HBM4EU science and policy for a healthy future

HORIZON2020 Programme
Contract No. 733032 HBM4EU

# ICI / EQUAS REPORT

## DINCH/round_01 (2018)

## DINCH biomarkers in urine

| | |
|---|---|
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**Appendices**

# 1 Summary

Within the frame of the HBM4EU project, an Interlaboratory Comparison Investigation (ICI) was organised on the determination of two DINCH biomarkers in urine.

In total 12 laboratories were invited for this first round, of which 11 laboratories from 9 EU countries (see Appendix 1) registered and submitted results.

In June 2018, each participant received two burdened control materials of human urine, A and B (single tube each), containing DINCH biomarkers at concentrations of approximately 3 and 15 µg/L, respectively.

Homogeneity assessment showed that both materials were sufficiently homogeneous for ICI testing. The stability test demonstrated no significant loss of the biomarkers during the course of the ICI.

For both biomarkers in both test materials, the variability was too high to calculate meaningful consensus values and Z-scores. The ICI interlaboratory relative standard deviation (ICI-$RSD_R$) varied from 46%-70%, exceeding the fit-for-purpose target RSD of 25% in all cases.

Inconsistencies in separation and/or inclusion of biomarker isomers, and in a number of cases the use of internal standards other than the isotope analogue are most likely major reasons for the high variability.

For improvement of DINCH biomarkers determination it is recommended that laboratories use the corresponding isotope labelled internal standard for each of the individual biomarker. Guidance/consensus is needed on the inclusion/quantification of the different isomers.

It had to be concluded that at the moment, classification of labs for HBM4EU determination of DINCH biomarkers through ICIs is not possible.

It is recommended that lab capabilities are improved before initiating the next ICI, and for the 2nd round to organise a combined ICI/EQUAS exercise to ensure a successful QA assessment of HBM4EU laboratories for the determination of DINCH biomarkers.

*Note: as an add-on to this ICI, laboratories were also asked to analyse the urine samples for creatinine. The creatinine concentrations were consistent, the assessment is provided in Appendix 8.*

# 2 Introduction

Interlaboratory Comparison Investigations (ICI) and External Quality Assurance Schemes (EQUAS) are tools to access the proficiency of laboratories, and the comparability and reliability of analytical methods. Participation in ICI / EQUAS forms an integral part of quality control, in addition to initial and on-going in-house method validation.

This ICI/EQUAS study has been organised within the frame of HBM4EU as part of the Quality Assurance program for biomonitoring analyses, following protocols HBM4EU-SOP-QA-001 to 004 which are available through the HBM4EU website. Within HBM4EU, participation in ICI/EQUAS exercises is mandatory for laboratories that will analyse HBM4EU samples.

This report describes the outcome of the 1st round for DINCH in urine and was organised by RIKILT – Wageningen University & Research in The Netherlands. RIKILT is ISO/IEC 17043 accredited for organisation of proficiency tests, but the specific substances in this ICI study were outside the specified scope of accreditation.

The selection of the most relevant/feasible biomarkers of DINCH was previously done in WP9, and has been described in Deliverable report 9.2 v1.1. Based on this, two target biomarkers were to be included in the ICI for DINCH biomarker analysis (see Table 1)..

**Table 1. Biomarkers for DINCH* included in the ICI.**

| Biomarker | |
| --- | --- |
| OH-MINCH | cyclohexane-1,2-dicarboxylate-mono-(7-hydroxy-4-methyl)octyl ester |
| cx-MINCH | cyclohexane-1,2-dicarboxylate-mono-(7-carboxylate-4-methyl)heptyl ester |

* Di-isononyl cyclohexane-1,2-dicarboylate

For this first ICI round, the anticipated target concentrations were relatively high (range 2-20 µg/L) to ensure laboratories would have no detectability issues.

The LOQs provided by the participants during registration for the ICI ranged from 0.05 to 1 µg/L).

## 2.1 Confidentiality

In this report the identity of the participants and the information provided by them is treated as confidential. However, lab codes of the participants will be disclosed to the HBM-QAU for performance assessments.

# 3 Control material

## 3.1 Preparation of control material

For this ICI two control materials, A and B, were prepared, one aiming at concentrations in the range 2-5 µg/L and one roughly five times higher. The control materials were prepared by blending aliquots of different burdened human urine samples. The burdened human urines (15 in total) were kindly provided by the Institute for Prevention and Occupational Medicine of the German Social Accident Insurance (IPA), with concentration estimates.

For blending, selected materials were thawed, the appropriate volumes taken and mixed. The blend (approx. 400 ml) was centrifuged to remove any precipitates. Then the urine was aliquoted (4 ml portions) into coded polypropylene tubes with screwcap. The tubes were stored in the freezer (16 May 2018, <-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. Ten tubes of control material A and ten tubes of control material B were randomly selected from the freezer and sent to IPA for analysis. Each sample was analysed in duplicate. In brief, after thawing/mixing, an aliquot of the urine was taken, isotope labels of the biomarkers were added as internal standard, and a deconjugation step using E. coli β-glucuronidase was performed. The deconjugated urine was analysed by on-line SPE coupled to LC-MS/MS. The analysis results were sent to the organiser and processed according to the SOP using an Excel macro ("HBM4EU macro homogeneity test v1.xlsm"). The mean concentrations and relative standard deviations as obtained during homogeneity testing are presented in Table 2. The statistical evaluation of materials A and B for each of the biomarkers are provided in Appendix 2. It was concluded that homogeneity was adequate for all biomarkers in both control materials.

**Table 2. Concentration of DINCH biomarkers as obtained during homogeneity testing (details see Appendix 2).**

| | material A | | material B | |
| --- | --- | --- | --- | --- |
| Biomarker | µg/L | $RSD_r$ | µg/L | $RSD_r$ |
| OH-MINCH | 3.28 | 1% | 19.1 | 2% |
| cx-MINCH | 3.16 | 3% | 14.6 | 2% |

## 3.3 Stability of control material

Stability testing was done according to HBM4EU-SOP-QA-002. At the day of preparation of the control materials, randomly selected test samples of material A and B were stored at -80°C. The assumption here is that under these conditions, the biomarkers are stable in urine. After the (extended) deadline of submission of analysis results by the participants (17 July 2018), six test samples of both material A and B stored at -80°C, and six samples of material A and B randomly selected from the -18°C freezer, where the ICI samples were stored, were sent to IPA for analysis (method same as described in 3.2). Results were received by RIKILT on 20th August 2018 and used to assess stability. The control materials were considered stable when the difference of the means of the -80°C and the -18°C samples was ≤0.3 $\sigma_T$. In both control materials this was the case for both biomarkers. The detailed data are provided in Appendix 3.

# 4 Organisational details

## 4.1 Participants

Participants for this ICI were laboratories from the HBM4EU consortium (including linked-third parties) that had been included as candidate laboratories for analyses in the frame of the HBM4EU project through WP9 (Task 9.2, Deliverable 9.3). A list of 9 eligible candidate laboratories was provided to RIKILT. As this number was considered relatively low for an ICI, the organiser requested the WP9 leader to verify whether there were any additional labs in the process of being included in the list of eligible labs. This resulted in three more candidate labs. Invitation letters were sent by e-mail on 12 April 2018 (see Appendix 4). For registration, each participant was asked to provide whether or not both biomarkers were included in their scope, and the LOQs in µg/L.

