HORIZON2020 Programme
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# ICI / EQUAS REPORT

## OPFRs/round_04 (2019/2020)

### OPFRs in urine

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# 1 Summary

Within the frame of the HBM4EU project, an External Quality Assurance Scheme (EQUAS) was organised on the determination of four OPFR biomarkers in urine. This was the 4th ICI/EQUAS round for this substance group within the HBM4EU program.

In total, 14 laboratories were invited for this 4th ICI/EQUAS and six laboratories (including three expert laboratories) submitted results. Four labs were able to report results for all four parameters and two labs reported results for three parameters.

In December 2019, each participant received one tube of burdened control materials of human urine (low level – level 1), one tube of burdened control materials of human urine (high level – level 2). The biomarker concentrations were approximately in the range of 2-6 µg/L and 8-25 µg/L for level 1 and level 2, respectively. The concentrations were chosen according to the review of relevant data on the occurrence of OPFRs in urine of the European population published mostly during the last five years.

A homogeneity assessment showed that both materials were sufficiently homogeneous for EQUAS testing. No issues with stability of testing materials occurred for OPFRs.

The determination of expert value based on results from expert laboratories was not possible only for BCEP, because the minimum number of expert results wasn´t reached for this biomarker.

The evaluation of laboratory performance using Z-scores was performed for DPHP, BDClPP and BClPP for the first time. Most laboratories showed well comparable results for BCEP too, although a regular evaluation was not possible. The achieved results for BCEP are present in the report for further comparison between participants and expert labs.

# 2 Introduction

Interlaboratory Comparison Investigations (ICI) and External Quality Assurance Schemes (EQUAS) are tools to assess 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 4th 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 (https://www.hbm4eu.eu/online-library/). Within HBM4EU, participation in ICI/EQUAS exercises is mandatory for laboratories that will analyse HBM4EU samples.

This report describes the 4th ICI/EQUAS for OPFRs in urine, which was conducted as EQUAS and was organised by UCT Prague (University of Chemistry and Technology, Prague; VŠCHT, Vysoká škola chemicko-technologická v Praze), Department of Food Analysis and Nutrition. The analyses for homogeneity and stability testing were performed by the partner laboratory IPASUM (Institut und Poliklinik für Arbeits-, Sozial- und Umweltmedizin der Universität Erlangen-Nürnberg).

For this 4th ICI/EQUAS, expert laboratories had to be selected according to the selection criteria described in HBM4EU-SOP-QA-001 and in agreement with the QAU.

The selection of the most relevant OPFRs was previously done in WP9, and has been described in Deliverable report 9.2 v1.1. Based on this, a set of four target biomarkers was compiled to be included in the EQUAS for OPFR analysis in urine.

EQUAS is similar to ICI but instead of using the consensus value as assigned value, the mean concentration as established from data generated by at least three designated expert laboratories is used. As in an ICI, Z-scores are calculated as a measure of proficiency.

## 2.1 Confidentiality

In this report the identity of the participants and the information provided by them are 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

The human urine was collected from one person during one day. A total of two litres were obtained. Urine was placed into the refrigerator at 7 °C overnight. The next day the sediment was centrifuged and filtrated. The whole procedure was repeated twice.

Before the spiking procedure, the urine was thawed at room temperature (20 °C). Then it was stirred for 30 min in a 3 L beaker using a magnetic stirrer. After that, three aliquots (500 mL in graduated cylinder) were transferred into the 1 L beaker (one aliquot for urine level 1 and one for urine level 2). Individual OPFR delivered as solids were dissolved with respect to the manufacturers' recommendations. Subsequently, each standard of the biomarker was appropriately diluted into methanol and individually spiked into the urine level 1 and urine level 2 using calibrated Eppendorf Multipette®. During the spiking procedure, the urine was mixed using a magnetic stirrer for the whole time, and when all compounds had been added, subsequent mixing for 30 minutes was performed. A total of 10 mL from "blank" urine, level 1 and level 2 urine was placed into the tube and later analysed for homogeneity testing. For the ICI/EQUAS and stability testing, a total of 5 mL was placed into the tube from each prepared material (urine level 1, urine level 2). All tubes were placed into the freezer at -18 °C before analysis / dispatch.

## 3.2 Homogeneity of control material

The homogeneity of the control material was tested according to HBM4EU-QA-002. Ten tubes of control material at level 1 and level 2 were randomly selected from the freezer and sent to IPASUM for analysis. The GC-MS/MS-based method for the detection of OPFR metabolites in human urine after solid phase extraction and derivatization with pentafluorobenzylbromide was used (Fromme et al. 2014).

The mean concentrations and relative standard deviations (RSDr) as obtained during a homogeneity testing are presented in **Table 1**. The statistical evaluation of level 1 and level 2 materials for each of the biomarkers is provided in **Appendix 1**. It was concluded that homogeneity was adequate for all quantified biomarkers at both levels.

