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# Interlaboratory comparison investigations (ICIs) and external quality assurance schemes (EQUASs) for flame retardant analysis in biological matrices: Results from the HBM4EU project☆

Darina Dvorakova [a,*], Jana Pulkrabova [a], Tomas Gramblicka [a], Andrea Polachova [a], Martina Buresova [a], Marta Esteban López [b], Argelia Castaño [b], Stefanie Nübler [c], Karin Haji-Abbas-Zarrabi [c], Nadine Klausner [c], Thomas Göen [c], Hans Mol [d], Holger M. Koch [e], Vincent Vaccher [f], Jean-Philippe Antignac [f], Line Småstuen Haug [g], Katrin Vorkamp [h], Jana Hajslova [a]

[a] University of Chemistry and Technology (UCT), Prague, Faculty of Food and Biochemical Technology, Department of Food Analysis and Nutrition, Technicka 5, Prague, 166 28, Czech Republic
[b] National Centre for Environmental Health, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain
[c] Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine (IPASUM), Friedrich-Alexander Universität Erlangen-Nürnberg, Henkestraße 9-11, 91054, Erlangen, Germany
[d] Wageningen Food Safety Research (WFSR), Part of Wageningen University & Research, Wageningen, Netherlands
[e] Institute for Prevention and Occupational Medicine of the German Social Accident Insurance, Institute of the Ruhr Universität Bochum (IPA), Bürkle-de-la-Camp-Platz 1, 44789, Bochum, Germany
[f] Oniris, INRAE, UMR 1329 Laboratoire d'Etude des Résidus et Contaminants dans les Aliments (LABERCA), F-44307, Nantes, France
[g] Department of Environmental Health, Norwegian Institute of Public Health, Oslo, Norway
[h] Aarhus University, Department of Environmental Science, Frederiksborgvej 399, 4000, Roskilde, Denmark

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ABSTRACT

The European Human Biomonitoring Initiative (HBM4EU) is coordinating and advancing human biomonitoring (HBM). For this purpose, a network of laboratories delivering reliable analytical data on human exposure is fundamental. The analytical comparability and accuracy of laboratories analysing flame retardants (FRs) in serum and urine were investigated by a quality assurance/quality control (QA/QC) scheme comprising interlaboratory comparison investigations (ICIs) and external quality assurance schemes (EQUASs).

This paper presents the evaluation process and discusses the results of four ICI/EQUAS rounds performed from 2018 to 2020 for the determination of ten halogenated flame retardants (HFRs) represented by three congeners of polybrominated diphenyl ethers (BDE-47, BDE-153 and BDE-209), two isomers of hexabromocyclododecane (α-HBCD and γ-HBCD), two dechloranes (anti-DP and syn-DP), tetrabromobisphenol A (TBBPA), decabromodiphenylethane (DBDPE), and 2,4,6-tribromophenol (2,4,6-TBP) in serum, and four metabolites of

*Abbreviations:* 2,4,6-TBP, 2,4,6-tribromophenol; BCEP, bis(2-chloroethyl) phosphate; BClPP, bis(1-chloro-2-propyl) phosphate; BDClPP, bis(1,3-dichloro-2-propyl) phosphate; BDE-47, 2,2',4,4'-tetrabromodiphenyl ether; BDE-153, 2,2',4,4',5,5'-hexabromodiphenyl ether; BDE-209, decabromodiphenyl ether (DecaBDE); BFRs, brominated flame retardants; CMs, control materials; CVs, coefficients of variation; DBDPE, decabromodiphenyl ethane; DPHP, diphenyl phosphate; DPs, dechloranes; EQUAS, external quality assurance scheme; FRs, flame retardants; GC, gas chromatography; HBCD, hexabromocyclododecane; HBM, human biomonitoring; HBM4EU, European Human Biomonitoring Initiative; HFRs, halogenated flame retardants; HL, high level; HRMS, high resolution mass spectrometry; ICI, interlaboratory comparison investigation; LC, liquid chromatography; LL, low level; LLE, liquid–liquid extraction; LOQ, limit of quantification; LRMS, low resolution mass spectrometry; MS, mass spectrometry; OPFRs, organophosphorus flame retardants; PBDEs, polybrominated diphenyl ethers; POPs, persistent organic pollutants; QA/QC, quality assurance/quality control; QuEChERS, Quick; Easy, Cheap; Effective, Rugged and Safe; RSD, relative standard deviation; SD, standard deviation; SOPs, standard operation procedures; SPE, solid phase extraction; TBBPA, tetrabromobisphenol A; TCEP, tris (2-chloroethyl) phosphate; TClPP, tris (2-chloroisopropyl) phosphate; TDClPP, tris(1,3-dichloro-2-propyl) phosphate; $u_{ICI}$, uncertainty of $X_P$; $X_E$, expert value derived from the experts' results; $X_P$, consensus value derived from the participants' results.

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* Corresponding author.
*E-mail address:* darina.dvorakova@vscht.cz (D. Dvorakova).

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organophosphorus flame retardants (OPFRs) in urine, at two concentration levels. The number of satisfactory results reported by laboratories increased during the four rounds. In the case of HFRs, the scope of the participating laboratories varied substantially (from two to ten) and in most cases did not cover the entire target spectrum of chemicals. The highest participation rate was reached for BDE-47 and BDE-153. The majority of participants achieved more than 70% satisfactory results for these two compounds over all rounds. For other HFRs, the percentage of successful laboratories varied from 44 to 100%. The evaluation of TBBPA, DBDPE, and 2,4,6-TBP was not possible because the number of participating laboratories was too small. Only seven laboratories participated in the ICI/EQUAS scheme for OPFR metabolites and five of them were successful for at least two biomarkers. Nevertheless, the evaluation of laboratory performance using Z-scores in the first three rounds required an alternative approach compared to HFRs because of the small number of participants and the high variability of experts' results. The obtained results within the ICI/EQUAS programme showed a significant core network of comparable European laboratories for HBM of BDE-47, BDE-153, BDE-209, α-HBCD, γ-HBCD, anti-DP, and syn-DP. On the other hand, the data revealed a critically low analytical capacity in Europe for HBM of TBBPA, DBDPE, and 2,4,6-TBP as well as for the OPFR biomarkers.

## 1. Introduction

Flame retardants (FRs) are a diverse group of chemicals that are added to consumer products or building materials to reduce their flammability and thus improve product safety. Most of these compounds are used as additives rather than being chemically bound to the product matrix, with the consequence of losses to the environment (De Wit, 2002). Human exposure to these substances, especially brominated flame retardants (BFRs), is of great concern due to the potential health risks in terms of endocrine disruption, neurodevelopment, hepatic and behavioural abnormality (Van der Veen and de Boer, 2012; Lyche et al., 2015). Such evidence has contributed to the inclusion of polybrominated diphenyl ethers (PBDEs) in the Stockholm Convention on Persistent Organic Pollutants (POPs), i.e. the addition of Penta- and OctaBDE mixtures in 2009 and the most recent addition of DecaBDE (BDE-209) in 2017, and the development of substitutes. Hexabromocyclododecane (HBCD) has been listed in the Convention since 2013 (Sharkey et al., 2020). The bans of PBDEs and HBCD have led to higher worldwide production of tetrabromobisphenol A (TBBPA) and to their replacement with alternative BFRs in manufacturing processes, for example decabromodiphenyl ethane (DBDPE) (Kierkegaard et al., 2004; Shaw et al., 2014). The highly chlorinated FR dechlorane plus (DP) has been on the market since the 1960s (Wang et al., 2016), but has been recently proposed for listing under the Stockholm Convention (UNEP, 2019).

The legacy BFRs have also been replaced by organophosphate esters (OPFRs, also used as plasticizers) (Lyche et al., 2015). Halogenated OPFRs, such as tris(2-chloroethyl) phosphate (TCEP), tris (2-chloroisopropyl) phosphate (TCIPP) and tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) are suspected to be carcinogenic (EU Risk Assessment Report, TCEP, 2009; EU Risk Assessment Report, TDClPP, 2008). TCEP has been phased out since the 1980s and is no longer produced within the European Union (EU) (EU Risk Assessment Report, TCEP, 2009). The other OPFRs are still used, but TCIPP and TDCIPP are not allowed to be used in toys produced in the EU (EC Directive, 2014/79/EU).

Despite the legislative restrictions, human exposure to BFRs and OPFRs is likely to continue for some time due to the persistence of some of these compounds in the environment and their presence in a number of consumer materials. Exposure sources of BFRs include fatty foods and sources in the indoor environment, such as dust. There is less information about exposure to DPs and OPFRs, but ingestion of dust and food as well as inhalation of air have been suggested to be important exposure sources to these chemicals as well (Ma et al., 2020).

PBDEs and HBCD are bioaccumulative and have long half-lives (weeks to years) in the human body, while OPFRs are rapidly metabolized with relatively short half-lives (hours to days) (Geyer et al., 2004; Hoffman et al., 2014). Therefore, BFRs are generally measured in human serum as biomarkers of exposure, while OPFR diester metabolites are generally analysed in urine as indicators of OPFR exposure (Vorkamp et al., 2021). Serum PBDE levels have been documented mostly in the range of ng/L (on a wet weight basis). Urinary OPFR metabolite levels have been reported in the low to mid μg/L range, with diphenyl phosphate (DPHP, a metabolite of multiple OPFRs), bis(1-chloro-2-propyl) phosphate (BCIPP, metabolite of TCIPP), bis(2-chloroethyl) phosphate (BCEP, metabolite of TCEP) and bis(1,3-dichloro-2-propyl) phosphate (BDCIPP, metabolite of TDCIPP) frequently being detected at higher levels compared to other urinary metabolites (Blum et al., 2019; Varshavsky et al., 2021).

