UNIVERSITY OF CHEMISTRY AND TECHNOLOGY PRAGUE
Faculty of Food and Biochemical Technology
Department of Food Analysis and Nutrition

Habilitation Thesis

BIOMONITORING: TOOL FOR THE ASSESSMENT OF HUMAN EXPOSURE TO ENVIRONMENTAL CONTAMINANTS

Ing. Darina Dvořáková, Ph.D.

Prague, 2022

FINANCIAL ACKNOWLEDGEMENTS

The research presented in this Habilitation Thesis was financially supported by:

- FP7-227525-CP - PERFOOD (PERFluorinated Organics in Our Diet) supported by the European Commission through 7th Framework Programme
- MultiCoop - Multidisciplinary approach to strengthen cooperation and establish novel platform for comprehensive assessment of food and feed safety (H2020-TWINN-2015-Twinning 692195)
- HBM4EU ("Human Biomonitoring for Europe") - European Union's Horizon 2020 research and innovation programme under grant agreement (No. 733032) supported by the European Commission
- MSMT 6046137305 and 7E08068 supported by the Ministry of Education, Youth and Sports, Czech Republic
- "National Programme of Sustainability I", NPU I, supported by Ministry of Education, Youth and Sports (MSMT-34870/2013, MSMT-43760/2015, LO1215 and LO1601)
- LT Inter-Excellence, Exposure biomarkers to selected air pollutants, health risk assessment in children population, specific university research project supported by Ministry of Education, Youth and Sports (LTAUSA17182)
- Impact of air pollution to genome of newborns, Czech Science Foundation Project (No. 13-13458S)
- "Operational Program Prague – Competitiveness" supported by the European Commission (CZ.2.16/3.1.00/22197, CZ.2.16/3.1.00/21537 and CZ.2.16/3.1.00/24503)

The financial support is gratefully acknowledged.

ACKNOWLEDGEMENTS

I would like to thank Prof. Jana Pulkrabová for her valuable advice, comments, and suggestions that helped me to accomplish this work.

My thanks also belong to my colleagues, especially Prof. Jana Hajslova, and friends from EnviroTeam, namely Martina, Tomáš, Káťa, Andrea, and other students and colleagues from the Department of Food Analysis and Nutrition for their help and friendship throughout all the years I have spent in the laboratory.

Last but not least, I would like to thank my husband Honzík and my family for the eternal support, patience and tolerance and staying by me all the time.

TABLE OF CONTENTS

PREFACE

People in modern societies are exposed to a broad spectrum of environmental chemicals. Exposure to some of them may be associated with adverse health effects. The knowledge of population exposure to these substances is therefore crucial for the estimation of associated health risks. An intensive anthropogenic activity and rapidly progressing industrial development have resulted in the production of various chemicals, which are used in many industrial and commercial applications. Pollution by man-made persistent organic pollutants (POPs) has been a serious global issue for over half a last century. POPs have become chemicals of concern due to their following features: a) considerable resistance to degradation, b) ability to be transported over long distances from sources by air and ocean currents, c) potential to bioaccumulate through terrestrial and aquatic food webs in amounts that may result in adverse health effects for animals and humans, and d) potential toxic effects such as immunotoxicity, neurotoxicity, developmental toxicity, carcinogenicity, mutagenicity and endocrine disruption. Typical substances of the POPs group are organohalogenated compounds represented by polychlorinated biphenyls (PCBs), brominated flame retardants (BFRs), organochlorinated pesticides (OCPs) and per- and polyfluoroalkyl substances (PFAS).

This Habilitation Theses is focused on the human biological monitoring (or the so-called human biomonitoring), which represents an effective tool for the assessment of population exposure to a broad spectrum of environmental chemicals. Special attention is paid to the development and validation of sample preparation procedures and possibility to combine determination of various groups of compounds into one "multi-analytes" method, thus decrease the requirements for the amount of biological material needed for the ultra-trace analysis.

The practical applications have been developed within the framework of the working group of Prof. Jana Pulkrabová at the Department of Food Analysis and Nutrition of the University of Chemistry and Technology, Prague, in the period September 2010 until February 2022.

This Habilitation Thesis documents author´s inputs to the development of analytical methods for the analysis of PCBs, OCPs, BFRs, PFAS and metabolites of polycyclic aromatic hydrocarbons (PAHs), phthalates and di-iso-nonyl cyclohexane-1,2-dicarboxylate (DINCH), and their further application for the investigation of biomonitoring samples represented by blood serum, breast milk and urine. The results obtained within the research activities were published in 21 original papers, 13 short papers (conference proceedings) and also presented in more than 100 contribution at scientific conferences both as oral presentations and posters.

1 MAIN OBJECTIVES OF THE PRESENTED HABILITATION THESIS

The presented Habilitation Thesis is dealing with human biomonitoring and novel analytical approaches for the simultaneous determination of various groups of environmental contaminants and their exposure markers (metabolites) in human biological matrices (blood serum, urine and breast milk). Experimental part focuses mainly on the development of analytical methods employing liquid chromatography (LC) and gas chromatography (GC) separation techniques coupled to (tandem) mass spectrometry (MS(/MS)). Special attention is paid to the validation of sample preparation procedures and their applications to real-life samples obtained primarily within the Czech human biomonitoring studies.

Following tasks are addressed:

(A) DEVELOPMENT OF THE NOVEL ANALYTICAL STRATEGIES

- Multi-analyte method for the analysis of five groups of persistent organic pollutants (POPs) in human blood serum (**Appendix I**)
- Method for the simultaneous determination of perfluoroalkyl substances (PFAS), brominated flame retardants (BFRs) and their metabolites in human breast milk (**Appendix II**)
- Simple method for the analysis of phthalate and di-iso-nonyl cyclohexane-1,2-dicarboxylate (DINCH) urinary metabolites (**Appendix III**)
- Novel strategy for the determination of polycyclic aromatic hydrocarbon monohydroxylated metabolites (OH-PAHs) in urine (**Appendix IV**)

(B) HUMAN BIOLOGICAL MONITORING (HBM)

- Introduction of the quality assurance/quality control programme for the analysis of selected priority chemicals within the European human biomonitoring platform (**Appendix V** and **Appendix VI**)
- Biomonitoring of POPs represented by flame retardants, PFAS, organochlorinated pesticides (OCPs) and polychlorinated biphenyls (PCBs) in serum of Czech population (**Appendix I** and **Appendix VII**)
- Determination of PFAS in serum of mother-child cohort in the Slovak Republic (**Appendix VIII**)
- Analysis of PFAS in breast milk collected within Czech human biomonitoring program (**Appendix II** and **Appendix IX**)
- Occurrence of OH-PAHs (**Appendix X** and **Appendix XI**), metabolites of phthalates and DINCH (**Appendix III**) in urine of Czech mothers and their newborns

2 HUMAN BIOLOGICAL MONITORING

2.1 Characterization of human biomonitoring

Exposure to environmental pollutants (*further described in Chapter 3*) may occur through different routes, such as inhalation, ingestion, and dermal absorption (**Figure 1**). The amount of pollutant uptake is often termed as the "absorbed dose". Thus, the body burden of a specific compound is determined by several factors, such as the contaminant's concentration in a specific environmental medium, its physical and chemical properties, and timing and duration of exposure, as well as individual factors, such as uptake, metabolism and excretion rates. Human bimonitoring (HBM) reflects all these factors by measuring the concentration of a chemical or its metabolites in human matrices. In contrast, for environmental pollutants with multiple exposure pathways, comprehensive exposure assessment based on environmental data requires quantitation of pollutant levels in multiple matrices and data on individual behavioral patterns affecting exposure, such as the consumption of contaminated foods (*HBM, facts and figures, WHO, 2015*).

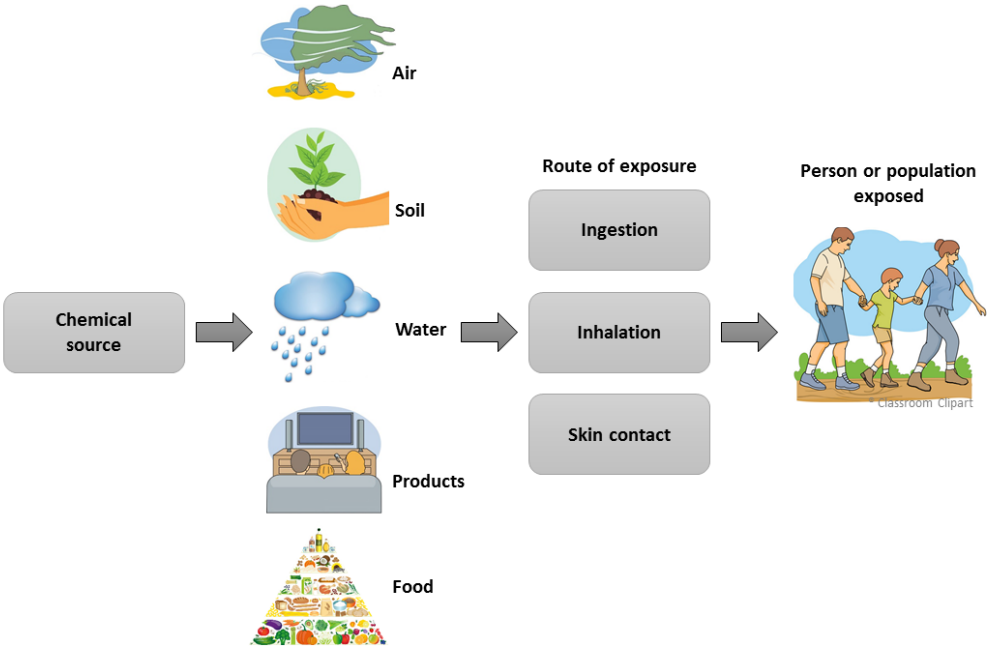


Figure 1 Assessment of human exposure to environmental pollutants (*created by author of this Habilitation Thesis using Classroom Clipart © 2021*)

HBM can be defined as *"the method for assessing human exposure to chemicals or their effects by measuring these chemicals, their metabolites, or reaction products in human specimens"* (*CDC, 2005*). Once the chemical is absorbed in the body, it can be excreted without transformation, excreted after metabolization, or stored in various tissues or bones. Biomonitoring involves measurement of biomarkers in bodily fluids, such as blood, urine, saliva, breast milk, sweat, and other specimens, such as faeces, hair, teeth, and nails. **Table 1** summarizes the advantages and limitations of biological matrices that are commonly used in HBM studies to assess environmental exposures in humans.

Table 1 Biological matrices used in HBM studies (*HBM, facts and figures, WHO, 2015*)

Matrix	Population	Advantages	Limitations	Example of compounds measured in the matrix
Blood, serum, plasma	General	○ In equilibrium with all organs and tissues ○ Well established SOPs for sampling	○ Invasive; trained staff and special materials required ○ Volume limitation. ○ Special conditions for transport and shipment	POPs, metals/trace elements, organic compounds, tobacco smoke
Cord blood	Specific (neonates)	○ Non-invasive; provides information about mother and child ○ Well defined SOP for peripheral blood can be used for cord blood	○ Only available at birth in maternity ward settings ○ Ethical constraints ○ Special conditions for transport and storage	POPs and other organic compounds, metals/trace elements, tobacco smoke
Urine	General	○ Non-invasive, easy collection, no volume limitation ○ Allows analysis of metabolite	○ Composition of urine varies over time	Metals/trace elements, organic compounds, tobacco smoke. Metabolites of environmental pollutants
Breast milk	Specific (mothers)	○ Provides information about mother and child ○ Enriched with lipophilic compounds	○ Somewhat invasive ○ Restricted period of availability ○ Depuration of chemicals during lactation should be considered	POPs, metals/trace elements, organic compounds, tobacco
Hair	General, with few exceptions (neonates)	○ Non-invasive; minimum training required for sampling ○ No special requirements for transport and storage ○ Information about cumulative exposure during previous months ○ Segmental analysis is possible	○ Hair is exposed to the environment and can be externally contaminated ○ Potential variations with subject's hair colour, hair care or race	Metals/trace elements, POPs
Nails	General	○ Non-invasive, easy collection ○ No special storage or transport requirements ○ Provide information about short and long term exposure	○ Exposed to the environment and can be externally contaminated (toenails are less exposed) ○ Less documented for HBM applications	Metals/trace elements, tobacco, e.g., arsenic, mercury, cadmium, lead
Saliva	General	○ Non-invasive ○ Easy collection	○ Lower concentrations of analytes than in blood; requires sensitive analytical techniques ○ Variation in flow rate and composition ○ The use of stimulant or absorbent pads can interfere with analysis ○ Less documented for HBM applications	Metals/trace elements, organic compounds, POPs, tobacco

Legend: HBM – human biomonitoring; POPs – persistent organic pollutants; SOP - standard operating procedure

Biomonitoring data directly reflect the total body burden or biological effect resulting from all routes of exposure, and interindividual variability in exposure levels, metabolism, and excretion rates. Such data are often the most relevant metric for health impact assessment, especially for bioaccumulation of persistent chemicals. For a given chemical, HBM can identify spatial and time trends, lifestyle contributing factors, and specific at-risk groups. HBM is an important tool to support environment protection and health policy-making because it can provide useful quantitative information regarding the actual exposure of a population to environmental pollutants including emerging ones, as well as data regarding the resulting health effects and/or population susceptibility to these xenobiotic compounds. Worth to notice, that biomonitoring usually does not reveal exposure sources and routes. Therefore, environmental monitoring remains crucial for the development of targeted policy actions (*Choi et al., 2015; HBM, facts and figures, WHO, 2015*).

2.2 European biomonitoring programs

A number of countries in the European Union (EU) including the Czech Republic have well-established HBM programs, but only at the national level (*Černá et al., 2017; Ganzleben et al., 2017*). The EU's Environment and Health Action Plan 2004–2010 recognized the value of HBM and called for a coherent approach to biomonitoring across Europe (*European Commission, 2004*). First steps in this direction were undertaken in COPHES/DEMOCOPHES EU funded project, which developed harmonized protocols for HBM in Europe to improve the comparability of generated data (*Schindler et al., 2014*). To build on the work of these programs, a framework for the coordination of human biomonitoring activities at EU level was required.

The European Commission launched a call in 2015 for a creating of European Joint programme for the monitoring and scientific assessment of human exposures to chemicals and potential health impacts in Europe, building on previous activities undertaken at the EU and national levels. As a result, in 2017, an initiative was started under the acronym HBM4EU ("Human Biomonitoring for Europe"; https://www.hbm4eu.eu/) that has brought together scientists from 30 countries and European Commission authorities (under the Horizon2020 Programme of the EU) to coordinate and advance HBM in Europe (*Vorkamp et al., 2021*). The prioritization strategy identified substances for research and survey under the HBM4EU project. The first list of HBM4EU priority compounds was published at website in 2016 and included: aniline family; bisphenols; cadmium and chromium VI; chemical mixtures; emerging substances; flame retardants (FRs); polycyclic aromatic hydrocarbons (PAHs); per- and polyfluoroalkyl substances (PFAS); phthalates and Hexamoll® di-iso-nonyl cyclohexane-1,2-dicarboxylate (DINCH). The second extended list of priority compounds from 2018 included: acrylamide; aprotic solvents; arsenic; diisocyanates; lead; mercury; mycotoxins; pesticides and benzophenones (*Vorkamp et al., 2021*).

The whole project was very ambitious, however all tasks defined in the HBM4EU have not been finished especially due to the restrictions related to COVID-19 pandemic in 2020-2021. From this reason the new Partnership for Chemicals Risk Assessment (PARC), which is currently under preparation by the EU Commission in cooperation with many of the HBM4EU partners will include a continuation of HBM4EU activities. In the PARC partnership, the plan is to join forces with European scientists to create the next generation of chemical risk assessment and to facilitate "The European Green Deal" (*European Commission,*

2019), which includes the sustainable management of chemicals for a non-toxic environment. An essential role of this partnership is to foster better use of existing knowledge and data, and better cooperation and coordination of research on the regulatory needs - all in order to improve risk assessment and management, including the development of an EU early warning system for emerging risks of chemicals occurring/entering in the environment (*Dulio et al., 2020*).

2.3 Quality assurance/quality control of human biomonitoring studies

For advancing HBM, a network of laboratories delivering reliable analytical data on human exposure is fundamental. In general, the chemical analysis of HBM samples involves a number of challenges, including trace levels of targeted analytes the variety of those to be included in various biological matrices, the risk of cross-contamination due to the omnipresence of some target contaminants (e.g., FRs and phthalates) and the limited availability of analytical standards and certified reference materials. Thus, HBM4EU implemented a complete quality assurance/quality control (QA/QC) scheme for the verification of analytical quality and comparability between laboratories for the biomonitoring analysis in the project.

Interlaboratory Comparison Investigations (ICIs) and External Quality Assurance Schemes (EQUASs) were organized and their results were critically evaluated (Appendix V).

University of Chemistry and Technology, Prague, Department of Food Analysis and Nutrition, was responsible for the realization of ICI/EQUAS programme (preparation, testing and distribution of control material, evaluation of results) for a complex spectrum FR biomarkers represented by three congeners of polybrominated diphenyl ethers (PBDEs; namely BDE-47, BDE-153 and BDE-209), two isomers of hexabromocyclododecane (α-HBCD and γ-HBCD), two dechlorane plus (anti-DP and syn-DP), tetrabromobisphenol A (TBBPA), decabromodiphenyl ethane (DBDPE), and 2,4,6-tribromophenol (2,4,6-TBP) in blood serum, and by four metabolites of organophosphorus flame retardants (OPFRs) in urine. As a measure of the laboratories' proficiency, Z-scores (Z) were calculated according to formula (1):

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

With x = participant´s result; X = assigned value derived from the participants' results (in ICI) or expert results (in EQUAS); σ_T = standard deviation for proficiency assessment, pre-set at = 0.25*X

Achievement of the study – HBM4EU QA/QC programme for FRs in serum and urine (Appendix VI)

- The results obtained within the four rounds of the ICI/EQUAS programme (2018-2020) for FR biomarkers revealed the benefits and needs to involve this activity into biomonitoring. It confirmed availability of a fairly significant network of European laboratories capable to report the data 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 fewer biomonitoring data have been published yet. The majority of participants achieved more than 70 % satisfactory results (|Z|≤2) for BDE-47 and BDE-153 overall

rounds. For other halogenated FRs, the percentage of successful laboratories varied from 44 to 100 %.

- Current 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 low participation rate for OPFR metabolites made it challenging to evaluate the results according to standardized operation procedures (SOPs), thus the search for alternative approaches for results evaluation had to be considered.
- The network of laboratories created under HBM4EU can be considered as the project's legacy for future HBM actions in Europe such as project PARC, which will start in 2022.

3 ENVIRONMENTAL CONTAMINANTS

Environmental contaminants are chemicals that accidentally or deliberately enter the environment, often, but not always, as a result of human activities. Some of these contaminants may have been produced for industrial use and because they are very stable, they do not break down easily. If released into the environment, these pollutants may enter a food chain. Other environmental contaminants are naturally-occurring chemicals, but industrial activity may increase their mobility or increase the amount available to circulate in the environment, allowing them to enter the food chain at higher levels than would otherwise occur. A wide variety of environmental contaminants have been detected in environment. These range from inorganic compounds represented by toxic metals and some of their biotranformation products (e.g., lead, mercury/methylmercury, arsenic, cadmium) and different types of salts, which generally occur in the form of dissolved anions and cations; then "ionic" species (e.g., perchlorate) and finally organic (carbon-based) substances including persistent organic pollutants (POPs).

POPs such as polychlorinated biphenyls (PCBs), organochlorinated pesticides (OCPs), chlorinated paraffins (CPs), brominated flame retardants (BFRs) and PFAS are a group of synthetic chemicals that have been intentionally or inadvertently produced and introduced into the environment. As mentioned before, due to their stability, persistence and long-range transport properties, they are ubiquitous around the world and are even found in places such as the arctic regions, far distant from the locality where they had been intensively used (**Figure 2**). Such chemicals tend to accumulate to different extents in food chain, especially in fat-containing foods (PCBs, OCPs, PCBs and CPs). Traces of POPs are found globally in the environment, animal and human organisms. This has led to the establishment of monitoring programs worldwide to determine prevailing levels of POPs in the population as well in the environmental compartments and to investigate the adverse health effects associated with background exposure. Some cancers, birth defects, dysfunctional immune and reproductive systems and even diminished intelligence are suspected to be related to an exposure to these chemicals (*Qing Li et al., 2006; Xu et al., 2013; UNEP, 2015*).

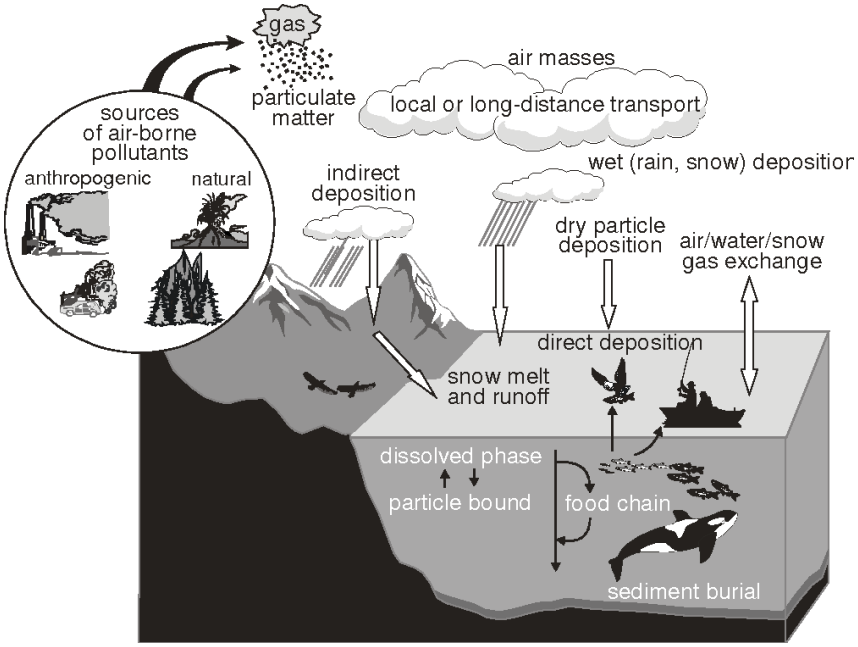


Figure 2 Global distribution of POPs through atmospheric processes (*Ross and Birnbaum, 2001*)

Protection of human health as well as the environment from the harmful effects of POPs is addressed at the international level through various agreements and programs. The Stockholm Convention on POPs (*UNEP 2001, UNEP 2018*), the largest international agreement in this field, was adopted in 2001 and entered into force in 2004 with twelve substances (called the "dirty dozen") listed in either three Annexes: A (parties must take measures to eliminate the production and use of the chemicals listed under Annex A), B (parties must take measures to restrict the production and use of the chemicals listed under Annex B), or C (parties must take measures to reduce the unintentional release of the chemicals listed under Annex C) (*Fiedler et al. 2019*). To date (January 2022), the Convention has 185 Parties and a total of 35 POPs slated for elimination or severe restrictions in the production and use and control of their disposal. Several other chemicals such as syn-DP, methoxychlor, UV-328 (substituted phenolic benzotriazole used as a UV absorber in many products), chlorpyrifos, CPs with carbon chain lengths in the range C14-17 and chlorination levels at or exceeding 45 per cent chlorine by weight and long-chain perfluorocarboxylic acids (PFCAs), their salts and related compounds have been proposed for the inclusion and are currently under a review process. Currently POPs Review Committe completed the risk profile and risk management evaluation for perfluorohexane sulfonic acid (PFHxS), its salts and PFHxS-related compounds. (*UNEP, 2021a*).

OCPs & PCBs (organochlorinated pesticides & polychlorinated biphenyls)

For many decades, OCPs have been applied to eradicate pests and vectors of diseases in agriculture (extensively from 1950s to the 1970s), homes and public health sector (*Shen & Wania, 2005*). PCBs were produced as a mixture (theoretically 209 congeners) with the trademark "Aroclor®". These compounds have been used since the 1930s as insulating fluids in electrical equipment and as additives in sealants (*Helou et al., 2019*). International bodies have identified seven PCBs, so-called indicator PCBs, which can be used to characterize the presence of PCB contamination. Six of these seven are non-dioxine like PCBs (# 28, 52, 101, 138, 153, and 180), and one is a dioxine-like PCB (# 118). Dioxine-like PCBs exhibit similar toxicological effects as polychlorinated dibenzo-p-dioxins/dibenzofurans with the most toxic representative 2,3,7,8-tetrachlorodibenzo-p-dioxin (*WHO, 2016*).

PCBs and major OCP representatives (e.g., aldrin, chlordane, dichloro-diphenyl-trichloroethane (DDT), dieldrin, endrin, heptachlor, mirex, toxaphene and hexachlorobenzene (HCB)) are on the "dirty dozen" list, thus the parties to the Stockholm Convention can no longer produce these chemicals since 2004. The European Commission set maximum levels for the sum of dioxin-like PCBs, six major (indicator) PCBs in food and feed in the *Commission Regulation No. 1881/2006* and its amendment (*Commission Regulations No. 1259/2011*).

CPs (chlorinated paraffins)

CPs, as technical mixtures of polychlorinated alkanes, have been produced since the 1930s for use as additives in lubricants and cutting fluids as well as FRs in plastics and sealants. They can be divided into three groups: short-chain CPs (SCCPs) comprising 10 to 13 carbon atoms, medium-chain CPs (MCCPs) comprising 14 to 17 carbon atoms and long-chain CPs (LCCPs) with more than 18 or more carbon atoms. The many possible positions for the chlorine atoms and presence of chiral carbon atoms lead to a large number of potential positional isomers, enantiomers and diastereoisomers (*van Mourik et al., 2016*). SCCPs

were classified as endocrine disruptors (*Li et al., 2020*) and possible carcinogens to humans (group 2B) (*IARC, 2020*) and were added to the Stockholm convention (Annex A) in 2017 (*UNEP, 2021b*).

FRs (flame retardants)

FRs are a class of chemicals utilized to provide fire safety performance to other materials, structures, and devices used in modern society. These chemicals are highly varied in molecular structure and chemistry. FRs are divided into three major categories depending on their chemical composition: inorganic FRs, halogenated FRs, with brominated and chlorinated FRs and OPFRs (*Beard & Angeler, 2010*). According to the European Flame Retardants Association (EFRA), the total consumption of FRs in Europe was estimated at 465,000 t in 2006, of which 54% were metal hydroxides, 20% OPFRs, 10% BFRs, 7% CPs, 3% borates and stannates, 3% antimony trioxide and 3% melamine based FRs (*van der Veen & de Boer, 2012; Pantelaki et al., 2019*).

BFRs have been extensively used as additives worldwide in products such as foams, resins, rubbers, adhesives, plastics, textiles, electronics, and construction materials to comply with fire safety standards and regulations (*Alaee et al., 2003*). In recent years, several traditional BFRs, such as some PBDEs, polybrominated biphenyls and HBCDs, have been banned or strictly phased out due to their persistent, bioaccumulative, and toxic effects on environmental and human health such as endocrine disruption, developmental and carcinogenic effects (*Covaci et al., 2011*; *Wu et al,. 2020*). In particular, octa-, penta-, and deca-brominated diphenyl ethers (octa-, penta-, or deca-BDEs) and HBCDs have been listed as POPs under Stockholm Convention during the last decade (*UNEP, 2021b*).

As a result, novel (or new) brominated flame retardants (NBFRs) have been introduced as alternatives, as well as OPFRs, DPs, and other FRs, which have been identified as "emerging" contaminants (*Covaci et al. 2011*, *Ezechiáš et al. 2014*). Highly chlorinated DPs has been recently proposed for listing under the Stockholm Convention (*UNEP, 2021a*).

NBFRs can be divided into three classes: (i) monoaromatic, which mainly includes polybromobenzene analogues; (ii) multi-aromatic, which includes 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE), DBDPE, and several TBBPA derivatives; and (iii) cycloaliphatic, which includes polybromocyclohexane analogues and 1,2-dibromo-4-(1,2-dibromoethyl) cyclohexane isomers. However, these chemicals share a similar structure with halogenic substitution in cyclic hydrocarbons, which can cause their physicochemical properties to be generally analogous to those of PBDEs and HBCDs. As reviewed by *Hou et al. (2021),* occurrence of NBFRs has been frequently reported in various environmental matrices globally including humans, thus numerous concerns have been expressed about their ubiquitous environmental occurrence. Additionally, TBBPA is one of the most prevalent BFRs in the world. Its main application is in epoxy resin used to produce printed circuit boards, in which the bromine content can reach 20% by weight. It is still unclear which potential health impacts of TBBPA on the general population. However, toxicological and human exposure data suggest a relationship between TBBPA exposure and human health injury (*Feiteiro et al., 2021*).