In HBM practise of urine analysis, µg/L results are often normalised to account for fluctuations in urine dilution, e.g. using the creatinine concentration, specific gravity or by other means. To gain insight in what is done amongst the participants, they were asked to specify what is used in their HBM practise.

Of the 12 invited laboratories, 11 laboratories from 9 countries registered (see Appendix 1). Each of the participants received a randomly assigned laboratory code, generated by the web application.

## 4.2 Dispatch and instructions

Test materials (one tube A and one tube B, with unique codes, containing approx. 4 ml urine each, frozen conditions) were dispatched to the participants on 11$^{th}$ June 2018. The samples were packed in an insulation box with dry ice and sent by courier. Instructions and an "acknowledgement of receipt form" were included in the box and also sent by e-mail at the day of shipment (see Appendix 5). Participants were asked to check the content of the box upon receipt, to store the samples in the freezer, and to analysis the samples according to their routine method. The deadline for submission of results was 10$^{th}$ July 2018. At request of some participants and after consultation with the QAU, the deadline was extended to 17$^{th}$ July 2018.

Based on the feedback on practise of normalisation of µg/L urine concentrations, and consultations with the QAU, it was decided to ask laboratories to also determine and report the creatinine concentrations in the urine samples.

An e-mail with a request to provide detailed method information in an Excel file was sent to the participants on 5 July 2018. A follow up e-mail for more specific method details was sent on 29/30 August 2018.

## 4.3 Deviations from ICI SOPs

There were no deviations from the HBM4EU-QA-SOPs.

# 5 Data evaluation

As mentioned in 4.2, laboratories were asked to also report the creatinine concentrations, if these were used in their HBM practise for normalisation of urine data. The creatinine results were evaluated separately and are provided here as additional information in Appendix 8. The biomarkers were evaluated on a µg/L basis only.

## 5.1 False negatives and false positives

Classification of results as false negatives or false positives was done as described in HBM4EU-SOP-QA-003.

A result was assigned as false negative when the following conditions all applied:

1) the biomarker was present in the control material (as established during the homogeneity/ stability assessment) and reported by the majority of the participants.

2) the biomarker was measured by the participant, but reported as below the specified LOQ value.

3) the participant's LOQ for the biomarker was below [assigned value - $3*\sigma_T$].

A result was assigned as false positive when the following conditions all applied:

1) the biomarker was not present in the control material, i.e. below the LOQ value as used by the organiser during the homogeneity/ stability assessment, and not reported by the majority of the participants.

2) the biomarker was reported by the participant.

In this ICI, both biomarkers were present in both control materials, and no 'blank' control material was provided.

## 5.2 Assigned value

For ICI studies, the consensus value is used as assigned value and calculated as described in HBM4EU-SOP-QA-003. In brief, the consensus values and its uncertainty are calculated from the results submitted by the participants using robust statistics to minimize the influence of outliers.

## 5.3 Target standard deviation ($\sigma_T$)

For calculation of the Z-scores, a fit-for-purpose relative target standard deviation (FFP-$RSD_R$) of 25% of the assigned value was used as target standard deviation. This was the default indicated in HBM4EU-SOP-QA-003 and used for this first ICI in lack of sufficient historical data for these biomarkers to set more stringent requirements while target standard deviations are not considered fit for purpose.

## 5.4 ICI standard deviation (ICI-$RSD_R$)

To gain insight in the actual variability of the biomarker determination in this study, the robust relative standard deviation (ICI-$RSD_R$) was calculated based on the participants' results, as described in HBM4EU-SOP-QA-003.

## 5.5 Z-scores

Z-scores are calculated according to SOP HBM4EU-SOP-QA-003, using an Excel macro ("HBM4EU macro ICI-evaluation_v1.xlsm"). The procedure in brief is as follows: first the consensus value and its uncertainty (u) are determined. A Z-score is provided when $u \leq 0.3*\sigma_T$, and a Z'-score when $0.3*\sigma_T < u \leq 0.7*\sigma_T$. In the latter case, the uncertainty of the consensus value is not considered negligible and taken into account in calculation of the Z-scores. Instability of the biomarkers in the control material, if applicable, is also taken into account in the calculation of the Z-scores. When $u > 0.7*\sigma_T$, or the number of results for a biomarker is <7, the data set is considered unfit for evaluating individual laboratory's performance and no Z-scores are provided.

In accordance with ISO 13528 and ISO 17043 and the deliverable D 9.4 "*The Quality Assurance/Quality Control Scheme in the HBM4EU project*, Z-scores are classified as presented in Table 4.

**Table 4: Classification of Z-scores**

| | |
| --- | --- |
| $\lvert Z \rvert \leq 2$ | Satisfactory |
| $2 < \lvert Z \rvert < 3$ | Questionable |
| $\lvert Z \rvert \geq 3$ | Unsatisfactory |

# 6 Results and discussion

## 6.1 Results submitted by participants

In total 11 laboratories from 9 countries agreed to participate in this ICI and submitted results (see appendix 1). One laboratory submitted their results one week after the deadline, these were nevertheless included in the data set.

During registration, participants were asked to indicate if their method included both biomarkers and to specify the LOQs. Except one lab that only determined OH-MINCH, all labs measured both biomarkers. The LOQs as provided by the labs are included in the method details in Appendix 7.

The LOQs were generally in the range 0.1-0.2 µg/L, for some laboratories higher (up to 1 µg/L). For the test materials of this ICI the participants' LOQs were adequate.

An overview of results as submitted by the participants is included in Appendix 6.

**False negatives/false positives:** the biomarkers were present in both control materials, and detected by all participants, hence, there were no false positives or false negatives.

Laboratories were asked to provide details on the method used for analysis. This information is compiled in appendix 7. Ten participants in this ICI also participated in the phthalate biomarkers ICI. Seven of these ten used the same method for the DINCH biomarkes as they used for determination of the phthalates biomarkers. Three labs used (slightly) different methods or conditions.

In general, the laboratories did not do any filtration/centrifugation after thawing the urine sample, added isotope label(s) to an aliquot of 0.2-1 ml urine, and adjusted the pH to values ranging from 5.5 to 6.5. All labs did an enzymatic deconjugation step, mostly using E. Coli β-glucuronidase (two labs used Helix Pomatia β-glucuronidase/aryl-sulfatase), at 37°C for 1.5-2.5 hours (1x 0.5 h, 1x overnight). The deconjugated urine was often acidified and then either extracted/preconcentrated using on-line or off-line SPE, or analysed directly. The biomarkers were measured using LC-MS/MS (triple quads; 1x single MS) with electrospray ionisation in negative mode. The number of transitions measured varied from 1 to 3, various criteria were used for identification (retention time/ion ratio tolerances). Quantification was mostly done against calibration standards prepared in solvent/eluent. It was noted that although isotope labels were used by all laboratories, there were several cases where the laboratory did not have the corresponding label for each individual biomarker (information included in Appendices 7). DINCH biomarkers in the burdened samples can be present as multiple isomers. Most labs integrated a single peak (the one corresponding to the labelled internal standard) and did not include isomers as recommended in the analytical comments included in HBM4EU Deliverable 9.2.

In addition to the table in Appendix 7, the analysis results of the labs are also graphically shown in Figure 1, from which it can be seen that the results are rather scattered. The two labs that included the isomers in the determination of OH-MINCH tended to have higher results for this biomarker. For cx-MINCH, where three labs indicated to include the isomers in the determination, this was not evident. However, in this case different internal standards than the isotope analogue had been used.