The European Human Biomonitoring Initiative (HBM4EU) is a joint effort of 30 countries and European Commission authorities under the Horizon2020 Programme of the EU. The main aim of this initiative is to harmonize and advance HBM, and support collaboration and knowledge exchange across Europe. HBM4EU targets the exposure of EU citizens to a variety of chemicals and their possible health effects to support policy-making (Ganzleben et al., 2017). FRs were included in the first priority substance list of HBM4EU, and 14 biomarkers were selected for chemical analysis, including ten halogenated flame retardants (HFRs; BDE-47, BDE-153, BDE-209, α-HBCD, γ-HBCD, TBBPA, 2,4,6-tribromophenol (2,4,6-TBP), DBDPE, anti-DP, and syn-DP) and four OPFR metabolites (DPHP, BCEP, BClPP, and BDClPP) (Louro et al., 2019).

In general, the chemical analysis of HBM samples involves a number of challenges, including low levels, the variety of compounds to be included in various biological matrices, the risk of contamination due to the omnipresence of FRs and the availability of analytical standards and certified reference materials. One of the objectives within the HBM4EU project is to establish a network of European laboratories for the realization of harmonized HBM analysis of prioritized groups of environmental contaminants. The generation of high-quality and comparable results is crucial for further data evaluation in the context of risk management and policy-making. Thus, HBM4EU implemented a complete quality assurance/quality control (QA/QC) scheme for the verification of analytical quality and comparability between candidate laboratories for the HBM analysis in the project (Nübler et al., 2021; Esteban López et al., 2021). Within the QA/QC scheme, interlaboratory comparison investigations (ICIs) and external quality assurance schemes (EQUASs) were organized and their results were evaluated.

This paper presents the ICI/EQUAS programme for ten HFRs in serum and four OPFR metabolites in urine, designed and conducted within HBM4EU, including the evaluation process, the main difficulties encountered and the results obtained.

## 2. Materials & method

### 2.1. QA/QC scheme and ICI/EQUAS programme

The objective of the QA/QC scheme was to identify laboratories that could analyse the HBM4EU samples in a comparable way and with a defined analytical quality. In this project, two different harmonized approaches were used for the organization and evaluation of

interlaboratory exercises. The first one is the ICI approach which principally assesses the comparability of results between equally ranked laboratories. For that purpose, two different control samples were analysed by all laboratories using their own method in the same time frame. As a measure of proficiency, Z-scores were calculated using the consensus value derived from the participants' results ($X_P$) and a pre-set target standard deviation. The other approach is the EQUAS which involves with a sufficient number of designated, international expert laboratories generating an assigned value ($X_E$) instead of $X_P$. As with the ICI, for all participating laboratories Z-scores are calculated as a measure of proficiency. The organizational processes and conditions of ICIs and EQUASs for all substance groups in the HBM4EU project are described in detail in Esteban López et al., 2021.

In total, four ICI/EQUAS rounds for both HFRs and OPFR metabolites were organized. The results and conclusions were presented to the participants at a web conference after round 1 for both HFRs and OPFR metabolites as well as by a report after each round. The information regarding the upcoming rounds was presented at the web conference and some analytical difficulties were discussed. A second web conference was conducted after round 3 for OPFR metabolites. The main aim was to identify critical analytical method steps and to propose improvements, which could support the comparability of participants' and expert laboratories' results in the final round 4.

### 2.2. Invitation of candidate laboratories

The registration procedure for candidate laboratories was described previously (Esteban López et al., 2021). In brief, two calls were made to identify candidate laboratories from European countries to perform HFRs and OPFR metabolite analysis in HBM4EU. Candidates were allowed to decide for which group of compounds they wanted to participate. The result after the first call was a list of 24 candidate laboratories from 16 countries for HFRs and 13 candidate laboratories from nine countries for OPFR biomarkers. These numbers increased to 31 laboratories for HFRs from 17 countries and 17 laboratories for OPFR metabolites from ten countries after the second call.

### 2.3. Selection of expert laboratories

For the interlaboratory exercises organized as EQUAS (rounds 2–4), five and three expert laboratories for HFRs and OPFR metabolites, respectively, were selected by the HBM4EU Quality Assurance Unit (Esteban López et al., 2021). Experts were laboratories with experience in the determination of FR HBM parameters documented in peer-reviewed publications. Additional criteria used to select experts included several years of experience in the analysis of these compounds, as well as application of highly sensitive and selective analytical techniques. Furthermore, the availability of in-house validation reports, data on on-going intra-laboratory performance (e.g., control charts), or ISO17025 accreditation for the biomarker of interest and successful participation in relevant commercial proficiency tests, or long-standing experience in FR HBM studies were also considered. For HFR analysis, two selected expert laboratories were from outside Europe, and three expert laboratories were from Europe, of which two already participated as candidate laboratories in the programme. For OPFR metabolites, all three expert laboratories were from Europe and these laboratories were already participated as candidates in the programme. After round 2 for OPFR metabolites, one expert laboratory was replaced by another expert laboratory.

### 2.4. Preparation and testing of CMs

The preparation of control materials (CMs) as well as the scheme for homogeneity and stability testing was realized according to HBM4EU standard operation procedures (SOPs) as explained in the paper of the QA/QC design (Esteban López et al., 2021). Serum and urine were spiked with HFRs or OPFR metabolites, respectively, at two concentration levels (low concentration level ($LL_{HFR}$ and $LL_{OPFR}$) and high concentration level ($HL_{HFR}$ and $HL_{OPFR}$)) (Tables S1A and S1B), which were in agreement with the range of concentrations and profiles commonly observed in the general European population, based on the relevant scientific literature (further details in **3.1**). For each ICI or EQUAS round, new CMs were prepared covering relevant concentration levels (Tables S1A and S1B).

#### 2.4.1. Standards of target biomarkers

Certified analytical standards of HFR biomarkers for PBDEs (BDE-47, BDE-153, and BDE-209), isomers of HBCD (α-HBCD and γ-HBCD), DBDPE, 2,4,6-TBP, and TBBPA were obtained from Wellington Laboratories (Guelph, Ontario, Canada). The standards of anti-DP and syn-DP were purchased from Accustandards®, Inc. (New Haven, Connecticut, USA). The purity of the individual HFR standards was at least 98% and they were obtained in toluene or nonane (except TBBPA, which was in methanol). Thus, for the preparation of working stock solutions for the fortification of serum, the nonpolar solvents were removed under a gentle stream of nitrogen and the residues were dissolved in acetone.

The analytical standards of OPFR metabolites (BCEP, BClPP, BDClPP, and DPHP) were supplied by Toronto Research Chemicals, Inc. (North York, Canada). The purity of BCEP, BClPP, and BDClPP was 95%, and it was 96% for DPHP. Individual standards delivered as solids were dissolved in compliance with the manufacturer's recommendations and then used to prepare working stock solutions in methanol for the fortification of urine.

#### 2.4.2. Fortification procedure

The CM for the analysis of HFR was sterile-filtered bovine serum obtained from Sigma Aldrich (USA). Before the fortification procedure at the expected concentration levels, the serum was thawed at room temperature and stirred on a magnetic stirrer for 30 min. An aliquot of 10 mL was removed and investigated using the method by Svarcova et al. (2019) for the background occurrence of target biomarkers. The rest of the serum was stored at −18 °C. For fortification, the serum was thawed again at room temperature (20 °C) and stirred on a magnetic stirrer for 30 min. After that, three aliquots of 500 mL were transferred into a beaker. One aliquot of serum was identified as $LL_{HFR}$, one as $HL_{HFR}$ and one as blank material. Each standard of the target HFRs was appropriately diluted in acetone and individually added into the serum according to each level.

The CM for the analysis of OPFR metabolites was human urine. The urine was placed in the refrigerator at 7 °C overnight. The next day, the urine was centrifuged and filtrated, which was repeated twice. Before the fortifying procedure, the urine was analysed by the method presented by (Fromme et al., 2014). In the meantime, the native urine was stored at −18 °C. After the investigation of background concentration, the urine was thawed at room temperature and stirred for 30 min using a magnetic stirrer. Three aliquots were transferred into a beaker for the fortifying procedure. One aliquot of urine was identified as $LL_{OPFR}$, one as $HL_{OPFR}$ and one as blank material. Each standard of the target OPFR metabolites was appropriately diluted in methanol and individually added to the urine according to each level.

During the fortifying procedure, the serum and urine, respectively, were mixed throughout, and when all compounds had been added, subsequent mixing was performed for 30 min. Aliquots of 10 mL of $LL_{HFR}/LL_{OPFR}$ and $HL_{HFR}/LL_{OPFR}$ were placed into polypropylene tubes with caps (Simport Scientific Inc., Quebec, Canada) for homogeneity assessment. For the participants' analysis and stability testing, aliquots of 5 mL from each prepared material ($LL_{HFR}$, $HL_{HFR}$, blank material/ $LL_{OPFR}$, $HL_{OPFR}$, blank material) were placed into a tube. All tubes were stored in the freezer at −18 °C before dispatch.

#### 2.4.3. Homogeneity tests of CMs

The homogeneity of CMs was tested according to the SOP developed

in HBM4EU (Esteban López et al., 2021). Ten tubes of the respective control serum and urine material (of each round) at both levels ($LL_{HFR}$, $LL_{OPFR}$, $HL_{HFR}$, $HL_{OPFR}$) were randomly selected from the freezer, thawed, re-homogenized by ultrasonication and each sample was analysed in duplicate. The analytical procedures used for the testing of CMs are described below in **2.4.5**.