**Table 1: Concentration of OPFRs as obtained during homogeneity testing (for details see Appendix 1)**

| Biomarker | Level 1 (low) | | Level 2 (high) | |
| --- | --- | --- | --- | --- |
| | Mean (µg/L) | RSDr (%) | Mean (µg/L) | RSDr (%) |
| BCPP | 5.915 | 3 | 29.342 | 4 |
| BCEP | 3.448 | 4 | 12.474 | 3 |
| DPHP | 2.426 | 7 | 8.484 | 5 |
| BDCPP | 4.833 | 10 | 16.262 | 8 |

## 3.3 Stability of control material

The stability of the control material was tested according to HBM4EU-QA-002. On the day of preparation of the control materials, randomly selected test urine samples of level 1 and level 2 were stored at -80 °C. After the deadline of submission of analysis results by the participants six test samples of both materials stored at -80 °C and six samples of both materials randomly selected from

the -18 °C freezer, where the ICI samples were stored, were selected for analysis by IPASUM. For the analysis the previously described methods were used (see 3.2 Homogeneity of control material). The stability was evaluated using the Excel-sheet “HBM4EU ICI-EQUAS stability test CM v1”. The results are presented in **Appendix 2**. In summary, no troubles with the stability were identified.

# 4 Organisational details

## 4.1 Participants

For the organisation of the 4th ICI/EQUAS, IPASUM conducted a survey to find expert laboratories for the analysis of OPFRs in urine willing to participate in the project. Then, IPASUM evaluated their eligibility and selected expert laboratories in agreement with the QAU and according to HBM4EU-SOP-QA-001.

UCT Prague contacted the selected expert laboratories and sent them invitation letters by e-mail. It was indicated that participation would be free of charge, and that those who subscribed to this EQUAS would receive a kit containing the test materials needed for analysis. The final number of expert labs was three, all of them from the HBM4EU consortium.

Participants of this 4th ICI/EQUAS 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). Invitation letters (**Appendix 3**) and registration forms (**Appendix 4**) were sent by e-mail on 14/10/2019 to 14 laboratories. For registration, each participant was asked to provide which of four biomarkers were included in their scope. The participants were informed that the participation will be free of charge. The deadline for registration was 10/11/2019. Out of 14 invited laboratories, six labs (including three expert labs) agreed to participate. All registered laboratories submitted results.

## 4.2 Dispatch and instructions

Test materials were dispatched on 02/12/2019. Each participant received one tube of burdened control materials of human urine (low level – level 1) and one tube of burdened control materials of human urine (high level – level 2). Each sample consisted of approximately 5 mL urine.

Moreover, a letter with instructions on sample handling (**Appendix 5**), a sample receipt form to be sent back to UCT Prague upon receipt of the test material (**Appendix 6**) as well as a result submission form and a method information form (**Appendix 7**) were sent to the participants by e-mail. The latter form was used to extract relevant information related to the analytical method used for quantification.

Test materials were dispatched to the expert laboratories under frozen conditions (on dry ice) on 02/12/2019. Each lab received six tubes of burdened control materials of urine (low level – level 1) and six tubes of burdened control materials of urine (high level – level 2). Each sample consisted of approximately 5 mL urine.

Participants and expert labs were asked to perform a single analysis of each sample using the same procedure as will be used for analysis of samples in the frame of HMB4EU and to report results following the instructions given. The deadline for submitting results was 20/01/2020.

## 4.3 Deviations from ICI/EQUAS SOPs

For this 4th ICI/EQUAS, the HBM4EU-QA-SOPs (version 2) were followed. There were no deviations from these SOPs.

# 5 Data evaluation

## 5.1 False positives and <LOQ

Classification of false positives and biomarkers reported as "<LOQ-value" or "not detected" is done as described in HBM4EU-SOP-QA-003.

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

1) the biomarker is below the LOQ value as applied by the organiser, the expert laboratories, and the majority of the participants.

2) the biomarker is reported by the participant at a level clearly exceeding the LOQs mentioned under 1).

When a biomarker is reported as "<LOQ-value", AND an assigned value could be established for the biomarker in the control material, a further assessment was done to verify whether this result might be a false negative and to judge whether the LOQ is considered adequate (low enough) for analysis in the frame of HBM4EU. A result is a false negative when the LOQ of a biomarker is well below the assigned value, but the laboratory did not report a quantitative value. The LOQ is considered not adequate (too high) when:

1) the LOQ is substantially above the assigned value

2) the assigned value represents a realistic concentration of real samples in the frame of HBM4EU

3) quantitative determination is feasible by the majority of laboratories

In order to judge "<LOQ" results in a quantitative way, 'proxy-Z-scores' are calculated as described in 5.6.

## 5.2 Assigned value

For EQUAS studies, the concentration as established by expert laboratories is used as assigned value. The expert-assigned value is the target value based on analysis results obtained from analysis of the control material by at least three expert laboratories (see HBM4EU-SOP-QA-001). In brief, using the individual means of the expert laboratories, the mean-of-means was calculated and its relative uncertainty. The mean of means is used as assigned value when the relative uncertainty was below $0.7*\sigma_T$. If this condition is not met, and no outliers could be identified, then the uncertainty of the expert-derived mean is considered too high to be used as assigned value. The other requirement to be met is that the number of (remaining) individual expert means had to be at least three.

In case no expert value could be obtained, the consensus value derived from the combined results from both participants and expert laboratories is used as an alternative, but this is subject to a minimum of seven results in total. In this case the consensus value is calculated using robust statistics as described for ICI in HBM4EU-SOP-QA-003.

