able to receive and analyse samples within three weeks' time. One laboratory re-confirmed withdrawal from the ICI.

Results were received within the deadline from the participating five laboratories.

4.2 Dispatch and instructions

The test materials for determination of DON (5 ml each) were dispatched to the participants on 23rd June. The samples were packed in an insulation box with dry ice and sent by courier. Instructions were sent by e-mail at the day of shipment (see **Appendix 3**). 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 13th July.

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, five laboratories from five European countries participated in this study. Quantitative results were reported by all five laboratories. The individual results of the laboratories are included in **Appendix 4**.

6.2 Analysis methods

The method details provided by the laboratories are included in **Appendix 5 and 6**.

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 four laboratories used ß-Glucuronidase from *E. coli*, and one laboratory used ß-Glucuronidase 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 6**). 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 4**. In the previous two rounds, a significant difference in total DON concentrations was observed between laboratories using enzymes from *Helix Pomatia* and laboratories using enzymes from *E.coli* for deconjugation. In this round, only one laboratory used enzymes from *Helix Pomatia*. The result obtained by that lab was not the lowest, and no indication for a difference could be observed in this round. Based on experience and the data from the previous two rounds, it is still believed that the enzymatic conditions matter. However, to make a conclusive statement, additional experiments with the control materials at the various deconjugation conditions by a single laboratory is required to eliminate interlaboratory variability. The organiser is planning to do this experiment.

For material C (R3A), a consensus value could be derived from the data, and it did not make a difference when all data (N=5) or only the data from the laboratories using *E.coli*-based enzymes for deconjugation were used (means were 3.08 and 3.10 ng/ml, respectively). For sake of consistency with previous round, the mean from E.coli was used.

For material F (R3B), a relatively high variability of results was observed. The relative uncertainty of the mean exceeded the 17.5% criterion, both when taking all five results into account, and also when using only the data from the four laboratories using E.coli-based enzymes for deconjugation. No Grubbs' outliers could be identified. Since statistical outliers are difficult to detect with such small data sets, the uncertainty was also calculated when eliminating the lowest (MEL5) or highest (MEL1) result. Elimination of MEL5 did not result in an acceptable uncertainty. Elimination of MEL1 did result in an acceptable uncertainty of the mean determined based on the remaining four values, but not when using the mean of the three remaining labs using E.coli. It appears that the result reported by

MEL1 was less comparable with the others, but this could not be made statistically sound. Therefore, at this stage, it was concluded that the variability of the data reported by the five labs for material F was too high to come to a consensus. For this sample, the labs were not providing sufficiently comparable results.

6.4 Assessment of laboratory performance

The performance of the individual laboratories for the biomarker was assessed by the determination of Z-scores based on the assigned value (consensus value), 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 4**.

For material C (R3A) comparable results were obtained for all five laboratories.

For material F (R3B) the variability of results reported by the labs was too high, no reliable consensus value could be established, and the performance of the individual labs could not be adequately assessed. As indicated in 6.3, the result reported by MEL1 appeared relatively high.

6.5 Conclusions and recommendations

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

Conclusions:

- Completion of stability test by the organiser: total DON in urine was demonstrated to be stable for at least 6 months when stored at -18°C.
- Comparable results were obtained for one sample (material C) by all five laboratories.
- For material F, the variability of results reported by the five labs was too high, i.e. results were not comparable.
- In this round, no indication for a difference between use of two enzymic deconjugation procedures was observed (more difficult to assess since only one lab used *Helix Pomatia* ß-Glucuronidase).

Recommendations

- For a final conclusion on the effect of deconjugation conditions on the total DON concentration measured, and to gain insight in the contribution of this in the interlab variability of results, additional experiments with the control materials at the various deconjugation conditions within the same lab is required.
- 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 C			Control material F	
	total DON			total DON	
	replicate-1	replicate-2		replicate-1	replicate-2
1	4.17	4.13		1.03	1.00
2	4.23	4.18		0.89	1.03
3	4.39	4.39		1.01	0.99
4	4.31	4.19		1.01	1.02
5	4.10	3.98		0.97	1.08
6					
7					
8					
9					
10					
grand mean	4.208			1.005	
Stdev	0.128			0.050	
VC%	3%			5%	
Cochran's test					
C	0.452			0.582	
Ccrit	0.841			0.841	
C < Ccrit →	No outliers detected			No outliers detected	
target σ_{FFP}	1.052			0.251	
s_x =	0.128			0.0253	
s_w =	0.058			0.0589	
s_s =	0.121			0.0000	
critical=0.3σ_{FFP}	0.316			0.0753	
s_s < critical?	Homogeneity adequate			Homogeneity adequate	
s_w< 0.5*σ_{FFP}?	Method suited			Method suited	

Appendix 2 Summary of results obtained during assessment of stability of total DON in urine.