Briefly, an assessment of whether or not the CMs were sufficiently homogenous for ICI/EQUAS was based on ISO 13528:2015 Fearn and Thompson (2001) and Thompson (2000), as also described by Esteban López et al. (2021). Firstly, the duplicate analysis results were tested for outliers using the Cochran's test. If an outlier result was identified, the duplicate result was discarded from the data set and further calculations of homogeneity were performed. Subsequently, the outlier test was repeated on the remaining data. If another outlier was detected, the homogeneity assessment had to be repeated because the data set was considered unfit (e.g., a problem occurred during the analysis which had to be resolved). Secondly, the assessment was made as to whether or not the analysis method used was suited to determine inhomogeneity. For this purpose, a standard deviation (SD) was compared to $0.5*\sigma_T$, where $\sigma_T$ is the target standard deviation calculated as 25% of the overall mean of the analysis results. For final consideration of whether the CMs were sufficiently homogenous, the between-sample SD was compared to the critical value, which corresponded to $0.3*\sigma_T$.

#### 2.4.4. Stability tests of CMs

The stability analyses were performed in line with the corresponding HBM4EU SOP (Esteban López et al., 2021). For stability assessment, the samples prepared for each test round were stored under conditions representative of storage at the participants' laboratories (−18 °C). The stability was determined by analysing six test samples ($LL_{HFR}$, $LL_{OPFR}$, $HL_{HFR}$, $HL_{OPFR}$) at a time interval covering the seven-week period between shipment and the deadline of submission of the results within each round. The results were evaluated according to ISO 13528 (Statistical methods for use in proficiency testing by interlaboratory comparison, 2015) and the International Harmonized Protocol for the Proficiency Testing of Analytical Laboratories (Thompson et al., 2006). First, the mean concentrations from replicate analysis at $t_0$ (date of shipment of samples) and $t_e$ (deadline of submission of results) were calculated. The biomarkers in the CMs were considered sufficiently stable if the difference between the means was $\leq 0.3*\sigma_T$. In case this criterion was not met, the statistical significance of the differences between the mean values at the different storage times was determined using an F-test.

#### 2.4.5. Analytical methods for the determination of homogeneity and stability

In brief, the sample preparation procedure for nonpolar compounds (BDE-47, BDE-153, BDE-209, anti-DP, syn-DP, and DBDPE) was based on a three-step solvent extraction of serum with an n-hexane:diethylether (9:1, *v/v*) mixture, followed by the purification step using a Florisil® column. The rest of the serum sample after removal of the nonpolar solvent, containing the nonpolar compounds, was further extracted by a modified QuEChERS extraction (Quick, Easy, Cheap, Effective, Rugged and Safe), when acetonitrile was used for the isolation of more polar compounds (α-HBCD, γ-HBCD, 2,4,6-TBP, and TBBPA) and the separation of organic and aqueous layers was induced by the addition of inorganic salts. Gas and liquid chromatography coupled to (tandem) mass spectrometry techniques (GC-MS(/MS) and LC-MS/MS, respectively) were used for the identification/quantification of the FRs in the nonpolar and the polar fractions, respectively (Svarcova et al., 2019).

For the determination of DPHP, BCEP, and BClPP in urine, a GC-MS/MS method with electron ionization was used after solid phase extraction (SPE) and derivatization with pentafluorobenzylbromide. The same sample preparation was applied for the determination of BDClPP in urine, but a GC-MS system with chemical ionization and detection in positive mode was used for quantification (Fromme et al., 2014).

### 2.5. Distribution of CMs

CMs were dispatched to the participants in a frozen state in polystyrene boxes. Each participant received samples for $LL_{HFR}$, $HL_{HFR}$ or $LL_{OPFR}$, $HL_{OPFR}$ according to their registration. Additionally, the laboratories obtained the blank serum or blank urine of the biological material used for the fortification procedure. In round 1 for HFRs, three samples of $LL_{HFR}$, three samples of $HL_{HFR}$, and three blank samples were sent to the participants. Likewise, three samples of $LL_{OPFR}$, three samples of $HL_{OPFR}$, and three blank samples were dispatched to the participants in round 1 for OPFR metabolites. From round 2, the participants received only one sample of each concentration ($LL_{HFR}$, $HL_{HFR}$, blank serum, or $LL_{OPFR}$, $HL_{OPFR}$, blank urine).

In round 2, round 3, and round 4 for both HFRs and OPFR metabolites, the selected expert laboratories received six samples of each CM ($LL_{HFR}$, $HL_{HFR}$, blank serum or $LL_{OPFR}$, $HL_{OPFR}$, blank urine) and were asked to perform a single analysis of each sample, so they would submit a total of 18 results. For further data evaluation, the results from the analysis of blank samples were not used.

At the time of shipment, a letter with instructions on sample handling, a sample receipt form, a result submission form and a method information form were e-mailed to the participants. Participants were asked to perform a single analysis of each sample using the same procedure intended to be used for the analysis of samples in the frame of HMB4EU and to submit their results via e-mail within seven weeks of sample delivery.

### 2.6. Assessment of laboratory performance

#### 2.6.1. HFRs in serum

Assessment of the laboratory performance was done as described in Esteban López et al., 2021. In case of a limited number of participants (ICI) and expert laboratories (EQUAS) as encountered in this study, these procedures were statistically not ideal (Rousseeuw and Verboven, 2002; Belli et al., 2007; Kuselman and Fajgelj, 2010). The datasets have been scrutinized by constructing kernel density plots that showed more or less symmetric plots with the maximum in a good agreement with $X_P$. Thus these procedures were considered to be acceptable for the first-time assessment of performance for these HBM parameters. In brief, for the ICI, the $X_P$ value (robust mean), uncertainty of $X_P$ ($u_{ICI}$) and ICI standard deviation of $X_P$ ($\sigma_{ICI}$) were calculated using robust statistics (Algoritm A in ISO 13528:2015) in accordance with Thompson et al. (2006) and Analytical Methods Committee (1989a, 1989b). The $u_{ICI}$ was calculated as follows:

$$u_{ICI} = 1.25\frac{\sigma_{ICI}}{\sqrt{n}} \quad (1)$$

with: n = number of results used for calculation of $X_P$ with $n \geq 7$.

The uncertainty of $X_P$ should be negligible, meaning not exceeding a value derived from the following equation:

$$u_{ICI} \leq 0.3^* \sigma_T \quad (2)$$

with: $\sigma_T$ = standard deviation for proficiency assessment with $\sigma_T = 0.25*X_P$ (Esteban López et al., 2021).

When the $u_{ICI}$ was not negligible, but not exceeding $0.7*\sigma_T$, the $X_P$ was still used for calculation of Z-scores, but the $u_{ICI}$ was taken into account using the formula (6).

In the EQUAS, the evaluation of the participants results was based on data generated by a minimum of three expert laboratories. Using the individual means of six replicate analysis of the CM by the expert laboratories, the mean of means and its relative standard deviation ($RSD_{mean\text{-}of\text{-}means}$) were calculated. The uncertainty ($u_{EQUAS}$) was defined as $RSD_{mean\text{-}of\text{-}means}$ divided by the square root of the number of expert

laboratories:

$$u_{EQUAS} = \frac{RSD_{mean-of-means}}{\sqrt{n}} \quad (3)$$

with: n = number of results used for calculation with n ≥ 3.

The mean of means was considered suitable as $X_E$ value in EQUAS studies if $u_{EQUAS}$ did not exceed a value of 17.5% derived from the following equation:

$$u_{EQUAS} \leq 0.7^* \sigma_T \quad (4)$$

with: $\sigma_T$ = standard deviation for proficiency assessment, pre-set at = 0.25*$X_E$ (Esteban López et al., 2021).

It should be noted that the determination of $u_{EQUAS}$ here might be an underestimation considering the low number of expert laboratories involved.

When $u_{EQUAS} > 0.7^*\sigma_T$, the individual means were checked for outliers. For this purpose, the Grubbs' outlier test was used. If an individual expert mean was identified as Grubbs' outlier, it was discarded from the data set and $u_{EQUAS}$ was recalculated. If the condition $u_{EQUAS} \leq 0.7^*\sigma_T$ was still not met, then the uncertainty of the expert-derived mean was too high to be used as $X_E$ value. In this case, no assessment of the participants' performance was possible for the biomarker in question.

The calculation of $X_P$ or $X_E$ values was not possible for all biomarkers because of the low number of reported results. In the case of EQUAS, when the number of expert results for a particular biomarker was < 3, $X_P$ value was determined according to the ICI approach for all data, i.e. a minimum of seven results from experts and participants combined together (Fig. 1).

As a measure of the participating laboratories' proficiency, Z-scores were calculated using the $X_P$ value derived from the participants' results (ICI) or $X_E$ value as the mean of means of expert results (EQUAS), and a pre-set relative standard deviation for proficiency of 25%.

In round 1 (conducted as ICI) the Z-scores (Z) of the results submitted by the participants (x) were calculated according to the equation:

$$Z = \frac{x - X_P}{0.25^*X_P} \quad (5)$$

As mentioned above, when the uncertainty of the $X_P$ was not negligible, but not exceeding $0.7^*\sigma_T$, the $X_P$ was still used for calculation of Z-scores, but the $u_{ICI}$ was taken into account for calculation of the Z-scores using the following formula:

$$Z' = \frac{x - X_P}{\sqrt{(0.25^*X_P)^2 + u_{ICI}^2}} \quad (6)$$

In rounds 2–4, when $X_E$ value was established, the Z-scores of the participants' results were calculated according to:

$$Z = \frac{x - X_E}{0.25^*X_E} \quad (7)$$

In rounds 2–4, when submitted expert results were < 3, the Z-scores of the participants' results were calculated according to formula (5) or (6), provided that the calculation of $X_P$ value was possible by combining the participants' and experts' results.