OPFRs are a group of compounds with the same phosphate base unit–a central phosphate molecular and heterogeneous substituents. These compounds are usually used as stabilizers for antifoaming and as additives to floor polishes, lubricants, lacquers, and hydraulic fluids. In addition, OPFRs are utilized as extreme pressure additives and antiwear agents in hydraulic fluids, lubricants, transmission

fluids and motor oils. As mentioned before, the legacy BFRs have been replaced by OPFRs. The effects of exposure to OPFRs have not been studied to the same extent as for the phased-out BFRs, and are therefore less well-known. Recent studies focus on the assessment of the toxicity of OPFRs and their possible negative effects. OPFRs have the potential to cause adverse development, neurotoxicity, and oxidative stress in animals as a result of short- and long-term exposure (*Du et al., 2019*). Moreover, tris(2-chloroethyl) phosphate (TCEP), tris(2-chloroisopropyl) phosphate (TClPP), and tris(1,3-dichloro-2-propyl) phosphate (TDClPP) have been proven to be neurotoxic and carcinogenic (*WHO, 1998*). TCEP has been phased out since the 1980s and is no longer produced within the 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 (*European Commission Directive, 2014/79/EU*).

PFAS (per-and polyfluoroalkyl substances)

PFAS are a broad class of chemicals with fluoroalkyl chains in their molecular structure (over 4 000 substances that contain at least one perfluoroalkyl moiety $-C_nF_{2n}-$). Low polarizability of these molecules, short C–F bond length, and its large binding energy contribute to a variety of useful and unique PFAS features, including oil and water repellency, physical and chemical stability, and reduced solvent surface tension of materials that employ PFAS as polymer building blocks. Because of these qualities, PFAS have been used since the 1950s in various applications: upholstery, textiles, fire-fighting materials, hydraulic fluids, photoresists, and emulsifying agents for polytetrafluoroethylene (*Zushi et al., 2012*). This widespread use has resulted in their global distribution into the environment either from direct sources such as the manufacturing of PFAS and the use of products containing these compounds, as well as from indirect sources like reaction impurities or degradation of precursors (*Lindstrom et al., 2011; Abunada et al., 2020*). As summarized recently by *Fenton et al. (2020),* epidemiological studies have revealed associations between exposure to specific PFAS (mostly to perfluorooctanoic and perfluorooctane sulfonic acid (PFOA and PFOS, respectively) and a variety of health effects, including altered immune response (e.g., vaccines can be rendered less effective) and thyroid function, liver disease, lipid and insulin dysregulation, kidney disease, adverse reproductive and developmental outcomes, and cancer. PFOS, PFOA, and their related compounds have been listed as POPs by the Stockholm Convention in 2009 (Annex A) and 2019 (Annex B), respectively; hence, their use is phased out globally.

Several actions spurred changes in PFAS manufacturing and treatment. Between 2000 and 2002, 3 M, the primary manufacturer of PFOS, voluntarily phased out its production. In 2006, eight additional manufacturers consensually agreed to stop global production of PFOA. In 2008, the European Food Safety Authority (EFSA) established tolerably daily intake values (TDIs) for PFOS and PFOA (1 500 and 150 ng/kg body weight (bw), respectively), and in their 2018 report, dramatically decreased their assessment to a tolerable weekly intake (TWI) of 13 ng/kg bw for PFOS and 6 ng/kg bw for PFOA (*EFSA Contam Panel, 2008, EFSA Contam Panel, 2018*). In February 2020, EFSA established a new TWI of 8 ng/kg bw per week for the sum of four major PFAS (PFOA, perfluorononanoic acid (PFNA), PFHxS and PFOS) and subsequently, in July 2020, the intake was reassessed again and a new TWI of 4.4 ng/kg bw (*EFSA Contam Panel, 2020*).

Despite the many measures taken across the world to limit PFAS use, there remain several exemptions for their production and use (e.g., metal plating, firefighting foams, incest baits, and photographic coating). In addition, PFAS production has shifted from North America, Europe and Japan to

emerging Asia countries. China has become the only producer of PFOS and the largest producer of PFOA (*Wang et al., 2020a*). Many more PFAS, which now number in the thousands, are being developed and produced to replace those that are phased out as well. The large market demand leads to the production of many alternatives, including short-chain PFAS (<C8 perfluorocarboxylic acids (PFCAs) and <C6 perfluorosulfonic acids (PFSAs)), some PFAS precursors (e.g., fluorotelomer sulfonates (FTSs); polyfluoroalkyl phosphate esters (PAPs) and fluorotelomer alcohols (FTOHs)) and alternative PFAS (e.g., chlorinated polyfluorinated ether sulfonate, namely F53B). Among them, PAPs are produced and used as oil- and water-repellent coating agents for food packaging materials as well as surfactants in personal care and cosmetic products (*Trier et al., 2011*). Additionally, scientists suspect they are just hazardous as their predecessors because of their higher mobility in aquatic ecosystem and it is very difficult to remove them from drinking water. Many studies indicated PFAS can be released from biosolids to soils and migrate into plants (*Wang et al., 2020b*).

PAHs (polycyclic aromatic hydrocarbons)

PAHs are also widely distributed ubiquitous environmental pollutants generated primarily during the incomplete combustion of organic materials. Emissions from anthropogenic activities predominate; nevertheless, some PAHs in the environment originate from natural sources such as open burning, natural loss or seepage of petroleum or coal deposits, and volcanic activities. Moreover, some improperly performed food processing practices, such smoking or grilling, may result in PAHs formation. In any case, contrary to above mentioned POPs, they have never been produced intentionally (*Samanta et al., 2002*). Many of them have detrimental biological effects, toxicity, mutagenicity, and carcinogenicity. Due to their occurrence, recalcitrance, bioaccumulation potential, and carcinogenic activity, PAHs as well as POPs have gathered significant environmental concern. Although PAH may undergo adsorption, volatilization, photolysis, and chemical degradation, microbial biotransformation is the major degradation process. PAH degradation depends on the environmental conditions, number and type of microorganisms, the nature, and chemical structure of the chemical compound being degraded (*Haritash & Kaushik, 2009*). Worth to be mentioned, that PAHs are not the subject of the international conventions on POPs, because their physicochemical properties do not suggest persistence or bioaccumulation potential (*Pribylova et al., 2012*).

Considering the toxicity of PAHs, several countries have drafted legislation to establish tolerable limits for PAHs in foods, food products, and beverages as well as to enforce monitoring strategies for the most relevant compounds. The European institutions have issued two regulations regarding presence of PAHs in food for human consumption: the *Commission Regulation No. 1881/2006* and its amendment (*Commission Regulation No. 835/2011*) that establishes the maximum levels in foodstuffs for four PAH compounds (benzo(a)pyrene (BaP), benzo(a)anthracene (BaA), benzo(b)fluoranthene (BbF) and chrysene (CHR)) and the *Commission Regulation No. 333/2007* and its amendment (*Commission Regulation No. 835/2011*) that defines the sampling and analytical methods approved for PAH detection in food products.

Furthermore, health agencies such as the World Health Organization (WHO) and European Commission have launched efforts to decrease the concentration of PAHs in food, especially through strategies to control the processes that induce their formation.

Phthalates and DINCH (di-iso-nonyl cyclohexane-1,2-dicarboxylate)

Plasticizers, such as phthalate esters, a group of several diesters of phthalic acid, are necessary additives in numerous consumer products due to their ability to make plastic materials flexible and durable. Phthalates are not covalently bound to the polymeric macromolecules. As a result, they can easily leach out and contaminate the surrounding environment, leading to human exposure with adverse health outcomes (*Wormuth et al., 2006*). There is a substantial evidence that phthalates can induce disruption in estrogenic activity and cause reproductive, developmental, and liver toxicity in experimental animals and in humans (*Giuliani et al., 2020*).

Phthalates can be classified into two groups, low molecular weight (LMW) phthalates, which are frequently added to various cosmetics personal care products to preserve scent, and high molecular weight (HMW) phthalates, which are used to produce vinyl plastics for diverse applications including flooring, clear food wrap, and intravenous tubing (*Schettler, 2006*). Until now, there were a number of regulations and restrictions. For example, HMW phthalates, such as di(2-ethylhexyl) phthalate (DEHP), diisononyl phthalate (DINP), and diisodecyl phthalate (DIDP) have been restricted due to their reproductive toxicity and endocrine disrupting properties. Certain phthalates, like diisobutyl phthalate (DiBP), di-n-butyl phthalate (DnBP), benzyl butyl phthalate (BBzP) and DEHP, have been prohibited in toys and childcare articles in the EU, the United States of America, and Canada (*Giovanoulis et al., 2018*). The recent restriction of phthalates concerns articles containing DEHP, DBP, DiBP, and BBzP in a concentration greater than or equal to 0.1% (individually or in combination) on the market in the European Union (*Fréry et al., 2020*).

Due to these strict regulations and the increasing evidence for adverse health effects on humans, there is a need for introduction of alternative plasticizers such as DINCH. This compound (marketed as Hexamoll® DINCH®) entered the market in 2002 as a substitute for the restricted high molecular weight phthalates DEHP and DINP (*Schütze et al., 2014*). DINCH is mainly used in polyvinyl chloride plastics, but its use is also authorized in sensitive applications such as toys, food contact materials, and medical devices (*Koch et al., 2013*) as current toxicological data suggest that DINCH does not exhibit similar toxic effects as phthalates (*Bui et al., 2016*).

In the paragraphs below, analytical approaches for the determination of various organohalogenated POPs and metabolites of phthalates, DINCH, and PAHs in different biological matrices (mainly blood serum, breast milk and urine) are discussed. Specifically, a lot of attention has been paid to integral sample preparation procedures which enable to increase the sample throughput and at the same time reduce requirements for the amount (volume) of biological samples, which is crucial for the realization of large HBM studies.

4 SAMPLE PREPARATION STRATEGIES IN THE ANALYSIS OF BIOLOGICAL MATRICES

Determination of various biomarkers of human exposure to environmental contaminants requires analytical methods of high selectivity and high sensitivity, due to low concentrations of target analytes and limited sample volume. Each method development for its application in large population studies should run in a fast routine and if possible, not be cost intensive. For QA/QC of developed methods, the availability of analytical standards and certified reference materials is crucial. The participation in interlaboratory exercises is important component of external quality control. Despite the fact that the possibility of participating in such tests is more accessible in the last years (often organized within the HBM projects – *described in Chapter 2*), the spectrum of substances/matrices does not often cover the whole scope of contaminants in the developed methods.

The most common matrices for the biomonitoring of environmental contaminants are urine and blood (serum), and for the latter case, in particular, lower limits of detection/quantification (LOD/LOQ) cannot always be achieved by increases amount of processed sample because of its limited availability. Therefore, emphasis should be placed on obtaining as much data as possible from a single sample for multiple groups of contaminants. The development of multi-analyte methods, mostly usage of gas chromatography (GC) or liquid chromatography (LC) with (tandem) mass spectrometry ((MS/)MS) is crucial for sufficient sensitivity in many fields of HBM.

4.1 Analysis of blood serum

Nowadays, 1–5 mL of samples are typically needed to achieve sufficient LOQs/LODs, which usually are 0.1-1 ng/g lipids, and extraction and cleanup procedures are simplified or partially automated in well-equipped labs (*reviewed in Covaci et al., 2007; Gao et al., 2016; Lu et al., 2017*). Nevertheless, due to the high lipophilicity of the analytes (e.g., most of BFRs and novel BFRs, PCBs, OCPs), the sample preparation usually still requires multistep purification for lipid and other interferences removal. The isolation of target analytes is typically based on liquid-liquid extraction (LLE; nonpolar solvents or their mixtures: hexane, dichloromethane and diethylether) or solid phase extraction (SPE; sorbents: e.g., C18 and Oasis Hydrophilic Lipophilic Balance (HLB)). If needed additional cleanup steps such gel permeation chromatography, acidic treatment, passing through multilayer silicagel, alumina, or Florisil, etc. are used (*Covaci et al., 2007; Gao et al., 2016; Lu et al., 2017; Pirard & Charlier 2018*). All these fastidious steps could be solvent and time consuming and increase the risk of external contamination of the sample (*Gao et al., 2016*). For the isolation of more polar PFAS from blood serum, mostly ion-pair extraction combined with tetra butyl ammonium hydrogen sulfate and methyl tert-butyl ether (MTBE), LLE (high organic polar solvent methanol or acetonitrile) or (on-line) SPE are commonly employed in the biomonitoring studies (*Nakayama et al., 2019; Almin et al., 2020*).

Achievement of the study – POPs in blood serum (Appendix I)

- Until now, various procedures have been available for the determination of a single group of POPs in serum. The study describes the development and validation of the method for the simultaneous analysis of 8 PCBs, 11 OCPs, 40 FRs, and 19 PFAS in human serum samples.
- Within the first step of this method nonpolar compounds were isolated by a three-step solvent extraction with *n*-hexane:diethylether (9:1, *v/v*) mixture, followed by the purification step using a Florisil® column. In the second step, the rest of the sample was further extracted by the modified QuEChERS-like extraction, when acetonitrile was used for the isolation of more polar compounds and then the separation of organic and aqueous layers was induced by the addition of inorganic salts.
- Depending on the polarity and volatility of the target compounds, either GC–MS(/MS), or ultra-high performance liquid chromatography (UHPLC) coupled to MS/MS, was employed for their final detection/quantification.
- The recoveries of compounds measured by GC–MS/MS at two different concentration levels ranged from 70 to 115% (repeatability of measurement expressed as RSD <18%) for FRs, 74 to 120% (RSD <20%) for OCPs and PCBs, with relevant LOQs reaching 0.05–2.5 ng/g lipid weight (lw). The recoveries of LC–MS/MS amenable analytes were in the range of 79–120% (RSD <20%) for PFAS and hydroxylated metabolites of polybrominated diphenyl ethers (OH-BDEs) 71–116% (RSD <19%) for HBCDs, TBBPA and brominated phenols and LOQs ranged from 0.01 to 0.3 ng/mL.
- The entire analytical method was also successfully verified on the SRM 1958 (Standard reference material, Organic Contaminants in Fortified Human Serum).
- Within human biomonitoring, the developed method contributes to the assessment of exposure to a broad range of 78 organohalogenated contaminants representing various classes.
- Worth to notice, that this method was further successfully validated for the analysis of SCCPs and MCCPs and was apply to the investigation of these contaminants not only in the Czech population, but in the whole Europe for the first time (*Tomasko et al., 2021*).

For instrumental analysis of POPs, GC–MS based techniques are most commonly used to detect organochlorinated POPs (electron ionization, EI) and PBDEs together with NBFRs (mostly in negative chemical ionization, NCI). Initially, total HBCD content was analyzed by GC–MS, together with the PBDEs. However, the interconversion of HBCD isomer occurring in the inlet system and in the MS source due to their high temperatures may give rise to misleading results. Therefore, reversed-phase LC–MS/MS is the preferred choice for these compounds, especially for the analysis of specific stereoisomers (α-, β- and γ-HBCDs) (*Xu et al., 2013*).

Ultra high performance liquid chromatography coupled to quadrupole tandem mass spectrometry applying negative electrospray ionization interface (UHPLC–ESI(-)–MS/MS) is the preferred instrumental method for the determination of ionic PFAS such as PFSAs and PFCAs. HRMS has been already proven as a powerful tool for the discovery and characterization of PFAS in environmental and human samples (*Liu et al., 2019*). The determination of neutral, volatile PFAS precursors such as FTOHs, perfluorooctane sulfonamides (FOSAs) and perfluorooctane sulfonamide ethanol (FOSEs) is usually done by GC–MS either

in combination with EI or chemical ionization (CI), nevertheless their LC–MS analysis is also reported (*Jahnke & Berger 2009; Almin 2020*).

4.2 Analysis of breast milk

Breast milk is a very commonly used matrix in human biomonitoring as its measurements give information concerning the exposure levels of both the mother and her child. Breast milk is usually employed for monitoring lipophilic chemicals due to its high fat content in which they accumulate. Besides lipophilic POPs (e.g., BFRs represented by HBCD and TBBPA), also PFAS are commonly measured in this matrix. Nevertheless, PFAS concentrations in milk are usually considerably lower than in human serum. As mentioned before, the analytical methods are rather uniform for all PFAS and include protein precipitation (e.g., addition of formic acid and acetonitrile), clean-up of supernatant (mostly based on SPE or dispersive SPE (d-SPE)) and LC–MS/MS analysis (*Macheka-Tendenguwo et al., 2018*). As reviewed by *Shi et al. (2013),* various extraction procedures have been published for the determination of HBCD and TBBPA in breast milk, e.g., Soxhlet extraction of freeze-dried samples or more recently accelerated solvent extraction. Due to the relatively low selectivity of the extraction techniques and the complexity of the matrix of biological samples, it is necessary to include a purification step in the analytical procedure, specifically gel permeation chromatography (GPC), sulfuric acid or acidified silica treatment for lipids removal.

Achievement of the study: PFAS & BFRs in breast milk (Appendix II)

- A new analytical approach for the simultaneous determination of 18 PFAS and 11 BFRs including HBCDs isomers, TBBPA, brominated phenols, and OH-BDEs has been developed and validated for breast milk and infant formula.
- The sample preparation procedure was based on extraction with acetonitrile and subsequent purification of a crude acetonitrile extract (obtained by partition induced by added inorganic salts) with d-SPE using a C18 sorbent.
- Good performance characteristics were achieved for all target analytes in both matrices, the method recoveries ranged from 80 to 117% with RSDs < 23% for all analytes and LOQs were in the range from 0.003–0.2 ng/mL for milk and 0.005–0.45 pg/g for infant formula.
- The benefits of the method are a rapid and simple sample extraction (ten samples/hour) with minimal solvent consumption and practically no need of expensive laboratory equipment.
- This was the very first study enabling simultaneous analysis of PFAS and BFRs including several metabolites in human milk and infant formulas, such analytical strategy has never been published before.

4.3 Analysis of urine

Urine is probably the second most common matrix for human biomonitoring, particularly for water-soluble chemicals. Generally, it is not a useful matrix for monitoring POPs. The creatinine values are commonly used to normalize the urine concentration/dilution in individual samples, thus ensuring improved data comparability. Commonly, various heavy metals, metabolites of PAHs and phthalates were measured in urine (*Esteban and Castaño, 2009; Vorkamp et al., 2021*).

Phthalates are best monitored as their monoester metabolites, in oxidized form or without oxidative modification. Uptake of phthalates is followed by their cleavage into monoesters, oxidation in phase I reactions, conjugation of this secondary metabolite with glucuronic acid in a phase II reaction and excretion in urine (*Koch & Calafat, 2009*). In the analysis, the conjugates are cleaved, and the released (oxidized) monoester is a suitable biomarker. In general, simple monoesters (without oxidative modification) will be the biomarkers of choice for LMW phthalates, such as DEP and DBP, whereas oxidized secondary metabolites are the biomarkers of choice for HMW phthalates, such as DEHP, DINP or DIDP (*Koch et al., 2003, Koch & Calafat, 2009*). This shift in target biomarkers (from simple to oxidized monoester metabolites) is mainly related to a lower water solubility of the monoesters with longer alkyl chains, which needs to be further increased in phase I reactions (*Koch and Calafat, 2009*). In addition, the oxidized metabolites are not prone to external contamination (*Wittassek et al., 2011*). Otherwise, the risk of external contamination during sampling and sample preparation is high, due to the wide use of phthalates as plasticizers, and needs to be considered in sampling and QA/QC strategies (*Koch & Calafat, 2009*). For some phthalates, such as DINP and DIDP, several isomers exist. Their quantification is typically based on analytical standards of single isomers, whereas all isomeric peaks are integrated in the urine sample. Having an isomeric composition of the alkyl side chain similar to DINP, the analysis of DINCH, simple monoester mono-isononyl-cyclohexane-1,2-dicarboxylate (MINCH) and its secondary oxidized metabolites cyclohexane-1,2-dicarboxylic mono hydroxyisononyl ester (OH-MINCH), cyclohexane-1, 2-dicarboxylic mono oxoisononyl ester (oxo-MINCH) and cyclohexane-1,2-diarboxylic mono carboxyisononyl ester (cx-MINCH) is comparable to that of DINP (*Koch et al., 2013*). Quantification should integrate overall isomers with the respective oxidative modifications, and the standards should be derived from the major alkyl chain isomer.

PAHs undergo oxidation and conjugation in phase I and phase II reactions in liver, similarly to the metabolism reactions described for phthalates. OH-PAH conjugates with two–three benzene rings (LMW OH-PAHs) are mostly excreted via urine and those with four or more benzene rings are mainly excreted via bile and faeces (HMW OH-PAHs) (*Onyemauwa et al., 2009, Zhong et al., 2011*). In older studies, roughly before 2007, recent exposure to PAHs was often determined by the presence of 1-hydroxypyrene (1-OHP) (*Esteban and Castaño, 2009*). Although 1-OHP is a metabolite of non-carcinogenic pyrene, it was considered a suitable surrogate marker of PAH exposure because this exposure frequently occurs with a mixture of PAHs where pyrene is present. On the other hand, PAHs compositional pattern in the environment and consequently in organisms varies largely depending on many factors. To obtain more accurate data, monitoring more than one OH-PAH marker, specifically OH-PAHs with two and three benzene rings, is needed.

Generally, the first step in sample preparation for both groups is an enzymatic hydrolysis of conjugated forms, using β-glucuronidase. Deconjugation of phthalate metabolites is best achieved by enzymatic hydrolysis with arylsulfatase-free *E.coli K12* β-glucuronidase. Some glucuronidase enzymes might hydrolyze phthalate ester bonds in the parent phthalates due to arylsulfatase or esterase side activities, which has to be avoided (*Feng et al., 2015, Koch et al., 2018*). Thus, the use of *Helix pomatia* β-glucuronidase, which is commonly used in for the hydrolysis of OH-PAHs conjugates, might lead to false positives when monoester metabolites are analyzed. As recently summarized by *Vorkamp et al. (2021)* for

the isolation of phthalate metabolites and OH-PAHs, mostly (online) SPE is most commonly applied, with the sorbent typically being silica C18 or HLB sorbent. Polar solvents such as water, methanol, acetonitrile, or their mixtures are then used for elution.

While high-performance liquid chromatography (HPLC) in combination with fluorescence detection is still applied in the analysis of PAH metabolites (*Yamamoto et al., 2015*), the current method of choice providing the highest sensitivity and selectivity is LC–MS/MS with electrospray ionization in negative mode. GC–based methods can offer comparable sensitivity, but require an additional derivatization step (*Li et al., 2014*). The analysis of the phthalate and DINCH metabolites is best performed by LC–MS/MS, offering sufficient sensitivity for biomonitoring of the general population (*Vorkamp et al., 2021*).

Achievement of the study: Metabolites of phthalates & DINCH in urine (Appendix III)

- In this study, a quick and easy sample preparation procedure for the simultaneous determination of eight phthalate and four DINCH metabolites in urine was implemented and validated.
- Sample preparation procedure was based on the enzymatic hydrolysis and dilution with methanol and for the instrumental analysis commonly used technique for these analytes, reversed phase UHPLC–MS/MS, was used.
- LOQs of the method for phthalate metabolites ranged from 0.15 to 0.4 ng/mL urine with recovery obtained by the analysis of SRM 3676 (urine sample of a non-smoker) in the range of 70–126% and repeatability in the range of 3–8%. Since DINCH metabolites were not certified in this material, the method validation was performed using artificial contamination (spiking) of a blank urine sample (concentration levels 0.7 and 7 ng/mL), which resulted in recoveries in the range of 60-104% with RSD < 11%.
- In follow-up experiments the originally developed method was successfully validated for ten additional phthalate metabolites and applied on the analysis of 315 paired urine samples collected from mothers and their newborn children living in the Czech Republic in 2016–2017 (*Urbancova et al., 2021*).

Achievement of the study: OH-PAHs in urine (Appendix IV)

- A novel analytical approach for the determination of 11 OH-PAHs in urine was developed and validated.
- The rapid, simple, and high-throughput sample preparation procedure based on ethyl acetate extraction and subsequent purification by d-SPE employing a Z-Sep sorbent was used for the first time in the analysis of PAH metabolites in biological matrices such as urine and substitutes demanding and time-consuming on SPE cartridges and thus significantly streamlines the analysis of urine in HBM studies.
- For the identification/quantification of target compounds, UHPLC–MS/MS was applied. The chromatographic separation of isomers of hydroxyphenanthrens was achieved using the pentafluorophenyl-bonded silica stationary phase.

- Great performance characteristics were obtained during the validation experiments with the SRM 3673 (urine of a non-smoker), and the method recoveries ranged from 77 to 114% with RSDs < 20% and LOQs in the range of 0.010–0.025 ng/mL (except for 3-OH-BaP with 0.9 ng/mL).

In the paragraphs below, analytical approaches for the determination of various organohalogenated POPs and metabolites of phthalates, DINCH and PAHs were used for the analysis of real samples obtained within the human biomonitoring studies in cooperation with the National Institute of Public Health, Prague; the Institute of Experimental Medicine, CAS; the Czech Academy of Science; and Slovak Medical University, Faculty of Public Health, Department of Environmental Medicine, Bratislava, Slovakia.

Worth to notice, that most of developed methods have been verified within the participation in four rounds of ICI/EQUAS exercises under HBM4EU project during 2018-2020. Successful Z-score values have been obtained at least two ICI/EQUAS rounds, thus above described methods were qualified for the analysis of 10 halogenated FRs and 12 PFAS in blood serum; and 9 OH-PAHs, 2 DINCH and 14 phthalate metabolites in urine. After method approval, a large sets of biological samples have been analyzed for project partners:

- *Santé publique France, Occupational and Environmental Health Division (analysis of halogenated FRs in blood serum)*
- *State General Laboratory, Ministry of Health, Cyprus (analysis of OH-PAHs in urine)*
- *National Institute of Health Dr. Ricardo Jorge, Portugal (analysis of OH-PAHs in urine)*
- *Nofer Institute of Occupational Medicine, Poland (analysis of phthalates and DINCH metabolites in urine)*
- *Cyprus State General Laboratory, Ministry of Health (analysis of phthalates and DINCH metabolites in urine)*

5 HUMAN BIOMONITORING STUDIES

5.1 Occurrence of POPs in blood serum

In total, three extensive studies have been realized and published on the biomonitoring of various POP groups: (i) biomonitoring of POPs in blood serum of Czech city policemen, (ii) analysis of POPs in blood serum of Czech general population and (iii) investigation of PFAS in mother-child cohort in the Slovak Republic. The obtained data contributed to the knowledge regarding the assessment of direct human body burden to possible harmful POPs. In the following bullet points, the major outcomes of these studies are summarized:

Achievement of the study (i) - Biomonitoring of POPs in blood serum of Czech city policemen (Appendix VII)

- The presented research was conducted in the cooperation with the Institute of Experimental Medicine, CAS in the frame of the project "Healthy Aging in Industrial Environment" (HAIE), which evaluates the effects of selected environmental and lifestyle risk factors on the health and aging of the population in the industrial region.
- A total of 274 serum samples obtained from policemen representing three Czech cities (Prague, Ostrava, and Ceske Budejovice) in spring and autumn 2019 was investigated. It should be noted that this is the first study performed on such a unique population group.
- The target spectrum of analytes included 33 BFRs, 7 novel FRs, 11 OCPs, 8 PCBs, and 30 PFAS.
- The most frequently detected pollutants were PFOA, PFNA, perfluorodecanoic acid (PFDA), PFOS, PFHxS, CB 138, CB 153, CB 170, CB 180, HCB and *p,p'*-dichlorodiphenyldichloroethylene (*p,p´*-DDE) quantified in 100% of samples. Concentrations of determined POPs were in the range of 0.108-900 ng/g lw for PCBs, 0.106-1 016 ng/g lw for OCPs, <0.01-18.3 ng/mL for PFAS and <0.1-618 ng/g lw for FRs, which were the minor contaminants, respectively.
- When comparing the concentrations of the main representatives of PCBs and OCPs in serum samples received from 2005 to 2019 (current study), a decreasing trend for these serum pollutants was confirmed.
- Concentrations of PFOS and PFOA are the lowest in comparison with the other countries in Europe (these contaminants have never been produced in the Czech Republic). Compared to studies from USA and China, results were approximately seven times lower for PFOS and sixteen times lower for PFOA.
- The most abundant representatives of FRs were BDE-47, BDE-209, and anti-DP. The congener BDE-209 was commonly detected in the highest levels in serum, but its concentration were lower compared to the European and Chinese studies.
- The data were also statistically processed and the results show statistically significant associations between selected POP concentrations and the sampling season, locality, and age of serum donors.