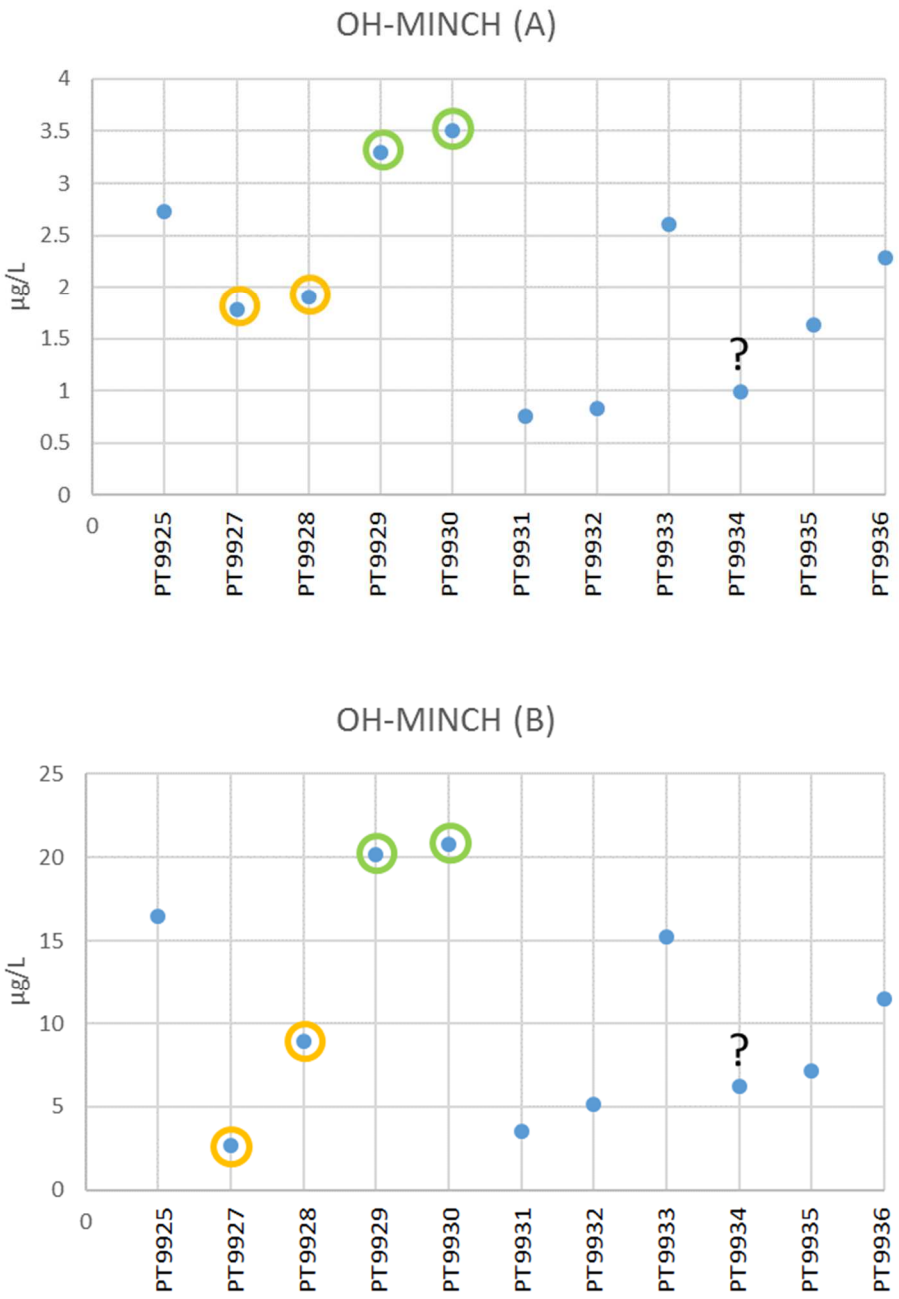


**Figure 1a.** Concentrations of OH-MINCH reported by the participants for test sample A and B. Green circle = sum of isomers integrated; Orange circle = no isotope analogue of biomarker used as internal standard.

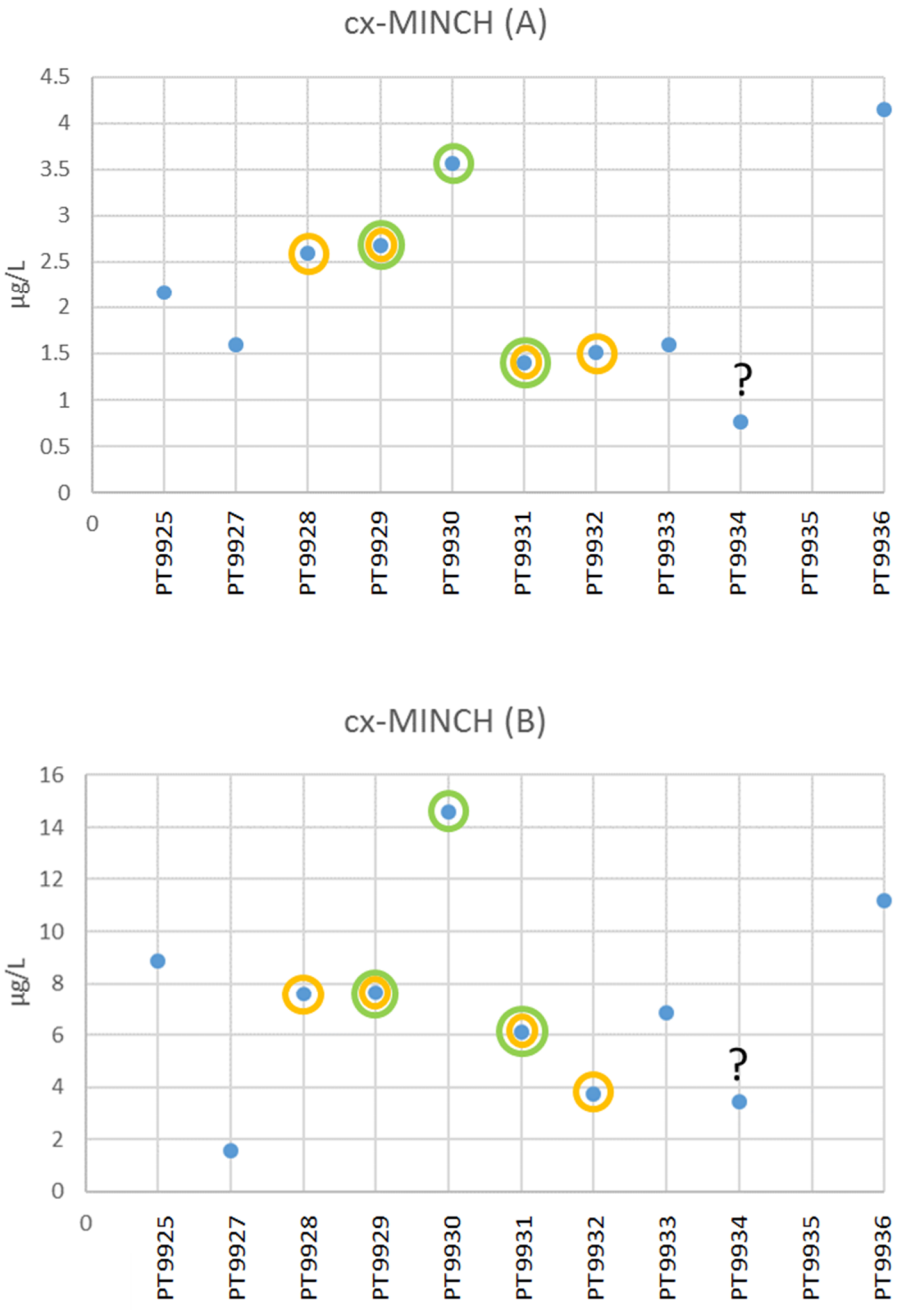


**Figure 1b.** Concentrations of cx-MINCH reported by the participants for test sample A and B. Green circle = sum of isomers integrated; Orange circle = no isotope analogue of biomarker used as internal standard.