In the ICI/EQUAS programme, Z-scores were classified into three categories: satisfactory (|Z| ≤ 2), questionable (2 < |Z| < 3), and unsatisfactory (|Z| ≥ 3). The results of the participating laboratories were evaluated on an individual biomarker/CM/concentration basis.

#### 2.6.2. OPFR metabolites in urine

Due to a small number of participants (n ≤ 7), the evaluation of the participating laboratory performance for OPFR metabolites using Z-scores according to the applied procedures was not possible in round 1. In round 2 (EQUAS), no $X_E$ value could be determined because either the number of experts was too small or the uncertainty of the mean-of-means was too high for the respective OPFR metabolites. A similar situation was observed in round 3, except for DPHP, for which the $X_E$ value was established for the first time. For this reason, an alternative approach was adopted. Briefly, all participant and expert results were used to calculate an $X_P$ value. The Grubb's outlier test was performed to identify and discard outliers. This $X_P$ value was accepted if it complied with a RSD of 17.5% or less and used to calculate the Z-scores of the participants' mean results according to the SOPs using $\sigma_T$ = 25%.

## 3. Results and discussion

### 3.1. Preparation of CMs

The choice of concentration levels for HFRs and OPFR metabolites that were used for the CMs of this ICI/EQUAS programme was based on the review of relevant scientific papers. Median and 95% percentile of reported concentrations were used for LL and HL for most of the target biomarkers, respectively. A summary of the concentration is presented in Table S1.

In the case of PBDEs and HBCDs, well-established analytical

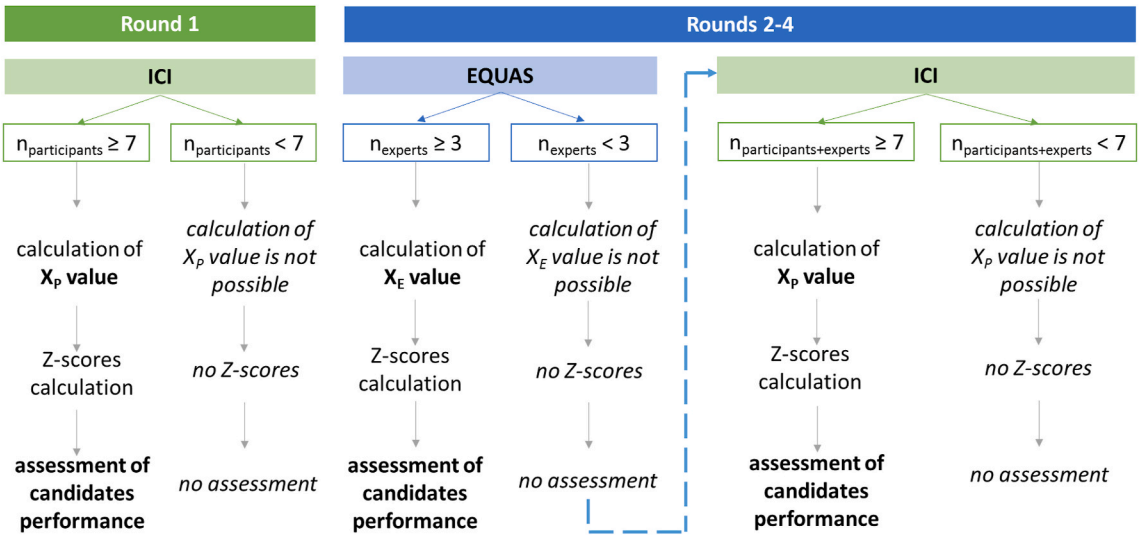


**Fig. 1.** ICI/EQUAS evaluation scheme for HFRs in serum

methods, a wide spectrum of analytical standards as well as certified reference materials and proficiency testing schemes are available. Therefore, biomonitoring data have been studied for these BFRs, including the description of time trends (Fängström et al., 2008; Darnerud et al., 2015, Bjermo et al., 2017). PBDEs occurrence in a wide range of human matrices (especially serum and breast milk) has been documented. Studies from Sweden (Sahlström et al., 2014; Darnerud et al., 2015; Bjermo et al., 2017), Norway (Cequier et al., 2013; Cequier et al., 2015a; Jansen et al., 2018), Germany (Fromme et al., 2016), France (Dereumeaux et al., 2016), Denmark (Frederiksen et al., 2010) and Czech Republic (Sochorová et al., 2017) were considered for setting target concentrations in serum. Regarding the HBCD isomers, biomonitoring data have been published primarily for serum (Roosens et al., 2009; Roze et al., 2009; Kalantzi et al., 2011; Sahlström et al., 2014; Fromme et al., 2016; Jansen et al., 2018) and human breast milk (Eljarrat et al., 2009; Thomsen et al., 2010; Abdallah and Harrad, 2011). Compared to the extent of biomonitoring studies dealing with PBDEs and HBCDs in serum, the number of relevant data published for DBDPE (Cequier et al., 2015a), TBBPA (Dufour et al., 2017), 2,4,6-TBP (Dufour et al., 2017; Sochorová et al., 2017) and anti-/syn-DP (Fromme et al., 2015; Sochorová et al., 2017) is much smaller. Comparing TBBPA and 2,4,6-TBP concentrations to nonpolar BFRs in serum is generally difficult because of the different ways of expressing results. Therefore, to be able to compare data, the results expressed on a lipid weight basis (μg/kg l.w.) were converted to μg/L using the specific lipid content of 0.6% by weight.

Compared to HFRs, analytical methods for OPFR metabolites are less established. Studies usually report a subset of OPFR metabolites and the methods vary widely between them. The occurrence of OPFR metabolites is predominantly described in urine. The choice of target levels was based mostly on data available from studies in Norway (Cequier et al., 2015b), Germany (Reemtsma et al., 2011; Schindler et al., 2013; Fromme et al., 2014) and Belgium (Van den Eede et al., 2013).

### 3.2. Homogeneity and stability testing

The results of the homogeneity testing for $LL_{HFR}$, $HL_{HFR}$ and $LL_{OPFR}$, $HL_{OPFR}$ are summarized in Table S2 in Supplementary data. No outliers were detected for any of the targeted compounds in any of the ICI/EQUAS rounds. The CMs showed sufficient homogeneity for both HFRs

**Table 1A**
Number of candidates and expert laboratories that participated for HFRs in serum.

| | | Round 1 | | Round 2 | | | | Round 3 | | | | Round 4 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **No. of invited laboratories (candidates)** | | **24** | | **31** | | | | **31** | | | | **31** | | | |
| **No. of registered/reporting laboratories** | | **11/11** | | **15/15** | | | | **15/15** | | | | **14/14** | | | |
| **No. of registered/reporting experts** | | **0/0** | | **5/4** | | | | **5/5** | | | | **5/5** | | | |
| **HFR** | | **$N_t$** | **$N_c$** | **$N_t$** | **$N_c$** | **$N_e$** | **$N_{ee}$** | **$N_t$** | **$N_c$** | **$N_e$** | **$N_{ee}$** | **$N_t$** | **$N_c$** | **$N_e$** | **$N_{ee}$** |
| BDE-47 | Registration | 10 | 10 | 16 | 11 | 3 | 2 | 16 | 11 | 3 | 2 | 13 | 8 | 3 | 2 |
| | Reporting | 10 | 10 | 15 | 11 | 3 | 1 | 16 | 11 | 3 | 2 | 13 | 9 | 2 | 2 |
| BDE-153 | Registration | 10 | 10 | 16 | 11 | 3 | 2 | 16 | 11 | 3 | 2 | 13 | 8 | 3 | 2 |
| | Reporting | 10 | 10 | 15 | 11 | 3 | 1 | 16 | 11 | 3 | 2 | 13 | 9 | 2 | 2 |
| BDE-209 | Registration | 9 | 9 | 15 | 10 | 3 | 2 | 15 | 10 | 3 | 2 | 11 | 6 | 3 | 2 |
| | Reporting | 9 | 9 | 13 | 9 | 3 | 1 | 13 | 8 | 3 | 2 | 10 | 6 | 2 | 2 |
| DBDPE | Registration | 6 | 6 | 9 | 6 | 3 | Did not | 8 | 6 | 2 | Did not | 6 | 4 | 2 | Did not |
| | Reporting | 6 | 6 | 6 | 4 | 2 | participate | 6 | 4 | 2 | participate | 3 | 1 | 2 | participate |
| Anti-DP | Registration | 4 | 4 | 8 | 5 | 3 | | 9 | 6 | 3 | | 8 | 5 | 3 | |
| | Reporting | 5 | 5 | 9 | 6 | 3 | | 9 | 6 | 3 | | 7 | 4 | 3 | |
| Syn-DP | Registration | 4 | 4 | 8 | 5 | 3 | | 9 | 6 | 3 | | 8 | 5 | 3 | |
| | Reporting | 5 | 5 | 9 | 6 | 3 | | 9 | 6 | 3 | | 7 | 4 | 3 | |
| α-HBCD | Registration | 6 | 6 | 8 | 6 | 2 | | 8 | 6 | 2 | | 8 | 6 | 2 | |
| | Reporting | 5 | 5 | 8 | 6 | 2 | | 8 | 6 | 2 | | 7 | 5 | 2 | |
| γ-HBCD | Registration | 6 | 6 | 8 | 6 | 2 | | 8 | 6 | 2 | | 8 | 6 | 2 | |
| | Reporting | 5 | 5 | 8 | 6 | 2 | | 8 | 6 | 2 | | 7 | 5 | 2 | |
| TBBPA | Registration | 2 | 2 | 5 | 3 | 2 | | 6 | 4 | 2 | | 5 | 3 | 2 | |
| | Reporting | 2 | 2 | 5 | 4 | 1 | | 6 | 3 | 3 | | 4 | 2 | 2 | |
| 2,4,6-TBP | Registration | 2 | 2 | 4 | 3 | 1 | | 5 | 4 | 1 | | 4 | 3 | 1 | |
| | Reporting | 2 | 2 | 5 | 4 | 1 | | 4 | 3 | 1 | | 3 | 2 | 1 | |