Achievement of the study (ii) - Analysis of POPs in blood serum of Czech general population (Appendix I)

- Within this survey, a total of 38 samples of human serum was obtained within cooperation with The National Institute of Public Health (Prague, Czech Republic) in 2015.
- The target spectrum of analytes included 40 BFRs, 7 novel FRs, 11 OCPs, 8 PCBs, and 19 PFAS.
- The most abundant contaminants were PFOA, PFNA, PFDA, perfluoroundecanoic acid (PFUndA), branched-PFOS (Br-PFOS), linear-PFOS (L-PFOS), HCB and *p,p′*-DDE found in all samples, followed by CB 138, CB 153 and CB 180; CB 170, α-hexachlorocyclohexan (α-HCH) and 1,1-bis(4-chlorophenyl)-2,2,2-trichloroethane (*p,p′*-DDT); CB 28; *o,p′*-dichlorodiphenyldichloroethane (*o,p′*-DDD); γ-HCH; β-HCH; *o,p′*-dichlorodiphenyldichloroethylene (*o,p′*-DDE); CB 118; *p,p′*-dichlorodiphenyldichloroethane (*p,p′*-DDD) and perfluoroheptanoic acid (PFHpA) quantified in > 50% of samples.
- The amounts of determined PFAS, which were the dominating group of contaminants, were in the range < 0.01–8.97 ng/mL (mean 0.631 ng/mL), followed by OCPs and PCBs ranging from < 0.1–1 626 ng/g lw (mean 40.0 ng/g lw) and < 0.1–481 ng/g lw (mean 63.3 ng/g lw), respectively. BFRs belonged to minor analytes found in serum.

Achievement of the study (iii) - Investigation of PFAS in mother-child cohort in the Slovak Republic (Appendix VIII)

- This study describes the determinants of prenatal exposure to PFAS in Slovakia, based on Slovak multicentric prospective mother-child cohort PRENATAL (project of Ministry of Health of the Slovak Republic; n = 796). Cord blood samples were collected within 2010–2012 and PFAS were analyzed in a subpopulation of 322 newborns.
- The highest concentration (geometric mean) in cord blood was observed for PFOA (0.79 ng/mL), followed by PFOS (0.36 ng/mL), PFNA (0.20 ng/mL) and PFHxS (0.07 ng/mL).
- Concentrations of all four target analytes (PFHxS, PFOS, PFOA, PFNA) to be highly correlated between PFAS cord blood concentration, with the strongest correlation between PFOA and PFNA ($r_s = 0.81$, $p < 0.001$).
- Prenatal PFASs concentrations vary across Europe due to a several factors (e.g., dietary exposure of mother during pregnancy, different time period when samples were taken, e.g., samples collected before and after implementation of restriction on PFOS production and usage in 2009). In general, PFASs cord blood concentrations in this study were lower compared to concentrations measured in Sweden and Spain but comparable with those in Norway or France.
- Parity was identified as the main determinant of PFAS exposure in Slovak population (multiparous women had significantly lower cord blood concentrations of all measured PFASs compared to primaparous).
- Higher age of mother was associated with higher levels of PFNA and PFHxS, interestingly, higher educational level was associated with higher PFNA levels and higher fish consumption with lower PFNA levels.

5.2 Occurrence of PFAS and BFRs in human breast milk

Human milk represents a unique food source for newborns at least in the first six months of life, as recommended by the WHO (*WHO, 2019*). However, as mentioned above, number of contaminants entering mother´s body is transferred into breast milk. Thus, the knowledge of contaminants occurrence in milk is crucial for the exposure estimation for breastfed infants, because it represents for most of them the key postnatal exposure pathway. It is well known that exposure to toxic contaminants in early life may impose greater health impacts than later in life (*Sly et al., 2016*). On the other hand, breastfeeding is still generally considered the optimal form of nutrition for infants (*Lyons et al., 2020*).Two studies have been conducted and published for the determination of selected pollutants in human breast milk of Czech women cohort: (i) biomonitoring of a broad spectrum of PFAS and estimation of infant's daily intake (DI) and (ii) analysis of PFAS, BFRs and their metabolites (pilot study). In the following bullet points, the major conclusions of these studies are mentioned:

Achievement of the study (i) - Biomonitoring of broad spectrum of PFAS and estimation of infant's daily intake (Appendix IX)

- PFAS were investigated in more than 600 human breast milk samples collected in the period 2006 through 2017 within the Czech human biomonitoring program (CZ-HBM) organized by The National Institute of Public Health (NIPH, Prague, Czech Republic).
- Five various sampling localities were selected to represent large agglomerations (Prague), industrial cities (Ostrava), smaller towns (Liberec), and rather rural municipalities (Žďár nad Sázavou and Uherské Hradiště).
- Only PFOS and PFOA (in 2017, also PFNA) were quantified in more than 90% of analyzed samples. In all sampling periods, the levels of PFOA were higher than those of PFOS (i.e., 1.2–1.9-fold higher depending on the sampling year).
- A significant downward temporal trend ($p < 0.001$) was observed for both PFOA and PFOS levels. The median concentrations in sampling years 2006, 2010/11, 2014, and 2017 were 0.075, 0.059, 0.035, and 0.023 ng/mL for PFOA and 0.045, 0.031, 0.029, and 0.020 ng/mL for PFOS, respectively.
- In 2017, PFNA was also quantified in 99% of samples with the median concentration of 0.007 ng/mL.
- The levels of PFAS correlated with maternal sea fish consumption.
- To quantify health risks from the exposure to PFOS and PFOA, the hazard quotient (HQ; a ratio between DI of PFOS/PFOA and TDI or Provisional Tolerably Weekly Intake (PTWI)) and hazard index (HI; sum of HQs) were calculated. Using TDI values for PFOS and PFOA established in 2008 (*EFSA, 2008*), the calculated DIs from breastfeeding were clearly below these limits. Using the new, more conservative PTWI values set in 2018 (*EFSA, 2018*), a considerable exceedance of PTWI was demonstrated with HQ and HI being substantially higher than 1 (median HI was 15.2 12.1, 8.09 and 5.33 in samples from 2006, 2010/11, 2014, and 2017, respectively).

Achievement of the study (ii) - Analysis of PFAS, BFRs and their metabolites (pilot study) (Appendix II)

- Altogether, 50 breast milk samples were obtained from mothers living in the Olomouc region in 2010.
- The target spectrum of analytes included 18 PFAS and 11 BFRs including 4 OH-BDEs, and it was measured in Czech women for the first time.
- The total concentration of PFAS ranged from 0.04 to 0.28 ng/mL (median 0.11 ng/mL). The most abundant substances were PFOA (median 0.04 ng/mL) and PFOS (median 0.03 ng/mL), which were detected in 100% of samples.
- Regarding BFRs, only TBBPA and α-HBCD were quantified in samples. TBBPA was found in 30 % of samples at a wide concentration range, <0.06–16.2 ng/mL (<2 to 688 ng/g lw), and α-HBCD was detected in 28% of samples at concentrations ranging from <0.030 to 16.6 ng/mL (<1 to 76 ng/g lw).

5.3 Occurrence of OH-PAHs, metabolites of phthalates and DINCH in urine

Three studies have been realized and summarized in publications for the biomonitoring of metabolites of emerging plasticizers (phthalates and DINCH) in the urine of Czech mothers and their newborns: (i) OH-PAHs in samples from Ceske Budejovice and Most, (ii) occurrence of OH-PAHs in samples from Ceske Budejovice and Karvina, followed by and (iii) determination of metabolites of phthalates and DINCH in urine obtained in Ceske Budejovice and Karvina. For surveys (i) and (ii), the determination of PAHs was realized to demonstrate the correlation between concentration of these pollutants in the ambient and their monohydroxylated metabolites measured in urine. In the bullet points, the major outcomes of these studies are mentioned:

Achievement of the study (ii) - OH-PAHs in samples from Ceske Budejovice and Most (Appendix X)

- The urine collection in (330 samples from mothers and 330 samples from their newborns, from July 2016 to August 2017) was carried out within the project funded by the Czech Academy of Sciences, Strategy AV21, Qualitas and EU Horizon 2020 HBM4EU.
- From 11 analyzed OH-PAHs, 2-OH-NAP was found in all measured samples at the highest concentrations in both mothers' and newborns' urine samples (median 5 150 and 3 580 ng/g creatinine, respectively); the results are in accordance with similar published studies worldwide.
- The profiles of other target OH-PAHs are variable between various countries/regions. This result indicates that people here are exposed to different mixtures of PAHs present in the air, diet and other sources.
- The median concentration of ΣOH-PAHs in all urine samples was approximately 1.7 times higher in mothers´ urine (median 8 960 ng/g creatinine) compared to their newborns (median 5 150 ng/g creatinine), while the amount of OH-PAHs from Most locality was almost 2 times higher compared

to the samples from Ceske Budejovice (median ΣOH-PAHs 9 280 ng/g creatinine and 4 920 ng/g creatinine, respectively).

- The results might indicate that the population in the previously highly air-polluted mining districts carries some long-term changes (maybe existing changes in genetic information), which also affect the metabolism of PAHs. It could be related to the long-lasting effect, and thus corresponding to the shortened life expectancy.

Achievement of the study (i) - OH-PAHs in samples from Ceske Budejovice and Karvina (Appendix XI)

- This study was realized under the national project "The impact of air pollution on the genome of newborns" (GA CR No. 13-13458S). A total of 265 samples from mothers and 266 samples from their newborn children was collected in two regions (Ceske Budejovice – control locality and Karvina – region with high atmospheric pollution because of heavy industry) in two rounds namely in August–September 2013 (summer period) representing the less air-polluted season for PAHs and in January–April 2014 (winter period) as environment is typically the more contaminated by these pollutants due to emissions associated with fossil fuels heating.
- From 11 targeted PAH biomarkers, 2-OH-NAP was the most abundant compound present in all samples of urine with the highest concentration (median 5 400 for mothers and 3 100 ng/g creatinine for newborns, respectively).
- The median concentration of ΣOH-PAHs in the children´s urine was 1.6 times lower compared to the respective mother which may correlates with higher inhalation and dietary intake of PAHs by mothers. Prenatal exposure via transfer of OH-PAHs metabolites from exposed mother need to be considered as well.
- ΣOH-PAHs concentrations determined in mothers' urine collected in the summer were comparable in both localities.
- No significant increase of ΣOH-PAHs amounts occurred in samples from Ceske Budejovice in winter, while in samples from the Karvina region a statistically significant difference ($\alpha = 0.05$) was observed. The median concentrations of ΣOH-PAHs in mothers' urine samples in the winter were 1.5 times higher than those measured in samples collected in the same locality in summer.
- The amounts of ΣOH-PAHs in newborns' urine from Karvina in the winter season were 1.5 times higher than in the summer collected in the same locality and 3.3 times higher when compared with the less polluted locality of Ceske Budejovice. This was probably due to the smog situation associated with emissions from heavy industry and local heating.

Achievement of the study (iii) - Metabolites of phthalates and DINCH in urine from Ceske Budejovice and Karvina (Appendix III).

- Within this study, total of 204 urine samples (102 samples from mothers and 102 samples from their newborns) were collected in Ceske Budejovice and Karvina in years 2013-2014.
- For the assessment of human exposure to hazardous plasticizers, concentrations of eight phthalate (monoethyl phthalate (MEP), mono-iso-butyl phthalate (MiBP), mono-n-butyl phthalate (MBP),

monobenzyl phthalate (MBzP), mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (OH-MEHP), mono(2-ethyl- 5-oxohexyl) phthalate (oxo-MEHP) and mono(2-ethyl-5-carboxypentyl) phthalate (cx-MEHP)) and four DINCH metabolites were investigated.

- The most contaminated samples were collected from children born in the Karvina industrial region, where the median concentration of phthalate metabolites was approx. 2 times higher compared to their mothers. The levels of phthalate metabolites found in the urine obtained from mothers and their newborn children in Ceske Budejovice.
- Metabolite cx-MEHP contributed by 43% to the overall contamination in newborns' urine samples from Karvina. The source of its parent (non-metabolized) compound (DEHP) will be investigated in a subsequent part of this study.
- Regarding DINCH metabolites, MINCH was not detected in any of samples. The other DINCH metabolites were found in only 7% of samples from mothers and in 1% of samples from children. Their median concentrations were very low (0.8–8.8 ng/mL urine), similarly to these biomarkers levels reported in other published papers.
- In most of the Czech samples, MBP was found at the highest concentration, while in other worldwide studies, MEP was typically the dominating metabolite. This result could be caused by the different exposure sources of phthalates in the Czech Republic and other countries and should be further investigated.

6 CONCLUSIONS

This Habilitation Thesis reflects the current requirements on ultratrace analysis of organic pollutants in HBM studies. The developed methods allows a more accurate assessment of the exposure and burden of Czech population which is crucial for the control and prevention of risks posed by these chemicals.

The first part is focused on the characterization of HBM including relevant biological matrices, which are analyzed in biomonitoring studies. In following part the main class of environmental pollutants are described. The second part is concerned with the development and validation of sample preparation procedures for various groups of POPs, and metabolites of phthalates, DINCH, and PAHs. Attention is paid to the possibility to integrate various groups of POPs with similar physico-chemical properties into one "multi-analyte" method or to simplify common procedures for the analysis of one group of contaminants, in particular of their metabolites. These approaches lead to reducing analysis time resulting in higher laboratory throughput and cost efficiency. In addition, the important outcome is a lowered need of the amount of biological materials, availability of which might be in some cases critical. For the identification/quantification of target compounds, both LC–MS and GC–MS techniques were used. The accuracy of most of the validated methods has been demonstrated by the analysis of certified materials and successful participation in ICI/EQUAS exercises within the HBM4EU project.

In the last part the main outcomes obtained within various HBM surveys (in which the Department of Food Analysis and Nutrition has been involved and their results were published in peer review journals) are summarized. These studies focused on the exposure assessment of specific cohorts, mostly mother-newborn, which represent one of the most vulnerable population groups.

The main outcomes of the realized studies for selected groups of contaminants in biological matrices obtained from specified cohorts of Czech population are summarized below:

POPs in blood serum

- In general, chlorinated POPs (mainly HCB, *p,p´*-DDE and highly chlorinated PCB congeners) belong to the frequently detected contaminants at the highest amounts, followed by PFAS (mainly with the length of chain C6-C10), while BFRs belong to the minor compounds.
- Exposure to POPs is highly variable worldwide and even within individual countries. The levels of these compounds are comparable or even lower (especially for PFAS) than those reported in other studies abroad. In spite to a declining trend in the concentrations of organochlorinated compounds was monitored, due to the historical production of PCBs and OCPs (DDTs and HCB) in the former Czechoslovakia, thus high population burden, the current levels are mostly higher than in similar studies.

PFAS in milk

- Significant time-related decreasing trends in the PFOS and PFOA levels in human milk between 2006 and 2017 were observed, probably due to a long term regulation of these compounds by authorities worldwide, especially in the frame of Stockholm Convention in 2009.

- Alike in case of other pollutants, infants´ exposure to PFAS through breast milk may pose some health risks, however, the benefits of breastfeeding should be always balanced against conceivable adverse effects resulting from body burden.

Metabolites of OH-PAHs in urine

- Higher amount of OH-PAHs was found in urine samples collected from population at highly industrialized areas in winter period, probably due to the smog situation associated with emissions from heavy industry (e.g. coke and steel production) and local heating.
- Amount of OH-PAHs did not correlate with PAHs measured in the ambient air, a possible explanation is the exposition of the population to PAHs from other sources, such as diet.
- Generally, the concentration of OH-PAHs in children´s urine is lower compared to their mothers.

Phthalate and DINCH metabolites in urine

- No correlation was found between the levels of target compounds in children's and mothers' urine samples, probably because of different exposure sources of phthalates.
- DINCH metabolites were found only in a low portion of urine samples, concentration on positive samples were comparable to those obtained in similar studies performed in other countries.
- The most often detected compound at the highest concentration was MBP. However, in similar worldwide studies, regarding the determined levels, MEP is commonly dominating. The explanation of this difference is needed and the exposure sources should be further investigated.

6.1 Plans and ideas for future experimental work

The future experimental work will be focused on the activities related to the PARC project, which is currently under development. Its beginning is planned in spring 2022 and will be held for seven years. Generally, PARC is a joint research and innovation programme to strengthen the scientific basis for chemical risk assessment in the EU. The plan is to bring chemical risk assessors and managers together with scientists to accelerate method development and the production of necessary data and knowledge, and to facilitate the transition to next-generation evidence-based risk assessment, a nontoxic environment and in line with "The European Green Deal" (*European Commission, 2019*). Based on my experiences obtained within previous Ph.D. studies and later during project HBM4EU, I would like to contribute to the activities in human biomonitoring, environmental and multisource monitoring, employing innovative methods and tools for monitoring and surveys. In this context, analysis of other emerging contaminants (e.g., UV filters, bisphenols, OPFRs) and their metabolites in both biological and environmental matrices will be subject of research focus.

Further research activities will be also concerned with the monitoring of PFAS groups, for which within the re-evaluation process the EFSA reduced the tolerable intake limit for certain representatives, as the knowledge on their harmful effects on human health is continuously expanding. The main route of human exposure is via diet including drinking water. Although in the latter case, PFAS are typically contained ultratrace levels, the daily intake makes drinking water worth of attention. The new European Union Directive 2020/2184 on the quality of water intended for human consumption from December 2020 defines two parameters for monitoring: 'sum of PFAS' (sum of ten PFCAs, and ten PFSAs, C4-13 limited up

to 0.1 μg/L) and 'PFAS total' with limit up to 0.5 μg/L (*Directive EU 2020/2184*). In addition, new EU legislation setting maximum levels of PFAS representatives for food (eggs, meat, crustaceans and bivalves, game, offal) can be expected in a close future. For the control purposes purpose, the development of new methods for the analysis of challenging matrices (e.g., baby food, fatty foods) meeting the QA/QC criteria including low LOQs will be urgently needed.

Needed to emphasize, that interdisciplinary cooperation with other research group, both at national and international level, will be further developed. At the same time, the contact and joint projects with other stakeholders concerned with mitigation of environmental pollution and food chain production will be strengthened.

7 REFERENCES

Abunada, Z., Alazaiza, M. Y., & Bashir, M. J. (2020). An Overview of Per-and Polyfluoroalkyl Substances (PFAS) in the Environment: Source, Fate, Risk and Regulations. *Water*, 12(12), 3590.

Al Amin, M., Sobhani, Z., Liu, Y., Dharmaraja, R., Chadalavada, S., Naidu, R., Chalker, J. M., & Fang, C. (2020). Recent advances in the analysis of per-and polyfluoroalkyl substances (PFAS)—A review. *Environmental Technology & Innovation*, 19, 100879.

Alaee, M., Arias, P., Sjödin, A., & Bergman, Å. (2003). An overview of commercially used brominated flame retardants, their applications, their use patterns in different countries/regions and possible modes of release. *Environment International*, 29(6), 683-689.

Beard, A., & Angeler, D. (2010). Flame retardants: chemistry, applications, and environmental impacts. *Handbook of Combustion: Online*, 415-439.

Bui, T. T., Giovanoulis, G., Cousins, A. P., Magnér, J., Cousins, I. T., & de Wit, C. A. (2016). Human exposure, hazard and risk of alternative plasticizers to phthalate esters. *Science of the Total Environmen*t, 541, 451-467.

CDC (2005) Third National Report on Human Exposure to Environmental Chemicals. *Centers for Disease Control and Prevention*, Atlanta, GA.

Choi, J., Aarøe Mørck, T., Polcher, A., Knudsen, L. E., & Joas, A. (2015). Review of the state of the art of human biomonitoring for chemical substances and its application to human exposure assessment for food safety. *EFSA Supporting Publications*, 12(2), 724E.

Commission Regulation (EC) 1881/2006 (2006) Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Off. J. Eur. Union L. 364:5–24

Commission Regulation (EC) 333/2007 (2007) Commission Regulation (EC) No 333/2007of 28 March 2007 laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs. Off. J. Eur. Union L. 88:29–38

Commission Regulation (EU) No 835/2011 of 19 August 2011 amending Regulation (EC) No 1881/2006 as regards maximum levels for polycyclic aromatic hydrocarbons in foodstuffs Text with EEA relevance.

Commission Regulation (EU) No. 1259/2011 of 2 December 2011 emending Regulation (EC) No 1881/2006 as regards maximum levels for dioxins, dioxin-like PCBs and non dioxin-like PCBs in foodstuffs. 2011, *Official Journal*, L-320:18-23.

Covaci, A., Harrad, S., Abdallah, M. A. E., Ali, N., Law, R. J., Herzke, D., & de Wit, C. A. (2011). Novel brominated flame retardants: a review of their analysis, environmental fate and behaviour. *Environment International*, 37(2), 532-556.

Covaci, A., Voorspoels, S., Ramos, L., Neels, H., & Blust, R. (2007). Recent developments in the analysis of brominated flame retardants and brominated natural compounds. *Journal of chromatography A*, 1153(1-2), 145-171.

Černá, M., Puklová, V., Hanzlíková, L., Sochorová, L., & Kubínová, R. (2017). 25 years of HBM in the Czech Republic. *International Journal of Hygiene and Environmental Health*, 220(2), 3-5.

Du, J., Li, H., Xu, S., Zhou, Q., Jin, M., & Tang, J. (2019). A review of organophosphorus flame retardants (OPFRs): occurrence, bioaccumulation, toxicity, and organism exposure. Environmental Science and Pollution Research, 26(22), 22126-22136.

Dulio, V., Koschorreck, J., Van Bavel, B., Van den Brink, P., Hollender, J., Munthe, J., Schlabach, M., Aalizadeh, R., Agerstrand, M., Ahrens, L., Allan, I., Alygizakis, N., Barceló, D., Bohlin-Nizzetto, P., Boutroup, S., Brack, W., Bressy., A., Christensen, J. H., Cirka, L., Covaci, A., Derksen, A., Deviller, G., Dingemans, M. M. L., Engwall, M., Fatta-Kassions, D., Gago-Ferrero, P., Hernández, F., Herzke, D., Hilscherová, K., Hollert, H., Junghans, M., Kasprzyl-Hordern, B., Keiter, S., Kools, S. A. E., Kruve, A., Lambropoulou, D., Lamoree, M., Leonards, P., Lopez., B., López de Alda, M., Lundy, L., Makovinská, J., Mrigómez, I., Martin, J. W., McHugh, B., Miége, C., O´Toole, S., Perkola, M., Polesello, S., Posthuma, L., Rodriguez-Mozaz, S., Roessink, I., Rostkowski, P., Ruedel, H., Samanipour, S., Schulze, T., Schymanski, E. L., Sengl, M., Tarábek, P., Ten Hulscher, D., Thomaidis, N., Togola, A., Valsecchi, S., van Leeuwen, S., von der Ohe, P., Vorkamp, K., Vrana, B., & Slobodnik, J. (2020). The NORMAN

Association and the European Partnership for Chemicals Risk Assessment (PARC): let's cooperate!. *Environmental Sciences Europe*, 32(1), 1-11.

EFSA Panel on Contaminants in the Food Chain, Knutsen, H. K., Alexander, J., Barregård, L., Bignami, M., Brüschweiler, B., Ceccatelli, S., Cottrill, B., Dinovi, M., Edler, L., Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L., Nebbia, C. S., Oswald, I. P., Petersen, A., Rose, M., Roudot, A.-C., Vleminckx, C., Vollmer, G., Wallace, H., Bodin, L., Cravedi, J.-P., Halldorsson, T. I., Haug, L. S., Johansson, N., van Loveren, H., Gergelova, P., Mackay, K., Levorato, S., van Manen, M., & Schwerdtle, T. Risk to human health related to the presence of perfluorooctane sulfonic acid and perfluorooctanoic acid in food. *EFSA Journal* 2018, 16 (12), e05194.

EFSA Panel on Contaminants in the Food Chain, Schrenk, D., Bignami, M., Bodin, L., Chipman, J. K., del Mazo, J., Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L., Leblanc, J.-C., Nebbia, C. S., Nielsen, E., Ntzani, E., Petersen, A., Sand, S., Vleminckx, C., Wallace, H., Barregård, L., Ceccatelli, S., Cravedi, J.-P., Halldorsson, T. I., Haug, L. S., Johansson, N., Knutsen, H. K., Rose, M., Roudot, A.-C., Van Loveren, H., Vollmer, G., Mackay, K., Riolo, F., & Schwerdtle, T. Risk to human health related to the presence of perfluoroalkyl substances in food. *EFSA Journal* 2020, 18 (9), e06223.

EFSA Panel on Contaminants in the Food Chain; Benford, D., De Boer, J., Carere, A., Di Domenico, A., Johansson, N., Schrenk, D., .Schoeters, G., de Voogt, P,. & Dellatte, E. (2008). Opinion of the scientific panel on contaminants in the food chain on perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and their salts. *EFSA Journal*, (653), 1-131.

Esteban, M., & Castaño, A. (2009). Non-invasive matrices in human biomonitoring: a review. *Environment International*, 35(2), 438-449.

European Commisssion. Commission of the European Communities. (2009). Communication from the commission to the council, the European parliament, the European economic and social committee, and the committee of the regions: a mid-term assessment of implementing the EC biodiversity action plan. *Journal of International Wildlife Law & Policy*, 12(1-2), 108-120.

European Commission: The European Green Deal-COM (2019) 640 final.

European Commission, European Union Risk Assessment Report. Tris (2-chloroethyl) Phosphate TCEP (2009) https://echa.europa.eu/documents/10162/2663989d-1795-44a1-8f50-153a81133258 (assessed 11/08/2021).

European Commission Directive 2014/79/EU of 20 june 2014 amending appendix C of annex II to directive 2009/48/EC of the European parliament and of the council on the safety of toys, as regards TCEP, TCPP and TDCP *The Official Journal of the European Union* (2014) L182/49–51.

European Parliament and of the Council, Directive (EU) 2020/2184 of the European Parliament and of the Council of 16 December 2020 on the quality of water intended for human consumption (recast). 2020.

Ezechiáš, M., Covino, S., & Cajthaml, T. (2014). Ecotoxicity and biodegradability of new brominated flame retardants: a review. *Ecotoxicology and Environmental safety*, 110, 153-167.

Feiteiro, J., Mariana, M., & Cairrão, E. (2021). Health toxicity effects of brominated flame retardants: From environmental to human exposure. *Environmental Pollution*, 117475.

Feng, Y. L., Liao, X., Grenier, G., Nguyen, N., & Chan, P. (2015). Determination of 18 phthalate metabolites in human urine using a liquid chromatography-tandem mass spectrometer equipped with a core–shell column for rapid separation. *Analytical Methods*, 7(19), 8048-8059.

Fenton, S. E., Ducatman, A., Boobis, A., DeWitt, J. C., Lau, C., Ng, C., Smith, J. S., & Roberts, S. M. (2021). Per-and polyfluoroalkyl substance toxicity and human health review: Current state of knowledge and strategies for informing future research. *Environmental Toxicology and Chemistry*, 40(3), 606-630.

Fiedler, H., Kallenborn, R., De Boer, J., & Sydnes, L. K. (2019). The Stockholm Convention: A tool for the global regulation of persistent organic pollutants. *Chemistry International*, 41(2), 4-11.

Fréry, N., Santonen, T., Porras, S. P., Fucic, A., Leso, V., Bousoumah, R., Duca, R. C., Yamani, M. E., Kolossa-Gehring, M., Ndaw, S., Viegas, S., & Iavicoli, I. (2020). Biomonitoring of occupational exposure to phthalates: A systematic review. *International Journal of Hygiene and Environmental Health*, 229, 113548.