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

For calculation of the Z-scores, a fit-for-purpose relative target standard deviation (FFP-RSDR) of 25% of the assigned value is used as target standard deviation. This was the default indicated in HBM4EU-SOP-QA-003 and considered appropriate based on the outcome of the 1$^{st}$, 2$^{nd}$ and 3$^{rd}$ rounds.

## 5.4 Z-scores

Z-scores are calculated according to SOP HBM4EU-SOP-QA-003.

$$Z = \frac{x - C}{\sigma_T} \qquad (1)$$

with: Z = Z-score for the submitted analysis result;
x = result submitted by the laboratory;
C = expert-assigned value;
$\sigma_T$ = target standard deviation, here 0.25*C

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 2**.

**Table 2: Classification of Z-scores**

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

## 5.5 Proxy-Z-scores

'Proxy-Z-scores' are used here to judge "<LOQ" results in a quantitative way (see 5.1). The proxy-Z-scores' are calculated using the LOQ-value as result and equation (1). If no LOQ is specified, zero is used.

Proxy-Z-scores are classified as follows:

| | |
| --- | --- |
| proxy-Z ≤ -3 | false negative. Based on the LOQ provided, the laboratory should have been able to detect and quantify the biomarker. Performance is considered 'unsatisfactory'. |
| proxy-Z ≥3 | the LOQ is considered too high to be fit-for-purpose in the frame of HBM4EU analysis. It also means that the LOQ is too high in comparison with other laboratories. (Note: proxy-Z can only be calculated when an assigned value could be established. If this is the case, this inherently means that reliable quantitative determination at a certain low level is feasible). Performance is considered 'unsatisfactory'. |
| -3 ≤ proxy-Z < -2 | possible false negative. Performance is considered 'questionable'. |
| 2 < proxy-Z ≤ 3 | the LOQ is relatively high in relation to HBM4EU analysis and compared to other laboratories. Performance is considered 'questionable'. |
| -2 ≤ proxy-Z ≤ 2 | LOQ is within an acceptable range relative to the assigned value, adequate for HBM4EU analysis, and in line with the LOQs of the majority of the participating laboratories. Performance is considered 'satisfactory'. |

# 6 Results and discussion

## 6.1 Results submitted by participants

In total, six laboratories including three expert labs agreed to participate in this study and all of them submitted results. Two expert labs reported six results for each analysed urine sample. As described above, the urine material was sent to IPASUM for homogeneity testing. This lab is also involved as an expert in this round.

Four labs were able to report results for all four parameters (DPHP, BDClPP, BClPP, BCEP) and two labs reported results for three parameters (DPHP, BDClPP, BClPP). The provided LOQs were comparable between participants (**Table 3**).

**Table 3: Scope and LOQs (µg/L) as provided in the method information submitted by the laboratories**

| Lab code | DPHP | BDCIPP | BCEP | BCIPP | Total |
|---|---|---|---|---|---|
| PT4OPFR01 (expert) | 0.05 | 0.02 | NA | 1 | 3 |
| PT4OPFR03 | 0.03 | 0.09 | 0.3 | 0.3 | 4 |
| PT4OPFR04 (expert) | 0.15 | 0.1 | 0.1 | 0.2 | 4 |
| PT4OPFR05 (expert) | 0.1 | 0.5 | NA | 6 | 3 |
| PT4OPFR06 | 0.1 | 1.3 | 2.3 | 0.2 | 4 |
| PT4OPFR07 | 0.3 | 0.02 | 0.5 | 0.2 | 4 |
| Total | 6 | 6 | 4 | 6 | |

**Table 4** gives an overview of all results reported by both expert and candidate laboratories. Regarding BClPP at level 2, five out of six labs provided comparable results. In the case of BCEP at level 2, three out of four labs reported similar results.

**Table 4: The comparison of results reported by participating laboratories**

| | DPHP level 1 | DPHP level 2 | BDCIPP level 1 | BDCIPP level 2 | BCIPP level 1 | BCIPP level 2 | BCEP level 1 | BCEP level 2 |
|---|---|---|---|---|---|---|---|---|
| No. of candidates + experts | 3+3 | 3+3 | 3+3 | 3+3 | 3+3 | 3+3 | 3+1 | 3+1 |
| No. of quantitative results | 6 | 6 | 6 | 6 | 6 | 6 | 4 | 4 |
| Study $RSD_R$ for all results (%) | 17 | 11 | 12 | 11 | 17 | 26 | 16 | 29 |
| Lab code | Results (µg/L) | | | | | | | |
| PT4OPFR01 (expert) | 2.295 | 8.089 | 4.386 | 13.592 | 5.668 | 23.673 | NA | NA |
| PT4OPFR03 | 2.370 | 8.600 | 3.950 | 12.000 | 5.350 | 24.300 | 3.290 | 11.640 |
| PT4OPFR04 (expert) | 2.468 | 8.454 | 5.170 | 16.993 | 5.903 | 29.851 | 3.448 | 12.474 |
| PT4OPFR05 (expert) | 2.552 | 8.864 | 4.434 | 14.180 | 4.864 | 26.671 | NA | NA |
| PT4OPFR06 | 3.572 | 11.074 | 5.763 | 14.636 | 3.344 | 10.733 | 4.140 | 9.253 |
| PT4OPFR07 | 2.346 | 8.254 | 4.754 | 15.759 | 4.912 | 21.840 | 4.912 | 19.644 |

## 6.2 Assigned values and (target) standard deviations

Using the individual means of the expert values, the mean-of-means was calculated and its relative uncertainty. The mean-of-means can be used as assigned value if the relative uncertainty is below 0.7*σT. This condition was met for DPHP, BDClPP and BClPP at both levels.