## 6.2 Assigned values and (target) standard deviations

For each of the biomarkers in both control materials, the assigned value, its uncertainty, and the relative standard deviation were determined. The data are included in Appendix 7.

In all four cases (two biomarkers in two materials), the uncertainly of the consensus value was too high to establish a reliable value, and the assigned value should be considered as indicative and

for information only. Consequently, it was not possible to provide a meaningful Z-score for the DINCH biomarkers. The Z-scores were therefore left out from Appendix 7.

A comparison between the fixed fit-for-purpose target standard deviation of 25% (FFP-$RSD_R$) used for calculation of the Z-scores, and the actual relative standard deviation as observed in this ICI (ICI-$RSD_R$), showed that in all four cases the ICI-$RSD_R$, ranging from 46%-70%, exceeded the FFP-$RSD_R$.

The cause of the high variability for the DINCH biomarkers most likely lies in the difficulty of recognising isomer peaks that were present in the burdened urine, and/or differences in the selection of the isomer peak used for quantification. In addition, the fact that in some cases internal standards other than the isotope analogues were used for quantification may have further contributed to the (very) high ICI-$RSD_R$s.

## 6.3 Assessment of laboratory performance

Although the number of laboratories submitting results for the two DINCH biomarkers was sufficient, and all laboratories were able detect and quantify them, the variability was too high to determine consensus values and Z-scores. Consequently, no assessment of laboratory performance could be made through this ICI.

## 6.4 Conclusions and recommendations

- in this first ICI on 2 DINCH biomarkers in urine, 12 laboratories were invited of which 11 registered and submitted results.

- two test materials were provided containing both DINCH biomarkers, at concentrations of approximately 3 and 15 µg/L respectively.

- all labs were capable of detecting and quantifying the two DINCH biomarkers, however, evaluation of lab performance was not possible due to too high variability of the participants' results.

- ICI-$RSD_R$ ranged from 46% to 70%, above the FFP-$RSD_R$ (25%) in all cases.

- the troublesome determination of the DINCH biomarkers was attributed to differences in recognising/inclusion of isomers, not always using the isotope analogues as internal standard, and most likely other not yet clearly identified causes.

- at the moment, classification of labs for determination of DINCH biomarkers in the frame of HBM4EU through ICIs is not possible.

- It is recommended that lab capabilities are improved before initiating the next ICI, and for the 2$^{nd}$ round to organise a combined ICI/EQUAS exercise to ensure a successful QA assessment of HBM4EU laboratories for the determination of DINCH biomarkers.

# 7 References

Analytical Methods Committee, 1989a, Robust statistics - How not to reject outliers Part 1. Basic concepts, Analyst, 114, 1693-1697.

Analytical Methods Committee, 1989b, Robust statistics - How not to reject outliers Part 2. Interlaboratory trials, Analyst, 114, 1699-1702.

HBM4EU-SOP-QA-001 "Organisation of Interlaboratory Comparison Investigations (ICI) and External Quality Assurance Schemes (EQUAS) of interlaboratory studies"

HBM4EU-SOP-QA-002 "Preparation of control materials for Interlaboratory Comparison Investigations (ICI) and External Quality Assurance Schemes (EQUAS)"

HBM4EU-SOP-QA-003 "Evaluation of results from Interlaboratory Comparison Investigations (ICI) and External Quality Assurance Schemes (EQUAS)"

HBM4EU-SOP-QA-004 "Reporting of results of Interlaboratory Comparison Investigations (ICI) and External Quality Assurance Schemes (EQUAS)"

*Note: the above mentioned SOPs can be found on the HBM4EU website:* https://www.hbm4eu.eu/online-library/?mdocs-cat=mdocs-cat-1&mdocs-att=null

HBM4EU Deliverable 9.2 Prioritised list of biomarkers, matrices and analytical methods for the 1st prioritisation round of substances. https://www.hbm4eu.eu/deliverables/

ISO/IEC 17043:2010, Conformity assessment – General requirements for proficiency testing

ISO 13528, 2015, Statistical methods for use in proficiency testing by interlaboratory comparison.

Official Methods of Analysis Program Manual, 2002, Appendix D: Guidelines for Collaborative Study Procedures To Validate Characteristics of a Method of Analysis. Association Of Analytical Communities International. http://www.aoac.org/vmeth/Manual_Part_6.pdf.

Thompson, M., 2000, Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations in relation to fitness for purpose criteria in proficiency testing, Analyst, 125, 385-386.

Thompson M., Ellison R. and Wood, R., 2006, The International Harmonized Protocol for the Proficiency Testing of Analytical Chemistry Laboratories, Pure Appl. Chem, 78(1), 145-196.

## Appendix 1. List of participants

| Country | Number of laboratories participating |
| --- | --- |
| Belgium | 2 |
| Czech Republic | 2 |
| Denmark | 1 |
| Germany | 1 |
| Greece | 1 |
| Hungary | 1 |
| Norway | 1 |
| Slovakia | 1 |
| Sweden | 1 |

## Appendix 2. Homogeneity data

| | Control material A | | | Control material A | |
| --- | --- | --- | --- | --- | --- |
| | OH-MINCH | | | cx-MINCH | |
| | replicate 1 | replicate 2 | | replicate 1 | replicate 2 |
| 1 | 3.29 | 3.32 | | 3.06 | 3.14 |
| 2 | 3.27 | 3.25 | | 3.00 | 3.13 |
| 3 | 3.30 | 3.24 | | 3.22 | 3.16 |
| 4 | 3.26 | 3.19 | | 3.12 | 3.13 |
| 5 | 3.28 | 3.32 | | 3.17 | 3.23 |
| 6 | 3.26 | 3.28 | | 3.22 | 3.13 |
| 7 | 3.23 | 3.35 | | 3.05 | 3.12 |
| 8 | 3.23 | 3.38 | | 3.18 | 3.44 |
| 9 | 3.27 | 3.30 | | 3.09 | 3.12 |
| 10 | 3.31 | 3.29 | | 3.28 | 3.15 |
| grand mean | 3.28 | 1% | | 3.157 | 3% |
| Cochran's test | | | | | |
| C | 0.450 | | | 0.524 | |
| Ccrit | 0.602 | | | 0.602 | |
| C < Ccrit? | No outliers detected | | | No outliers detected | |
| target $\sigma_{FFP}$ | 0.820 | | | 0.789 | |
| sx= | 0.025 | | | 0.075 | |
| sw= | 0.050 | | | 0.080 | |
| ss= | 0.000 | | | 0.049 | |
| critical=0.3$\sigma_{FFP}$ | 0.246 | | | 0.237 | |
| ss < critical? | Homogeneity adequate | | | Homogeneity adequate | |
| sw< 0.5*$\sigma_{FFP}$ | Method suited | | | Method suited | |