Ganzleben, C., Antignac, J. P., Barouki, R., Castaño, A., Fiddicke, U., Klánová, J., Lebret, E., Olea, N., Sarigiannis, D., Schoeters, G. R., Sepai, O., Tolonen, H., & Kolossa-Gehring, M. (2017). Human biomonitoring as a tool to

support chemicals regulation in the European Union. *International Journal of Hygiene and Environmental Health*, 220(2 Pt A), 94-97.

Gao, L., Li, J., Wu, Y., Yu, M., Chen, T., Shi, Z., Zhou, X., & Sun, Z. (2016). Determination of novel brominated flame retardants and polybrominated diphenyl ethers in serum using gas chromatography–mass spectrometry with two simplified sample preparation procedures. *Analytical and Bioanalytical Chemistry*, 408(27), 7835-7844.

Giovanoulis, G., Bui, T., Xu, F., Papadopoulou, E., Padilla-Sanchez, J. A., Covaci, A., .Haug, L. S., Cousins, A. P., Magnér, J., Cousin, I. T., & de Wit, C. A. (2018). Multi-pathway human exposure assessment of phthalate esters and DINCH. *Environment International*, 112, 115-126.

Giuliani, A., Zuccarini, M., Cichelli, A., Khan, H., & Reale, M. (2020). Critical review on the presence of phthalates in food and evidence of their biological impact. *International Journal of Environmental Research and Public Health*, 17(16), 5655.

Haritash, A. K., & Kaushik, C. P. (2009). Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. *Journal of Hazardous Materials*, 169(1-3), 1-15.

Helou, K., Harmouche-Karaki, M., Karake, S., & Narbonne, J. F. (2019). A review of organochlorine pesticides and polychlorinated biphenyls in Lebanon: Environmental and human contaminants. Chemosphere, 231, 357-368.

Hou, R., Lin, L., Li, H., Liu, S., Xu, X., Xu, Y., Jin, X., Yuan, Y., & Wang, Z. (2021). Occurrence, bioaccumulation, fate, and risk assessment of novel brominated flame retardants (NBFRs) in aquatic environments—A critical review. *Water Research*, 117168.

Human biomonitoring: facts and figures. Copenhagen: *WHO Regional Office for Europe*, 2015.

IARC, Some Flame Retardants and Textile Chemicals, IARC Monographs, vol. 48, IARC, Lyon (1990), pp. 55-72; https://publications.iarc.fr/66 (assessed 11/08/2021)

Jahnke, A., & Berger, U. (2009). Trace analysis of per-and polyfluorinated alkyl substances in various matrices—how do current methods perform? *Journal of Chromatography A*, 1216(3), 410-421.

Koch, H. M., & Calafat, A. M. (2009). Human body burdens of chemicals used in plastic manufacture. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364(1526), 2063-2078.

Koch, H. M., Lessmann, F., Swan, S. H., Hauser, R., Kolossa-Gehring, M., Frederiksen, H., Andersson, A.-M., Thomsen, C., Sakhi, A. K., Bornehag, C.-G., Mueller, J. F., Rudel, R. A., Braun, J. M., Harth, V., & Brüning, T. (2018). Analyzing terephthalate metabolites in human urine as biomarkers of exposure: Importance of selection of metabolites and deconjugation enzyme. *Journal of Chromatography. B, Analytical technologies in the biomedical and life sciences*, 1100, 91-92.

Koch, H. M., Rossbach, B., Drexler, H., & Angerer, J. (2003). Internal exposure of the general population to DEHP and other phthalates-determination of secondary and primary phthalate monoester metabolites in urine. *Environmental Research*, 93(2), 177-185.

Koch, H. M., Schütze, A., Pälmke, C., Angerer, J., & Brüning, T. (2013). Metabolism of the plasticizer and phthalate substitute diisononyl-cyclohexane-1, 2-dicarboxylate (DINCH®) in humans after single oral doses. *Archives of Toxicology*, 87(5), 799-806.

Li, H., Gao, S., Yang, M., Zhang, F., Cao, L., Xie, H., Chen, X., & Cai, Z. (2020). Dietary exposure and risk assessment of short-chain chlorinated paraffins in supermarket fresh products in Jinan, China. *Chemosphere*, 244, 125393.

Li, Z., Romanoff, L. C., Trinidad, D. A., Pittman, E. N., Hilton, D., Hubbard, K., Carmichael, H., Parker, J., Calafat, A. M. & Sjödin, A. (2014). Quantification of 21 metabolites of methylnaphthalenes and polycyclic aromatic hydrocarbons in human urine. *Analytical and Bioanalytical Chemistry*, 406(13), 3119-3129.

Lindstrom, A. B., Strynar, M. J., & Libelo, E. L. (2011). Polyfluorinated compounds: past, present, and future. *Environmental Science & Technology*, 45(19), 7954-7961.

Liu, Y., D'Agostino, L. A., Qu, G., Jiang, G., & Martin, J. W. (2019). High-resolution mass spectrometry (HRMS) methods for nontarget discovery and characterization of poly-and per-fluoroalkyl substances (PFAS) in environmental and human samples. *TrAC Trends in Analytical Chemistry*, 121, 115420.

Lu, D., Feng, C., Wang, D., Lin, Y., Qiu, X., Xu, Q., Wen, Y., She, J., Wang, G., & Zhou, Z. (2017). Multi-analyte method development for analysis of brominated flame retardants (BFRs) and PBDE metabolites in human serum. *Analytical and Bioanalytical Chemistry*, 409(22), 5307-5317.

Lyons, K. E., Ryan, C. A., Dempsey, E. M., Ross, R. P., & Stanton, C. (2020). Breast milk, a source of beneficial microbes and associated benefits for infant health. *Nutrients*, 12(4), 1039.

Macheka-Tendenguwo, L. R., Olowoyo, J. O., Mugivhisa, L. L., & Abafe, O. A. (2018). Per-and polyfluoroalkyl substances in human breast milk and current analytical methods. *Environmental Science and Pollution Research*, 25(36), 36064-36086.

Nakayama, S. F., Yoshikane, M., Onoda, Y., Nishihama, Y., Iwai-Shimada, M., Takagi, M., Kobayashi, Y., & Isobe, T. (2019). Worldwide trends in tracing poly-and perfluoroalkyl substances (PFAS) in the environment. *TrAC Trends in Analytical Chemistry*, 121, 115410.

Onyemauwa, F., Rappaport, S. M., Sobus, J. R., Gajdošová, D., Wu, R. A., & Waidyanatha, S. (2009). Using liquid chromatography–tandem mass spectrometry to quantify monohydroxylated metabolites of polycyclic aromatic hydrocarbons in urine. *Journal of Chromatography B*, 877(11-12), 1117-1125.

Panel, E. C. (2008). Opinion of the Scientific Panel on Contaminants in the Food chain on Perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and their salts. *The EFSA Journal*, 653, 1-131.

Pirard, C., & Charlier, C. (2018). Simple and fast method for the measurement of legacy and novel brominated flame retardants in human serum. *Chemosphere*, 211, 918-925.

Pribylova, P., Kares, R., Boruvkova, J., Cupr, P., Prokes, R., Kohoutek, J., Holoubek, I., & Klanova, J. (2012). Levels of persistent organic pollutants and polycyclic aromatic hydrocarbons in ambient air of Central and Eastern Europe. *Atmospheric Pollution Research*, 3(4), 494-505.

Qing Li, Q., Loganath, A., Seng Chong, Y., Tan, J., & Philip Obbard, J. (2006). Persistent organic pollutants and adverse health effects in humans. *Journal of Toxicology and Environmental Health*, Part A, 69(21), 1987-2005.

Ross, P. S., & Birnbaum, L. S. (2001). A. Persistent Organic Pollutants (POPs) in humans and wildlife. Integrated Risk Assessment. Report prepared for the WHO/UNEP/ILO International Programme on Chemical Safety.

Samanta, S. K., Singh, O. V., & Jain, R. K. (2002). Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. *TRENDS in Biotechnology*, 20(6), 243-248.

Schindler, B.K., Esteban, M., Koch, H.M., Castano, A., Koslitz, S., Canas, A., Casteleyn, L., Kolossa-Gehring, M., Schwedler, G., Schoeters, G., Hond, E.D., Sepai, O., Exley, K., Bloemen, L., Horvat, M., Knudsen, L.E., Joas, A., Biot, P., Aerts, D., Lopez, A., Huetos, O., Katsonouri, A., Maurer-Chronakis, K., Kasparova, L., Vrbík, K., Rudnai, P., Naray, M., Guignard, C., Fischer M.E., Ligocka, D., Janasik, B., Reis, M.F., Namorado, S., Pop, C., Dumitrascu, I., Halzlova, K., Fabianova, E., Mazej, D., Tratnik, J.S., Berglund, M., Jönsson, B., Lehman, A., Crettaz, P., Frederiksen, H., Nielsen, F., McGrath, H., Nesbitt, I., De Cremer, K., Vanermen, G., Koppen, G., Wilhelm, M., Becker, K., Angerer, J. (2014). The European COPHES/DEMOCOPHES project: Towards transnational comparability and reliability of human biomonitoring results. *International Journal of Hygiene and Environmental Health*, 2017(6), 653-661.

Shen, L., & Wania, F. (2005). Compilation, evaluation, and selection of physical− chemical property data for organochlorine pesticides. *Journal of Chemical & Engineering Data*, 50(3), 742-768.

Shi, Z., Wang, Y., Niu, P., Wang, J., Sun, Z., Zhang, S., & Wu, Y. (2013). Concurrent extraction, clean-up, and analysis of polybrominated diphenyl ethers, hexabromocyclododecane isomers, and tetrabromobisphenol A in human milk and serum. *Journal of Separation Science*, 36(20), 3402-3410.

Schettler, T. E. D. (2006). Human exposure to phthalates via consumer products. *International Journal of Andrology*, 29(1), 134-139.

Schütze, A., Kolossa-Gehring, M., Apel, P., Brüning, T., & Koch, H. M. (2014). Entering markets and bodies: increasing levels of the novel plasticizer Hexamoll® DINCH® in 24 h urine samples from the German Environmental Specimen Bank. *International Journal of Hygiene and Environmental Health*, 217(2-3), 421-426.

Sly, P. D., Carpenter, D. O., Van den Berg, M., Stein, R. T., Landrigan, P. J., Brune-Drisse, M. N., & Suk, W. (2016). Health consequences of environmental exposures: causal thinking in global environmental epidemiology. *Annals of Global Health*, 82(1), 3-9.

Tomasko, J., Stupak, M., Parizkova, D., Polachova, A., Sram, R. J., Topinka, J., & Pulkrabova, J. (2021). Short- and medium-chain chlorinated paraffins in human blood serum of Czech population. *Science of The Total Environment*, 149126.

Trier, X., Granby, K., & Christensen, J. H. (2011). Polyfluorinated surfactants (PFS) in paper and board coatings for food packaging. *Environmental Science and Pollution Research*, 18(7), 1108-1120.

UNEP Stockholm Convention on Persistent Organic Pollutants; United Nations Environment Programme, Geneva, Switzerland (2001).

UNEP Stockholm Convention on Persistent Organic Pollutants (2015). Risk profile on short-chained chlorinated paraffins, UNEP/POPS/POPRC.11/10/Add.2.

UNEP Stockholm Convention on Persistent Organic Pollutants, Basel, Rotterdam and Stockholm conventions United Nations Environment Programme (UNEP), Geneva, Switzerland (2018), p. 78.

UNEP Stockholm Convention on Persistent Organic Pollutant, Chemicals proposed for listing under the Convention (2021a); http://www.pops.int/TheConvention/ThePOPs/ChemicalsProposedforListing/tabid/2510/Default.aspx (assessed 18/01/2022).

UNEP Stockholm Convention on Persistent Organic Pollutant, All POPs listed in the Stockholm Convention (2021b); http://chm.pops.int/TheConvention/ThePOPs/AllPOPs/tabid/2509/Default.aspx (assessed 20/07/2021).

Urbancova, K., Sram, R. J., Hajslova, J., & Pulkrabova, J. (2021). Concentrations of Phthalate and DINCH Metabolites in Urine Samples from Czech Mothers and Newborns. *Exposure and Health*, 1-11.

Van der Veen, J., de Boer, J. (2012). Phosphorus flame retardants: Properties, production, environmental occurrence, toxicity and analysis. *Chemosphere*, 88, 1119-1153.

Van Mourik, L. M., Gaus, C., Leonards, P. E., & de Boer, J. (2016). Chlorinated paraffins in the environment: A review on their production, fate, levels and trends between 2010 and 2015. *Chemosphere*, 155, 415-428.

Vorkamp, K., Castaño, A., Antignac, J. P., Boada, L. D., Cequier, E., Covaci, A., Esteban López, M., Haug, L. S., Kasper-Sonnenberg, M., Koch, H. M., Luzardo, O. P., Osite, A., Rambaud, L., Pinorini, M.-T., Sabbioni, G., & Thomsen, C. (2021). Biomarkers, matrices and analytical methods targeting human exposure to chemicals selected for a European human biomonitoring initiative. *Environment International*, 146, 106082.

Wang, Q., Ruan, Y., Lin, H., & Lam, P. K. (2020a). Review on perfluoroalkyl and polyfluoroalkyl substances (PFAS) in the Chinese atmospheric environment. *Science of the Total Environment*, 737, 139804.

Wang, W., Rhodes, G., Ge, J., Yu, X., & Li, H. (2020b). Uptake and accumulation of per-and polyfluoroalkyl substances in plants. *Chemosphere*, 261, 127584.

WHO, Environmental Health Criteria 198, International Program on Chemical Safety, United Nations Environmental Program. World Health Organization, Geneva, Switzerland (1998).

WHO, World Health Organization. Exclusive Breastfeeding for Optimal Growth, Development and Health of Infants (2019).

Wittassek, M., Koch, H. M., Angerer, J., & Brüning, T. (2011). Assessing exposure to phthalates–the human biomonitoring approach. Molecular *Nutrition & Food Research*, 55(1), 7-31.

World Health Organization. (2016). Safety evaluation of certain food additives and contaminants, supplement 1: non-dioxin-like polychlorinated biphenyls, prepared by the eightieth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

Wormuth, M., Scheringer, M., Vollenweider, M., & Hungerbühler, K. (2006). What are the sources of exposure to eight frequently used phthalic acid esters in Europeans *Risk Analysis*, 26(3), 803-824.

Wu, Z., He, C., Han, W., Song, J., Li, H., Zhang, Y., Jing, X., & Wu, W. (2020). Exposure pathways, levels and toxicity of polybrominated diphenyl ethers in humans: A review. *Environmental Research*, 187, 109531.

Xu, W., Wang, X., & Cai, Z. (2013). Analytical chemistry of the persistent organic pollutants identified in the Stockholm Convention: A review. *Analytica Chimica Acta*, 790, 1-13.

Yamano, Y., Hara, K., Ichiba, M., Hanaoka, T., Pan, G., & Nakadate, T. (2014). Urinary 1-hydroxypyrene as a comprehensive carcinogenic biomarker of exposure to polycyclic aromatic hydrocarbons: a cross-sectional study of coke oven workers in China. *International Archives of Occupational and Environmental Health*, 87(7), 705-713.

Zhong, Y., Carmella, S. G., Hochalter, J. B., Balbo, S., & Hecht, S. S. (2011). Analysis of r-7, t-8, 9, c-10-tetrahydroxy-7, 8, 9, 10-tetrahydrobenzo[a]pyrene in human urine: a biomarker for directly assessing carcinogenic polycyclic aromatic hydrocarbon exposure plus metabolic activation. *Chemical Research in Toxicology*, 24(1), 73-80.

Zushi, Y., Hogarh, J. N., & Masunaga, S. (2012). Progress and perspective of perfluorinated compound risk assessment and management in various countries and institutes. *Clean Technologies and Environmental Policy*, 14(1), 9-20.

8 SYMBOLS AND ABBREVIATIONS

1-OHP	1-hydroxypyrene
2,4,6-TBP	2,4,6-tribromophenol
2-OH-NAP	2-hydroxynaphthalene
BaA	benz(a)anthracene
BaP	benzo(a)pyrene
BbF	benzo(b)fluoranthene
BBzP	benzyl butyl phthalate
BFRs	brominated flame retardants
Br-PFOS	branched-PFOS
BTBPE	1,2-bis(2,4,6-tribromophenoxy)ethane
BTBPE	1,2-bis(2,4,6-tribromophenoxy)ethane
bw	body weight
CI	chemical ionization
CPs	chlorinated paraffins
cx-MEHP	mono (2-ethyl-5-carboxypentyl) phthalate
cx-MINCH	cyclohexane-1,2-diarboxylic mono carboxyisononyl ester
CZ-HBM	Czech human biomonitoring program
DBDPE	decabromodiphenyl ethane
DBP	dibutyl phthalate
DDT	dichloro-diphenyl-trichloroethane
DEHP	di(2-ethylhexyl) phthalate
DiBP	diisobutyl phthalate
DIDP	diisodecyl phthalate
DINCH	di-iso-nonyl cyclohexane-1,2-dicarboxylate
DINP	diisononyl phthalate
DnBP	di-n-butyl phthalate
DP	Dechlorane Plus
d-SPE	dispersive solid phase extraction
EFRA	European Flame Retardants Association
EFSA	European Food Safety Authority
EI	electron ionization
EQUAS	external quality assessment scheme
ESI-	electrospray ionisation in negative mode
EU	the European Union
FOSAs	perfluorooctane sulfonamides
FOSEs	perfluorooctane sulfonamide ethanols
FRs	flame retardants
FTOHs	fluorotelomer alcohols
FTSs	fluorotelomer sulfonates
GC	gas chromatography
GPC	gel permeation chromatography
HAIE	Healthy Aging in Industrial Environment
HBCDs	hexabromocyclododecanes
HBM	human biomonitoring
HCB	hexachlorobenzen

HCH	α-hexachlorocyclohexan
HI	hazard index
HLB	hydrophilic lipophilic balance
HMW	high molecular weight
HPLC	high-performance liquid chromatography
HQ	hazard quotient
CHR	chrysene
ICI	interlaboratory comparison investigation
LC	liquid chromatography
LCCPs	long-chain chlorinated paraffins
LLE	liquid-liquid extraction
LMW	low molecular weight
LOD	limit of detection
LOQ	limit of quantification
L-PFOS	linear-PFOS
lw	lipid weight
MBP	monobutyl phthalate
MBzP	monobenzyl phthalate
MCCPs	medium-chain chlorinated paraffins
MEHP	mono(2-ethylhexyl) phthalate
MEP	monoethyl phthalate
MEP	monoethyl phthalate
MiBP	mono-iso-butyl phthalate
MINCH	mono-isononyl-cyclohexane-1,2-dicarboxylate
MnBP	mono-n-butyl phthalate
MS(/MS)	(tandem) mass spectrometry
MTBE	methyl tert-butyl ether
NBFRs	novel (or new) brominated flame retardants
NCI	negative chemical ionization
NIPH	The National Institute of Public Health
o,p′-DDD	*o,p′*-dichlorodiphenyldichloroethane
o,p′-DDE	*o,p′*-dichlorodiphenyldichloroethylene
OCPs	organochlorine pesticides
OH-BDEs	hydroxylated metabolites of polybrominated diphenyl ethers
OH-MEHP	mono(2-ethyl-5-hydroxyhexyl) phthalate
OH-MINCH	cyclohexane-1,2-dicarboxylic mono hydroxyisononyl ester
OH-PAHs	monohydroxylate metabolites of polycyclic aromatic hydrocarbons
OPFRs	organophosphorus flame retardants
oxo-MEHP	mono(2-ethyl-5-oxohexyl) phthalate
oxo-MINCH	cyclohexane-1, 2-dicarboxylic mono oxoisononyl ester
p,p′-DDE	*p,p′*-dichlorodiphenyldichloroethylene
p,p′-DDT	1,1-bis(4-chlorophenyl)-2,2,2-trichloroethane
PAHs	polycyclic aromatic hydrocarbons
PAPs	polyfluoroalkyl phosphate esters
PARC	Partnership for Chemicals Risk Assessment
PBDEs	polybrominated diphenyl ethers

PCBs	polychlorinated biphenyls
PFAS	per- and polyfluoroalkyl substances
PFCAs	perfluorocarboxylic acids
PFDA	perfluorodecanoic acid
PFHpA	perfluoroheptanoic acid
PFHxS	perfluorohexane sulfonic acid
PFNA	perfluorononanoic acid
PFNA	perfluorononanoic acid
PFOA	perfluorooctanoic acid
PFOS	perfluorooctane sulfonic acid
PFSAs	perfluorosulfonic acids
PFUnDA	perfluoroundecanoic acid
POPs	persistent organic pollutants
PTWI	provisional tolerably weekly intake
QA/QC	quality assurance/quality control
RSD	relative standard deviation
SCCPs	short-chain chlorinated paraffins
SOP	standard operation procedure
SPE	solid phase extraction
SRM	standard reference material
TBBPA	tetrabromobisphenol A
TCEP	tris(2-chloroethyl) phosphate
TClPP	tris(2-chloroisopropyl) phosphate
TDClPP	tris(1,3-dichloro-2-propyl) phosphate
TDI	tolerably daily intake
TWI	tolerably weekly intake
UHPLC	ultra-high performance liquid chromatography
WHO	World Health Organization
Z	Z-scores

9 LIST OF PUBLICATIONS

9.1 Journal Papers

[23] Jurikova M., **Dvorakova D.**, Pulkrabova J.: The occurrence of perfluoroalkylated substances (PFAS) in drinking water in the Czech Republic: a pilot study. Submitted to *Environmental Science and Pollution Research* on 15/11/2021, minor revision.

[22] Vaccher V., Esteban López M., Castaño A., Mol H., Zarrabi K., Bury D., Koch H., **Dvorakova D.**, Hajslova J., Nübler S., Sakhi A.K., Thomsen C., Vorkamp K., Göen T., Antignac J.P.: European interlaboratory comparison investigations (ICI) and external quality assurance schemes (EQUAS) for the analysis of bisphenol A, S and F in human urine: results from the HBM4EU project. *Environmental Research* (2022): In Press: 112933 (doi: https://doi.org/10.1016/j.envres.2022.112933). IF 6.498

[21] Mol H., Elbers I., Pälmke C., Bury D., Göen T., Esteban López M., Nübler S., Vaccher V., Antignac J.P., **Dvorakova D.**, Hajslova J., Thomsen C., Vorkamp K., Castaño A., Koch H.M.: Proficiency and interlaboratory variability for the determination of phthalate and DINCH biomarkers in human urine: results from the HBM4EU project. *Toxics* (2022) 10(2): 57 (doi: https://doi.org/10.3390/toxics10020057). IF 4.816

[20] Drabova L., **Dvorakova D.**, Urbancova K., Gramblicka T., Hajslova J., Pulkrabova J.: Critical assessment of clean-up techniques employed in simultaneous analysis of persistent organic pollutants and polycyclic aromatic hydrocarbons in fatty samples. *Toxics* (2022) 10(1): 12 (doi: https://doi.org/10.3390/toxics10010012). IF 4.816

[19] Polachova A., Gramblicka T., Bechynska K., Parizek O., Turnerova D., **Dvorakova D.**, Honkova K., Rossnerova A., Rossner P., Sram R. J., Topinka J., Pulkrabova J.: Biomonitoring of 89 POPs in blood serum samples of Czech city policemen. *Environmental Pollution* (2021) 291: 118140 (doi: https://doi.org/10.1016/j.envpol.2021.118140). IF 8.071

[18] **Dvorakova D.**, Pulkrabova J., Nübler S., Haji-Abbas-Zarrabi K., Göen T., Mol H., Koch H. M., Vaccher V., Antignac J.P., Haug L. S., Vorkamp K., López M.E., Castaño A., Hajslova J.: Interlaboratory Comparison Investigations (ICIs) and External Quality Assurance Schemes (EQUASs) for Flame Retardants Analysis in Biological Matrices: Results from the HBM4EU Project. *Environmental Research* (2021) 202: 111705 (doi: 10.1016/j.envres.2021.111705). IF 6.498

[17] López M.A., Göen T., Mol H., Nübler S., Haji-Abbas-Zarrabi K., Koch H.M., Kasper-Sonnenberg M., **Dvorakova D.**, Hajslova J., Antignac J.P., Vaccher V., Elbers I., Thomsen C., Vorkamp K., Pedraza – Díaz S., Kolossa-Gehring M., Castaño A.: The European human biomonitoring platform - Design and implementation of a laboratory quality assurance/quality control (QA/QC) programme for selected priority chemicals. *International Journal of Hygiene and Environmental Health* (2021) 234: 113740. (doi:10.1016/j.ijheh.2021.113740). IF 5.840

[16] Nübler S., López M.E., Castaño A., Mol H., Schäfer M., Haji-Abbas-Zarrabi K., Bury D., Koch H.M., Vaccher V., Antignac J.-P., **Dvorakova D.**, Hajslova J., Thomsen C., Vorkamp K., Göen T.: Interlaboratory comparison investigations (ICI) and external quality assurance schemes (EQUAS) for cadmium in urine and blood: Results from the HBM4EU project. *International Journal of Hygiene and Environmental Health* (2021) 234, 113711 (doi: 10.1016/j.ijheh.2021.113711). IF 5.840

[15] Cerna M., Grafnetterova A.P., **Dvorakova D.**, Pulkrabova J., Maly M., Janos T., Vodrazkova N., Tupa Z., Puklova V.: Biomonitoring of PFOA, PFOS and PFNA in human milk from Czech Republic, time trends and estimation of infant's daily intake. *Environmental Research* (2020) 188: 109763. (doi: 10.1016/j.envres.2020.109763). IF 6.498

[14] Urbancova K., **Dvorakova D.**, Gramblicka T., Sram R.J., Hajslova J., Pulkrabova J.: Comparison of polycyclic aromatic hydrocarbon metabolite concentrations in urine of mothers and their newborns. *Science of the Total Environment* (2020) 723:138116 (doi: 10.1016/j.scitotenv.2020.138116). IF 7.963

[13] Svarcova A., **Lankova D.**, Gramblicka T., Stupak M., Hajslova J., Pulkrabova J.: Integration of five groups of POPs into one multi-analyte method for human blood serum analysis: An innovative approach within biomonitoring studies. *Science of the Total Environment* (2019) 667: 701-709. (doi: 10.1016/j.scitotenv.2019.02.336). IF 7.963

[12] Urbancova K., **Lankova D.**, Sram R.J., Hajslova J., Pulkrabova J.: Urinary metabolites of phthalates and di-iso-nonyl cyclohexane-1,2-dicarboxylate (DINCH) – Czech mothers´ and newborns´ exposure biomarkers. *Environmental Research* (2019), 173: 342-348. (doi: 10.1016/j.envres.2019.03.067). IF 6.498

[11] Richterova D., Fabelova L., Patayova H., Pulkrabova J., **Lankova D.**, Rausova K., Sovcikova E., Stencl J., Hajslova J., Trnovec T., Palkovicova Murinova L.: Determinants of prenatal exposure to perfluoroalkyl substances in the Slovak birth cohort. *Environment International* (2018) 121 (2):1304-1310. (doi:10.1016/j.envint.2018.10.051). IF 9.621

[10] Socas-Rodríguez B., **Lankova D.**, Urbancova K., Krtkova V., Hernández-Borges J., Rodríguez-Delgado M. A., Pulkrabova J., Hajslova J.: Multiclass analytical method for the determination of natural/synthetic steroid hormones, phytoestrogens, and mycoestrogens in milk and yogurt. *Analytical and Bioanalytical Chemistry* (2017) 409 (18): 4467-4477. (doi: 10.1007/s00216-017-0391-x). IF 4.142

[9] Urbancova K., **Lankova D.**, Rossner P., Rossnerova A., Svecova V., Tomaniova M., Veleminsky M., Jr., Sram R. J., Hajslova J., Pulkrabova J.: Evaluation of 11 polycyclic aromatic hydrocarbon metabolites in urine of Czech mothers and newborns. *Science of the Total Environment* (2017) 577: 212–219. (doi: 10.1016/j.scitotenv.2016.10.165). IF 7.963

[8] **Lankova D.**, Urbancova K., Sram R.J., Hajslova J., Pulkrabova J.: A novel strategy for the determination of polycyclic aromatic hydrocarbon monohydroxylated metabolites in urine using ultra-high-performance liquid chromatography with tandem mass spectrometry. *Analytical and Bioanalytical Chemistry* (2016) 408: 2515–25. (doi: 10.1007/s00216-016-9350-1). IF 4.142