Calculation of expert value for BCEP was not possible because of the small number of experts' results (minimum is three expert labs). Alternative approach based on calculation of the consensus value derived from the combined results from both participants and expert laboratories was also not possible, because the number of labs' results needed for the robust statistic was smaller than seven.

## 6.3 Assessment of laboratory performance

The assessment of laboratory performance was possible for most biomarkers and it is summarized in **Table 5, Table 6** and **Table 7**. A graphical presentation of the Z-scores is provided in **Appendix 8**. The number of satisfactory scores (-2 < Z-score < 2) was 100 % for DPHP and BDClPP at both levels. Regarding BClPP the number of satisfactory scores was 100 % at level 1 and 83 % at level 2.

**Table 5: Assigned values and participant´s performance for DPHP**

| | DPHP: Level 1 | | | DPHP: Level 2 | | |
|---|---|---|---|---|---|---|
| **Z-score based on** | **expert value ($n_{expert\ labs}$=3)** | | | **expert value ($n_{expert\ labs}$=3)** | | |
| **No. of participants** | **6** | | | **6** | | |
| **No. of quantitative results** | **6** | | | **6** | | |
| **Expert value (ng/ml)** | **2.438** | | | **8.469** | | |
| **Uncertainty of assigned value (ng/ml)** | **0.062** | | | **0.183** | | |
| **Relative uncertainty (%)** | **2.5** | | | **2.2** | | |
| **Relative FFP-target standard deviation (%)** | **25** | | | **25** | | |
| **Study RSDr (%)** | **19** | | | **12** | | |
| | **Value** | **Z-score** | **Classification** | **Value** | **Z-score** | **Classification** |
| **PT4OPFR01** | 2.295 | -0.24 | satisfactory | 8.089 | -0.18 | satisfactory |
| **PT4OPFR03** | 2.370 | -0.11 | satisfactory | 8.600 | 0.06 | satisfactory |
| **PT4OPFR04** | 2.468 | 0.05 | satisfactory | 8.454 | -0.01 | satisfactory |
| **PT4OPFR05** | 2.552 | 0.19 | satisfactory | 8.864 | 0.19 | satisfactory |
| **PT4OPFR06** | 3.572 | 1.86 | satisfactory | 11.074 | 1.23 | satisfactory |
| **PT4OPFR07** | 2.346 | -0.15 | satisfactory | 8.254 | -0.10 | satisfactory |

**Table 6: Assigned values and participant´s performance for BDCIPP**

| | BDCIPP: Level 1 | | | BDCIPP: Level 2 | | |
|---|---|---|---|---|---|---|
| **Z-score based on** | **expert value ($n_{expert\ labs}$=3)** | | | **expert value ($n_{expert\ labs}$=3)** | | |
| **No. of participants** | **6** | | | **6** | | |
| **No. of quantitative results** | **6** | | | **6** | | |
| **Expert value (ng/ml)** | **4.663** | | | **14.922** | | |
| **Uncertainty of assigned value (ng/ml)** | **0.207** | | | **0.857** | | |
| **Relative uncertainty (%)** | **4.4** | | | **5.7** | | |
| **Relative FFP-target standard deviation (%)** | **25** | | | **25** | | |
| **Study RSDr (%)** | **14** | | | **12** | | |
| | **Value** | **Z-score** | **Classification** | **Value** | **Z-score** | **Classification** |
| **PT4OPFR01** | 4.386 | -0.24 | satisfactory | 13.592 | -0.36 | satisfactory |
| **PT4OPFR03** | 3.950 | -0.61 | satisfactory | 12.000 | -0.78 | satisfactory |
| **PT4OPFR04** | 5.170 | 0.43 | satisfactory | 16.993 | 0.56 | satisfactory |
| **PT4OPFR05** | 4.434 | -0.20 | satisfactory | 14.180 | -0.20 | satisfactory |
| **PT4OPFR06** | 5.763 | 0.94 | satisfactory | 14.636 | -0.08 | satisfactory |
| **PT4OPFR07** | 4.754 | 0.08 | satisfactory | 15.759 | 0.22 | satisfactory |