| | Control material B | | | Control material B | |
| --- | --- | --- | --- | --- | --- |
| | OH-MINCH | | | cx-MINCH | |
| | replicate 1 | replicate 2 | | replicate 1 | replicate 2 |
| 1 | 18.7 | 19.3 | | 14.68 | 15.12 |
| 2 | 19.0 | 18.3 | | 14.38 | 14.37 |
| 3 | 19.1 | 18.7 | | 14.93 | 14.39 |
| 4 | 19.1 | 19.2 | | 14.35 | 14.42 |
| 5 | 19.5 | 19.1 | | 14.60 | 14.16 |
| 6 | 18.7 | 19.7 | | 14.13 | 14.65 |
| 7 | 19.4 | 18.9 | | 14.62 | 14.66 |
| 8 | 19.0 | 19.0 | | 14.70 | 14.42 |
| 9 | 19.6 | 19.6 | | 15.03 | 14.69 |
| 10 | 19.3 | 19.4 | | 14.86 | 15.00 |
| grand mean | 19.1 | 2% | | 14.61 | 2% |
| Cochran's test | | | | | |
| C | 0.420 | | | 0.249 | |
| Ccrit | 0.602 | | | 0.602 | |
| C < Ccrit? | No outliers detected | | | No outliers detected | |
| target $\sigma_{FFP}$ | 4.782 | | | 3.652 | |
| sx= | 0.269 | | | 0.227 | |
| sw= | 0.345 | | | 0.242 | |
| ss= | 0.114 | | | 0.149 | |
| critical=0.3$\sigma_{FFP}$ | 1.434 | | | 1.096 | |
| ss < critical? | Homogeneity adequate | | | Homogeneity adequate | |
| sw< 0.5*$\sigma_{FFP}$ | Method suited | | | Method suited | |

## Appendix 3. Stability data

| | Control material A | | | Control material A | |
| --- | --- | --- | --- | --- | --- |
| | OH-MINCH | 96 days | | cx-MINCH | 96 days |
| Sample | -80°C | -18°C | | -80°C | -18°C |
| 1 | 3.61 | 3.70 | | 3.55 | 3.63 |
| 2 | 3.66 | 3.53 | | 3.56 | 3.45 |
| 3 | 3.58 | 3.51 | | 3.56 | 3.47 |
| 4 | 3.65 | 3.73 | | 3.6 | 3.57 |
| 5 | 3.60 | 3.56 | | 3.56 | 3.55 |
| 6 | 3.60 | 3.49 | | 3.51 | 3.39 |
| average | 3.617 | 3.587 | | 3.557 | 3.510 |
| stdev | 0.031 | 0.103 | | 0.029 | 0.089 |
| difference | | -0.03 | | | -0.047 |
| critical=0.3$\sigma_{FFP}$ | | 0.27 | | | 0.267 |
| consequential instability | | no | | | no |
| t | | 0.685 | | | 1.228 |
| tcrit | | 2.228 | | | 2.228 |
| statistical difference | | no | | | no |

| | Control material B | | | Control material B | |
| --- | --- | --- | --- | --- | --- |
| | OH-MINCH | 96 days | | cx-MINCH | 96 days |
| Sample | -80°C | -18°C | | -80°C | -18°C |
| 1 | 20.7 | 20.8 | | 14.10 | 13.94 |
| 2 | 21.0 | 20.3 | | 14.32 | 14.68 |
| 3 | 19.9 | 20.8 | | 14.15 | 13.76 |
| 4 | 19.7 | 20.6 | | 13.77 | 14.37 |
| 5 | 21.0 | 20.6 | | 14.35 | 14.32 |
| 6 | 20.6 | 20.2 | | 13.86 | 14.34 |
| average | 20.50 | 20.53 | | 14.09 | 14.24 |
| stdev | 0.548 | 0.253 | | 0.236 | 0.331 |
| difference | | 0.04 | | | 0.143 |
| critical=0.3$\sigma_{FFP}$ | | 1.54 | | | 1.057 |
| consequential instability | | no | | | no |
| t | | 0.156 | | | 0.863 |
| tcrit | | 2.228 | | | 2.228 |
| statistical difference | | no | | | no |

## Appendix 4. Copy of letter of invitation

WAGENINGEN UNIVERSITY & RESEARCH

HBM4EU science and policy for a healthy future

Postbus 230 | 6700 AE WAGENINGEN The Netherlands

**HBM4EU: Announcement / invitation to participate in ICI study DINCH/round_01**

**Title of ICI:** DINCH biomarkers in urine

Dear colleague,

Within the frame of HBM4EU, RIKILT – Wageningen University & Research announces the first round of ICI/EQUAS for the determination of DINCH-biomarkers in urine. The aim of ICI/EQUAS exercises is to provide laboratories with an assessment of their analytical performance and reliability of their data in comparison with other laboratories and/or expert laboratories. This will aid in the quality improvement of analysis in human biomonitoring at each of the laboratories.

Participation is mandatory for laboratories analysing samples in the frame of HBM4EU.

**1. Samples**

- Two urine samples (approximately 2-4 ml) will be supplied for the analysis of DINCH biomarkers. See the registration form for potentially present biomarkers.
- Besides the biomarkers, we also ask you to provide the result for the parameter that you use for normalisation of urine concentrations.
- The samples are expected to be sent in second half of May 2018. The distribution of the samples will be announced by e-mail.

**2. Quantitative analysis**

- The results have to be reported within 4 weeks after shipment of the samples.
- Results must be submitted via the electronic submission form for which each participant must register, as explained in the document "Instruction registration login account RIKILT Laboratory Quality Services".

**3. Report**

- A report of the proficiency test will be dispatched in July/August 2018.
- Results of the proficiency test will be presented anonymously.

**4. Additional information**

- All laboratory specific information will be treated confidentially, and will never be disclosed to third parties (government, accreditation bodies) except the HBM4EU QAU, without permission of the laboratory.

100years 1918 — 2018

RIKILT

DATE
April 12, 2018

SUBJECT
Invitation for ICI study DINCH biomarkers in urine

POSTAL ADDRESS
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HANDLED BY
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## Appendix 4. Copy of letter of invitation (continued)

DATE
April 12, 2018

PAGE
2 of 2

**5. Costs**

- For partners and linked-third parties of HBM4EU, participation is free of charge. Please note that the participant is responsible for custom clearance and associated costs if applicable.

**6. Calender**

- Deadline registration: 26th April 2018
- Distribution of samples: second half of May 2018
- Deadline submission of results: 4 weeks after distribution

If you would like to participate, please fill out the registration form and send it to me before April 27 2018 by e-mail (pt.rikilt@wur.nl). Please indicate on the registration form which biomarkers are within the scope of your method, and the estimated LOQ.