[7] **Lankova D.**, Svarcova A., Kalachova K., Lacina O., Pulkrabova J., Hajslova J.: Multi-analyte method for the analysis of various organohalogen compounds in house dust. *Analytica Chimica Acta* (2015) 854: 61–69. (doi: 10.1016/j.aca.2014.11.007). IF 6.558

[6] Svihlikova V., **Lankova D.**, Poustka J., Tomaniova M., Hajslova J., Pulkrabova J.: Perfluoroalkyl substances (PFAS) and other halogenated compounds in fish from the upper Labe River basin. *Chemosphere* (2015) 129: 170–178. (doi: 10.1016/j.chemosphere.2014.09.096). IF 7.086

[5] Hlouskova V., **Lankova D.**, Kalachova K., Hradkova P., Poustka J., Hajslova J., Pulkrabova J.: Brominated flame retardants and perfluoroalkyl substances in sediments from the Czech aquatic ecosystem. *Science of the Total Environment* (2014) 470–471:407–416. (doi: 10.1016/j.scitotenv.2013.09.074). IF 7.963

[4] Hlouskova V., **Lankova D**., Kalachova K., Hrádkova P., Poustka J., Hajslova J., Pulkrabova J.: Occurrence of brominated flame retardants and perfluoroalkyl substances in fish from the Czech aquatic ecosystem. *Science of the Total Environment* (2013) 461–462:88–98. (doi:10.1016/j.scitotenv.2013.04.081). IF 7.963

[3] **Lankova D.**, Kockovska M., Lacina O., Kalachova K., Pulkrabova J., Hajslova J.: Rapid and simple method for determination of hexabromocyclododecanes and other LC–MS/MS-amenable brominated flame retardants in fish. *Analytical and Bioanalytical Chemistry*, (2013) 405:7829–7839 (doi: 10.1007/s00216-013-7076-x). IF 4.142

[2] **Lankova D.**, Lacina O., Pulkrabova J., Hajslova J.: The determination of perfluoroalkyl substances, brominated flame retardants and their metabolites in human breast milk and infant formula. *Talanta* (2013), 117: 318–325. (doi: 10.1016/j.talanta.2013.08.040). IF 6.057

[1] Kalachova K., Hradkova P., **Lankova D.**, Hajslova J., Pulkrabova J.: Occurrence of brominated flame retardants in household and car dust from the Czech Republic. *Science of the Total Environment* (2012) 441:182–193. (doi: 10.1016/j.scitotenv.2012.09.061). IF 7.963

9.2 Short Papers

[13] **Lankova D**., Lacina O., Pulkrabova J., Hajslova J.: UHPLC–MS based analysis of polyfluorinated surfactants and related fluorinated contaminants in food contact material. Proceedings of the 5th MS Food Day, Bologna, Italy, October 11–13, 2017, 64–66. ISBN 9788890738838

[12] Pulkrabova J., **Lankova D.**, Urbancova K., Svarcova A., Stupak M., Hajslova J. Assessment of body burden of Czech population to polycyclic aromatic hydrocarbons. Environmental Technology and Innovations. CRC Press (2016) 251–254. Proceedings of the 1st International Conference on Environmental Technology and Innovations. ISBN 9781138029965

[11] **Lankova D.**, Vincikova A., Pulkrabova J., Hajslova J.: Halogenované kontaminanty a jejich deriváty v mořských plodech dostupných na českém trhu, Sborník příspěvků XLV. Symposium o nových směrech výroby a hodnocení potravin (2015) 104–108. ISBN 978-80-7080-937-2

[10] **Lankova D.**, Odlerova S., Socas-Rodríguez B., Pulkrabova J., Hajslova J.: Výskyt estrogenních látek v mléce a mléčných výrobcích z obchodní sítě České republiky, Sborník příspěvků XLIV. Symposium o nových směrech výroby a hodnocení potravin (2014) 43–47. ISBN 978-80-7080-903-7

[9] Urbancova K., **Lankova D.**, Pulkrabova J., Hajslova J.: Fast procedure for the monitoring of organohalogenated contaminants in breast milk and blood plasma, Chemické listy 108 (2014) S266–269. ISSN 0009-2770

[8] **Lankova D.**, Kalachova K., Lacina O., Pulkrabova J., Hajslova J.: Multi-analyte method for the determination of organohalogen contaminants in dust, Proceedings of 9th ISC Modern Analytical Chemistry, Prague, Czech Republic, September 23–24, 2013, 91–94. ISBN 978-80-7444-023-6

[7] Slovakova M., Pulkrabova J., **Lankova D.**, Kalachova K., Hajslova J.: Determination of fluorotelomer alcohols in food contact materials and food, Proceedings of 9th ISC Modern Analytical Chemistry, Prague, Czech Republic, September 23–24, 2013, 17–20. ISBN 978-80-7444-023-6

[6] **Lankova D.**, Kockovska M., Kalachova K., Pulkrabova J., Hajslova J.: Dust as an indicator of the environmental pollution with halogenated compounds, Zborník príspevkov doktorandov z 10. ročníka Študentskej vedeckej konferencie, Zvolen, Slovakia, May 23, 2013, 59–67. ISBN 978-80-228-2528-3

[5] Kockovska K., **Lankova D.**, Kalachova K., Pulkrabova J., Hajslova J.: Case study: Contamination of terrestric environment by industrial fire, Zborník príspevkov doktorandov z 10. ročníka Študentskej vedeckej konferencie, Zvolen, Slovakia, May 23, 2013, 49–58. ISBN 978-80-228-2528-3

[4] Papez M., Lacina O., **Lankova D.**, Pulkrabova J., Hajslova J.: "Nové" organohalogenované kontaminanty ve vodním ekosystému, Chemické listy 105 (2011) 51–54. ISSN 0009-2770

[3] Pulkrabova J, Hlouskova V, **Lankova D**: Halogenované látky v rybách a sedimentech, Projekt ''Živá voda'', Arnika (2011), ISBN 978-80-904686-6-6.

[2] Pulkrabova J., Lacina O., **Lankova D.**, Kalachova K., Hajslova J.: Novel LC–MS strategy to monitor brominated flame retardants together with their metabolites in food matrices, *Organohalogen Compounds* 72 (2010) 1205–1208. ISSN 1026-4892

[1] Pulkrabova J, Hradkova P, **Lankova D**, Hlouskova V, Hajslova J: Výskyt perfluoroalkylovaných a polybromovaných polutantů v rybách z českých řek. Statistická ročenka životního prostředí České Republiky (2010), ISBN 978-80-7212-489-3.

9.3 Oral Presentations

In the English Language

[32] Buresova M., **Dvorakova D.**, Pulkrabova J.; An investigation of various sources of background contamination in PFAS trace analysis – a troubleshooting study; 16th International Students Conference “Modern Analytical Chemistry”; Prague, Czech Republic, September 17–18, 2020.

[31] Gramblicka T., **Dvorakova D.**, Urbancova K.; Pulkrabova J.; Urinary metabolites of organophosphorus flame retardants: A pilot study assessing exposure of Czech population; Student conference "Chemie je život", Brno, Czech Republic, November 21–22, 2019.

[30] **Dvorakova D.**, Buresova M., Pulkrabova J., Hajslova J.: Perfluoroalkylated substances (PFAS) in drinking water: a pilot study from the Czech Republic, ENDWARE 46th meeting, European Network of Drinking Water Regulators, Prague, Czech Republic, October 29–30, 2019.

[29] Pulkrabova J., Buresova M., **Lankova D.**, Hajslova K.: Occurrence of perfluoroalkylated substances (PFAS) in drinking water in Czech Republic; 4th IMEKOFOODS; Brussel, Belgium, September 16–18, 2019.

[28] **Lankova D.**: Current trends in human biomonitoring, Training school on challenges in food and feed safety research, Prague, Czech Republic, November 27–29, 2018.

[27] **Lankova D.**: Sample preparation strategies - Choice of isolation purification, pre-concentration strategy for analysis of biologically active compounds, Training school on challenges in food and feed safety research, Prague, Czech Republic, November 27–29, 2018.

[26] **Lankova D.**, Pulkrabova J., Hajslova J.: Current trends in human biomonitoring, International Conference on Environmental Technology and Innovations, Prague, Czech Republic, November 21–22, 2018.

[25] Buresova M., **Lankova D.**, Pulkrabova J: Cocktail of perfluoroalkylated substances in drinking water and soft drinks; 3rd MultiCoop integration workshop on "Support of young scientists in their research project proposals ideas", Prague, Czech Republic, November 26, 2018.

[24] **Lankova D.**, Lacina O., Pulkrabova J., Hajslova J.: UHPLC–MS based analysis of polyfluorinated surfactants and related fluorinated contaminants in food contact material. 5th MS Food Day, Bologna, Italy, October 11–13, 2017.

[23] Hajslova J., **Lankova D.**, Urbancova K., Pulkrabova J.: Using hair for biomonitoring studies: analytical challenges to be addressed, Dioxin 2017, Vancouver, Canada, August 20–25, 2017.

[22] Pulkrabova J., **Lankova D.**, Urbancova K., Svarcova A., Gramblicka T., Sram R.J., Hajslova J.: Comprehensive assessment of exposure of Czech population to polycyclic aromatic hydrocarbons, 2nd International Conference on Food Contaminants, Braga, Portugal, July 13–14, 2017.

[21] Urbancova K., Lankova D., Tomaniova M., Sram R.J., Hajslova J., Pulkrabova J.: Critical assessment of 11 polycyclic aromatic hydrocarbon metabolites in urine of Czech mothers and newborns. 12th International Students Conference 'Modern Analytical Chemistry', Prague, Czech Republic, September 22–23, 2016.

[20] **Lankova D.**, Urbancova K., Sram R. J., Pulkrabova J., Hajslova J.: The assessment of mothers´/newborns´ exposure pathways to various PAHs including the analysis of their metabolites, Central and Eastern European Conference on Health and the Environment, Prague, Czech Republic, April 10–14, 2016.

[19] Svarcova A., Gramblicka T., **Lankova D.**, Hajslova J., Pulkrabova J.: Ultra-trace analysis of persistent halogenated contaminants in human blood serum, 11th ISC Modern Analytical Chemistry, Prague, Czech Republic, November 22–23, 2015.

[18] **Lankova D.**, Urbancova K., Sram R., Pulkrabova J., Hajslova J.: Critical assessment of mothers´/newborns´ exposure pathways to carcinogenic PAHs through analysis of their metabolites, 7th International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic, November 3–6, 2015.

[17] **Lankova D.**, Pulkrabova J., Hajslova J.: The analysis of organohalogenated contaminants in biological samples, Seminar "Advanced analysis of food and biological samples" at National University of Littoral, Chemical Engineering Faculty, Program of Research and Analysis of Chemical Residues and Contaminants, Santa Fe, Argentina, September 25, 2015.

[16] Pulkrabova J., **Lankova D.**, Urbancova K., Svarcova A., Stupak M., Hajslova J.: Assessment of body burden of Czech population to polycyclic aromatic hydrocarbons, Chemistry & Life 2015, Brno, September 2–4, 2015.

[15] **Lankova D.**, Urbancova K., Pulkrabova J., Hajslova J.: A novel strategy for the determination of polycyclic aromatic hydrocarbons (PAHs) metabolites in urine, Chemistry & Life 2015, Brno, September 2–4, 2015.

[14] **Lankova D.**, Stahl-Zeng J., Hajslova J.: The Application of TripleTOF® Systems for Residue Analysis of Veterinary Drugs in Products of Animal Origin, Sciex Workshop TripleTOF® in Food Analysis, Prague, April 1, 2015.

[13] Hajslova J., Pulkrabova J., **Lankova D.**: Sources and transfer of emerging halogenated contaminants across the food chain, 5th European Chemistry Congress, Istanbul, Turkey, August 31–September 4, 2014.

[12] Pulkrabova J., **Lankova D.**, Hajslova J.: Multi-analyte method for the analysis of various organohalogen compounds in indoor dust, 16th International symposium on advances in extraction technologies, Crete, Greece, May 25–28, 2014.

[11] Slovakova M., Pulkrabova J., **Lankova D.**, Kalachova K., Hajslova J.: Determination of fluorotelomer alcohols in food contact materials and food, Proceedings of 9th ISC Modern Analytical Chemistry, Prague, Czech Republic, September 23–24, 2013.

[10] **Lankova D.**, Kalachova K., Lacina O., Pulkrabova J., Hajslova J.: Multi-analyte method for the determination of organohalogen contaminants in dust, Proceedings of 9th ISC Modern Analytical Chemistry, Prague, Czech Republic, September 23–24, 2013.

[9] **Lankova D.**, Kockovska M., Kalachova K., Pulkrabova J., Hajslova J.: Dust as an indicator of the environmental pollution with halogenated compounds, 10. ročník Študentskej vedeckej konferencie, Zvolen, Slovakia, May 23, 2013.

[8] Kockovska M., **Lankova D.**, Kalachova K., Pulkrabova J., Hajslova J: Case study: Contamination of terrestric environment by industrial fire, 10. ročník Študentskej vedeckej konferencie, Zvolen, Slovakia, May 23, 2013.

[7] Hajslova J., Lacina O., Pulkrabova J., **Lankova D.**: High resolution mass spectrometry: an efficient tool to monitor polyfluorinated surfactants (PFSs) in food contact materials and their migration into foodstuffs, 7th International Symposium MS and Related Techniques in Advanced Analytical Science, Manchester, United Kingdom, April 23–24, 2013.

[6] Kockovska M., Pulkrabova J., **Lankova D.**, Hajslova J.: Analysis of Polyfluorinated Surfactants in Food Contact Materials, Chemie je život, Brno, Czech Republic, December 7, 2012.

[5] Hajslova J., Lacina O., Pulkrabova J., Hradkova P., **Lankova D.**, Zachariasova M.: Environmental contaminants and toxicants in food chain, AOAC Annual Meeting & Exposition, Las Vegas, Nevada, USA, September 30–October 3, 2012.

[4] Lacina O., Pulkrabova J., **Lankova D.**, Jandova R., Hajslova J.: Sources and analysis of polyfluorinated surfactants and related "emerging" fluorinated contaminants, 8th Annual LC/MS/MS Workshop on Environmental Applications and Food Safety, Barcelona, Spain, July 2–4, 2012.

[3] Lacina O., Pulkrabova J., Hradkova P., Hlouskova V., **Lankova D.**, Hajslova J.: Simple, high throughput UHPLC–MS/MS ultra trace analysis of perfluorinated compounds in food of animal origin: milk and fish, 3rd International Workshop Anthropogenic Perfluorinated Compounds, Amsterdam, The Netherlands, June 15–17, 2011.

[2] Pulkrabova J., Lacina O., **Lankova D.**, Papez M., Hajslova J.: Analysis of Various PFC Groups in Czech Household Dust, 3rd International Workshop Anthropogenic Perfluorinated Compounds, Amsterdam, The Netherlands, June 15–17, 2011.

[1] Hajslova J., Lacina O., Pulkrabova J., Hradkova P., **Lankova D.**, Korba T.: Overcoming of challenges in trace analysis of perfluorinated and other halogenated persistent food contaminants: Solutions offered by cutting edge MS equipment, 1st AB SCIEX ECMSMS European Conference on MS/MS, Noordwijkerhout, The Netherlands, October 6–7, 2010.

In the Czech Language

[21] **Dvorakova D.**, Jurikova M., Pulkrabova J.: Stanovení per- a polyfluoralkylovaných sloučenin (PFAS) ve vodě - shrnutí studií zaměřených na koncentrace PFAS ve vodách. Provoz vodovodů a kanalizací, SOVAK, Prague, Czech Republic, November 3, 2021.

[20] **Dvorakova D.**, Buresova M., Pulkrabova J.: Stanovení per- a polyfluoralkylovaných sloučenin (PFAS) ve vodě, Seminář „Nová směnice EU pro pitnou vodu 2020/2184/ES“, Prague, Czech Republic, September 21, 2021.

[19] Buresova M., **Lankova D.**, Pulkrabova J.: Pitná voda – zdroj expozice perfluoralkylovaným sloučeninám, Výživa, potraviny a zdraví 2018, Prague, Czech Republic, December 5, 2018.

[18] Urbancova K., **Lankova D.**, Tomaniova M., Sram R.J., Hajslova J., Pulkrabova J.: Biomarkery expozice PAU u dětské populace –hodnocení rizik. Odborný pracovní seminář Znečištění ovzduší 2016 – důsledky pro zdraví naší populace, Czech Academy of Sciences, Prague, Czech Republic, December 12, 2016.

[17] Urbancova K., **Lankova D.**, Tomaniova M., Sram R.J., Hajslova J., Pulkrabova J.: Metabolity polycyklických aromatických uhlovodíků v moči jako markery lidské expozice, 18. Chromatografický seminář – Moderní analytické strategie pro ověření autenticity a kvality potravin, podporu zdraví a ochranu životního prostředí, Prague, Czech Republic, June 2, 2016.

[16] Drabova L., Pulkrabova J., **Lankova D.**, Urbancova K., Gramblicka T., Hajslova J.: Nové možnosti přečištění extraktů při analýze organických polutantů vpotravinách s vysokým obsahem tuku, Novinky a trendy Agilent Technologies, Prague, Czech Republic, April 12, 2016.

[15] **Lankova D.**, Vincikova A., Pulkrabova J., Hajslova J.: Halogenované kontaminanty a jejich deriváty v mořských plodech dostupných na českém trhu, XLIV. Symposium o nových směrech výroby a hodnocení potravin, Skalský Dvůr u Bystřice nad Pernštejnem, Czech Republic, May 25–27, 2015.

[14] **Lankova D.**, Urbancova K., Pulkrabova J., Hajslova J.: Nový analytický postup pro stanovení metabolitů PAH v moči, Seminář: Necílový screening: nová strategie pro laboratoře zaměřené na složky prostředí, potraviny a krmiva, Prague, Czech Republic, February 13, 2015.

[13] **Lankova D.**, Rodríguez B. S., Pulkrabova J., Hajslova J.: Výskyt estrogenních látek v mléce a mléčných výrobcích z obchodní sítě České republiky, Studentská konference výživa, potraviny a zdraví, Prague, Czech Republic, November 20, 2014.

[12] **Lankova D.**, Lacina O., Jandova R., Pulkrabova J., Hajslova J.: PFS v potravinách a potravinářských obalech, IV. mezinárodní konference Materiály a předměty určené pro styk potravinami, aktuální legislativní požadavky a úřední kontrola, Prague, Czech Republic, October 23, 2014.

[11] **Lankova D.**, Odlerova S., Rodríguez B S., Pulkrabova J., Hajslova J.: Výskyt estrogenních látek v mléce a mléčných výrobcích z obchodní sítě České republiky, XLIV. Symposium o nových směrech výroby a hodnocení potravin, Skalský Dvůr u Bystřice nad Pernštejnem, Czech Republic, May 26–28, 2014.

[10] **Lankova D.**, Rodríguez B. S., Pulkrabova J., Hajslova J.: Nová analytická strategie sledování širokého spektra přírodních a syntetických estrogenů v mléce a mléčných výrobcích, Toxiny v potravinách a prostředí: "Moderní koncepty pro kontrolu a výzkum", Prague, Czech Republic, February 14, 2014.

[9] Kalachova K., **Lankova D.**, Pulkrabova J., Lacina O., Hajslova J.: Prach v domácnostech: co všechno odhalí chromatografie?, Seminář: Chemie v potravinách a životním prostředí: "Jak nám chromatografie pomáhá v poznání?", Prague, Czech Republic, February 1, 2013.

[8] Lacina O., **Lankova D.**, Hlouskova V., Pulkrabova J., Hradkova P., Hajslova J.: Analýza poly- a perfluorovaných látek v potravinách a environmentálních matricích technikou LC/MS, Waters LC/MS seminar, Prague, Czech Republic, June 27, 2012.

[7] Lacina O., Pulkrabova J., Hradkova P., **Lankova D.**, Hajslova J.: Trace analysis of halogenated contaminants in food and environment by UHPLC—MS/MS, Prague, Czech Republic, June 6, 2012.

[6] Hradkova P., Lacina O., **Lankova D.**, Hlouskova V., Pulkrabova J., Hajslova J.: Použití metody LC–MS pro stanovení perfluorovaných a bromovaných kontaminantů ve vzorcích životního prostředí, Analýza organických látek, Valtice, Czech Republic, October 17–20, 2011.

[5] Pulkrabova J., Lacina O., **Lankova D.**, Hlouskova V., Pulkrabova J., Hajslova J.: Výskyt perfluorovaných látek v potravinách živočišného původu v ČR a Evropě, XLI. Symposium o nových směrech výroby a hodnocení potravin, Skalský Dvůr, Czech Republic, May 23–25, 2011.

[4] Lacina O., Pulkrabova J., Hradkova P., **Lankova D.**, Hajslova J.: LC—MS analýza organohalogenovaných kontaminantů, Seminář Nová témata v oblasti analýzy potravin a kontroly složek životního prostředí: „Požadavky na inovativní strategie v chromatografii", Prague, Czech Republic, February 10, 2011.

[3] Pulkrabova J., Lacina O., **Lankova D.**, Hajslova J.: Kontaminace bioindikátorových matric perfluorovanými a dalšími halogenovanými sloučeninami, Seminář: Nová témata v oblasti analýzy potravin a kontroly složek životního prostředí: „Požadavky na inovativní strategie v chromatografii", Prague, Czech Republic, February 10, 2011.

[2] **Lankova D.**: Analysis of PFCs in human breast milk, WORKSHOP on Perfluorinated compounds (PFCs) and their occurrence in food and environmental matrices, Prague, Czech Republic November 25, 2010.

[1] Kalachova K., Pulkrabova J., Drabova L., **Lankova D.**, Cajka T., Hajslova J.: Nová integrální strategie sledování organických polutantů v potravinách živočišného původu, XL. Symposium o nových směrech výroby a hodnocení potravin, Skalský Dvůr u Bystřice nad Pernštejnem, Czech Republic, May 3–5, 2010.

9.4 Poster Presentations

In the English Language

[50] Parizek O., Gramblicka T., Parizkova D., Polachova A., **Dvorakova D.**, Topinka J., Sram R., Pulkrabova J.: Assessment of 75 persistent organic pollutants in breast milk from two urban areas of the Czech Republic, 41st International Symposium on Halogenated Persistent Organic Pollutants, DIOXIN 2021, Xi´an, China, November 8–11, 2021.

[49] Tomasko J., Parizek O., Jurikova M., **Dvorakova D.**, Pulkrabova J.: Simultaneous sample-preparation method for the determination of endocrine disruptors (chlorinated paraffins, phthalates and bisphenols) in textile, 41st International Symposium on Halogenated Persistent Organic Pollutants, DIOXIN 2021, Xi´an, China, November 8–11, 2021.

[48] **Dvorakova D.**, Buresova M., Kotal F., Kozisek F., Pulkrabova J.: Monitoring of perfluoroalkylated substances in tap drinking water in the Czech Republic, 41st International Symposium on Halogenated Persistent Organic Pollutants, DIOXIN 2021, Xi´an, China, November 8–11, 2021.

[47] Polachova A., Gramblicka T., **Dvorakova D.**, Hajslova J., Pulkrabova J.: Assessment of body burden of Czech population to various groups of POPs, 9th International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic, November 5–8, 2019.

[46] **Lankova D.**, Buresova M., Hajslova J., Pulkrabova J.: Analysis of perfluoroalkylated substances in water, part B: Occurrence in tap water in the Czech Republic, 9th International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic, November 5–8, 2019.

[45] Gramblicka T., **Dvorakova D.**, Urbancova K., Pulkrabova J.: Urinary metabolites of organophosphorus flame retardants: A pilot study assessing exposure of Czech population, 9th International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic, November 5–8, 2019.

[44] Buresova M., **Lankova D.**, Pulkrabova J., Hajslova J.: Analysis of perfluoroalkylated substances (PFAS) in water, part A: SPE columns as a source of background contamination in PFAS trace analysis, 9th International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic, November 5–8, 2019.

[43] Buresova M., **Lankova D.**, Pulkrabova J.: UHPLC–MS/MS method for the determination of bisphenol A and its structural analogues in human hair, 11th International Symposium on Biological Monitoring in Occupational and Environmental Health, Leuven, Belgium, August 28–30, 2019.

[42] **Lankova D.**, Buresova M., Hajslova J., Pulkrabova J.: Perfluoroalkylated compounds in tap drinking water in Czech Republic, 39th International Symposium on Halogenated Persistent Organic Pollutants, DIOXIN 2019, Kyoto, Japan, August 25–30, 2019.

[41] Gramblicka T., Tomasko J., **Lankova D.**, Stupak M., Pulkrabova J.: Monitoring of multiple flame retardants in the black kitchen utensils, plastic toys and objects of everyday use, 19th International Conference on Chemistry & the Environment (ICCE), Thessaloniki, Greece, June 16–20, 2019.

[40] Urbancova K., **Lankova D.**, Sram R.J., Hajslova J., Pulkrabova J.: Comparison of monohydroxylated metabolites of polycyclic aromatic hydrocarbon concentrations in urine samples collected from mothers and their newborns living in two localities of the Czech Republic. 38th International Symposium on Halogenated Persistent Organic Pollutants, Krakow, Poland, August 26–31, 2018.

[39] Buresova M., **Lankova D.**, Pulkrabova J.: The LC–MS/MS analysis of bisphenol A and its structural analogues (BPB, BPF and BPS) in human urine and hair samples; 14th Annual LC/MS/MS Workshop on Environmental Applications and Food Safety; Barcelona, Spain, June 26–27, 2018.

[38] Urbancova K., **Lankova D.**, Hajslova J., Pulkrabova J.: Levels of phthalate and DINCH metabolites in urine samples from Czech mothers and newborns. 8th International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic, November 7–10, 2017.

[37] Svarcova A., **Lankova D.**, Hajslova J., Pulkrabova J.: The assessment of human dietary exposure to organic contaminants based on the total diet study, 8th International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic, November 7–10, 2017.

[36] Urbancova K., **Lankova D.**, Hajslova J., Pulkrabova J.: Levels of phthalate metabolites and bisphenol A in urine samples from Czech mothers and newborns, Dioxin 2017, Vancouver, Canada, August 20–25, 2017.

[35] Pulkrabova J., Gramblicka T., Jerabkova M., Svarcova A., **Lankova D.**, Hajslova J.: The new method for analysis of selected organophosphorus flame retardants in indoor dust, 37th International Symposium on Halogenated Environmental Pollutants and POPs (DIOXIN 2017), Vancouver, Canada, August 20–25, 2017.

[34] Pulkrabova J., Svarcova A., Gramblicka T., **Lankova D.**, Szakova J., Cerny J., Hajslova J.: The impact of the different fertilization on the soil contamination by organic pollutants, 37th International Symposium on Halogenated Environmental Pollutants and POPs (DIOXIN 2017),Vancouver, Canada, August 20–25, 2017.

[33] Gramblicka T., Jerabkova M., **Lankova D.**, Hajslova J., Pulkrabova J.: Simultaneous determination of perfluoroalkylated substances (PFAS) and organophosphorus flame retardants (OPFR) in dust; 54th North American Chemical Residue Workshop, Naples, Miami, USA, July 23–26, 2017.

[32] Gramblicka T., Jerabkova M., **Lankova D.**, Svarcova A., Hajslova J., Pulkrabova J.: Development of a multi-analytical method for the simultaneous determination of selected organophosphorus flame retardants (OPFRs) and perfluoroalkyl substances (PFAS) in indoor dust, ICCE 2017 16th International Conference on Chemistry and the Environment, Oslo, Norway, June 18–22, 2017.

[31] Pulkrabova J., Svarcova A., Gramblicka T., **Lankova D.**, Szakova J., Cerny J., Hajslova J.: Assessment of the impact of the different fertilization on the soil contamination by organic pollutants, 16th International Conference on Chemistry and the Environment, Oslo, Norway, June 18–22, 2017.

[30] Pulkrabova J., Urbancova K., **Lankova D.**, Hajslova J.: A novel strategy for determining metabolites of multiple-pollutants in urine, 130th Annual Meeting & Exposition, Dallas, Texas, USA, September 18–21, 2016.