**Table 7: Assigned values and participant´s performance for BCIPP**

| | BCIPP: Level 1 | | | BCIPP: Level 2 | | |
|---|---|---|---|---|---|---|
| **Z-score based on** | **expert value ($n_{expert\ labs}$=3)** | | | **expert value ($n_{expert\ labs}$=3)** | | |
| **No. of participants** | **6** | | | **6** | | |
| **No. of quantitative results** | **6** | | | **6** | | |
| **Expert value (ng/ml)** | **5.478** | | | **26.732** | | |
| **Uncertainty of assigned value (ng/ml)** | **0.257** | | | **1.456** | | |
| **Relative uncertainty (%)** | **4.7** | | | **5.4** | | |
| **Relative FFP-target standard deviation (%)** | **25** | | | **25** | | |
| **Study RSDr (%)** | **18** | | | **29** | | |
| | **Value** | **Z-score** | **Classification** | **Value** | **Z-score** | **Classification** |
| **PT4OPFR01** | 5.668 | 0.14 | satisfactory | 23.673 | -0.46 | satisfactory |
| **PT4OPFR03** | 5.350 | -0.09 | satisfactory | 24.300 | -0.36 | satisfactory |
| **PT4OPFR04** | 5.903 | 0.31 | satisfactory | 29.851 | 0.47 | satisfactory |
| **PT4OPFR05** | 4.864 | -0.45 | satisfactory | 26.671 | -0.01 | satisfactory |
| **PT4OPFR06** | 3.344 | -1.56 | satisfactory | 10.733 | -2.39 | questionable |
| **PT4OPFR07** | 4.912 | -0.41 | satisfactory | 21.840 | -0.73 | satisfactory |

## 6.4 Conclusions and recommendations

In this HBM4EU 4th ICI/EQUAS on OPFR biomarkers in urine, 14 laboratories were invited, of which six submitted results. The overall participation rate was 43%. Two test materials were provided to each participant (spiked material at low level 1 and spiked material at high level 2).

Quantitative performance using Z-scores was assessed for DPHP, BClPP and BDClPP at both levels. The number of satisfactory scores (-2 < Z-score < 2) was 100 % for DPHP and BDClPP at both levels. Regarding BClPP the number of satisfactory scores was 100 % at level 1 and 83 % at level 2.

As explained above, the determination of assigned value was not possible for BCEP, for which the number of expert labs was smaller than three and there was no possibility to calculate a consensus value due to the small number of participants. Nevertheless, most labs showed well comparable results.

In conclusion, the calculation of the assigned value for three biomarkers (DPHP, BDClPP and BClPP) and Z-score determination was realized for the first time. As recognized in the previous three ICI/EQUAS, the determination of OPFR metabolites in urine is very challenging. For this reason a Webex meeting had been organized by UCT Prague and IPASUM after the 3rd Round. The discussion with candidate labs proposed several steps towards the harmonization of the analytical method which can increase the result quality. This 4th ICI/EQUAS documents great improvement of participants' performance for three OPFR biomarkers. Finally, a quite significant core network of satisfactory laboratories is qualified for the analysis of real samples.

# 7 References

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)"

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## Appendix 1: Homogeneity data