**Table 1B**
Number of candidates and expert laboratories that participated for OPFR biomarkers in urine.

| | | Round 1 | | Round 2 | | | | Round 3 | | | | Round 4 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **No. of invited laboratories (candidates)** | | **13** | | **17** | | | | **17** | | | | **17** | | | |
| **No. of registered/reporting laboratories** | | **7/5** | | **6/5** | | | | **5/5** | | | | **6/6** | | | |
| **No. of registered/reporting experts** | | **0/0** | | **3/3** | | | | **3/3** | | | | **3/3** | | | |
| **OPFR metabolites** | | **$N_t$** | **$N_c$** | **$N_t$** | **$N_c$** | **$N_e$** | **$N_{ee}$** | **$N_t$** | **$N_c$** | **$N_e$** | **$N_{ee}$** | **$N_t$** | **$N_c$** | **$N_e$** | **$N_{ee}$** |
| BCEP | Registration | 7 | 7 | 4 | 2 | 2 | Did not participate | 2 | 1 | 1 | Did not participate | 3 | 2 | 1 | Did not participate |
| | Reporting | 1 | 1 | 1 | 0 | 1 | | 2 | 1 | 1 | | 3 | 2 | 1 | |
| BClPP | Registration | 7 | 7 | 5 | 3 | 2 | | 4 | 2 | 2 | | 6 | 3 | 3 | |
| | Reporting | 3 | 3 | 4 | 2 | 2 | | 4 | 2 | 2 | | 6 | 3 | 3 | |
| BDClPP | Registration | 7 | 7 | 6 | 3 | 3 | | 5 | 2 | 3 | | 6 | 3 | 3 | |
| | Reporting | 5 | 5 | 5 | 2 | 3 | | 5 | 2 | 3 | | 6 | 3 | 3 | |
| DPHP | Registration | 7 | 7 | 6 | 3 | 3 | | 5 | 2 | 3 | | 6 | 3 | 3 | |
| | Reporting | 5 | 5 | 5 | 2 | 3 | | 5 | 2 | 3 | | 6 | 3 | 3 | |

Legend: $N_t$ - total number of all participants (candidates, expert laboratories within the HBM4EU consortium and external expert laboratories outside the HBM4EU consortium); $N_c$ - total number of participants; $N_e$ - total number of expert laboratories that were from the HBM4EU consortium and participated as candidate laboratories; $N_{ee}$-total number of external experts outside the HBM4EU consortium.

and OPFR metabolites in all rounds.

The results of the stability testing for $LL_{HFR}$, $HL_{HFR}$, $LL_{OPFR}$ and $HL_{OPFR}$ in the four ICI/EQUAS rounds are shown in Table S3 in Supplementary data. In all rounds, sufficient stability was found for all HFRs. Regarding OPFR metabolites, the means of the results obtained from the time interval were significantly different in several cases. However the differences were still in the range of what is to be expected from intermediate precision data (<20%). Thus the CMs were considered sufficiently stable.

### 3.3. Establishment of $X_P$ or $X_E$ values for HFRs

The established $X_P$ or $X_E$ values for HFRs are shown in Table S4 (round 1) and Table S5 (round 2–4) in upplementary data. The corresponding numerical values can also be found in the lower part in Table 2.

In round 1 (ICI), $X_P$ values were established only for BDE-47 (n = 10), BDE-153 (n = 10) and BDE-209 (n = 9). For other HFRs, the calculation of $X_P$ values was not possible because the number of results required for their determination was < 7.

In the following three rounds (2–4), which were organized as EQUAS, each expert laboratory analysed six samples of each CM ($LL_{HFR}$, $HL_{HFR}$) for a single analysis. In round 2, only four out of five registered expert laboratories submitted results. In the third and fourth rounds, all five and four expert laboratories, respectively, reported results. Since not all experts covered all ten HFR biomarkers, determination of $X_E$ values were again only possible for BDE-47, BDE-153, and BDE-209 in all three EQUAS. The criterion of a minimum of three expert laboratories was also met for anti-DP and syn-DP, but establishment of $X_E$ values was only possible in round 2 and round 4. In round 3, the uncertainty of the $X_E$ value, $u_{EQUAS}$, was too high (higher than 17.5%). In general, $RSD_{mean\text{-}of\text{-}means}$ for specific HFRs in $LL_{HFR}$ and $HL_{HFR}$ varied from 5% to 40%. The highest $RSD_{mean\text{-}of\text{-}means}$ was observed for BDE-209. This was probably related to the small number of expert laboratories.

As mentioned above, calculation of $X_E$ values was not possible for all HFRs. The main reasons were the limited scope of reported experts' results or too high uncertainty of the $X_E$ value. In this case, the possibility of using $X_P$ as an alternative to the $X_E$ value was investigated. For the determination of a robust mean, the results of all participants were evaluated together with the expert laboratories, resulting in a total of results ≥7. For anti-DP and syn-DP in round 3, α-HBCD and γ-HBCD in round 2, round 3, and round 4, this resulted in a sufficiently reliable $X_P$ value suitable for the determination of Z-scores and evaluation of laboratory performance.

### 3.4. Establishment of $X_P$ or $X_E$ values for OPFR metabolites

The OPFR biomarker group posed more difficulties due to the small number of participants and high variability of results. A similarly high variability, especially for BCEP and BClPP, was described in a recent comparative study of nine laboratories determining OPFR metabolites (including DPHP, BDCIPP, BCEP, and BCIPP) in the certified reference material SRM 3673 (Organic contaminants in non-smokers' urine) (Bastiaensen et al., 2019).

The calculation of $X_P$ or $X_E$ values according to the standardized ICI/EQUAS approach was not possible at all for BDClPP, BClPP and BCEP in the first three rounds or only to a limited extent for DPHP (in round 2). Thus, it was necessary to apply a more flexible approach to evaluate the results from these rounds and draw conclusions. It is worth noting that the last round was very successful due the effort of participants following discussions of main analytical difficulties in web conferences after round 1 and round 3. Consequently, the calculation of the $X_E$ value using the EQUAS approach was possible for BDClPP, BClPP, and DPHP. The overview of $X_E$ values is shown in Table S6 and details of the $X_E$ value calculations are provided in Table S7 in Supplementary data. The corresponding numerical values can be found in the lower part in Table 3.

#### 3.4.1. Alternative evaluation approach in rounds 1, 2, and 3

For DPHP and BDClPP, the permissible relative uncertainty of the mean of means (RSD < 17.5%) was exceeded for all samples in all three rounds, except for BDCIPP in the $LL_{OPFR}$ within round 3. In this case, the RSD of 17.6% was only very slightly above 17.5%, so that the calculated $X_E$ value was accepted.

For BClPP, the uncertainty of the $X_E$ was too high in round 1 and round 2. This was partly influenced by the fact that there were only three to four results. Therefore, in most cases, an obvious outlier could not be removed. Nevertheless, Z-scores were calculated in these cases as well, using the mean based on the data from all laboratories. The apparent outliers then obtained questionable or unsatisfactory Z-scores in agreement with a more subjective assessment of the data.

For BCEP, there was too little data to apply the alternative approach for the calculation of the $X_E$ value. In round 1 and round 2, only one laboratory reported results, in round 3 two participants submitted concentrations.

#### 3.4.2. Evaluation procedure in round 4

In round 4, all three registered expert laboratories all reported results for DPHP, BDClPP, and BClPP, so that the $X_E$ value determination was possible. The $RSD_{mean\text{-}of\text{-}means}$ significantly decreased for all these OPFR metabolites compared to the value calculated in the previous three rounds using the alternative approach. Specifically, the $RSD_{mean\text{-}of\text{-}means}$ was in the wide range of 6–66% and in round 4 in the range of 4–10%.

#### 3.4.3. Comparison of alternative evaluation approach and EQUAS

For $X_E$ values obtained by EQUAS the evaluation using alternative approach was also done (DPHP in round 3 and round 4; BDClPP, and BClPP in round 4). Comparison of $X_E$ from both approaches showed comparable $X_E$ with the exception of BClPP at HL in round 4 (Fig. S1).

### 3.5. Participation and method characteristics

Table 1 provides an overview of the number of participating and expert laboratories. For HFRs, 24 laboratories were invited to round 1 (ICI), eleven of which agreed to participate. In the following three rounds, the number of invited laboratories increased to 31, of which 15 participated. The scope of biomarkers measured by the participants varied substantially in all rounds: from two to all ten HFRs. Over all rounds, the highest average participation rate was achieved for BDE-47, BDE-153, and BDE-209 (more than 73%), followed by α-HBCD, γ-HBCD, anti-DP and syn-DP (more than 50%). In contrast, the lowest average percentage of participants was for DBDPE (39%), TBBPA (30%), and 2,4,6-TBP (25%).