[29] Pulkrabova J., **Lankova D.**, Urbancova A., Svarcova A., Stupak M., Sram R.J., Hajslova J.: The critical assessment of mothers´/newborns´ exposure pathway to PAHs, DIOXIN 2016 FIRENZE The 36th International Symposium on Halogenated Persistent Organic Pollutants, Florence, Italy, August 28–September 2, 2016.

[28] Svarcova A., Gramblicka T., **Lankova D.**, Vincikova A., Hajslova J., Pulkrabova J.: Flame retardants in human blood serum: integration of "novel" halogenated flame retardants into multi-analyte method, DIOXIN 2016 FIRENZE 36th International Symposium on Halogenated Persistent Organic Pollutants, Florence, Italy, August 28–September 2, 2016.

[27] Urbancova K., **Lankova D.**, Bacova K., Hajslova J., Pulkrabova J.: Simultaneous analysis of multiple contaminants in urine: bisphenol A, metabolites of phthalates and polycyclic aromatic hydrocarbons, DIOXIN 2016 FIRENZE 36th International Symposium on Halogenated Persistent Organic Pollutants, Florence, Italy, August 28–September 2, 2016.

[26] **Lankova D.**, Urbancova K., Rossner P., Rossnerova A., Ambroz A., Svecova V., Tomaniova M., Veleminsky M., Sram R.J., Hajslova J., Pulkrabova J.: Metabolites of polycyclic aromatic hydrocarbons metabolites (OH-PAHs) in urine collected from women and their newborns in the Czech Republic, DIOXIN 2016 FIRENZE 36th International Symposium on Halogenated Persistent Organic Pollutants, Florence, Italy, August 28–September 2, 2016.

[25] Drabova L., Pulkrabova J., **Lankova D.**, Gramblicka T., Lacina O., Hajslova J.: Critical assessment of clean-up techniques employed in analysis of organochlorine pesticides and persistent organic pollutants in fatty samples, 11th European Pesticide Residue Workshop, Limassol, Cyprus, May 24–27, 2016.

[24] Cerna M., Sochorova L., Hanzlikova L., Cejchanova M., **Lankova D.**, Maly M.: Czech human biomonitoring system enters the third decade of its activity. 2nd International Conference on Human Biomonitoring, Berlin, Germany, April 17–19, 2016.

[23] Hanzlikova L., Sochorova L., Cerna M., Drgacova A., Fialova A., **Lankova D.**, Pulkrabova J.: Biomonitoring of perfluorinated alkylated substances (PFAS) in human milk, Central and Eastern European Conference on Health and the Environment, Prague, Czech Republic, April 10–14, 2016.

[22] Urbancova K., **Lankova D.**, Sram J., Rossner P., Rossnerova A., Ambroz A., Hajslova J., Pulkrabova J.: Critical assessment of 11 polycyclic aromatic hydrocarbon metabolites in urine of Czech mothers and newborns, Central and Eastern European Conference on Health and the Environment, Prague, Czech Republic, April 10–14, 2016.

[21] Stahl-Zeng J., Jasak J., Sage A., Eilfeld A., **Lankova D.**, Hajslova J.: Screening and quantitation of veterinary drugs in food of animal origin by high sensitive triple TOF system, 7th International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic, November 3–6, 2015.

[20] Drabova L., **Lankova D.**, Gramblicka T., Stupak M., Pulkrabova J., Hajslova J.: Comparison of clean-up techniques for the determination of polycyclic aromatic hydrocarbons and persistent organic pollutants in fatty samples, 7th International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic, November 3–6, 2015.

[19] Urbancova K., **Lankova D.**, Gramblicka T., Drabova L., Pulkrabova J., Hajslova J.: "Loose the lipids, find your analytes" – the implementation of new sorbent for d-SPE in analysis of organohalogen compounds in food and complex biological matrices, 7th International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic, November 3–6, 2015.

[18] Svarcova A., Gramblicka T., **Lankova D.**, Hajslova J., Pulkrabova J.: A new multi-class method for determination of halogenated contaminants in human serum, 7th International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic, November 3–6, 2015.

[17] Svarcova .A, **Lankova D.**, Pulkrabova J., Hajslova J.: Development of analytical method for the determination of organophospate flame retardants and phthalates in dust, EMEC15 The 15th European Meeting on Environmental Chemistry, Brno, Czech Republic, December 3–6, 2014.

[16] **Lankova D.**, Urbancova K., Zuzankova J., Pulkrabova J., Hajslova J.: A liquid chromatography/tandem mass spectrometry (LC–MS/MS) method for determination of monohydroxylated polycyclic aromatic hydrocarbons metabolites (OH-PAHs) in human urine, EMEC15 The 15th European Meeting on Environmental Chemistry, Brno, Czech Republic, December 3–6, 2014.

[15] Pulkrabova J., **Lankova D.**, Socas-Rodríguez B. S., Hajslova J.: New approach for determination of different groups of estrogenic compounds in milk and milk products, AOAC Annual Meeting & Exposition, Boca Raton, Florida, USA, September 7–10, 2014.

[14] Slovakova M., **Lankova D.**, Kalachova K., Lacina O., Pulkrabova J., Hajslova J.: A novel method for evaluation of migration of fluorotelomer alcohols from food contact materials to food, 6th International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic, November 5–8, 2013.

[13] **Lankova D.**, Kalachova K., Svarcova A., Pulkrabova J., Hajslova J.: Occurence of organohalogenated compounds and PAHs in fish from Turkey, PART B: GC–MS/MS Determination of halogenated POPs and PAHs in fish, 6th International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic, November 5–8, 2013.

[12] **Lankova D.**, Svarcova A., Pulkrabova J., Hajslova J.: Occurrence of organohalogenated compounds and PAHs in fish from Turkey, PART A: UHPLC–MS/MS Determination of PFAS, BFRs and their hydroxylated metabolites in fish, 6th International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic, November 5–8, 2013.

[11] Hlouskova V., Hradkova .P, **Lankova D.**, Poustka J. Pulkrabova J., Hajslova J.: Rapid procedure for determination of 25 perfluorinated alkylated substances (PFAS) in sediment and soil, 4th International Workshop "Per- and Polyfluorinated Alkyl Substances – PFAS", Idstein, Germany, November 7–9, 2012.

[10] Slovakova M., **Lankova D.**, Pulkrabova J., Hajslova J.: The new effective method for determination of fluorotelomer alcohols in paper and board food contact materials, 5th International Workshop on Per- and Polyfluorinated Alkyl Substances-PFAS, Helsingør, Denmark, October 27–29, 2013.

[9] **Lankova D.**, Svarcova A., Pulkrabova J., Hajslova J.: The occurrence of perfluoroalkyl substances (PFAS) and other organohalogen pollutants in fish and seafood products, 5th International Workshop on Per- and Polyfluorinated Alkyl Substances-PFAS, Helsingør, Denmark, October 27–29, 2013.

[8] **Lankova D.**, Urbancova K., Pulkrabova J., Hajslova J.: UHPLC–MS/MS determination of perfluoroalkyl substances (PFAS) and other emerging contaminants in human serum and breast milk, 5th International Workshop on Per- and Polyfluorinated Alkyl Substances-PFAS, Helsingør, Denmark, October 27–29, 2013.

[7] Pulkrabova J., **Lankova D.**, Kalachova K., Hajslova J., Lacina O.: Rapid Method for Determination of HBCD Isomers in Fish and Fish Feed, 8th Annual LC/MS/MS Workshop on Environmental Applications and Food Safety, Barcelona, Spain, July 2–4, 2012.

[6] **Lankova D.**, Pulkrabova J., Lacina O., Kockovska M., Hajslova J.: Analysis of Fluorotelomer Alcohols (FTOHs) in Food Contact Materials, 8th Annual LC/MS/MS Workshop on Environmental Applications and Food Safety, Barcelona, Spain, July 2–4, 2012.

[5] **Lankova D.**, Lacina O., Pulkrabova J., Fischerova B., Hajslova J.: Perfluorinated Compounds in Human Breast Milk from the Czech Republic: Results of a Pilot Study, 1st FOODSEG Symposium, Berlin, Germany, June 21–22, 2012.

[4] **Lankova D.**, Pulkrabova J., Lacina O., Kockovska M., Hajslova J.: Critical comparison of two instrumental techniques, GC–MS and LC–MS, for the FTOHs determination in food contact materials, 5th International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic, November 1–4, 2011.

[3] Lacina O., Vaclavik L., **Lankova D.**, Pulkrabova J., Hradkova P., Hajslova J.: Analysis of polyfluorinated surfactants in food and food contact materials, 5th International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic, November 1–4, 2011.

[2] **Lankova D.**, Lacina O., Pulkrabova J., Fischerova B., Hajslova J.: Perfluorinated compounds in human breast milk from the Czech Republic: Results of a pilot study, 3rd International Workshop Anthropogenic Perfluorinated Compounds, Amsterdam, The Netherlands, June 15–17, 2011.

[1] Pulkrabova J., Lacina O., **Lankova D.**, Kalachova K., Hajslova J.: Novel LC–MS strategy to monitore brominated flame retardants together with their metabolites in food matrices, Dioxin 2010, San Antonio, USA, September 12–17, 2010.

10 APPENDICES

Appendix I

Svarcova A., **Lankova D.**, Gramblicka T., Stupak M., Hajslova J., Pulkrabova J.: Integration of five groups of POPs into one multi-analyte method for human blood serum analysis: An innovative approach within biomonitoring studies. *Science of the Total Environment* (2019) 667: 701–709.

Appendix II

Lankova D., Lacina O., Pulkrabova J., Hajslova J.: The determination of perfluoroalkyl substances, brominated flame retardants and their metabolites in human breast milk and infant formula. *Talanta* (2013) 117: 318–325.

Appendix III

Urbancova K., **Lankova D.**, Sram R.J., Hajslova J., Pulkrabova J.: Urinary metabolites of phthalates and di-iso-nonyl cyclohexane-1,2-dicarboxylate (DINCH) – Czech mothers´ and newborns´ exposure biomarkers. *Environmental Research* (2019) 173: 342–348.

Appendix IV

Lankova D., Urbancova K., Sram R.J., Hajslova J., Pulkrabova J.: A novel strategy for the determination of polycyclic aromatic hydrocarbon monohydroxylated metabolites in urine using ultra-high-performance liquid chromatography with tandem mass spectrometry. *Analytical and Bioanalytical Chemistry* (2016) 408: 2515–2125.

Appendix V

López M.A., Göen T., Mol H., Nübler S., Haji-Abbas-Zarrabi K., Koch H.M., Kasper-Sonnenberg M., **Dvorakova D.**, Hajslova J., Antignac J.P., Vaccher V., Elbers I., Thomsen C., Vorkamp K., Pedraza – Díaz S., Kolossa-Gehring M., Castaño A.: The European human biomonitoring platform - Design and implementation of a laboratory quality assurance/quality control (QA/QC) programme for selected priority chemicals. *International Journal of Hygiene and Environmental Health* (2021) 234: 113740.

Appendix VI

Dvorakova D., Pulkrabova J., Nübler S., Haji-Abbas-Zarrabi K., Göen T., Mol H., Koch H. M., Vaccher V., Antignac J.P., Haug L. S., Vorkamp K., López M.E., Castaño A., Hajslova J.: Interlaboratory Comparison Investigations (ICIs) and External Quality Assurance Schemes (EQUASs) for Flame Retardants Analysis in Biological Matrices: Results from the HBM4EU Project. *Environmental Research* (2021) 202: 111705.

Appendix VII

Polachova A., Gramblicka T., Bechynska K., Parizek O., Turnerova D., **Dvorakova D.**, Honkova K., Rossnerova A., Rossner P., Sram R. J., Topinka J., Pulkrabova J.: Biomonitoring of 89 POPs in blood serum samples of Czech city policemen. *Environmental Pollution* (2021) 291: 118140.

Appendix VIII

Richterova D., Fabelova L., Patayova H., Pulkrabova J., **Lankova D.**, Rausova K., Sovcikova E., Stencl J., Hajslova J., Trnovec T., Palkovicova Murinova L.: Determinants of prenatal exposure to perfluoroalkyl substances in the Slovak birth cohort. *Environment International* (2018) 121 (2):1304–1310.

Appendix IX

Cerna M., Grafnetterova A.P., **Dvorakova D.**, Pulkrabova J., Maly M., Janos T., Vodrazkova N., Tupa Z., Puklova V.: Biomonitoring of PFOA, PFOS and PNFNA in human milk from Czech Republic, time trends and estimation of infant's daily intake. *Environmental Research* (2020) 188: 109763.

Appendix X

Urbancova K., **Dvorakova D.**, Gramblicka T., Sram R.J., Hajslova J., Pulkrabova J.: Comparison of polycyclic aromatic hydrocarbon metabolite concentrations in urine of mothers and their newborns. *Science of the Total Environment* (2020) 723:138116.

Appendix XI

Urbancova K., **Lankova D.**, Rossner P., Rossnerova A., Svecova V., Tomaniova M., Veleminsky M., Jr., Sram R. J., Hajslova J., Pulkrabova J.: Evaluation of 11 polycyclic aromatic hydrocarbon metabolites in urine of Czech mothers and newborns. *Science of the Total Environment* (2017) 577: 212–219.

APPENDIX I

Svarcova A., Lankova D., Gramblicka T., Stupak M., Hajslova J., Pulkrabova J.

Integration of five groups of POPs into one multi-analyte method for human blood serum analysis: An innovative approach within biomonitoring studies

Analytical and Bioanalytical Chemistry 405 (2013) 7829–7839

doi: 10.1016/j.scitotenv.2019.02.336

Science of the Total Environment 667 (2019) 701–709

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Integration of five groups of POPs into one multi-analyte method for human blood serum analysis: An innovative approach within biomonitoring studies

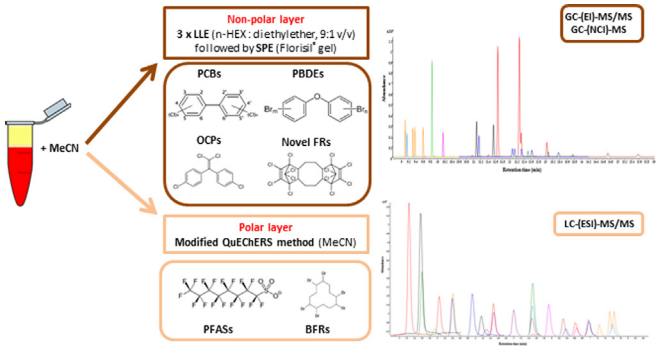
Andrea Svarcova, Darina Lankova, Tomas Gramblicka, Michal Stupak, Jana Hajslova, Jana Pulkrabova *

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

HIGHLIGHTS

- A novel multi-analyte procedure for assessment of human blood serum was developed.
- Only 3 mL of sample for determination of all 78 various analytes is needed.
- Benefit of this approach is analysis of FRs, PFASs, OCPs, PCBs in a single sample.
- 10 PFASs, 10 OCPs, 8 PCBs and 6 PBDEs were detected in human serum.
- PFASs were the dominating group of all monitored contaminants in human serum.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 4 December 2018
Received in revised form 21 February 2019
Accepted 21 February 2019
Available online 22 February 2019

Editor: Damia Barcelo

Keywords:
Human blood serum
Organohalogenated contaminants
Sample preparation
GC–MS
LC-MS

ABSTRACT

Within this study, a new analytical strategy was developed and validated for the simultaneous determination of 78 organohalogenated contaminants in human blood serum, namely 40 flame retardants (FRs) including 7 "novel" brominated and chlorinated FRs (novel FRs), 19 perfluoroalkylated substances (PFASs), 11 organochlorine pesticides (OCPs) and 8 polychlorinated biphenyls (PCBs). The integral sample preparation procedure was implemented for the isolation of non-polar compounds, based on three-step solvent extraction using a mixture of *n*-hexane:diethylether (9:1, *v*/*v*), followed by purification using a solid-phase extraction (SPE) on a Florisil® column. For isolation of more polar and lipophobic analytes, the remaining fraction from the first extraction step was further processed, using a modified QuEChERS method. Depending on the polarity and volatility of target compounds, either gas chromatography coupled to (tandem) mass spectrometry (GC–MS/(MS)), or ultra-high performance liquid chromatography coupled to triple quadrupole tandem mass spectrometry (UHPLC-MS/MS), was employed for their identification/quantification. Within the subsequent pilot study, the new validated procedure was successfully applied to the monitoring of organohalogenated contaminants in 38 samples of human blood serum obtained from Prague, Czech Republic. From 78 targeted analytes, 10 PFASs, 10 OCPs, 8 PCBs and 6 BFRs were detected in serum at concentrations above method quantification limits (MQLs). In the serum samples, the amounts of determined PFASs were in the range < 0.01–8.97 ng mL^{-1} (mean 0.631 ng mL^{-1}), OCPs and PCBs ranged from <0.1–1626 ng g^{-1} lw (mean 40.0 ng g^{-1} lw) and < 0.1–481 ng g^{-1} lw (mean 63.3 ng g^{-1} lw), respectively.

* Corresponding author.
E-mail address: jana.pulkrabova@vscht.cz (J. Pulkrabova).

https://doi.org/10.1016/j.scitotenv.2019.02.336
0048-9697/

1. Introduction

The key objective of a biological monitoring (biomonitoring) is the assessment of human exposure to various groups of environmental chemicals. The target analytes, which could be either the parent compounds or their metabolites, are considered as 'biomarkers' of exposure and are linked to the concentration of their internal dose. The most commonly analysed matrices are blood (serum or plasma), human milk, urine, adipose or other tissues (Needham et al., 2007).

As regards organohalogenated pollutants, polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), brominated flame retardants (BFRs) represented mainly by polybrominated diphenyl ethers (PBDEs) have been widely studied in human biological samples, specifically in their lipid fraction. In the recent decade, the list of priority has been extended by perfluoroalkylated substances (PFASs), mainly to perfluorooctane sulphonate (PFOS) and perfluorooctanoic acid (PFOA), which are due to their affinity to proteins widespread in breast milk and blood serum (Ben Hassine et al., 2014; Mørck et al., 2015; Whitehead et al., 2015). While assessing human exposure based on the analysis of contaminants in breast milk (i.e. matrix which, due to its non-invasive collection, is fairly popular) provides information on the body burden of only limited population groups, the analysis of blood serum/plasma samples enables the exposure of all population groups to be monitored. It is worth noting that, to date, many biomonitoring studies have only been focused on one or a few groups of pollutants possessing the same or a very similar mode of action. However, the health risks to humans are commonly derived from exposure to multiple chemicals – 'chemical mixtures' – thus their 'cocktail effects' should be considered (Gribble et al., 2015; Nøst et al., 2017; Salihovic et al., 2013b). Recently, the extensive Human Biomonitoring project HBM4EU (https://www.hbm4eu.eu/) as a joint effort of 28 countries, the European Environment Agency and the European Commission, has been initiated. The key objective is a better understanding of the dose–response relationship and the associated health impacts. In this context, the improvement of chemical risk assessment and the support of policy making should be achieved.

Under these conditions, the implementation of high throughput reliable methods for multiple contaminants' analysis is obviously a challenging task. In general terms, the most critical step in analysis of the above-mentioned organohalogenated pollutants in blood serum is the sample processing procedure, since the volume of available biological fluid is usually rather limited. In general, the sample quantity needed for the analysis is variable, ranging from 0.2 to 40 mL (Coakley et al., 2018; Flores-Ramírez et al., 2017; Nøst et al., 2017; Thuresson et al., 2006), depending on the target compounds (concentrations, polarity) and the selected analytical method. Focusing on studies from the past three years, the most common quantity of sample for analysis is 0.2–2 mL (Cequier et al., 2015; Gribble et al., 2015; Moon et al., 2017; Salihovic et al., 2015; Whitehead et al., 2015). In most cases, the denaturing and precipitating agents (formic and hydrochloric acid, methanol, ethanol, acetonitrile and 2-propanol) are added to the serum samples before the extraction process. Typical isolation methods for PCBs, OCPs, BFRs and novel FRs are liquid-liquid extraction (LLE) and solid-phase extraction (SPE; sorbents: C_{18} and Oasis HLB), employing non-polar solvents or their mixtures: hexane, dichloromethane and diethylether. For the extraction of PFASs, polar solvents e.g. methanol and acetonitrile are applied. The purification process of the crude extracts is often performed by SPE columns employing the sorbents silica, C_{18} and HLB (Salihovic et al., 2013a).

Currently, there is no published analytical procedure for the simultaneous determination of PCBs, OCPs, FRs and PFASs in human serum samples. Typically, one or two groups of contaminants (PFASs; PCBs and OCPs; PCBs and BFRs) are most often determined in serum (Porta et al., 2012; Ulutaş et al., 2015; Wang et al., 2018). Only one study, published by Long et al., 2015, deals with an occurrence such as a broader spectrum of organohalogenated contaminants ($n = 45$; PFASs, OCPs, PCBs and PBDEs) in human blood samples. However, within the isolation of target compounds, the serum sample was separated into several parts and the extraction methods were used separately for OCPs and PCBs; PBDEs and PFASs. This study mainly summarises the findings of monitored contaminants in serum samples of pregnant women (Long et al., 2015).

The main aims of this study were (i) to develop an innovative sample preparation procedure, enabling the isolation of the precedence of multiple organohalogenated contaminants represented by PCBs, OCPs, FRs and PFASs from a single 3 mL human serum sample, and (ii) to apply the new strategy for the analysis of biomonitoring samples aimed at the assessment of human exposure to multiple organohalogenated contaminants.

2. Materials and methods

2.1. Standards

The target analytes including CAS number, concentration and other information are summarised in Tables S1 and S2 in the Supplementary data. Certified standards of BFRs, represented by 16 PBDE congeners and other BFRs (PBEB, HBB, PBT, OBIND, BTBPE and DBDPE), HBCD isomers (α-, β- and γ-), TBBPA were obtained from Wellington Laboratories (Guelph, Ontario, Canada). Three brominated phenols and individual standards of 4 OH-PBDEs and 7 novel FRs (DBE-DBCH, TBCO, DPTE, EH-TBB, HCDBCO, DP syn and DP anti) were purchased from AccuStandard (New Haven, CT, USA). The individual standards of 19 PFASs were purchased from Wellington Laboratories. A standard mixture of PCBs consisting of 6 congeners (No. 28, 52, 101, 138, 153 and 180), the individual standards of CB 170 and CB 118, standards of 11 OCPs were obtained from Dr. Ehrenstorfer (Augsburg, Germany). The other individual PCB standards of CB 65 and 166 were purchased from Absolute Standards, Inc. (Hamden, USA). Isotopically labelled internal standards of PFASs and BFRs were supplied by Wellington Laboratories. All standards were obtained with the highest available purity (>97%).

The working solutions of standard mixtures of PFASs and OH-PBDEs were prepared in methanol at concentrations of 0.25–100 ng mL^{-1}; the concentrations of HBCD isomers, TBBPA and brominated phenols were 5 times higher. Each calibration standard contained labelled internal standards of ^{13}C-BFRs at 10 ng mL^{-1} and ^{13}C-PFASs at 1 ng mL^{-1}. Similarly, calibration solutions with PCBs, OCPs, novel FRs and BDE 28-203, PBT, PBEB, HBB, OBIND and BTBPE at concentration levels of 0.05–500 ng mL^{-1} and BDE 206-209 and DBDPE at 0.25–2500 ng mL^{-1} were prepared in isooctane. Each calibration level of novel FRs, BFRs and OCPs, PCBs contained surrogate standards, such as BDE 37, BDE 77 at 5 ng mL^{-1}, $^{13}C_{12}$-BDE 209 at 50 ng mL^{-1} and CB 65, CB 166 at 5 ng mL^{-1}, respectively. All solutions were stored at 5 °C in the refrigerator.

The standard reference material of fortified human blood serum SRM 1958 used for the validation experiments was obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, Maryland, USA).

2.2. Chemicals, reagents and other materials

Diethylether, dichloromethane, *n*-hexane, methanol, sulphuric acid (98%) and isooctane were supplied by Merck (Germany); acetonitrile, formic acid (98%) and magnesium sulphate (98%) from Sigma-Aldrich (USA); sodium chloride (99.9%) and acetone from Penta (Czech Republic). Polypropylene (PP) centrifuge tube filters (nylon, pore size 0.22 μm) were obtained from Sigma-Aldrich. Silica (0.15–0.25 mm) supplied by Merck was activated by heating at 180 °C for 5 h, then deactivated by adding 2% of deionised water, shaken for 3 h and stored in a desiccator for 12 h before use. Florisil® for residual analysis (0.15–0.25 mm) provided by Merck was activated by heating at 600

°C for 4 h, then at 130 °C for 5 h and finally stored in a desiccator. The human blood serum used for the method development and validation experiments was supplied by Sigma-Aldrich (USA).

2.3. Sample collection

All 38 samples of human blood serum were obtained within cooperation with The National Institute of Public Health (Prague, Czech Republic). The sampling was carried out in Prague in 2015. The serum samples (2–4 mL) were stored in the freezer at −80 °C until analysis.

2.4. Optimisation of the sample preparation procedure

Within the optimisation experiments, several parameters had to be tested. The sample preparation procedure for the isolation of non-polar analytes amenable to GC–MS (PBDEs, PBEB, HBB, PBT, OBIND, BTBPE, DBDPE; novel FRs; OCPs and PCBs) was inspired by the method described in the Swedish study concerned with PBDEs (Darnerud et al., 2015), in which serum was mixed with methanol, then followed by the repeated extraction of analytes with a mixture of *n*-hexane:diethylether (1:1, *v*/*v*).

Our method is described as follows: Firstly, there is an integrated step for all targeted compounds based on the weighing of 3 mL serum and the addition of 3 mL acetonitrile for protein precipitation. For the isolation of non-polar compounds, the time of shaking (1 min, 2 min and 3 min), a number of repeated extraction steps (2 or 3) and the various ratios of *n*-hexane:diethylether mixtures (1:1; 4:1 and 9:1, *v*/*v*) were tested. As a purification step of a non-polar extract intended mainly for removing lipids, an SPE column (0.5 g) with 2 different sorbents, namely Florisil® and silica, were tested. Before the application of a sample extract, the sorbent was conditioned by 4 mL of *n*-hexane: dichloromethane (3:1, *v*/*v*), followed by 3 mL of *n*-hexane. The elution profile of target non-polar compounds from SPE columns was verified by loading 1 mL of standard solution (mixture of FRs, PCBs and OCPs at concentration 50 ng mL^{-1} in hexane). Thereafter, fractions of 1 mL (10 mL in total) of *n*-hexane:dichloromethane (3:1, *v*/*v*) were collected, then evaporated (BüchiRotavapor R-114, R-200, Büchi, Switzerland) and re-dissolved in 0.2 mL of isooctane for GC–MS analysis.

For the more polar and lipophobic compounds amenable to LC-MS (HBCD isomers (α-, β- and γ), TBBPA, OH-PBDEs, brominated phenols and PFASs) which remain associated with the residues after the first extraction step, the modified QuEChERS-like extraction with acetonitrile, water and added inorganic salts (sodium chloride 1 g and magnesium sulphate 4 g) was tested. After evaporation of the MeCN fraction, the residues were dissolved in 0.25 mL of methanol and prepared for measurement by ultra-high performance liquid chromatography coupled to triple quadrupole tandem mass spectrometry (UHPLC-MS/MS). To avoid losses of the individual analytes, the distribution of all target compounds between individual fractions (non-polar or more polar) was also verified. For this purpose, before a purification step (SPE columns) performed within the extraction of non-polar compounds measured by GC–MS, 100 μl of non-polar extract was taken and was measured by LC-MS/MS.

2.4.1. Final sample preparation procedure

3 mL of thawed serum was weighed into the polypropylene tube and then the surrogate standards were added, specifically 0.04 mL of a mixture of ^{13}C-PFASs and ^{13}C-HBCDs, ^{13}C-TBBPA at the concentration 10 ng mL^{-1} and 100 ng mL^{-1} and 0.03 mL of a mixture of BDE 37, BDE 77, CB 65, CB 166 and $^{13}C_{12}$-BDE 209 at the concentration 50 ng mL^{-1} and 500 ng mL^{-1}. The sample was mixed with 3 mL acetonitrile for the protein precipitation and the tube was shaken for 2 min.