| | BCPP - level 1 | | BCEP - level 1 | | DPP - level 1 | | BDCPP - level 1 | |
|---|---|---|---|---|---|---|---|---|
| | Replicate 1 | Replicate 2 | Replicate 1 | Replicate 2 | Replicate 1 | Replicate 2 | Replicate 1 | Replicate 2 |
| 1 | 5.798 | 5.863 | 3.478 | 3.315 | 2.605 | 2.622 | 5.230 | 5.380 |
| 2 | 5.915 | 5.966 | 3.493 | 3.477 | 2.573 | 2.381 | 4.870 | 5.380 |
| 3 | 5.870 | 5.979 | 3.538 | 3.533 | 2.467 | 2.730 | 5.340 | 5.300 |
| 4 | 5.825 | 5.911 | 3.410 | 3.393 | 2.474 | 2.322 | 4.930 | 4.260 |
| 5 | 6.195 | 6.207 | 3.334 | 3.500 | 2.446 | 2.454 | 4.280 | 4.280 |
| 6 | 6.086 | 6.244 | 3.567 | 3.372 | 2.568 | 2.484 | 4.740 | 5.050 |
| 7 | 5.930 | 5.947 | 3.297 | 3.230 | 2.348 | 2.279 | 5.290 | 4.470 |
| 8 | 5.811 | 5.890 | 3.658 | 3.674 | 2.117 | 2.378 | 4.560 | 4.680 |
| 9 | 5.711 | 5.686 | 3.278 | 3.613 | 2.511 | 2.083 | 4.420 | 4.370 |
| 10 | 5.616 | 5.844 | 3.352 | 3.438 | 2.238 | 2.441 | 4.680 | 4.440 |
| Grand mean | 5.915 | | 3.448 | | 2.426 | | 4.798 | |
| Cochran´s test | | | | | | | | |
| C | 0.4701 | | 0.5158 | | 0.4220 | | 0.4266 | |
| C crit | 0.8674 | | 0.8674 | | 0.8674 | | 0.8674 | |
| C < Ccrit ? | no outliers detected | | no outliers detected | | no outliers detected | | no outliers detected | |
| $\sigma_T$ | 1.4787 | | 0.8619 | | 0.6065 | | 1.1994 | |
| $s_x$ | 0.1634 | | 0.1052 | | 0.1281 | | 0.3671 | |
| $s_w$ | 0.0743 | | 0.1041 | | 0.1473 | | 0.2807 | |
| $s_s$ | 0.1547 | | 0.0751 | | 0.0745 | | 0.3088 | |
| c crit | 0.3904 | | 0.2275 | | 0.0998 | | 0.3166 | |
| Ss < c | homogeneity adequate | | homogeneity adequate | | homogeneity adequate | | homogeneity adequate | |
| sw < 0.5 σT ? | method suited | | method suited | | method suited | | method suited | |
| | | | | | | | | |
| | BCPP - level 2 | | BCEP - level 2 | | DPP - level 2 | | BDCPP - level 2 | |
| | | | | | | | | |
| 1 | 29.783 | 30.100 | 12.124 | 12.486 | 8.564 | 8.290 | 16.050 | 15.140 |
| 2 | 29.349 | 30.391 | 12.595 | 12.266 | 8.471 | 8.187 | 17.640 | 17.750 |
| 3 | 30.022 | 29.463 | 12.438 | 12.548 | 8.277 | 8.935 | 17.780 | 17.600 |
| 4 | 29.907 | 30.098 | 12.503 | 12.988 | 9.067 | 8.477 | 16.130 | 17.700 |
| 5 | 29.957 | 29.192 | 12.449 | 12.801 | 8.722 | 8.969 | 16.060 | 16.210 |
| 6 | 29.928 | 29.073 | 12.459 | 12.376 | 8.251 | 8.461 | 17.430 | 17.100 |
| 7 | 29.814 | 30.463 | 12.491 | 12.313 | 7.849 | 8.395 | 16.590 | 17.300 |
| 8 | 28.152 | 28.930 | 11.961 | 12.476 | 7.669 | 8.944 | 14.000 | 15.040 |
| 9 | 27.307 | 29.215 | 12.279 | 12.766 | 8.233 | 9.156 | 14.230 | 15.510 |
| 10 | 26.179 | 29.522 | 11.753 | 13.414 | 7.866 | 8.889 | 15.030 | 15.940 |
| Grand mean | 29.342 | | 12.474 | | 8.484 | | 16.312 | |
| Cochran´s test | | | | | | | | |
| C | 0.5979 | | 0.7056 | | 0.3342 | | 0.3277 | |
| C crit | 0.8674 | | 0.8674 | | 0.8674 | | 0.8674 | |
| C < Ccrit ? | no outliers detected | | no outliers detected | | no outliers detected | | no outliers detected | |
| $\sigma_T$ | 7.3356 | | 3.1186 | | 2.1209 | | 4.0779 | |
| $s_x$ | 0.8153 | | 0.1548 | | 0.2335 | | 1.1549 | |
| $s_w$ | 0.9668 | | 0.4422 | | 0.4932 | | 0.6132 | |
| $s_s$ | 0.4444 | | 0.2717 | | 0.2591 | | 1.0704 | |
| c crit | 1.9366 | | 0.8233 | | 0.5599 | | 1.0766 | |
| Ss < c | homogeneity adequate | | homogeneity adequate | | homogeneity adequate | | homogeneity adequate | |
| sw < 0.5 σT ? | method suited | | method suited | | method suited | | method suited | |

## Appendix 2: Stability data

| Biomarker | **BCPP level 1** | | **BCPP level 2** | | **BCEP level 1** | | **BCEP level 2** | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | | | | | | | |
| time (days) | 0 | 40 | 0 | 40 | 0 | 40 | 0 | 40 |
| | 5.947 | 6.220 | 30.100 | 29.510 | 3.478 | 3.530 | 12.124 | 11.890 |
| | 6.195 | 6.070 | 30.022 | 31.080 | 3.315 | 3.540 | 11.266 | 11.580 |
| | 6.207 | 5.870 | 30.098 | 29.420 | 3.297 | 3.320 | 12.376 | 11.750 |
| | 6.086 | 5.950 | 29.192 | 29.360 | 3.230 | 3.260 | 11.961 | 11.740 |
| | 6.244 | 6.280 | 29.073 | 30.680 | 3.493 | 3.250 | 12.279 | 11.830 |
| | 5.890 | 5.860 | 30.463 | 30.430 | 3.477 | 3.350 | 11.753 | 12.170 |
| Average | 6.095 | 6.042 | 29.825 | 30.080 | 3.382 | 3.375 | 11.960 | 11.827 |
| Std dev | 0.147 | 0.179 | 0.559 | 0.743 | 0.115 | 0.129 | 0.407 | 0.198 |
| x0-xa (difference) | 0.053 | | -0.255 | | 0.007 | | 0.133 | |
| Test 'consequential instability': | | | | | | | | |
| σH | 1.34 | | 6.56 | | 0.74 | | 2.63 | |
| 0,3*σH | 0.40 | | 1.97 | | 0.22 | | 0.79 | |
| x0-xa<0,3*σH? (consequential instability) | NO | | NO | | NO | | NO | |
| Test 'significant difference': | | | | | | | | |
| t | 0.56 | | 0.67 | | 0.09 | | 0.72 | |
| t-crit | 2.23 | | 2.23 | | 2.23 | | 2.23 | |
| Significant difference | NO | | NO | | NO | | NO | |
| | | | | | | | | |
| Biomarker | **BDCPP level 1** | | **BDCPP level 2** | | **DPP level 1** | | **DPP level 2** | |
| | | | | | | | | |
| time (days) | 0 | 40 | 0 | 40 | 0 | 40 | 0 | 40 |
| | 4.280 | 5.080 | 15.140 | 16.080 | 2.605 | 2.440 | 8.564 | 7.840 |
| | 4.260 | 4.590 | 16.130 | 15.740 | 2.622 | 2.520 | 8.290 | 8.550 |
| | 4.560 | 4.730 | 17.700 | 15.550 | 2.348 | 2.530 | 8.471 | 8.950 |
| | 4.680 | 4.440 | 16.590 | 15.590 | 2.279 | 2.560 | 8.187 | 8.220 |
| | 4.420 | 4.660 | 15.040 | 15.140 | 2.573 | 2.830 | 8.277 | 7.870 |
| | 4.370 | 4.560 | 14.230 | 15.860 | 2.381 | 2.560 | 8.935 | 9.160 |
| Average | 4.428 | 4.677 | 15.805 | 15.660 | 2.468 | 2.573 | 8.454 | 8.432 |
| Std dev | 0.164 | 0.220 | 1.251 | 0.319 | 0.149 | 0.133 | 0.273 | 0.552 |
| x0-xa (difference) | -0.248 | | 0.145 | | -0.105 | | 0.022 | |
| Test 'consequential instability': | | | | | | | | |
| σH | 0.97 | | 3.48 | | 0.54 | | 1.86 | |
| 0,3*σH | 0.29 | | 1.04 | | 0.16 | | 0.56 | |
| x0-xa<0,3*σH? (consequential instability) | NO | | NO | | NO | | NO | |
| Test 'significant difference': | | | | | | | | |
| t | 2.21 | | 0.28 | | 1.29 | | 0.09 | |
| t-crit | 2.23 | | 2.23 | | 2.23 | | 2.23 | |
| Significant difference | NO | | NO | | NO | | NO | |