Regarding OPFR metabolites, 13 laboratories were invited to round 1, seven of which announced their participation. After round 1, the number of invited laboratories was 17, but the number of laboratories responding positively did not increase. The scope of target OPFR metabolites varied among the participants: from two (DPHP and BDClPP) to all four biomarkers. During the ICI/EQUAS programme, the laboratories were encouraged to analyse as many biomarkers as possible. The response from participants was generally positive, resulting in the highest number of analysed OPFR metabolites in the last round.

The LOQs reported by the participants in the four rounds for HFRs and OPFR metabolites are shown in Table S8. No specific LOQ values were required for participation. The high variability of LOQ values (3-4 orders of magnitude) for HFRs determination among laboratories was observed in all rounds. For all OPFR metabolites, relatively comparable LOQs were submitted by the participants, differing by a maximum of one order of magnitude.

Details of the analytical methods used by participants and experts for the analysis of HFRs and OPFR metabolites are shown in Table S9. For HFRs, approximately 25% of the laboratories over all rounds reported

**Table 2**
Summary of HFRs results evaluation in each round of the QA/QC programme.

| HFRs | Round | CMs | $X_P$ (ICI)/$X_E$ (EQUAS) (μg/L) | | Uncertainty (μg/L) | Study $RSD_R$ | No. of participants reporting results | Performance (Z-scores) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Satisfactory (%) | Questionable (%) | Unsatisfactory (%) |
| BDE-47 | 1 | $LL_{HFR}$ | $X_P$ | 0.098 | 0.005 | 31% | 10 | 90 | 0 | 10 |
| | | $HL_{HFR}$ | $X_P$ | 0.298 | 0.014 | 27% | 10 | 90 | 0 | 10 |
| | 2 | $LL_{HFR}$ | $X_E$ | 0.196 | 0.020 | 27% | 14 | 93 | 0 | 7 |
| | | $HL_{HFR}$ | $X_E$ | 0.996 | 0.177 | 21% | 14 | 93 | 7 | 0 |
| | 3 | $LL_{HFR}$ | $X_E$ | 0.151 | 0.013 | 24% | 14 | 100 | 0 | 0 |
| | | $HL_{HFR}$ | $X_E$ | 0.644 | 0.098 | 23% | 14 | 100 | 0 | 0 |
| | 4 | $LL_{HFR}$ | $X_E$ | 0.162 | 0.009 | 31% | 11 | 82 | 18 | 0 |
| | | $HL_{HFR}$ | $X_E$ | 0.554 | 0.044 | 27% | 11 | 91 | 9 | 0 |
| BDE-153 | 1 | $LL_{HFR}$ | $X_P$ | 0.071 | 0.004 | 159% | 10 (1*) | 80 | 0 | 20 |
| | | $HL_{HFR}$ | $X_P$ | 0.409 | 0.021 | 35% | 10 | 80 | 10 | 10 |
| | 2 | $LL_{HFR}$ | $X_E$ | 0.268 | 0.024 | 19% | 14 | 100 | 0 | 0 |
| | | $HL_{HFR}$ | $X_E$ | 0.808 | 0.068 | 17% | 14 | 93 | 0 | 7 |
| | 3 | $LL_{HFR}$ | $X_E$ | 0.184 | 0.019 | 24% | 13 (1*) | 92 | 8 | 7 |
| | | $HL_{HFR}$ | $X_E$ | 0.549 | 0.059 | 33% | 14 | 86 | 0 | 14 |
| | 4 | $LL_{HFR}$ | $X_E$ | 0.177 | 0.009 | 38% | 10 (1*) | 73 | 9 | 18 |
| | | $HL_{HFR}$ | $X_E$ | 0.605 | 0.065 | 37% | 11 | 82 | 9 | 9 |
| BDE-209 | 1 | $LL_{HFR}$ | $X_P$ | 0.105 | 0.008 | 70% | 8 | 67 | 0 | 33 |
| | | $HL_{HFR}$ | $X_P$ | 0.966 | 0.097 | 55% | 9 | 89 | 0 | 11 |
| | 2 | $LL_{HFR}$ | $X_E$ | 0.709 | 0.105 | 40% | 11 (1*) | 67 | 8 | 25 |
| | | $HL_{HFR}$ | $X_E$ | 2.09 | 0.31 | 43% | 12 | 75 | 17 | 8 |
| | 3 | $LL_{HFR}$ | $X_E$ | 1.12 | 0.12 | 61% | 11 | 64 | 9 | 27 |
| | | $HL_{HFR}$ | $X_E$ | 1.78 | 0.32 | 54% | 11 | 64 | 9 | 27 |
| | 4 | $LL_{HFR}$ | $X_E$ | 0.901 | 0.100 | 43% | 8 | 75 | 13 | 13 |
| | | $HL_{HFR}$ | $X_E$ | 1.65 | 0.15 | 43% | 8 | 50 | 38 | 13 |
| anti-DP | 1 | $LL_{HFR}$ | n.c. | (1) | n.c. | n.c. | 5 | n.c. | n.c. | n.c. |
| | | $HL_{HFR}$ | n.c. | (1) | n.c. | n.c. | 4 | n.c. | n.c. | n.c. |
| | 2 | $LL_{HFR}$ | $X_E$ | 0.297 | 0.026 | 44% | 9 | 67 | 11 | 22 |
| | | $HL_{HFR}$ | $X_E$ | 1.23 | 0.07 | 45% | 9 | 67 | 22 | 11 |
| | 3 | $LL_{HFR}$ | $X_P$ | 0.134 | 0.032 | 34% | 9 | 89 | 0 | 11 |
| | | $HL_{HFR}$ | $X_P$ | (2) | n.c. | n.c. | 9 | n.c. | n.c. | n.c. |
| | 4 | $LL_{HFR}$ | $X_E$ | 0.292 | 0.014 | 19% | 7 | 100 | 0 | 0 |
| | | $HL_{HFR}$ | $X_E$ | 1.21 | 0.09 | 25% | 7 | 100 | 0 | 0 |
| syn-DP | 1 | $LL_{HFR}$ | n.c. | (1) | n.c. | n.c. | 5 | n.c. | n.c. | n.c. |
| | | $HL_{HFR}$ | n.c. | (1) | n.c. | n.c. | 4 | n.c. | n.c. | n.c. |
| | 2 | $LL_{HFR}$ | $X_E$ | 0.375 | 0.022 | 48% | 9 | 56 | 11 | 33 |
| | | $HL_{HFR}$ | $X_E$ | 1.06 | 0.03 | 44% | 9 | 44 | 11 | 44 |
| | 3 | $LL_{HFR}$ | $X_P$ | 0.313 | 0.045 | 33% | 9 | 89 | 11 | 0 |
| | | $HL_{HFR}$ | $X_P$ | 0.764 | 0.31 | 43% | 9 | 89 | 11 | 0 |
| | 4 | $LL_{HFR}$ | $X_E$ | 0.47 | 0.037 | 22% | 7 | 100 | 0 | 0 |
| | | $HL_{HFR}$ | $X_E$ | 1.22 | 0.08 | 29% | 7 | 86 | 14 | 0 |
| α-HBCD | 1 | $LL_{HFR}$ | n.c. | (1) | n.c. | n.c. | 5 | n.c. | n.c. | n.c. |
| | | $HL_{HFR}$ | n.c. | (1) | n.c. | n.c. | 5 | n.c. | n.c. | n.c. |
| | 2 | $LL_{HFR}$ | $X_P$ | 0.560 | 0.054 | 19% | 8 | 100 | 0 | 0 |
| | | $HL_{HFR}$ | $X_P$ | 5.19 | 0.35 | 13% | 8 | 100 | 0 | 0 |
| | 3 | $LL_{HFR}$ | $X_P$ | 0.583 | 0.051 | 29% | 8 | 88 | 13 | 0 |
| | | $HL_{HFR}$ | $X_P$ | 4.88 | 0.42 | 17% | 8 | 100 | 0 | 0 |
| | 4 | $LL_{HFR}$ | $X_P$ | 0.501 | 0.067 | 32% | 7 | 86 | 14 | 0 |
| | | $HL_{HFR}$ | $X_P$ | 5.17 | 0.59 | 38% | 7 | 86 | 0 | 14 |
| γ-HBCD | 1 | $LL_{HFR}$ | n.c. | (1) | n.c. | n.c. | 5 | n.c. | n.c. | n.c. |
| | | $HL_{HFR}$ | n.c. | (1) | n.c. | n.c. | 5 | n.c. | n.c. | n.c. |
| | 2 | $LL_{HFR}$ | $X_P$ | 0.338 | 0.027 | 18% | 8 | 100 | 0 | 0 |
| | | $HL_{HFR}$ | $X_P$ | 7.64 | 0.50 | 15% | 8 | 63 | 13 | 25 |
| | 3 | $LL_{HFR}$ | $X_P$ | 0.321 | 0.013 | 59% | 8 | 88 | 0 | 13 |
| | | $HL_{HFR}$ | $X_P$ | 5.91 | 0.26 | 9% | 8 | 100 | 0 | 0 |
| | 4 | $LL_{HFR}$ | $X_P$ | 0.396 | 0.034 | 20% | 7 | 100 | 0 | 0 |
| | | $HL_{HFR}$ | $X_P$ | 6.14 | 0.48 | 18% | 7 | 100 | 0 | 0 |

Legend: (1) no result because the uncertainty of XP or XE was too high; (2) no result because n < 7; * number of laboratories reporting "<LOQ"; n.c. - not calculated.