Hoping to welcome you for this ICI/EQUAS round,

Yours sincerely,

IJ.Elbers

Ingrid Elbers (organiser proficiency test/ICI/EQUAS)
Hans Mol (scientific expert)

## Appendix 4. Copy of letter of invitation (continued)

WAGENINGEN UNIVERSITY & RESEARCH

HBM4EU science and policy for a healthy future

**Registration form ICI study DINCH/round_01** (RIKILT code 2018-06)

| **Contact person:** | |
| --- | --- |
| E-mail: | |
| Username for RIKILT web application:<br>(needed for reporting results) | |
| | |
| No username? Please register at: | Register (Ctrl + click) |

Please indicate below the biomarkers that are within the scope of your method, and provide an estimate of your LOQ. We would appreciate your registration for both biomarkers.

| Biomarkers included in the scope of your method (please tick) | Abbreviation | LOQ (µg/l urine) |
| --- | --- | --- |
| ▫ cyclohexane-1,2-dicarboxylic mono hydroxyisononyl ester | OH-MINCH | |
| ▫ cyclohexane-1,2-dicarboxylic mono carboxyisooctyl ester | cx-MINCH | |

**How do you normalise your µg/l urinary concentrations:**

▫ Creatinine ▫ Specific gravity ▫ other:_____

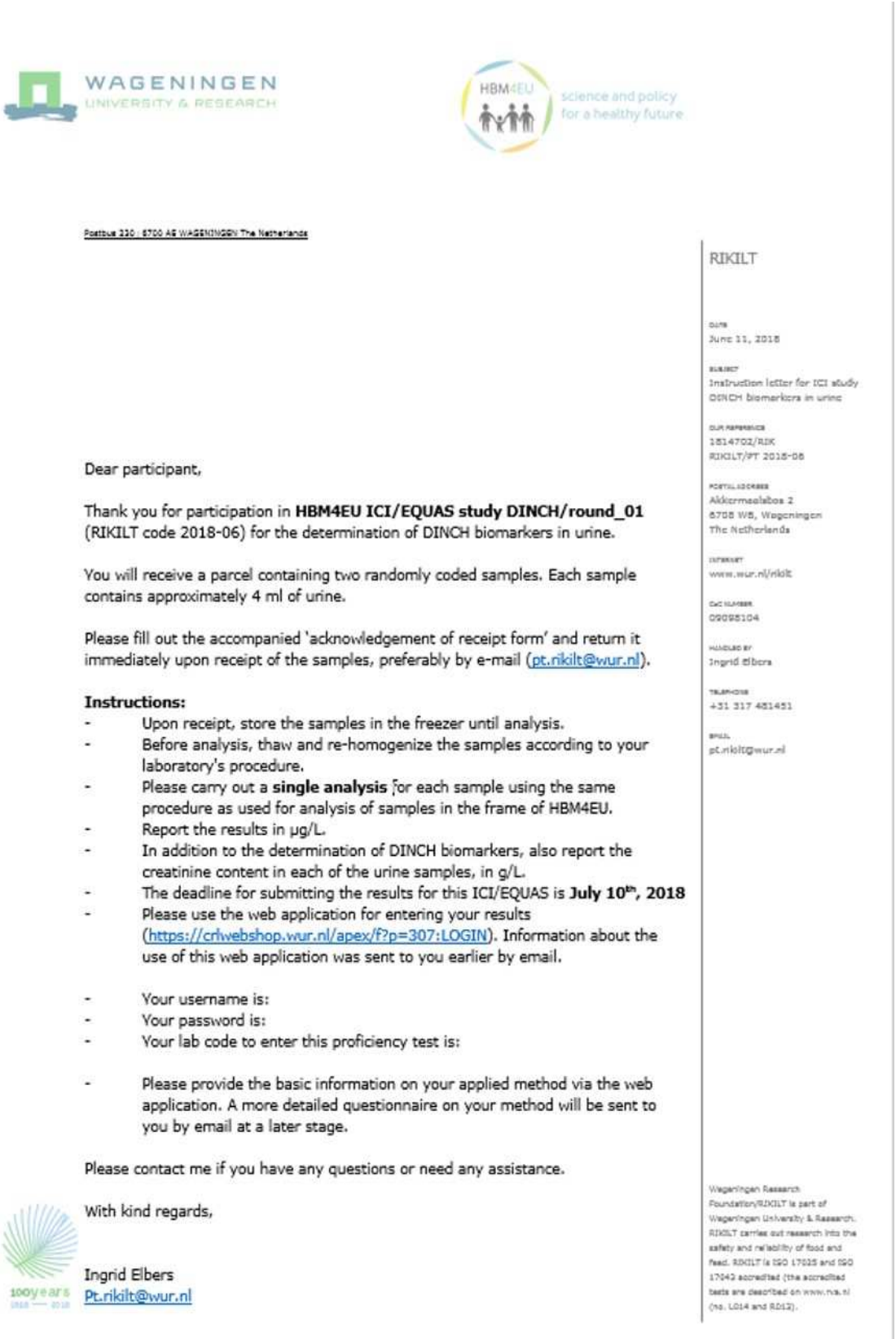
I hereby accept the conditions for participation as outlined in the letter accompanying this form.

Date / Signature:

Please sign a print of this document and e-mail a scan to pt.rikilt@wur.nl.
**Please subscribe before April 27, 2018**

Ingrid Elbers (organiser proficiency test/ICI/EQUAS)
Hans Mol (scientific expert)
RIKILT - Wageningen University & Research
The Netherlands

## Appendix 5. Copy of letter/instructions sent together with test samples



WAGENINGEN UNIVERSITY & RESEARCH

HBM4EU science and policy for a healthy future

Postbus 230 | 6700 AE WAGENINGEN The Netherlands

RIKILT

DATE
June 11, 2018

SUBJECT
Instruction letter for ICI study DINCH biomarkers in urine

OUR REFERENCE
1814702/RIK
RIKILT/PT 2018-06

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CoC NUMBER
09098104

HANDLED BY
Ingrid Elbers

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pt.rikilt@wur.nl

Dear participant,

Thank you for participation in **HBM4EU ICI/EQUAS study DINCH/round_01** (RIKILT code 2018-06) for the determination of DINCH biomarkers in urine.

You will receive a parcel containing two randomly coded samples. Each sample contains approximately 4 ml of urine.

Please fill out the accompanied 'acknowledgement of receipt form' and return it immediately upon receipt of the samples, preferably by e-mail (pt.rikilt@wur.nl).

**Instructions:**

- Upon receipt, store the samples in the freezer until analysis.
- Before analysis, thaw and re-homogenize the samples according to your laboratory's procedure.
- Please carry out a **single analysis** for each sample using the same procedure as used for analysis of samples in the frame of HBM4EU.
- Report the results in µg/L.
- In addition to the determination of DINCH biomarkers, also report the creatinine content in each of the urine samples, in g/L.
- The deadline for submitting the results for this ICI/EQUAS is **July 10th, 2018**
- Please use the web application for entering your results (https://crlwebshop.wur.nl/apex/f?p=307:LOGIN). Information about the use of this web application was sent to you earlier by email.

- Your username is:
- Your password is:
- Your lab code to enter this proficiency test is:

- Please provide the basic information on your applied method via the web application. A more detailed questionnaire on your method will be sent to you by email at a later stage.

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

With kind regards,

Ingrid Elbers
Pt.rikilt@wur.nl

100 years 1918 — 2018

Wageningen Research Foundation/RIKILT is part of Wageningen University & Research. RIKILT carries out research into the safety and reliability of food and feed. RIKILT is ISO 17025 and ISO 17043 accredited (the accredited tests are described on www.rva.nl (no. L014 and R012).