# Appendix 3: Copy of letter of invitation

HBM4EU science and policy for a healthy future

**HBM4EU: Announcement / invitation to participate in ICI / EQUAS study**
**OPFR/Round 4**

**Title of ICI/EQUAS:** OPFR in urine

Dear Colleagues,

within the frame of HBM4EU,

Prof. Dr. Jana Hajslova & Dr. Darina Dvorakova

University of Chemistry and Technology, Prague
Department of Food Analysis and Nutrition
Technicka 3
166 28 Prague 6
Czech Republic

announce the 4th round of ICI/EQUAS for the determination of OPFR 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.

**Test samples**
The matrix will be human urine. The participants will receive:

- 2 different materials of urine (1 sample of 5 ml each) for determination of OPFR in urine

**Target biomarkers**
Please see registration form for OPFR/Round 4 for the biomarkers potentially present in the test samples. We would be pleased if your laboratory could participate with the analysis of as most as possible OPFR.
LOQs should allow the analysis of OPFR in samples of the general population.

**Calendar:**

| | |
|---|---|
| Deadline for registration | 10-11-2019 |
| Distribution of test samples (projected) | 12-11-2019 |
| Deadline for reporting the results (projected) | 20-12-2019 |

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**Registration**
For registration, please find attached a registration form for OPFR in serum. Please send it back to us by mail in case you want to register.
Upon registration, the participant will receive a lab-code to be used for submission of results.

**Fee**
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.

**Confidentiality:**
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.

**Contact information organiser:**

Dr. Darina Dvorakova
University of Chemistry and Technology, Prague
Department of Food Analysis and Nutrition
Technicka 3
166 28 Prague 6
Czech Republic

Email of organiser: darina.lankova@vscht.cz
**Email for registration: VSCHT-hbm4EU@vscht.cz**
Phone: 00420 22044 4312

## Appendix 4: Copy of registration form for participation

HBM4EU science and policy for a healthy future

**HBM4EU: Registration form for participation in ICI / EQUAS study OPFR/Round 4.**

**Title of ICI/EQUAS:** OPFR in urine

Please choose the OPFR you want to participate with.
We would appreciate your registration for as much biomarkers as possible.

| Parameter | Participation | |
|---|---|---|
| | Yes | No |
| DPHP | | |
| BDCIPP | | |
| BCEP | | |
| BCIPP | | |

**Participating laboratory:**
name of institution

address of the laboratory

name of 1st contact person, telephone number and email address

name of 2nd contact person, telephone number and email address

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**Address for delivery of the test samples**
name of institution

address of the laboratory

The above laboratory will participate in the ICI/EQUAS study **OPFR/Round 4**.
I agree with the conditions mentioned in the invitation letter, and that the laboratory will analyse the ICI/EQUAS samples using the same procedure as will be used for analysis of samples in the frame of HBM4EU, and submit results before the indicated deadline.