the use of a deconjugation step in the sample process procedure. The further steps included SPE (25–36% of participants in four rounds) or liquid-liquid extraction (LLE) followed by SPE (64–75% of participants in four rounds). For the LLE, mostly hexane, dichloromethane, acetone, diethylether, or methyl-tert-butyl ether were used, or solvent mixtures. The most common SPE sorbents consisted of silica, acid silica, florisil, or alumina. Due to the largely differing physicochemical properties of the target HFRs, laboratories used both the instrumental techniques GC coupled to low resolution mass spectrometry (LRMS) with electron capture negative ionization, GC with high resolution mass spectrometry

**Table 3**
Summary of OPFR metabolites results assessment in each round of the QA/QC programme.

| OPFR metabolites | Round | CMs | Approach | $X_E$ (μg/L) | Uncertainty (μg/L) | Study $RSD_R$ | No. of participant reporting results | Performance (Z-scores) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | % satisfactory | % questionable | % unsatisfactory |
| DPHP | 1 | $LL_{OPFR}$ | alternative | 1.72 | 0.14 | 18% | 5 | 100 | 0 | 0 |
| | | $HL_{OPFR}$ | alternative | 11.1 | 0.4 | 10% | 5 | 100 | 0 | 0 |
| | 2 | $LL_{OPFR}$ | alternative | 2.75 | 0.30 | 81% | 5 | 80 | 0 | 20 |
| | | $HL_{OPFR}$ | alternative | 8.34 | 0.83 | 87% | 5 | 80 | 0 | 20 |
| | 3 | $LL_{OPFR}$ | EQUAS | 1.91 | 0.30 | 30% | 5 | 80 | 20 | 0 |
| | | $HL_{OPFR}$ | EQUAS | 8.49 | 0.30 | 6% | 5 | 100 | 0 | 0 |
| | 4 | $LL_{OPFR}$ | EQUAS | 2.44 | 0.06 | 19% | 6 | 100 | 0 | 0 |
| | | $HL_{OPFR}$ | EQUAS | 8.47 | 0.19 | 12% | 6 | 100 | 0 | 0 |
| BDClPP | 1 | $LL_{OPFR}$ | alternative | 1.81 | 0.07 | 45% | 5 | 80 | 0 | 20 |
| | | $HL_{OPFR}$ | alternative | 10.5 | 0.6 | 42% | 5 | 80 | 0 | 20 |
| | 2 | $LL_{OPFR}$ | alternative | 3.03 | 0.30 | 72% | 5 | 80 | 0 | 20 |
| | | $HL_{OPFR}$ | alternative | 10.3 | 0.4 | 75% | 5 | 80 | 0 | 20 |
| | 3 | $LL_{OPFR}$ | alternative | 2.49 | 0.44 | 39% | 5 | 80 | 20 | 0 |
| | | $HL_{OPFR}$ | alternative | 9.20 | 1.38 | 35% | 5 | 80 | 20 | 0 |
| | 4 | $LL_{OPFR}$ | EQUAS | 4.66 | 0.21 | 14% | 6 | 100 | 0 | 0 |
| | | $HL_{OPFR}$ | EQUAS | 14.9 | 0.9 | 12% | 6 | 100 | 0 | 0 |
| BClPP | 1 | $LL_{OPFR}$ | alternative | 2.48 | 0.94 | 59% | 3 | 67 | 33 | 0 |
| | | $HL_{OPFR}$ | alternative | 17.2 | 6.0 | 53% | 3 | 67 | 33 | 0 |
| | 2 | $LL_{OPFR}$ | alternative | 5.70 | 0.34 | 57% | 4 | 75 | 0 | 25 |
| | | $HL_{OPFR}$ | alternative | 32.6 | 9.8 | 53% | 4 | 75 | 0 | 25 |
| | 3 | $LL_{OPFR}$ | alternative | 5.66 | 0.96 | 35% | 4 | 75 | 25 | 0 |
| | | $HL_{OPFR}$ | alternative | 20.2 | 2.8 | 27% | 4 | 100 | 0 | 0 |
| | 4 | $LL_{OPFR}$ | EQUAS | 5.48 | 0.44 | 18% | 6 | 100 | 0 | 0 |
| | | $HL_{OPFR}$ | EQUAS | 26.7 | 2.5 | 29% | 6 | 83 | 17 | 0 |
| BCEP | 1 | $LL_{OPFR}$ | (1) | n.c. | n.c. | n.c. | 1 | n.c. | n.c. | n.c. |
| | | $HL_{OPFR}$ | (1) | n.c. | n.c. | n.c. | 1 | n.c. | n.c. | n.c. |
| | 2 | $LL_{OPFR}$ | (1) | n.c. | n.c. | n.c. | 1 | n.c. | n.c. | n.c. |
| | | $HL_{OPFR}$ | (1) | n.c. | n.c. | n.c. | 1 | n.c. | n.c. | n.c. |
| | 3 | $LL_{OPFR}$ | (1) | n.c. | n.c. | n.c. | 2 | n.c. | n.c. | n.c. |
| | | $HL_{OPFR}$ | (1) | n.c. | n.c. | n.c. | 2 | n.c. | n.c. | n.c. |
| | 4 | $LL_{OPFR}$ | (1) | n.c. | n.c. | n.c. | 4 | n.c. | n.c. | n.c. |
| | | $HL_{OPFR}$ | (1) | n.c. | n.c. | n.c. | 4 | n.c. | n.c. | n.c. |

Legend: (1) no result because the uncertainty of $X_P$ or $X_E$ was too high. n.c. – not calculated.

(HRMS) and GC-MS/MS with electron ionization (for BDE-47, BDE-153, BDE-209, DBDPE, anti-DP, syn-DP, and DBDPE) and LC-MS/MS (for α-HBCD, γ-HBCD, TBBPA, and 2,4,6-TBP). Only one laboratory used a GC-MS/MS analysis of TBBPA and 2,4,6-TBP, following a derivatization step. Both isotope-labelled internal standards (mainly $^{13}$C-BDE 209 and $^{13}$C-HBCD) as well as native BDEs (BDE-51, BDE-71, BDE-77, BDE-128, BDE-156 or BDE-181) were used for normalization by all laboratories. In a few cases, a correction for recovery was applied over all rounds.

In the case of OPFR metabolites, around 50% of the laboratories applied enzymatic deconjugation using beta-glucuronidase in all four rounds. The isolation of the target compounds was mainly done by SPE extraction using nonpolar or weak anion exchange sorbents. One laboratory participating only in round 4 applied QuEChERS-based extraction prior to SPE. Another laboratory measured OPFR metabolites after their derivatization by GC-MS, while the other laboratories used LC-MS/MS. From the beginning of the ICI/EQUAS programme, this was the only participant, who later also participated as an expert, who was able to analyse all biomarkers. The greatest challenge was the determination of BCEP and BClPP. Although they are structurally similar to BDCIPP and DPHP, their lower hydrophobicity and different ionization potential make it difficult to analyse all target OPFR metabolites in urine samples with sufficient sensitivity using the same technique (Van den Eede et al., 2013). Over time, other participants incorporated BCEP into their LC-MS methods. The use of isotope-labelled internal standards (d10-DPHP, d8-BCEP, d10-BDClPP, and d12-BClPP) was reported by all laboratories. In more than 67%, the responses were normalised to internal standards. One laboratory corrected the results for recovery.

### 3.6. Assessment of laboratory performance

#### 3.6.1. HFRs in serum

The outcome of the four ICI/EQUAS rounds for HFRs is shown in Table 2. The participants' performance was only assessed for BDE-47, BDE-153, BDE-209, anti-DP, syn-DP, α-HBCD, and γ-HBCD. Due to the small number of participant and expert results ($n < 7$ and $n < 3$, respectively), it was not possible to calculate the $X_P$ or $X_E$ value for DBDPE, TBBPA, and 2,4,6-TBP. Thus, the Z-scores were not established for these three biomarkers in any of the ICI/EQUAS rounds.

In general, the highest number of satisfactory results was obtained for BDE-47 (82–100%) and BDE-153 (73–100%) within the four rounds of ICI/EQUAS. The number of participants for BDE-209 was slightly smaller than for the above-mentioned BDE congeners, but the success rate was not as high (50–89%). The satisfactory performance of the participants over rounds 2–4 for anti-DP, syn-DP, α-HBCD, and γ-HBCD was quite similar, in the range of 67–100%, 44–100%, 86–100%, and 63–100%, respectively. The poorest performance was achieved for syn-DP in round 2, when only 56% (for $LL_{HFR}$) and 44% (for $HL_{HFR}$) of participants achieved satisfactory results. In the following rounds 3 and 4, significant improvement was achieved not only for syn-DP (satisfactory Z-scores 86–100%) but also for anti-DP (satisfactory Z-scores 89–100%). Participant performances for α-HBCD and γ-HBCD were quite consistent (satisfactory results were in the range of 63–100%); in most cases, all participants achieved satisfactory Z-scores.

The number of participating laboratories that could not detect the HFRs in serum and thus indicated "<LOQ" in their report was very small and only for $LL_{HFR}$ samples (numbers in parentheses in Table 2). The performance of these participants was assessed using LOQ-Z-scores.

Their LOQs were above the $X_E$ or $X_P$, thus they were not able to detect the biomarkers. These "<LOQ results" were not considered false negatives.