## Appendix 5. Copy of letter/instructions sent together with test samples (continued)



**HBM4EU: Acknowledgement of receipt form ICI / EQUAS study DINCH/round_01**

**Title of ICI/EQUAS:** DINCH biomarkers in urine

**Laboratory code:**

Contact person:

**Contents of parcel:**
- 2 containers/tubes with ~4 ml urine
- Sample receipt form

Please verify that the items listed below have been received and provide the information requested below:

Date of receipt (dd-mm-yyyy):

Conditions: ambient / partly thawed / frozen / dry-ice still present

| Code on container | Damaged/leakage | Remarks |
| --- | --- | --- |
| | Yes / No | |
| | Yes / No | |

Name: Signature

Date:

After signing this form, please scan and send the pdf to: pt.rikilt@wur.nl

**Contact information organiser:**
Ingrid Elbers (organiser proficiency test/ICI/EQUAS) tel: +31 317 481451 email: pt.rikilt@wur.nl
Hans Mol (scientific expert) tel: +31 317 480318 email: hans.mol@wur.nl
RIKILT - Wageningen University & Research
The Netherlands

## Appendix 6. Assigned values and Z-scores

| Control material | test sample A | | | | test sample B | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Biomarker | OH-MINCH | | cx-MINCH | | OH-MINCH | | cx-MINCH | |
| Assigned value (µg/L)* | ***2.03*** | | ***2.06*** | | ***10.67*** | | ***6.99*** | |
| Uncertainty (µg/L) | ***0.415*** | | ***0.371*** | | ***2.81*** | | ***1.56*** | |
| Target FFP-RSD | 25% | | 25% | | 25% | | 25% | |
| ICI RSDR (%) | **54%** | | **46%** | | **70%** | | **57%** | |
| Laboratory code | µg/L | Z-score* | µg/L | Z-score* | µg/L | Z-score* | µg/L | Z-score* |
| PT9925 | 2.73 | | 2.16 | | 16.5 | | 8.89 | |
| PT9927 | 1.78 | | 1.59 | | 2.71 | | 1.57 | |
| PT9928 | 1.9 | | 2.6 | | 8.9 | | 7.6 | |
| PT9929 | 3.3 | | 2.68 | | 20.2 | | 7.65 | |
| PT9930 | 3.51 | | 3.57 | | 20.8 | | 14.6 | |
| PT9931 | 0.76 | | 1.4 | | 3.56 | | 6.11 | |
| PT9932 | 0.825 | | 1.51 | | 5.17 | | 3.732 | |
| PT9933 | 2.61 | | 1.6 | | 15.27 | | 6.86 | |
| PT9934 | 0.99 | | 0.76 | | 6.23 | | 3.42 | |
| PT9935 | 1.6363 | | | | 7.1658 | | | |
| PT9936 | 2.28 | | 4.15 | | 11.47 | | 11.2 | |

* assigned values for information only, the uncertainty is too high to determine a consensus value and to provide meaningful Z-scores.

## Appendix 7. Details of analysis methods used by the participants

| | PRETREATMENT | | | | | |
|---|---|---|---|---|---|---|
| Labcode | Pre-treatment | urine aliquot used (ml) | pH adjustment (provide buffer and pH) | Deconjugation | time(hrs) / temp (°C) | post deconjugation adjustment of sample |
| PT9925 | none | 0.5 | Acetate buffer, pH 6.5 | Helix Pomatia | 1.5 h / 37°C | acetic acid |
| PT9927 | none | 0.3 | Acetate buffer, pH 6 | E. coli B-glucuronidase | 2.5 h / 37°C | 10 µl acetic acid |
| PT9928 | none | 0.5 | Acetate buffer, pH 6.5 | E. coli B-glucuronidase | 2 h / 37°C | 50 µL acetic acid, 10 µL ACN |
| PT9929 | none | 0.3 | Acetate buffer, pH 6 | E. coli B-glucuronidase | 2h / 37°C | acetic acid |
| PT9930 | none | 0.3 | Acetate buffer, pH 6.0-6.4 | E. coli B-glucuronidase | 2.5 h / 37°C | 10 µL acetic acid |
| PT9931 | none | 0.3 | Acetate buffer, pH 5 | Helix Pomatia (HP-2) | 1.5 h / 37 °C | formic acid / MeOH; centrifugation |
| PT9932 | none | 0.2 | <not specified>, pH6.5 | E. coli B-glucuronidase | 0.5 h / 37°C | |
| PT9933 | none | 0.2 | Acetate buffer, pH 5.5 | E. coli B-glucuronidase | 1.5 h / 37°C | 20 µL formic acid, 50 µL MeOH, centrif. |
| PT9934 | none | 1.0 | Acetate buffer, pH 6.5 | E. coli B-glucuronidase | overnight, 37°C | none |
| PT9935 | none | 0.3 | Acetate buffer, pH 6.5 | E. coli B-glucuronidase | 1.5 h / 37°C | Formic acid, centrifugation |
| PT9936 | none | 1.0 | Phosphate buffer pH 6 | E. coli B-glucuronidase | 1.5h / 37°C | none |

| | EXTRACTION & CLEANUP | | INSTRUMENTAL ANALYSIS | | |
|---|---|---|---|---|---|
| Labcode | Technique | SPE column or LLE solvent | Separation technique | inj. vol. (µl) | Column |
| PT9925 | SPE (off-line) | Oasis HLB | (U)HPLC | 5 | phenomenex Luna c18(2) |
| PT9927 | freeze out (3 hrs), centrifugation | | (U)HPLC | 10 | Zorbax Eclipse Plus C18, 2.1x50mm, 1.8 µm |
| PT9928 | SPE (on-line) | | (U)HPLC | 100 | YMC Triart C18 5.0×2.0, 1.9 um |
| PT9929 | C-18 precolumn | | (U)HPLC | 200 | 150mmx2.1mm 3.5um C-18 |
| PT9930 | SPE (on-line) | 5 µm C18-MG-II (10 x 4mm) | (U)HPLC | 25 | Atlantis dC18 (2.1 x 150mm; 3µm; Waters) |
| PT9931 | none | – | (U)HPLC | 5 | Acquity HSS T3 (100×2.1 mm; 1.8 µm), Waters |
| PT9932 | dilute and shoot | | (U)HPLC | 4 | Fortis C18 1.7µm, 100x2.1mm |
| PT9933 | SPE (on-line) | TurboFlow Cyclone P column | (U)HPLC | 100 | Hypersil Gold aQ (0.4x50 mm, 3 µm) |
| PT9934 | | | (U)HPLC | 10 | ACQUITY BEH Phenyl Column, 1.7 µm, 2.1 X 100 mm |
| PT9935 | SPE (on-line) | Betasil C18 (10x3 mm, 5 µm) | (U)HPLC | 240 | Kinetex C18 (100x2.1 mm, 2.6 µm) |
| PT9936 | SPE (off-line) | Oasis HLB | (U)HPLC | 5 | Kinetex biphenyl 2.6 µm |