Name: Signature

Date:

**After signing this form, please scan and send the pdf to:**

**VSCHT-hbm4EU@vscht.cz**

**Contact information organiser:**
Dr. Darina Dvorakova
University of Chemistry and Technology, Prague
Department of Food Analysis and Nutrition
Technicka 3
166 28 Prague 6
Czech Republic

Email: darina.lankova@vscht.cz
Phone: 00420 22044 4312

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

HBM4EU science and policy for a healthy future

**HBM4EU: Result submission form ICI/EQUAS study OPFR/ Round 4.**

**Title of ICI/EQUAS:** OPFR in urine

Laboratory name & code:

Contact person:

Institute:

Address:

Country:

For each of the analytes from this ICI/EQUAS round:
- provide the LOQ
- report NA for not analysed/not included in the scope of the method used
- report ND when not detected or detected below LOQ
- report a numerical value when found above LOQ, express to three significant figures (e.g. 0.543)

Date:

Signature:

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**Please send the filled in result table together with this signed result submission form back to:**

**VSCHT-hbm4EU@vscht.cz**

If you have any questions or need any assistance, please contact:

Darina Dvorakova
Email: darina.dvorakova@vscht.cz
Phone: 00420 22044 4312

Jana Hajslova
Email: jana.hajslova@vscht.cz
Phone 00420602833424

**Contact information organiser:**
Dr. Darina Dvorakova
University of Chemistry and Technology, Prague
Department of Food Analysis and Nutrition
Technicka 3
166 28 Prague 6
Czech Republic

Prof. Ing. Jana Hajslova, CSc.
(for the ICI/EQUAS organisers)

## Appendix 6: Copy of acknowledgement of receipt sent together with test samples

HBM4EU science and policy for a healthy future

**HBM4EU: Acknowledgement of receipt form ICI/EQUAS study OPFR/Round 4.**

**Title of ICI/EQUAS:** OPFR in urine

Laboratory name:

Contact person:

Contents of parcel:
- 2 tubes with 5 ml urine

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

Date of receipt (dd-mm-yyyy):

| **Code on tube** | Damage/leakage | Remarks |
|---|---|---|
| | | |
| | | |

Name: Signature:

Date:

After signing this form, please scan and send the pdf. to: **VSCHT-hbm4EU@vscht.cz**

HBM4EU science and policy for a healthy future

If you have any questions or need any assistance, please contact:

Darina Dvorakova
Email: darina.dvorakova@vscht.cz
Phone: 00420 22044 4312

Jana Hajslova
Email: jana.hajslova@vscht.cz
Phone 00420 602 833 424

**Contact information organiser:**
Dr. Darina Dvorakova
University of Chemistry and Technology, Prague
Department of Food Analysis and Nutrition
Technicka 3
166 28 Prague 6
Czech Republic

Prof. Ing. Jana Hajslova, CSc.
(for the ICI/EQUAS organisers)

# Appendix 7: Copy of method information form for participation in ICI/EQUAS

HBM4EU science and policy for a healthy future

**Method information form for participation in ICI/EQUAS OPFR in urine**
**OPFR/Round 4.**

| **Laboratory name & code** | |
|---|---|
| ISO17025 accredited | yes / no |
| **SAMPLE PREPARATION** | |
| amount sample extracted (ml) | |
| **Deconjugation** | yes / no |
| chemical (reagent / pH / temp / time) | |
| enzymatic (enzyme / pH / temp / time) | |
| **Extraction** | |
| pH adjustment | |
| LLE (solvent(s) / time / shaking) | |
| SPE (material) | |
| **Cleanup** | |
| LLE (solvent(s)) | |
| SPE (material) | |
| **Derivatisation** | |
| reagent | |
| **INSTRUMENTAL ANALYSIS** | |
| **HPLC** | |
| injection volume (µl) | |
| column stationary phase | |
| column L (mm) x ID (mm); dp (µm) | |
| temperature | |
| mobile phase A (specify the mobile additives) | |
| mobile phase B (specify the mobile additives) | |
| flow rate (ml/min) | |
| **GC** | |
| injector (splitless/PTV/…) | |
| injection volume | |
| column stationary phase | |
| column L (m) x ID (mm) df (µm) | |
| carrier | |
| flow rate / inlet pressure | |
| **Detection** | |
| MS (single quad/triple quad/Q-Orbitrap/Q-TOF) | |
| MS manufacturer (system specification) | |
| Other | |

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| **QUANTIFICATION** | |
|---|---|
| **Use of internal standard (IS)** | |
| isotopic label yes/no | yes / no |
| please specifify the IS including purity used for each OPFRs | |
| DPHP | |
| BCEP | |
| BDCIPP | |
| BCIPP | |
| moment of addition (e.g. before deconjugation, to final extract..) | |
| response normalised to IS | yes / no |
| **Calibration** | isotope dilution (addition to sample before extraction) |
| | isotope dilution (addition to final extract) |
| | standard addition (addition to sample before extraction) |
| | standard addition (addition to final extract) |
| | matrix-matched (addition to blank matrix before extraction) |
| | matrix-matched (addition to blank extract) |
| | solvent standards |
| | single level / multi level |
| **Correction for recovery** | yes / no |
| **Recovery value (%) (if applicable specify for each analytes)** | |
| DPHP | |
| BCEP | |
| BDCIPP | |
| BCIPP | |
| **Identification criteria used** | |
| retention time tolerance (min or % deviation from reference standard) | |
| number of ions/transitions required for identification | |
| specify ions/transitions used for the quantification and confirmation | |
| DPHP - quantification | |
| DPHP - confirmation | |
| BCEP - quantification | |
| BCEP - confirmation | |
| BDCIPP - quantification | |
| BDCIPP - confirmation | |
| BCIPP - quantification | |
| BCIPP - confirmation | |
| ion ratio tolerance (% relative/absolute deviation from reference standard) | |

## Appendix 8: Graphical representation of the Z-scores