Within the first step, 6 mL *n*-hexane:diethylether (9:1, *v*/*v*) was added to the tube containing 3 mL serum with the surrogate standards plus 3 mL acetonitrile. This solvent mixture was used for the extraction of non-polar compounds (PCBs, OCPs, Novel FRs, PBDEs and BFRs) and the tube was shaken again for 2 min. The tube was centrifuged for 5 min at a speed of 5000 rpm. The upper organic layer was transferred into a new flask. The extraction step was repeated twice with an additional 3 mL *n*-hexane:diethylether (9:1,*v*/*v*) to the original tube, and the organic layers were combined (altogether 10.5 mL obtained). To remove water residues, the combined organic phases were filtered through the anhydrous sodium sulphate into the pre-weighed flask. Extracts were concentrated using a vacuum rotary evaporator. The solvent residue was evaporated using a gentle stream of nitrogen, and the lipid weight was determined gravimetrically. The lipids were dissolved in 0.5 mL of *n*-hexane for the subsequent clean-up step. An aliquot of 0.1 mL was transferred into the vial for the LC–MS analysis of N-MeFOSA, N-EtFOSA, 2,4-DBP, HBCD isomers and OH-PBDEs. The remaining 0.4 mL was purified using solid-phase extraction on a Florisil® column (0.5 g). The column was conditioned with 4 mL of *n*-hexane:dichloromethane (3:1,*v*/*v*) and then 3 mL *n*-hexane. After that, the sample was loaded on to the column, and target analytes were eluted by 6 mL of the mixture *n*-hexane:dichloromethane (3:1, *v*/*v*). The obtained eluate was evaporated using the vacuum rotary evaporator, and the remaining solvent was removed by a gentle stream of nitrogen. The residue was dissolved in 0.2 mL of isooctane.

In the second step, the extraction of more polar substances (PFSAs, PFCAs, FOSA, TBBPA, PBP and 2,4,6-TBP) was performed by the modified QuEChERS-like extraction, when 5 mL acetonitrile and 5 mL deionised water were added to the serum sample (mixture of 3 mL serum and 3 mL acetonitrile prepared at the beginning of the extraction), which remained in the tube after removal of non-polar solvents in the previous extraction step. Then, the tube was shaken (1 min). Subsequently, NaCl (1 g) and $MgSO_4$ (4 g) were added, followed by further shaking for 1 min. After shaking, the extract was centrifuged and 5 mL of the organic (acetonitrile) phase was transferred into the flask and evaporated. Finally, the residues were dissolved in 0.25 mL of methanol. The extract was filtrated through a nylon filter (0.22 μm) and transferred into the vial for the LC–MS analysis. For the compounds determined by the LC-MS/MS, the step of purification was not implemented in the analytical process. Only minor impurities were removed by the aforementioned microfiltration.

The scheme of the novel sample preparation procedure employed for the determination of the set of organohalogenated pollutants method is shown in Fig. 1.

2.4.2. Validation experiments

The validation experiments were performed on the artificially contaminated human blood serum ($n = 6$), in which the occurrence of target compounds was previously examined and subtracted from the determined concentrations. The repeatabilities (expressed as relative standard deviations, RSD %) and recoveries (REC %) were obtained at two levels (high and low level), namely 1 and 10 ng g^{-1} lw for PCBs, OCPs, novel FRs; 0.2 and 1 ng g^{-1} lw for BFRs; 0.05 and 0.3 ng mL^{-1} for PFASs and OH-BDEs and 0.3 and 1.5 ng mL^{-1} for HBCDs, TBBPA and brominated phenols. To verify the applicability of the new analytical procedure, the validation experiments were also performed on the SRM 1958 (the measured and calculated concentrations and standard deviations were compared to the certified concentration values for PCBs, OCPs, PBDEs and PFASs). To control background contamination by target compounds, a procedural blank (the same sample preparation, only instead of the serum, deionised water was used) was prepared together with each batch of samples. The method quantification limits (MQLs) for all compounds were characterised as the lowest calibration standard at which the quantification and identity confirmation transitions or ions provided signal-to-noise ratio (S/N) > 10 for the quantitative transition/ion and $S/N > 3$ for at least one confirmation transition/ion.

However, for the control of the extraction efficiency (recoveries of target analytes) and correction of possible matrix effects, the isotopically labelled surrogates (^{13}C-PFASs, ^{13}C-HBCDs and ^{13}C-TBBPA) were

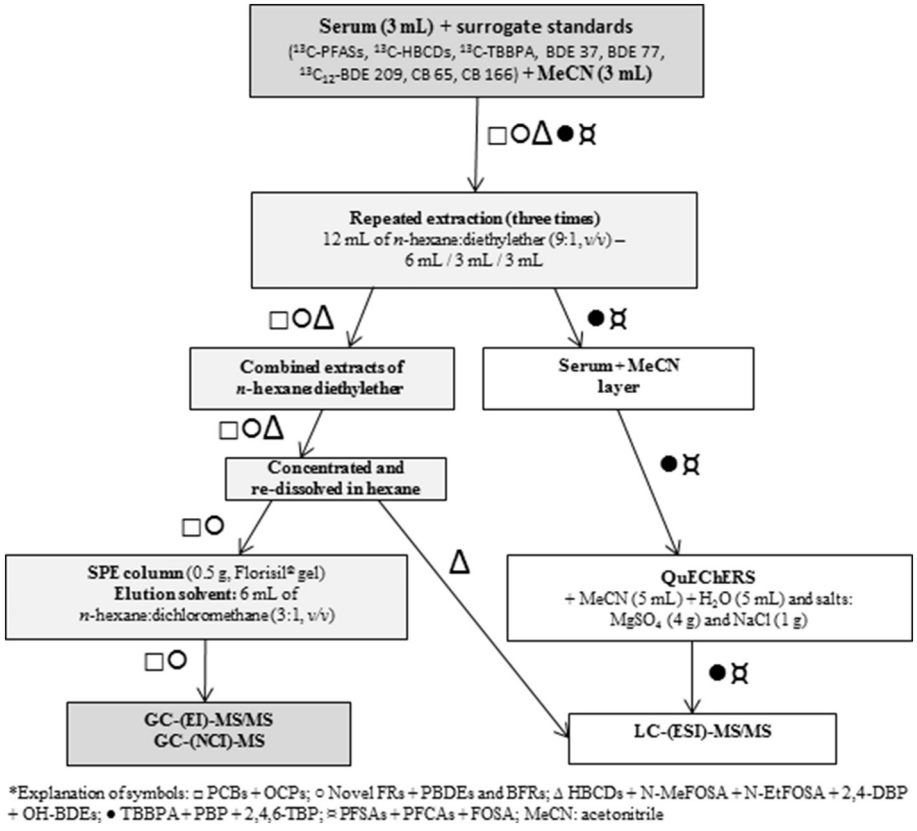


Fig. 1. A scheme of analytical method for the determination of PCBs, OCPs, BFRs, novel FRs and PFASs in serum.

used. Moreover, the standards of BFR (BDE 37, BDE 77 and ^{13}C-BDE 209) and PCB (CB 65 and CB 166) were also applied for the compensation of matrix effects mainly within the elimination of negative influences in the GC injector transferred into the vial for the GC analysis.

2.5. Instrumental analysis

The analysis of PCBs, OCPs, novel FRs and BFRs (PBDEs, PBEB, HBB, PBT, OBIND, BTBPE, DBDPE) was carried out on GC–MS/(MS). These measurements were performed using a gas chromatograph Agilent 7890A GC (Agilent Technologies, USA), coupled to a triple quadrupole mass spectrometer Agilent 7000B MS (Agilent Technologies, USA) operated in an electron ionisation (EI) mode for PCBs and OCPs. Within PCBs' and OCPs' measurements, more detailed information is summarised in the study published by (Kalachova et al., 2013b). During the MRM optimisation, the quantification transition *m/z* 292 → 220 and two confirmatory transitions *m/z* 292 → 257, 292 → 222 for CB 65 and the quantification transition *m/z* 360 → 290 and two confirmatory transitions *m/z* 360 → 325, 362 → 290 for CB 166 were optimised and applied.

A GC–MS method, employing the triple quadrupole mass spectrometer (QqQ) as a single quadrupole in a negative chemical ionisation (NCI) for BFRs and novel FRs, was also used. The GC–MS method applied for analysis of BFRs is described in detail by the study (Kalachova et al., 2013a). Regarding the analysis of novel FRs, the ion *m/z* 81 and the ions *m/z* 79 and 160 were used for quantification and confirmation purposes for DBE-DBCH, TBCO, EH-TBB and DPTE. For the determination of HCDBCO, the ions *m/z* 79 and *m/z* 542 were also used. The ion *m/z* 654 and the ions *m/z* 652, 656 were applied for the quantification/confirmation of DP syn and DP anti.

The determination of PFASs, HBCDs, TBBPA, OH-PBDEs and brominated phenols was performed by UHPLC–MS/MS. The Acquity Ultra-Performance LC system (Waters, USA) was used, hyphenated with a tandem quadrupole mass spectrometer XEVO TQ-S (Waters), operated in the multiple reaction monitoring mode (MRM) with electrospray in a negative ionisation mode (ESI). Further information about the LC–MS method is described in detail by the study (Lankova et al., 2015).

Examples of the GC and LC chromatograms of the serum sample, spiked with the mixture of all target contaminants at the higher level, are shown in Fig. S1 in the Supplementary data.

3. Results and discussion

3.1. Optimisation of the sample preparation procedure

As mentioned in the Introduction, monitoring of multiple environmental pollutants in biological matrices, such as human blood serum, is commonly involved in current biomonitoring studies (HBM4EU). With regard to a rather limited volume of available biological fluid and considering the (ultra)trace concentrations of target analytes, an effective sample processing procedure has to be selected. This task becomes even more demanding when several groups of pollutants differing largely in their physico-chemical properties are targeted. That was the case of our study, in which both lipophilic analytes represented by FRs, PCBs and OCPs, and lipophobic PFASs were to be monitored. In most monitoring surveys, the sample has to be split and processed separately using different procedures (Coakley et al., 2018; Nøst et al., 2017). To date, only the study (Long et al., 2015) reporting the analysis of both groups of these pollutants, namely POPs (11 OCPs, 14 PCBs and 5 PBDEs) and 14 PFASs, in blood serum has been published. Nevertheless, the extraction methods for analysis of these two groups of contaminants are also performed separately in two isolation steps, not in one weighted sample. In the latest study (Koponen et al., 2013), POPs (6 OCPs, 6 PCBs and BDE 47) and 13 PFASs were analysed, but the range of pollutants was not as extensive as in our study. In addition, within these studies, the novel FRs were not included. On this account, we decided to implement an optimised sample handling strategy enabling

high recoveries of various pollutants, based on the set of consecutive steps performed on a single sample.

In the first phase, the extraction procedure originally developed for PBDEs analysis in blood serum was employed to test whether it is also applicable to other lipophilic analytes. The isolation of non-polar compounds was inspired by the two-times repeated extraction of PBDEs with *n*-hexane:diethylether (1:1, *v*/*v*), followed by the gravimetric determination of lipids (Darnerud et al., 2015). Firstly, the original procedure was tested for the isolation of BFRs, PCBs and OCPs. However, insufficient recoveries (33–56%), especially for non-PBDE BFRs, PCBs (42–72%) and OCPs (40–69%), were achieved. For this reason, the extraction time and number of extraction cycles were modified. The times of extraction/shaking were extended from 1 to 2 min. Three minute shaking was also tested, but did not provide better results. Moreover, a third repetition of extraction with 3 mL of *n*-hexane:diethylether (1:1, *v*/*v*) was involved in the procedure. Compared to the original method (2 × 1 min extraction), fairly higher recoveries for BFRs, PCBs and OCPs (59–74%) were obtained when applying a three-step solvent extraction with 2 minute shaking. For the testing of isolation efficiency of non-polar analytes, various compositions of extraction solvent mixture using *n*-hexane:diethylether (1:1, 4:1 and 9:1; *v*/*v*) were compared. The best extraction efficiency was achieved with the most non-polar solvent mixture, *n*-hexane:diethylether (9:1, *v*/*v*); the recoveries of selected PCBs, OCPs and BFRs ranged from 76 to 111%. It is worth noting that the most significant improvement of extraction efficiency was achieved for PCBs.

Within the following experiment, for all target compounds isolated in the non-polar extract, the elution profiles on silica and Florisil® SPE columns were tested by applying 1 mL of standard solution. All monitored substances were eluted with 6 mL of the mixture of *n*-hexane:dichloromethane (3:1, *v*/*v*). The clean-up step on a silica and Florisil® SPE column was further verified by the purification of a spiked serum extract at concentration level 10 ng g^{-1} lw (six replicate analysis). Within an optimisation of the purification process for novel FRs, better recoveries 70–113% (RSD < 22%) for all analytes ($n = 7$) were obtained when the Florisil® SPE column was used. Regarding the silica SPE column, recoveries varied in a wide range 56–150% (RSD < 8%). Lower recoveries were obtained mainly for novel FRs, such as DBE-DBCH, TBCO, DPTE, DP syn and DP anti, and higher recoveries for EH-TBB and HCDBCO.

Finally, the newly developed method (three-step extraction with *n*-hexane:diethylether (9:1, *v*/*v*); clean-up step using Florisil® SPE column) was validated for the determination of PCBs, OCPs, BFRs and also for novel FRs in human blood serum.

The isolation of more polar/lipophobic compounds, represented primarily by HBCD isomers (α-, β- and γ), TBBPA, OH-PBDEs, brominated phenols and PFASs, was performed by the procedure, when the residual fraction obtained after the isolation of non-polar compounds was extracted by the mixture of acetonitrile and deionised water and the separation of aqueous and organic layers was induced by the addition of inorganic salts. The partition of all analytes between both *n*-hexane:diethylether (9:1, *v*/*v*) and acetonitrile layers was also investigated. In the case of LC–MS amenable analytes present in the first non-polar fraction, namely N-MeFOSA, N-EtFOSA, 2,4-DBP, HBCD isomers and OH-PBDEs, the clean-up step using a Florisil SPE column could not be used, because these substances were bound to the sorbent and could not be eluted with the other compounds. Therefore, an aliquot of 100 μL of hexane was transferred to the vial before purification on the Florisil column and directly measured using LC-MS/MS.

In summary, the sample preparation procedure consisted of the three-step solvent extraction with *n*-hexane:diethylether (9:1, *v*/*v*), followed by the purification on the SPE column (0.5 g, Florisil®). The modified QuEChERS-like extraction, employing acetonitrile applied for the isolation of the most polar compounds from the aqueous phase from the first extraction step, was used for the final validation of the new multi-analyte procedure.

3.2. Method validation

As described in the *Experimental part*, the method validation was performed using blood serum spiked at two concentration levels, adjusted to typical concentrations reported for the respective groups of pollutants in other studies. In Table S3 in the Supplementary data, recoveries and repeatabilities (expressed as a relative standard deviation, RSD, %) and MQLs of the newly validated method for the determination of various groups of environmental contaminants in blood serum are summarised. The recoveries of compounds measured by GC–MS/MS at both concentration levels ranged from 70 to 115% (RSD <18%) for FRs, 74 to 120% (RSD <20%) for OCPs and PCBs, with relevant MQLs reaching 0.05–2.5 ng g^{-1} lw (Table S3). The recoveries of LC–MS/MS amenable analytes were in the range of 79–120% (RSD <20%) for PFASs and OH-BDEs, 71–116% (RSD <19%) for HBCDs, TBBPA and brominated phenols and MQLs ranged from 0.01 to 0.3 ng mL^{-1}.

Moreover, the entire analytical method was also tested on the SRM 1958. The performance characteristics as recoveries and repeatabilities were calculated from the 7 replicate analyses of the SRM 1958 (Table 1). The appropriate recoveries ranged from 97 to 100% (RSD <7%) for PFASs; from 83 to 112% (RSD <18%) for OCPs; from 86 to 113% (RSD <10%) for PCBs, and from 72 to 119% (RSD <17%) for PBDEs. For more details, see Table 1. The results of all monitored organohalogenated contaminants (PFASs, PBDEs, OCPs and PCBs) were in accordance with the certified values.

3.3. Application of the newly developed multi-analyte method

The new multi-analyte approach was finally applied within a pilot monitoring survey, aiming to determine the concentrations of different groups of organohalogenated pollutants in the human blood serum samples ($n = 38$) which were collected in Prague (Czech Republic). The obtained results are summarised in Table 2. 50% of 78 target compounds were present above the MQLs (PFASs = 10, OCPs = 10; PCBs = 8 and BFRs = 6). The most abundant contaminants were PFOA, PFNA, PFDA, PFUndA, Br-PFOS, L-PFOS, HCB and *p*,*p*′-DDE found in all examined samples, followed by CB 138, CB 153 and CB 180; CB 170, α-HCH and *p*,*p*′-DDT; CB 28; *o*,*p*′-DDD; γ-HCH; β-HCH; *o*,*p*′-DDE; CB 118; *p*,*p*′-DDD and PFHpA quantified in >50% of human blood serum samples.

The dominating group of all monitored contaminants in serum was PFASs. The total concentrations of PFASs (expressed as a sum of quantified compounds, ΣPFASs) ranged from <0.01 to 8.97 ng mL^{-1} (mean 0.631 ng mL^{-1}). In detail, the highest concentrations were found for PFOA 0.280–8.97 ng mL^{-1} (mean 1.68, median 0.956 ng mL^{-1}), L-PFOS 0.335–6.68 ng mL^{-1} (mean 1.73, median 1.47 ng mL^{-1}) and PFNA 0.117–6.55 ng mL^{-1} (mean 0.554, median 0.366 ng mL^{-1}). As documented in Fig. 2, the measured concentrations of PFOA and PFOS were lower by approximately one order of magnitude than in other studies realised in Australia and USA (median: PFOA (6.40 ng mL^{-1}) and PFOS (14.8 ng mL^{-1}) (Toms et al., 2009); PFOA (3.30 ng mL^{-1}) and PFOS (28.5 ng mL^{-1}) (Kuklenyik et al., 2005) (Fig. 2). Nevertheless, compared to the other studies from Denmark (Joensen et al., 2013; Mørck et al., 2015), our values for PFOS and PFOA were approximately three times lower. Within the Italian studies (De Felip et al., 2015; Ingelido et al., 2010), during five years (2000–2005 and 2007–2012) the concentrations of PFOS and PFOA in human blood serum decreased to one half (from 6.31 to 2.43 ng g^{-1} for PFOS and from 3.59 to 1.55 ng g^{-1} for PFOA). The highest concentrations of PFOS (median 28.5 ng mL^{-1}) (Fig. 2) were detected within the American study (Kuklenyik et al., 2005), which is probably a consequence of the previous high production of these chemicals in the USA, since the American company 3 M was the biggest producer of PFOS.

Within the group of non-polar analytes, specifically PCBs dominated in the tested serum samples, the total amounts of ΣPCBs ranging from <0.1 to 481 ng g^{-1} lw (mean 63.3 ng g^{-1} lw). The highest

Table 1
The newly validated analytical method applied to the analysis of PCBs, OCPs, PBDEs and PFASs in seven replicates of the SRM 1958.

	Analyte	MQLs (ng g^{-1})	Certified concentration values in SRM 1958 (ng g^{-1})		Measured concentrations in SRM 1958 (ng g^{-1})		REC (%)	RSD (%)
			Mean	SD	Mean	SD		
PFASs	PFOA	0.010	4.11	0.170	3.98	0.214	97	5
	PFNA	0.010	0.660	0.130	0.657	0.016	100	3
	PFOS	0.010	16.6	0.900	16.2	0.733	98	5
	PFHxS	0.010	2.66	0.070	2.66	0.197	100	7
OCPs	HCB	0.005	0.442	0.046	0.486	0.047	110	18
	α-HCH	0.005	0.260	0.044	0.216	0.007	83	8
	β-HCH	0.005	0.278	0.047	0.311	0.010	112	3
	γ-HCH	0.005	0.315	0.043	0.317	0.014	99	12
	o,p′-DDE	0.005	0.450	0.020	0.413	0.022	92	6
	p,p′-DDE	0.005	1.25	0.130	1.07	0.059	85	6
	o,p′-DDD	0.005	0.347	0.046	0.287	0.019	83	7
	p,p′-DDD	0.005	0.416	0.017	0.407	0.009	98	5
	o,p′-DDT	0.010	0.313	0.043	0.267	0.025	85	15
	p,p′-DDT	0.010	0.293	0.012	0.268	0.014	91	12
PCBs	CB 28	0.005	0.402	0.012	0.424	0.012	106	10
	CB 52	0.005	0.401	0.014	0.406	0.009	101	5
	CB 101	0.005	0.409	0.027	0.430	0.018	105	6
	CB 118	0.005	0.412	0.035	0.396	0.015	96	4
	CB 138	0.005	0.473	0.054	0.535	0.012	113	4
	CB 153	0.005	0.457	0.036	0.410	0.021	90	6
	CB 170	0.010	0.422	0.023	0.388	0.020	92	8
	CB 180	0.010	0.459	0.049	0.397	0.014	86	7
PBDEs	BDE 28	0.005	0.462	0.019	0.392	0.057	72	17
	BDE 47	0.005	0.651	0.029	0.567	0.071	85	13
	BDE 66	0.005	0.440	0.041	0.396	0.018	90	6
	BDE 85	0.005	0.475	0.039	0.467	0.037	98	8
	BDE 99	0.005	0.492	0.015	0.498	0.028	101	7
	BDE 100	0.005	0.475	0.027	0.434	0.028	91	8
	BDE 153	0.005	0.455	0.054	0.478	0.025	105	5
	BDE 154	0.005	0.441	0.039	0.423	0.028	85	7
	BDE 183	0.005	0.453	0.042	0.399	0.020	85	6
	BDE 206	0.010	0.426	0.004	0.471	0.046	119	9
	BDE 209	0.050	0.417	0.005	0.461	0.073	111	16

Table 2
Concentrations of detected OCPs, PCBs, BFRs and PFASs in human blood serum (n = 38).[a,b]

	Analyte	Samples > MQL (%)	MQL	Median	Mean	Minimum	Maximum
ng mL^{-1}	PFOA	100	0.013	0.956	1.68	0.280	8.97
	PFNA	100	0.013	0.366	0.554	0.117	6.55
	PFDA	100	0.013	0.144	0.170	0.042	0.497
	Br-PFOS	100	0.004	1.35	1.44	0.290	3.38
	L-PFOS	100	0.005	1.47	1.73	0.335	6.68
	PFUndA	100	0.013	0.052	0.057	0.014	0.183
	PFHpA	53	0.013	0.015	0.021	<0.01	0.074
	PFDoDA	37	0.013			<0.01	0.121
	PFTrDA	29	0.013			<0.01	0.034
ng g^{-1} lw	HCB	100	0.1	29.6	64.8	7.64	638
	α-HCH	95	0.1	2.65	4.25	<0.1	19.9
	β-HCH	74	0.1	3.97	7.58	<0.1	92.4
	γ-HCH	76	0.1	1.02	1.03	<0.1	5.14
	o,p′-DDE	71	0.1	0.45	0.72	<0.1	3.44
	p,p′-DDE	100	0.1	207	278	40.2	1626
	o,p′-DDD	84	0.1	2.45	4.15	<0.1	37.4
	p,p′-DDD	58	0.1	3.84	23.9	<0.1	161
	o,p′-DDT	26	0.5	3.31	3.15	<0.5	4.64
	p,p′-DDT	95	0.5	13.5	14.5	<0.5	54.6
	CB 28	87	0.1	1.88	2.25	<0.1	10.7
	CB 52	5	0.1			<0.1	1.72
	CB 101	37	0.1			<0.1	2.43
	CB 118	61	0.1	3.18	4.73	<0.1	22.8
	CB 138	97	0.1	71.0	87.5	<0.1	332
	CB 153	97	0.1	137	168	<0.1	481
	CB 170	95	0.5	73.2	80.6	<0.5	225
	CB 180	97	0.5	158	163	<0.5	446
	BDE 47	8	0.1			<0.1	1.83
	BDE 99	5	0.1			<0.1	5.60
	BDE 153	13	0.1			<0.1	1.63
	BDE 196	3	0.1			<0.1	1.13
	BDE 197	3	0.1			<0.1	1.91
	BDE 209	13	2.5			<2.5	67.8

[a] Mean and median values were calculated when in >50% of samples compound was positively detected in concentration above the MQL.
[b] For results below MQL one-half the MQL value was used.

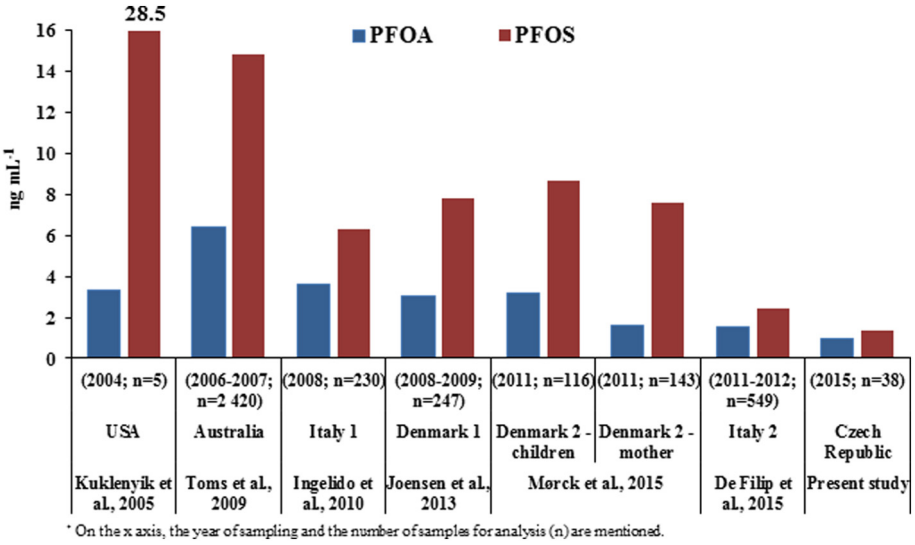


Fig. 2. Median concentrations of major PFASs (PFOS and PFAS) in human blood serum compared with studies conducted in other countries.*

concentrations were determined for hexa- and hepta-chlorinated congeners, represented by CB 138 < 0.1–332 ng g^{-1} lw (mean 87.5, median 71 ng g^{-1} lw), CB 153 < 0.1–481 ng g^{-1} lw (mean 168, median 137 ng g^{-1} lw) and CB 180 < 0.5–446 ng g^{-1} lw (mean 163, median 158 ng g^{-1} lw). In the present study, the concentrations of the most often detected CB 138, 153 and 180, were comparable with the data from the Romanian study (Dirtu et al., 2006) (CB 138 – median 38 ng g^{-1} lw; CB 153 – median 102 ng g^{-1} lw and CB 180 – median 107 ng g^{-1} lw). However, the levels of the major PCB congener in this study, CB 153 (<0.1–481 ng g^{-1} lw, median 137 ng g^{-1} lw) were slightly lower than the amount of this analyte (16.2–628 ng g^{-1} lw, median 187 ng g^{-1} lw) reported by the Slovak study (Chovancová et al., 2014). However, compared to the other studies from Italy, Korea and Greece (Amodio et al., 2012; Esposito et al., 2014; Kalantzi et al., 2011; Kang et al., 2008), our results were approximately four times higher (Fig. 3). Fig. 3 also shows that the lowest levels of medians CB 138 (6.4 ng g^{-1} lw), CB 153 (9.7 ng g^{-1} lw) and CB 180 (9.3 ng g^{-1} lw) were measured in the American study (Whitehead et al., 2015).

The total amounts of ΣOCPs in serum ranged from <0.1 to 1626 ng g^{-1} lw (mean 40.0 ng g^{-1} lw). The highest concentrations were determined for *p,p′*-DDE 40.2–1626 ng g^{-1} lw (mean 278, median 207 ng g^{-1} lw), HCB 7.64–638 ng g^{-1} lw (mean 64.8, median 29.6 ng g^{-1} lw) and *p,p′*-DDD <0.1–161 ng g^{-1} lw (mean 23.9, median 3.84 ng g^{-1} lw). The concentrations of major OCPs, *p,p′*-DDE (median 207 ng g^{-1} lw) and HCB (median 29.6 ng g^{-1} lw) were in accordance with the other mentioned studies from the USA, Italy and Korea (Fig. 4). The lowest concentrations of *p,p′*-DDE (median 122 ng g^{-1} lw) were detected in the study from Tunisia (Ben Hassine et al., 2014). The highest medians of *p,p′*-DDE were determined within the Polish (343 ng g^{-1} lw) and Greek (268 ng g^{-1} lw) studies (Jaraczewska et al., 2006; Kalantzi et al., 2011). The analyte *p,p′*-DDE is a degradation product of *p,p′*-DDT (main insecticidal activity), which was banned >30 years ago. These findings (relatively high levels of *p,p′*-DDE compared to the other OCPs in serum; degradation product of *p,p′*-DDT) are in accordance with the high biological persistence and stability (specifically *p,p′*-DDE has a median half-life of >8.6 years in serum) of organochlorine compounds that can accumulate in various environmental compartments, such as the atmosphere, water sediments, soil and also the food chain (main source through eating food containing small amounts of these contaminants) (Amodio et al., 2012; Kang et al., 2008).