| | INSTR. ANALYSIS | | QUANTIFICATION | | | | |
|---|---|---|---|---|---|---|---|
| Labcode | Detection technique | for MS(/MS): ionisation | internal standard added for calculation of the concentration? OH-MINCH | cx-MINCH) | moment of addition of internal standard to sample? | Preparation of calibration standards | were results correct for recovery? |
| PT9925 | MS/MS | ESI neg | D4 OH-MINCH | D2 cx-MINCH | before deconjugation | in solvent/eluent | inherent |
| PT9927 | MS (single) | ESI neg | *13C4 cx-MINCH* | 13C4 cx-MINCH | before deconjugation | in solvent/eluent | inherent |
| PT9928 | MS/MS | ESI neg | *13C4-MBzP* | *13C4-MBzP* | before deconjugation | in solvent/eluent | inherent |
| PT9929 | MS/MS | ESI neg | D8-OH-MINCH | *D8-OH-MINCH* | before deconjugation | in solvent/eluent | inherent |
| PT9930 | MS/MS | ESI neg | D4-OH-MINCH | D2-cx-MINCH | before deconjugation | in solvent/eluent | inherent |
| PT9931 | MS/MS | ESI neg | d8 OH-MINCH | *d8 oxo-MINCH* | before deconjugation | in solvent/eluent | inherent |
| PT9932 | MS/MS | ESI neg | D8-OH-MINCH | *no IS* | after deconjugation | In urine and surine | no(inherent) |
| PT9933 | MS/MS | ESI neg | D4-OH-MINCH | D2-cx-MINCH | before deconjugation | in solvent/eluent | inherent |
| PT9934 | MS/MS | ESI neg | *no details provided* | | before deconjugation | in solvent/eluent | inherent |
| PT9935 | MS/MS | ESI neg | Deuterium label | not in scope | before deconjugation | in water as samples | inherent |
| PT9936 | MS/MS | ESI neg | d4 OH-MINCH | d2 cx-MINCH | before deconjugation | in solvent/eluent | inherent |

## Appendix 7. Details of analysis methods used by the participants (continued)

| | **IDENTIFICATION** | | | **LOQs** | |
| --- | --- | --- | --- | --- | --- |
| Labcode | **retention time tolerance (min or % from ref. std)** | **number of ions/transitions required for identification** | **tolerance (% relative or** | OH-MINCH | cx-MINCH |
| PT9925 | 0.1 min | 2 | 20 | 1 | 1 |
| PT9927 | 2 s | 1 | | 0.4 | 0.2 |
| PT9928 | 0.1 min | 2 | 20 | under development | |
| PT9929 | 0.2 % (±0.03 min) | 2 | - | 0.1 | 0.1 |
| PT9930 | n.a. | 2 | 20 | 0.1 | 0.1 |
| PT9931 | ± 2.5% | 3 | ± 30 | 0.15 | 0.15 |
| PT9932 | manually judged | 2 if avail (only 1 eval.) | anually judg | not given | not given |
| PT9933 | 0.05 min | 1 | ot calculate | 0.05 | 0.06 |
| PT9934 | 10 | 2 | 40 | 0.05-1 | 0.05-1 |
| PT9935 | 0.1 min (rel to ISTD) | 1 | - | 0.2 | not tested |
| PT9936 | 0.1 | 2 | 20 | 0.2 | 0.2 |

### Information on integration of biomarker isomers

| **Lab code** | **Integration of isomer peaks** |
| --- | --- |
| PT9925 | only one isomer peak was integrated |
| PT9927 | intagrated one isomer peak accordninng to the retention time |
| PT9928 | OH-MINCH and cx-MINCH were quantified separately with different MRM transitions (313.4->153.1 and 327.4->173.1 respectively). Baseline separation was not a goal as the selected transitions were selective for these componds (see the overlay MRM chromatogram below) |
| PT9929 | integrated sum of isomers |
| PT9930 | integrated sum of isomers |
| PT9931 | OH-MINCH - integrated one isomer peak; cx-MINCH - integrated sum of isomers (in calibration standards and in urine samples) |
| PT9932 | We intregrate a broad peak that we don´t separate. OH-MINCH standard from TRC canada is a mix of diastereomerer - we use a sum of areas of both peaks and calibrate against the OH-MINCH isomers in the samples |
| PT9933 | The isomer peak nearest to our internal standards was integrated, however for both DINCH metabolites, 1-2 more metabolites could probably have been included in a summed integration of isomers |
| PT9934 | no information provided |
| PT9935 | Integrated the peak that corresponds to the internal standard peak (time and width), although the peak is not always a single one and seems to have more than one isomer. |
| PT9936 | integrated one isomer peak corresponding to the retention time of the standard used for quantification |

## Appendix 8. ICI add-on: Assessment of comparability of creatinine determination.

In HBM analysis of urinary biomarkers, the µg/L concentrations are often normalised to account for fluctuations in urine dilution. During registration for this ICI, the laboratories were asked which procedure they use for this. Ten out of 11 do this based on creatinine, of which three also use specific gravity as alternative approach. One lab uses osmolality. Based on the responses, the laboratories were asked to also analyse the urine samples for creatinine. This was done to gain insight in the variability of creatinine determinations as this may affect the biomarker data when expressing the urine concentrations on a creatinine basis.

Out of the 10 laboratories, 9 submitted results for creatinine which was either determined by themselves or outsourced to another lab (typically hospital lab). Because the concentration of creatinine is in the g/L range and classical spectrometric techniques are mostly used, Z-scores were determined using a target standard deviation based on Horwitz (see HBM4EU-QA-SOP-003).

The results are summarized in the table and the figures below.

| Control material | Test sample A | | Test sample B | | |
| --- | --- | --- | --- | --- | --- |
| Biomarker | Creatinine | | Creatinine | | |
| Assigned value (g/L) | 0.901 | | 2.24 | | |
| Uncertainty (g/L) | 0.0129 | | 0.050 | | |
| Target RSD (Horwitz) | 5.7% | | 5.0% | | |
| ICI $RSD_R$ (%) | 3.2% | | 4.8% | | |
| Lab code | g/L | Z-score | g/L | Z'-score | Method used |
| PT9925 | 0.842 | -1.1 | 2.045 | -1.4 | LC-MS/MS |
| PT9927 | 0.086* | **-15.7** | 0.221* | **-18.2** | ARCHITECT ci8200 int. system |
| PT9928 | 0.92 | 0.4 | 2.25 | 0.3 | Jaffe method |
| PT9929 | 0.9 | 0.0 | 2.28 | 0.5 | enzymatic assay with photom. det. |
| PT9930 | 0.92 | 0.4 | 2.14 | -0.6 | Jaffe |
| PT9931 | 1.09 | **3.6** | 2.47 | **2.1** | Jaffe method |
| PT9932 | 0.871 | -0.6 | 2.183 | -0.3 | Jaffe (hospital clinical chem. dept) |
| PT9933 | | | | | |
| PT9934 | | | | | |
| PT9935 | 0.905 | 0.1 | 2.1493 | -0.6 | DRI® Creatinine-Detect® Test |
| PT9936 | 0.89 | -0.2 | 2.31 | 0.8 | colorimetric (Jaffe variant) |

* suspect gross errors (10-fold calculation error, unit, decimal point), excluded from calculation of assigned value

From the table it can be seen that 7 out of the 9 laboratories produced acceptable results for creatinine for test sample A and B. For lab PT9927 a possible unit error or calculation error (factor 10) may have occurred. The results indicated that apart from the suspected gross errors the results for creatinine determination are very comparable. When excluding the suspect gross errors, the maximum difference between lowest and highest reported concentration (including the results with Z-scores >2) was a factor of 1.2-1.3.