The comparison of mean of participants' results and relevant $X_E$ or $X_P$ is illustrated in Fig. 2. The study $RSD_R$s across all rounds for seven HFRs (BDE-47, BDE-153, BDE-209, anti-DP, syn-DP, α-HBCD, and γ-HBCD) were in the range of 27–60% for $LL_{HFR}$ and 14–49% for $HL_{HFR}$ (Table 2). The highest variability of results within four rounds was for BDE-209, with the study $RSD_R$ being in the range of 64–75% and 50–89% for $LL_{HFR}$ and $HL_{HFR}$, respectively. Comparable average $RSD_R$s (expressed as a mean of study $RSD_R$s from four rounds for BDE-47, BDE-153, DPs, and HBCDs) were in the 14–39% range, except for 60% for BDE-153 at $LL_{HFR}$ in round 1.

The first reports on interlaboratory comparability on PBDEs (de Boer and Cofino et al., 2002; de Boer and Wells et al., 2006) showed the increasing agreement among laboratories over time, especially for BDE-209 reaching coefficients of variation of 20% and less (Duffek et al., 2008). No such trend was observed for BDE-209 over all rounds. Further studies presented results from interlaboratory comparisons on the analysis of BFRs in solvent mixtures (Melymuk et al., 2015) and biota and sediment samples (Ricci et al., 2020). Significantly poorer accuracy and precision for DBDPE, TBBPA, and HBCD isomers (>50% RSDs among measured values) and large deviations from the reference values (>25% bias in accuracy) suggest potential problems for comparability of the results (Melymuk et al., 2015). In the most recent study, RSDs among expert laboratories in the certification exercise for the testing of fish tissue and sediment were in the range of 9–13% (for BDE-47, BDE-153, and BDE-209) and 8–9% (for BDE-47 and BDE-153), respectively. The RSD of HBCD data (17%) reveals that they are more challenging analytes compared to PBDEs (Ricci et al., 2020). In general, $RSD_R$s achieved for BDE-47, BDE-153, and HBCDs within the presented study were quite comparable, showing no significant differences in data comparability. On the other hand, to compare the published data with the presented $RSD_R$s, various interlaboratory study designs need to be considered (different matrices, different concentration levels, pre-selection of laboratories etc.).

### 3.6.2. *OPFR metabolites in urine*

Table 3 provides an overview of the evaluation of participant performance for OPFR metabolites after four rounds. The Z-score

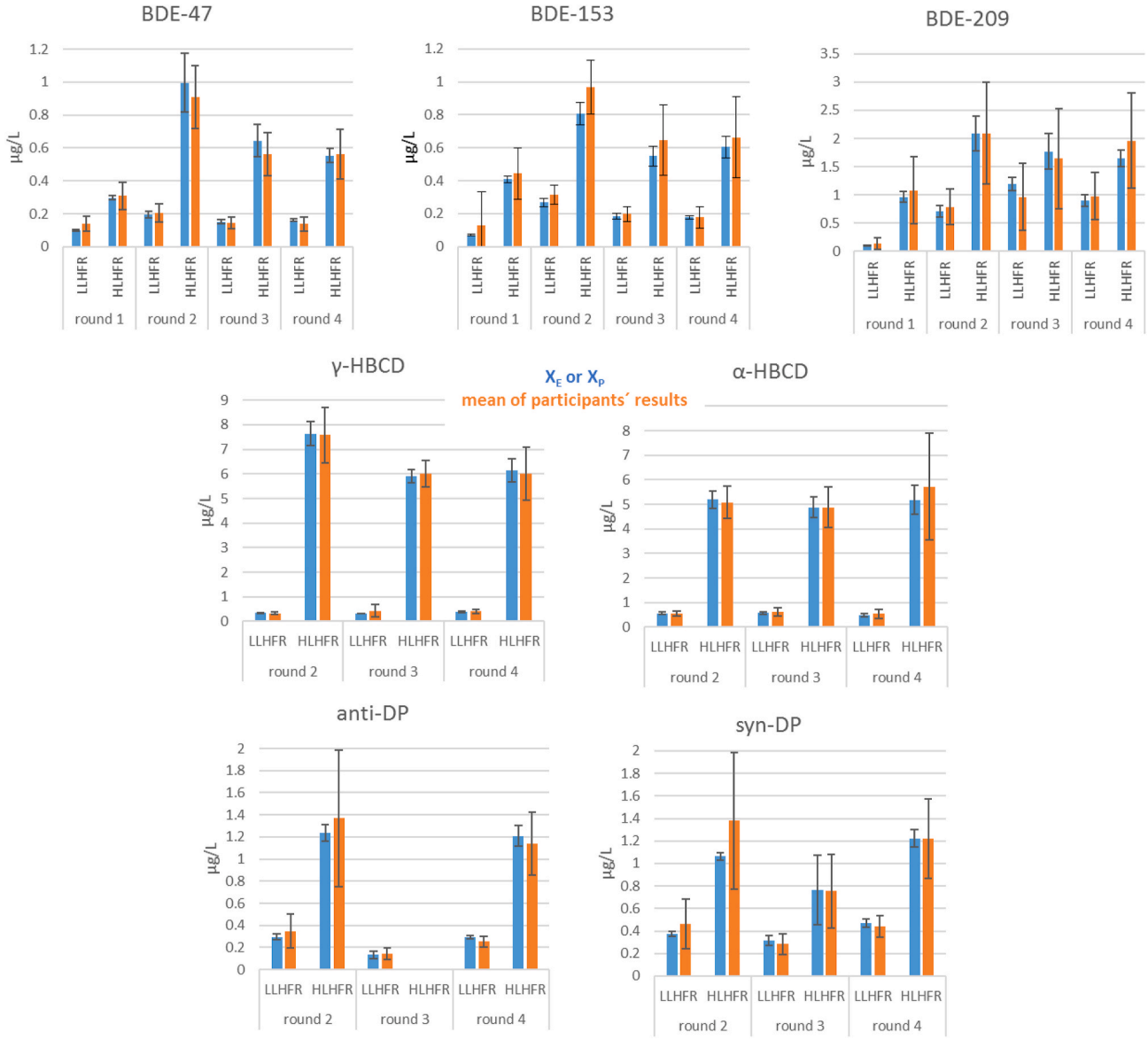


**Fig. 2.** The comparison of mean of participants results and relevant $X_E$ or $X_P$ for BDEs, HBCDs and DPs ($X_E$ – BDEs in rounds 2-4, DPs in rounds 2 and 4, $X_P$ – BDEs in round 1, DPs in round 3 and HBCDs in rounds 2-4; error bars indicate uncertainty for $X_E/X_P$ and $RSD_R$ for mean of participants results)

calculation was possible for DPHP, BClPP, and BDClPP. The rate of satisfactory results was relatively high in all four rounds for these OPFR metabolites, ranging from 67 to 100%. For BCEP only, the calculation of $X_E$ was not possible, mainly due to the limited number of results submitted by experts and participants.

The average study $RSD_R$ across all rounds for OPFR metabolites was in a similar range of 37–42% for $LL_{OPFR}$ and 29–41% for $HL_{OPFR}$. The highest $RSD_R$ was achieved in round 2 for DPHP, BDClPP, and BClPP (84%, 74%, and 55%, respectively). In contrast, the highest comparability of the submitted results was obtained in the fourth round, when a substantial reduction of $RSD_R$ values was observed for DPHP, BDClPP, and BClPP (16%, 13%, and 24%, respectively).

## 4. Conclusions

The QA/QC programme within the HBM4EU project was designed and implemented for the complex spectrum of biomarkers of human exposure to HFRs. Among target compounds, not only common BFRs (e.g., PBDEs, HBCDs, and TBBPA), but also other recently monitored compounds (e.g., DPs) and OPFR metabolites were included. Altogether ten HFRs and four OPFR metabolites in serum and urine, respectively, were targeted in the QA/QC programme. The interlaboratory comparability of these biomarkers at levels of the general European population was assessed.

The results obtained within the ICI/EQUAS programme for FR HBM parameters confirmed a fairly significant network of European laboratories not only for routinely measured BDE-47, BDE-153, BDE-209, α-HBCD, and γ-HBCD but also for anti-DP and syn-DP, for which less biomonitoring data are published. On the other hand, the data revealed critically low analytical capacity in Europe for HBM of TBBPA, DBDPE, and 2,4,6-TBP as well as of OPFR biomarkers. The poor participation rate for OPFR metabolites made it challenging to evaluate the results according to SOPs. To overcome these difficulties, additional tools had to be used, especially web conferences with participants, discussions within the HBM4EU Quality Assurance Unit and the search for alternative approaches for results evaluation.

Biological material in HBM surveys is considered valuable in terms of sample amount available for the analysis, and therefore emphasis should be placed on obtaining as much data as possible from a single sample. In this study, the scope of the participanting laboratories varied substantially and in some cases did not cover all target biomarkers (e.g., analysis of PBDEs or HBCD only). On the other hand, the FR group is very diverse in its physicochemical properties and its potential for bioaccumulation. The analysis of both serum and urine, as well as the use of GC and LC instrumentation (e.g., analysis of PBDEs and HBCD) is required. The laboratories should demonstrate the ability to extend the spectrum of substances analysed, not only in response to HBM project requirements, but also to consider the possibility of combining methods for other halogenated compounds with similar properties, e.g. simultaneous determination of GC-MS amenable HFRs with polychlorinated bisphenols.

The HBM4EU QA/QC programme has revealed the benefits of and need for a European network of analytical laboratories for human biomonitoring of FRs and other priority chemicals. This network would support the increasing HBM and risk assessment studies by providing high-quality analytical results as well as expertise for new method development and their implementation, which is necessary for TBBPA, DBDPE, 2,4,6-TBP, and most OPFR metabolites. The network of laboratories created under HBM4EU can be considered as the project's legacy for future human biomonitoring actions in Europe.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2021.111705.

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