		Homogeneity test (N=10)				Analysis RIKILT		Stability measurements (N=6)				
			average						stored -80°C		stored -18°C	
Round	CM	Date	ng/ml	RSD	remark	Date	ng/ml	Date	ng/ml	RSD	ng/ml	RSD
R1A	B	17.12.2019	9.41	4%	w/o ILIS	08.01.2020	11.2	24.07.2020	12.2	3%	12.0	3%
R1B	D	17.12.2019	0.481	9%	w/o ILIS	08.01.2020	0.707	24.07.2020	0.793	3%	0.806	3%
R2A	A	07.01.2020	33.2	2%	with ILIS	08.01.2020	31.8	24.07.2020	33.6	2%	32.8	5%
R2B	E	17.12.2019	2.16	5%	w/o ILIS	08.01.2020	2.71	24.07.2020	2.86	3%	2.88	3%
R3A	C	07.01.2020	4.21	3%	with ILIS	08.01.2020	3.98	24.07.2020	4.29	3%	4.30	2%
R3B	F	07.01.2020	1.00	5%	with ILIS	08.01.2020	1.08	24.07.2020	1.07	4%	1.09	7%

Appendix 3 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
June 23rd, 2020

SUBJECT
Instruction letter for ICI study mycotoxin biomarkers in urine

OUR REFERENCE
HBM4EU ICI-DON R3

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_03** 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_03_results and method information v1.xlsx'. Also provide your method details through this excel sheet.
- The deadline for submission of results is **13th July 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

Wageningen Research Foundation/WFSR is part of Wageningen University & Research. WFSR carries out research into the safety and reliability of food and feed. WFSR is ISO 17025 and ISO 17043 accredited (the accredited tests are described on www.rva.nl (no. L014 and R013).

Appendix 4: Overview results DON biomarker Round-3.

Biomarker	total DON					
Control material	R3A (material C)			R3B (material F)		
Conc. hom. test (ng/ml)	4.21			1.00		
Assigned value (ng/ml)	3.08			*		
Rel. uncertainty	13%			27%		
Lab code	ng/ml	%DA	Z	ng/ml	%DA	Z
MEL1 (a)	2.75	-11%	-0.4	1.87	*	*
MEL2	did not participate			did not participate		
MEL3 (a)	3.98	29%	1.2	1.08	*	*
MEL4 (b)	3.20	4%	0.2	0.886	*	*
MEL5 (a)	2.19	-29%	-1.2	0.492	*	*
MEL6 (a)	3.39	10%	0.4	0.849	*	*
N	5			5		
mean	3.10			1.04		
SD	0.67			0.51		
RSD	22%			50%		
Rel. uncertainty	10%			22%		
N (a)	4			4		
mean (a)	3.08			1.07		
SD (a)	0.78			0.58		
RSD (a)	25%			54%		
Rel. uncertainty (a)	13%			27%		
N (b)						
mean (b)						
SD (b)						
RSD (b)						
Rel. uncertainty (b)						

%DA = percent deviation from consensus value

Z = Z-score

(a) deconjugation using E. Coli ß-Glucuronidase

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

mean (a) was used for consensus for sake of consistency with the previous rounds. Taking mean of all did not make a difference for comparability.

Appendix 5: 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	did not participate						
MEL3	0.5	none	1.0	pH 6.8, phosphate buffer (75 mM)	E. coli B-glucuronidase	over night / 37 °C	none
MEL4	0.2	none	2.0	pH 4.7, acetate buffer (10 mM)	Helix Pomatia (B-glucuronidase)	24 / 37	-
MEL5	0.3	centrifugation	2.5	PH 6.8 phosphate buffer	E. coli B-glucuronidase	20h, 37°C	-
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 5 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 6. Details on enzyme/conditions used for deconjugation.

	MEL1	MEL2	MEL3	MEL4	MEL5	MEL6
Enzyme	β-Glucuronidase from Escherichia coli	did not participate	β-Glucuronidase from Escherichia coli	Beta-glucuronidase from Helix pomatia	β-glucuronidase from E-coli K12	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		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 supplier/vendor)	Sigma-Aldrich		Sigma-Aldrich	Sigma Aldrich / Merck	Roche	Sigma-Aldrich
Amount	727 mg			25 mL	40 µL	250 KU
Supplier	Sigma-Aldrich		Sigma-Aldrich	Sigma Aldrich / Merck	Sigma Aldrich	Sigma-Aldrich
Article number	G7396 500KU		G7396 250KU	G7017	3707580001	G7396-250KU
Batch number	SLBQ5263V		028M4113V	SLCB5079 (exp. 01/2021)	34341224	028M4113V
Units	688100 U/mg			≥ 100 000 units/mL	≥ 140 U/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		