The last targeted group of pollutants was BFRs, which belong to minor contaminants in human blood serum. Only six PBDE congeners namely, BDE 47 (<0.1–1.83 ng g^{-1} lw), BDE 99 (<0.1–5.60 ng g^{-1} lw), BDE 153 (<0.1–1.63 ng g^{-1} lw), BDE 196 (<0.1–1.13 ng g^{-1} lw), BDE 197 (<0.1–1.91 ng g^{-1} lw) and BDE 209 (<2.5–67.8 ng g^{-1} lw), were

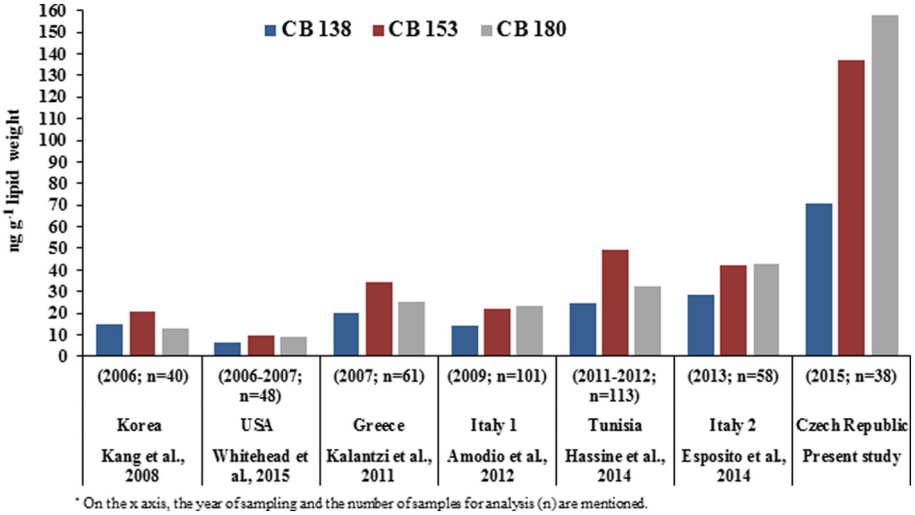


Fig. 3. Median concentrations of major PCBs (CB 138, 153 and 180) in human blood serum compared with studies conducted in other countries.*

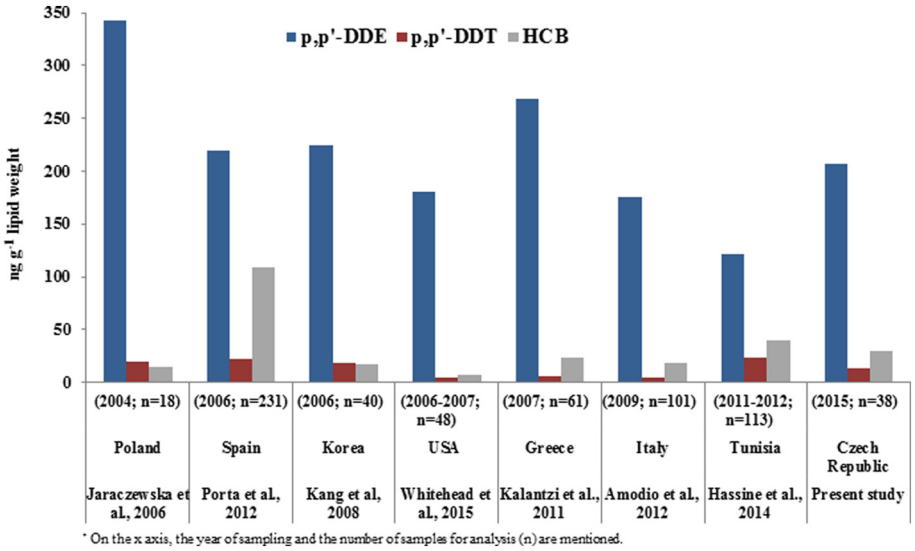


Fig. 4. Median concentrations of major OCPs (*p,p′*-DDE; *p,p′*-DDT and HCB) in human blood serum compared with data of similar studies conducted in other countries.*

detected in serum samples. The most frequently found representatives of this group were BDE 153, 209 (in 13% of samples) and 47 (in 8% of samples). The concentrations of detected BDE 47, 99 and 153 (<0.1–5.60 ng g^{-1} lw) were two times lower than the other data from the Norwegian study (<MQL-11 ng g^{-1} lw) and the Greek study (<0.3–10.2 ng g^{-1} lw), summarised by (Cequier et al., 2015; Kalantzi et al., 2011). The highest concentrations of BDE 209 (<2.5–67.8 ng g^{-1} lw) detected in our study were comparable with the Swedish study (<0.7–78 ng g^{-1} lw) (Bjermo et al., 2017). However, our values were approximately three times higher than the concentrations (<9–19.1 ng g^{-1} lw) reported in the Greek study (Kalantzi et al., 2011). Within the occurrence of PBDEs in human serum, there is a variable content of individual congeners in many studies, depending mostly on the region where different types of technical mixtures are used. In the case of novel FRs, targeted analytes ($n = 7$) were detected below the MQLs.

4. Conclusions

Within this study, the novel multi-analyte method for the simultaneous determination of 40 FRs, 19 PFASs, 11 OCPs and 8 PCBs in human blood serum was developed and validated. The sample preparation procedure for non-polar compounds was based on a three-step solvent extraction with *n*-hexane:diethylether (9:1, *v/v*) mixture, followed by the purification step using a Florisil® column. The rest of the sample after removal of non-polar solvent containing the non-polar compounds was further extracted by the modified QuEChERS-like extraction, when acetonitrile was used for the isolation of more polar compounds and the separation of organic and aqueous layers was induced by the addition of inorganic salts. For the identification/quantification of target analytes, both GC–MS(/MS) and LC–MS/MS techniques were employed. Good performance characteristics were achieved for all target analytes in human blood serum. The method recoveries were in the range of 70–115% (RSD <18%) for FRs; 74–120% (RSD <20%) for OCPs and PCBs; 79–120% (RSD <20%) for PFASs and OH-BDEs; 71–116% (RSD <19%) for HBCDs, TBBPA and brominated phenols. The trueness of the multi-analyte procedure was demonstrated by testing SRM 1958 for selected PFASs, PCBs, OCPs and PBDEs. The determined concentrations for all targeted analytes in the SRM 1958 were in good agreement with the reference values. The benefit of the presented method is the simultaneous analysis of such a large number of various environmental contaminants ($n = 78$) in one method, from only 3 mL of serum sample.

This newly validated method was successfully applied for the monitoring of organohalogenated contaminants in 38 samples of human blood serum obtained from Prague, Czech Republic. From 78 target analytes, 10 PFASs, 10 OCPs, 8 PCBs and 6 PBDEs were detected in serum above MQLs. The total concentrations of PFASs in serum were in the range < MQL-8.97 ng mL^{-1} (mean 0.631 ng mL^{-1}), <MQL-1626 ng g^{-1} lw (mean 40 ng g^{-1} lw) for OCPs and <MQL-481 ng g^{-1} lw (mean 63.3 ng g^{-1} lw) for PCBs. Regarding BFRs, only 6 PBDEs, namely BDE 47, 99, 153, 196, 197 and 209 (<MQL-67.8 ng g^{-1} lw), were detected in serum. The most abundant contaminants in serum were PFASs represented by PFOA, PFNA, PFDA, Br-PFOS, L-PFOS and PFUndA in all samples, followed by PFHpA detected in 20 samples. The major OCPs' analytes, HCB and *p,p′*-DDE, were quantified in all samples. From PCBs, the most abundant analytes were CB 138, CB 153 and CB 180, determined in 37 samples.

Within human biomonitoring, the development of the new method contributes to the assessment of exposure to a broad range of organohalogenated contaminants (n = 78; novel FRs, BFRs, PFASs, OCPs and PCBs).

Acknowledgments

This work was supported by the “National Programme of Sustainability I” - NPU I (LO1601 - No MSMT-43760/2015) and the specific university research (MSMT No 21-SVV/2018). Last but not least, we would like to thank The National Institute of Public Health (Prague, Czech Republic) for providing samples of human blood serum.

Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.02.336.

References

Amodio, E., Turci, R., Massenti, M.F., Di Gaudio, F., Minoia, C., Vitale, F., Firenze, A., Calamusa, G., 2012. Serum concentrations of persistent organic pollutants (POPs) in the inhabitants of a Sicilian city. Chemosphere 89, 970–974. https://doi.org/10.1016/j.chemosphere.2012.06.054.

Ben Hassine, S., Hammami, B., Ben Ameur, W., El Megdiche, Y., Barhoumi, B., El Abidi, R., Driss, M.R., 2014. Concentrations of organochlorine pesticides and polychlorinated biphenyls in human serum and their relation with age, gender, and BMI for the general population of Bizerte, Tunisia. Environ. Sci. Pollut. Res. 21, 6303–6313. https://doi.org/10.1007/s11356-013-1480-9.

Bjermo, H., Aune, M., Cantillana, T., Glynn, A., Lind, P.M., Ridefelt, P., Darnerud, P.O., 2017. Serum levels of brominated flame retardants (BFRs: PBDE, HBCD) and influence of dietary factors in a population-based study on Swedish adults. Chemosphere 167, 485–491. https://doi.org/10.1016/j.chemosphere.2016.10.008.

Cequier, E., Marcé, R.M., Becher, G., Thomsen, C., 2015. Comparing human exposure to emerging and legacy flame retardants from the indoor environment and diet with concentrations measured in serum. Environ. Int. 74, 54–59. https://doi.org/10.1016/j.envint.2014.10.003.

Chovancová, J., Drobná, B., Fabišiková, A., Čonka, K., Wimmerová, S., Pavuk, M., 2014. Polychlorinated biphenyls and selected organochlorine pesticides in serum of Slovak population from industrial and non-industrial areas. Environ. Monit. Assess. 186, 7643–7653. https://doi.org/10.1007/s10661-014-3956-6.

Coakley, J., Bridgen, P., Bates, M.N., Douwes, J., 't Mannetje, A., 2018. Chlorinated persistent organic pollutants in serum of New Zealand adults, 2011–2013. Sci. Total Environ. 615, 624–631. https://doi.org/10.1016/j.scitotenv.2017.09.331.

Darnerud, P.O., Lignell, S., Aune, M., Isaksson, M., Cantillana, T., Redeby, J., Glynn, A., 2015. Time trends of polybrominated diphenylether (PBDE) congeners in serum of Swedish mothers and comparisons to breast milk data. Environ. Res. 138, 352–360. https://doi.org/10.1016/j.envres.2015.02.031.

De Felip, E., Abballe, A., Albano, F.L., Battista, T., Carraro, V., Conversano, M., Franchini, S., Giambanco, L., Iacovella, N., Ingelido, A.M., Maiorana, A., Maneschi, F., Marra, V., Mercurio, A., Nale, R., Nucci, B., Panella, V., Pirola, F., Porpora, M.G., Procopio, E., Suma, N., Valentini, S., Valsenti, L., Vecchiè, V., 2015. Current exposure of Italian women of reproductive age to PFOS and PFOA: a human biomonitoring study. Chemosphere 137, 1–8. https://doi.org/10.1016/j.chemosphere.2015.03.046.

Dirtu, A.C., Cernat, R., Dragan, D., Mocanu, R., Van Grieken, R., Neels, H., Covaci, A., 2006. Organohalogenated pollutants in human serum from lassy, Romania and their relation with age and gender. Environ. Int. 32, 797–803. https://doi.org/10.1016/j.envint.2006.04.002.

Esposito, M., Serpe, F.P., Diletti, G., Messina, G., Scortichini, G., La Rocca, C., Baldi, L., Amorena, M., Monda, M., 2014. Serum levels of polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans and polychlorinated biphenyls in a population living in the Naples area, southern Italy. Chemosphere 94, 62–69. https://doi.org/10.1016/j.chemosphere.2013.09.013.

Flores-Ramírez, R., Pérez-Vázquez, F., Rodríguez-Aguilar, M., Medellín-Garibay, S., Van Brussel, E., Cubillas-Tejeda, A., Carrizales-Yáñez, L., Díaz-Barriga, F., 2017. Biomonitoring of persistent organic pollutants (POPs) in child populations living near contaminated sites in Mexico. Sci. Total Environ. 579, 1120–1126. https://doi.org/10.1016/j.scitotenv.2016.11.087.

Gribble, M.O., Bartell, S.M., Kannan, K., Wu, Q., Fair, P.A., Kamen, D.L., 2015. Longitudinal measures of perfluoroalkyl substances (PFAS) in serum of Gullah African Americans in South Carolina: 2003–2013. Environ. Res. 143, 82–88. https://doi.org/10.1016/j.envres.2015.03.012.

Ingelido, A.M., Marra, V., Abballe, A., Valentini, S., Iacovella, N., Barbieri, P., Porpora, M.G., di Domenico, A., Felip, E.D., 2010. Perfluorooctanesulfonate and perfluorooctanoic acid exposures of the Italian general population. Chemosphere 80, 1125–1130. https://doi.org/10.1016/j.chemosphere.2010.06.025.

Jaraczewska, K., Lulek, J., Covaci, A., Voorspoels, S., Kaluba-Skotarczak, A., Drews, K., Schepens, P., 2006. Distribution of polychlorinated biphenyls, organochlorine pesticides and polybrominated diphenyl ethers in human umbilical cord serum, maternal serum and milk from Wielkopolska region, Poland. Sci. Total Environ. 372, 20–31. https://doi.org/10.1016/j.scitotenv.2006.03.030.

Joensen, U.N., Veyrand, B., Antignac, J.-P., Blomberg Jensen, M., Petersen, J.H., Marchand, P., Skakkebæk, N.E., Andersson, A.-M., Le Bizec, B., Jørgensen, N., 2013. PFOS (perfluorooctanesulfonate) in serum is negatively associated with testosterone levels, but not with semen quality, in healthy men. Hum. Reprod. 28, 599–608. https://doi.org/10.1093/humrep/des425.

Kalachova, K., Cajka, T., Sandy, C., Hajslova, J., Pulkrabova, J., 2013a. High throughput sample preparation in combination with gas chromatography coupled to triple quadrupole tandem mass spectrometry (GC–MS/MS): a smart procedure for (ultra)trace analysis of brominated flame retardants in fish. Talanta 105, 109–116. https://doi.org/10.1016/j.talanta.2012.11.073.

Kalachova, K., Pulkrabova, J., Cajka, T., Drabova, L., Stupak, M., Hajslova, J., 2013b. Gas chromatography–triple quadrupole tandem mass spectrometry: a powerful tool for the (ultra)trace analysis of multiclass environmental contaminants in fish and fish feed. Anal. Bioanal. Chem. 405, 7803–7815. https://doi.org/10.1007/s00216-013-7000-4.

Kalantzi, O.I., Geens, T., Covaci, A., Siskos, P.A., 2011. Distribution of polybrominated diphenyl ethers (PBDEs) and other persistent organic pollutants in human serum from Greece. Environ. Int. 37, 349–353. https://doi.org/10.1016/j.envint.2010.10.005.

Kang, J.-H., Park, H., Chang, Y.-S., Choi, J.-W., 2008. Distribution of organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) in human serum from urban areas in Korea. Chemosphere 73, 1625–1631. https://doi.org/10.1016/j.chemosphere.2008.07.087.

Koponen, J., Rantakokko, P., Airaksinen, R., Kiviranta, H., 2013. Determination of selected perfluorinated alkyl acids and persistent organic pollutants from a small volume human serum sample relevant for epidemiological studies. J. Chromatogr. A 1309, 48–55. https://doi.org/10.1016/j.chroma.2013.07.064.

Kuklenyik, Z., Needham, L.L., Calafat, A.M., 2005. Measurement of 18 perfluorinated organic acids and amides in human serum using on-line solid-phase extraction. Anal. Chem. 77, 6085–6091. https://doi.org/10.1021/ac050671l.

Lankova, D., Svarcova, A., Kalachova, K., Lacina, O., Pulkrabova, J., Hajslova, J., 2015. Multianalyte method for the analysis of various organohalogen compounds in house dust. Anal. Chim. Acta 854, 61–69. https://doi.org/10.1016/j.aca.2014.11.007.

Long, M., Knudsen, A.-K.S., Pedersen, H.S., Bonefeld-Jørgensen, E.C., 2015. Food intake and serum persistent organic pollutants in the Greenlandic pregnant women: the ACCEPT sub-study. Sci. Total Environ. 529, 198–212. https://doi.org/10.1016/j.scitotenv.2015.05.022.

Moon, H.J., Lim, J., Jee, S.H., 2017. Association between serum concentrations of persistent organic pollutants and smoking in Koreans: a cross-sectional study. J. Epidemiol. 27, 63–68. https://doi.org/10.1016/j.je.2016.09.006.

Mørck, T.A., Nielsen, F., Nielsen, J.K.S., Siersma, V.D., Grandjean, P., Knudsen, L.E., 2015. PFAS concentrations in plasma samples from Danish school children and their mothers. Chemosphere 129, 203–209. https://doi.org/10.1016/j.chemosphere.2014.07.018.

Needham, L.L., Calafat, A.M., Barr, D.B., 2007. Uses and issues of biomonitoring. Int. J. Hyg. Environ. Health 210, 229–238. https://doi.org/10.1016/j.ijheh.2006.11.002.

Nøst, T.H., Sandanger, T.M., Nieboer, E., Odland, J.Ø., Breivik, K., 2017. The impacts of emission trends of POPs on human concentration dynamics: lessons learned from a longitudinal study in Norway (1979–2007). Int. J. Hyg. Environ. Health 220, 776–781. https://doi.org/10.1016/j.ijheh.2017.01.015.

Porta, M., López, T., Gasull, M., Rodríguez-Sanz, M., Garí, M., Pumarega, J., Borrell, C., Grimalt, J.O., 2012. Distribution of blood concentrations of persistent organic pollutants in a representative sample of the population of Barcelona in 2006, and comparison with levels in 2002. Sci. Total Environ. 423, 151–161. https://doi.org/10.1016/j.scitotenv.2012.02.001.

Salihovic, S., Kärrman, A., Lindström, G., Lind, P.M., Lind, L., van Bavel, B., 2013a. A rapid method for the determination of perfluoroalkyl substances including structural isomers of perfluorooctane sulfonic acid in human serum using 96-well plates and column-switching ultra-high performance liquid chromatography tandem mass spectrometry. J. Chromatogr. A 1305, 164–170. https://doi.org/10.1016/j.chroma.2013.07.026.

Salihovic, S., Nilsson, H., Hagberg, J., Lindström, G., 2013b. Trends in the analysis of persistent organic pollutants (POPs) in human blood. TrAC Trends Anal. Chem. 46, 129–138. https://doi.org/10.1016/j.trac.2012.06.009.

Salihovic, S., Kärrman, A., Lind, L., Lind, P.M., Lindström, G., van Bavel, B., 2015. Perfluoroalkyl substances (PFAS) including structural PFOS isomers in plasma from elderly men and women from Sweden: results from the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS). Environ. Int. 82, 21–27. https://doi.org/10.1016/j.envint.2015.05.003.

Thuresson, K., Höglund, P., Hagmar, L., Sjödin, A., Bergman, Å., Jakobsson, K., 2006. Apparent half-lives of hepta- to decabrominated diphenyl ethers in human serum as determined in occupationally exposed workers. Environ. Health Perspect. 114, 176–181. https://doi.org/10.1289/ehp.8350.

Toms, L.-M.L., Calafat, A.M., Kato, K., Thompson, J., Harden, F., Hobson, P., Sjödin, A., Mueller, J.F., 2009. Polyfluoroalkyl chemicals in pooled blood serum from infants, children, and adults in Australia. Environ. Sci. Technol. 43, 4194–4199. https://doi.org/10.1021/es900272u.

Ulutaş, O.K., Çok, I., Darendeliler, F., Aydin, B., Çoban, A., Henkelmann, B., Schramm, K.-W., 2015. Blood levels of polychlorinated biphenlys and organochlorinated pesticides in women from Istanbul, Turkey. Environ. Monit. Assess. 187. https://doi.org/10.1007/s10661-015-4358-0.

Wang, Y., Zhong, Y., Li, J., Zhang, J., Lyu, B., Zhao, Y., Wu, Y., 2018. Occurrence of perfluoroalkyl substances in matched human serum, urine, hair and nail. J. Environ. Sci. 67, 191–197. https://doi.org/10.1016/j.jes.2017.08.017.

Whitehead, T.P., Crispo Smith, S., Park, J.-S., Petreas, M.X., Rappaport, S.M., Metayer, C., 2015. Concentrations of persistent organic pollutants in California women's serum and residential dust. Environ. Res. 136, 57–66. https://doi.org/10.1016/j.envres.2014.10.009.

APPENDIX II

Lankova D., Lacina O., Pulkrabova J., Hajslova J.

The determination of perfluoroalkyl substances, brominated flame retardants and their metabolites in human breast milk and infant formula

Talanta (2013) 117: 318–325

doi: 10.1016/j.talanta.2013.08.040

Talanta 117 (2013) 318–325

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

The determination of perfluoroalkyl substances, brominated flame retardants and their metabolites in human breast milk and infant formula

Darina Lankova, Ondrej Lacina, Jana Pulkrabova, Jana Hajslova *

Institute of Chemical Technology, Prague, Faculty of Food and Biochemical Technology, Department of Analysis and Nutrition, Technicka 3, 166 28 Prague, Czech Republic

ARTICLE INFO

Article history:
Received 31 May 2013
Received in revised form
22 August 2013
Accepted 25 August 2013
Available online 30 August 2013

Keywords:
PFASs
BFRs
HBCDs
TBBPA
Human milk
LC–MS/MS

ABSTRACT

In the present study, a novel analytical approach for the simultaneous determination of 18 perfluoroalkyl substances (PFASs) and 11 brominated flame retardants (BFRs) including their hydroxylated metabolites and brominated phenols has been developed and validated for breast milk and infant formula. The sample preparation procedure based on extraction using acetonitrile and subsequent purification by dispersive solid-phase extraction (d-SPE) employing C18 sorbent is rapid, simple and high-throughput. Ultra-high performance liquid chromatography (UHPLC) interfaced with a tandem mass spectrometry (MS/MS) was employed for the identification/quantification of these compounds. The method recoveries of target compounds for both matrices ranged from 80% to 117% with relative standard deviations lower than 28% and quantification limits in the range of 3–200 pg/mL for milk and 5–450 pg/g for infant formula. Within the pilot study, the new method was used for the analysis of PFASs and BFRs in 50 human breast milks and six infant formulas. In the breast milk samples the total contents of PFASs and BFRs were in the range of 38–279 and 45–16,200 pg/mL, respectively. The most abundant PFASs detected in all tested breast milk samples were perfluorooctanoic acid (PFOA) and perfluorooctanesulfonate (PFOS), the latter contaminant was present not only as a linear form but also as a branched isomers. The incidence of BFRs was lower, the only representatives of this group, tetrabromobiphenol A (TBBPA) and α-hexabromocyclododecane (α-HBCD), were detected in less than 30% of breast milk samples. None of the infant formulas contained BFRs, traces of either PFOS, PFOA or PFNA were found in three samples.

1. Introduction

A wide range of halogenated contaminants are present in the human environment. While most of the early monitoring studies focused on various chlorinated persistent organic pollutants (POPs), in recent decades, toxicological concerns emerged on the ubiquitous occurrence of fluorine and bromine containing compounds that may also accumulate in food chains. The high volume production of perfluoroalkyl substances (PFASs) and brominated flame retardants (BFRs), the latter represented mainly by polybrominated diphenyl ethers (PBDEs), hexabromocyclododecanes (HBCDs) and tetrabromobisphenol A (TBBPA), have led to their widespread distribution in the environment. As regards to PFASs, due to their unique characteristics such as chemical inertness, stability, hydrophobicity and lipophobicity, they are used in a variety of industrial and consumer applications while BFRs are used to reduce the flammability of treated materials [1,2].

Non-occupational human exposure to PFASs and BFRs, that may occur through a variety of pathways including inhalation of contaminated dust particles [3,4] or food [5,6]/drinking water ingestion [7], has been clearly documented by findings of these chemicals and their (bio)transformation products in human tissues and fluids including plasma and breast milk [8–10]. The latter matrix is a widely used bioindicator that can be used to assess the body burden of these environmental pollutants especially with regard to its importance as the first food for the newborn.

Recently, the European Food Safety Authority (EFSA) has outlined European Union (EU) framework and respective activities of the Panel on Contaminants in the Food Chain (CONTAM Panel) in the field of BFRs. Six Scientific Opinions on the main classes of these contaminants completed between October 2010 and October 2012 have been presented [11]. It is worthy to note that the very recent Scientific Opinion is also concerned with brominated phenols and their derivatives. With the exception of 2,4,6-tribromophenol (2,4,6-TBP), the data for risk assessment are lacking. In

* Corresponding author. Tel.: +420 220 443 185; fax: +420 220 443 180.
E-mail address: jana.hajslova@vscht.cz (J. Hajslova).

0039-9140/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.08.040

general, the requirement for the continuation of BFRs surveillance and the need to fill in the gaps in occurrence data on emerging/novel BFRs has been emphasized by EFSA [12]. Similarly, in the last few years, the CONTAM Panel has paid a great deal of attention to public health concerns for a wide range of PFASs entering human food chain [13]. The occurrence of PFASs in various food commodities and estimation of dietary exposure has been recently reviewed by EFSA within a comprehensive scientific report [14]. Nevertheless, the CONTAM Panel has acknowledged the limitation in information available on other PFASs and recommended further monitoring of food contamination. The use of analytical methods with improved sensitivity are needed to increase the proportion of quantified results and thereby the reliability of exposure assessments that has been highlighted.

In our study, we attempted to respond to the EFSA requirement and to implement a highly sensitive method for selected representatives of both the above discussed POP groups. Since the only data on exposure to halogenated POPs in the Czech Republic is available for organochlorine pesticides (OCPs) [15], polychlorinated biphenyls (PCBs) [16], polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) [17] and PBDEs [18], we decided to provide a complementary information on two other groups of halogenated POPS. For this reason a set of breast milk samples for PFASs and polar BFRs represented by brominated phenols and hydroxylated derivatives of PBDEs (OH-PBDEs) was examined for the first time in the Czech Republic. Considering the fact that some newborns have to be fed by infant formula, we also included this matrix in our experiments as another potential exposure source to fluorine/bromine containing POPs.

Although the current trend in food contaminants control is to integrate various contaminants groups into a single procedure, the review of the available scientific literature did not reveal a procedure that was applied for analysis of BFRs and PFASs in breast milk/infant formula. In some studies, both groups of contaminants were monitored [19–21] by using two alternative methods for each compound class. As regards BFRs, gas chromatography coupled to mass spectrometry (GC–MS) is the method of choice for the most often monitored representatives-PBDEs [22]. This approach can also be employed for the determination of other widely used BFRs, nevertheless, some limitations need to be taken into consideration: the separation of HBCD isomers is hardly feasible by common GC capillaries [23], and in the case of rather more polar TBBPA, derivatization of the hydroxyl group is needed prior to injection [24]. Similarly, brominated phenols or OH-PBDEs are not amenable to GC–MS analysis without derivatization [25]. As a result, high performance liquid chromatography (HPLC) based methods represent a more convenient option. Several methods have been recently published describing the use of this approach for examination of polar BFRs, their metabolites [26], HBCD isomers or TBBPA in body fluids [27,28]. Regarding PFASs, the other group of contaminants involved in our study, HPLC–MS(/MS) is also the key technique used for their quantification in biotic matrices [9].

The objectives of our study were: (i) to implement an solution for the simultaneous analysis of PFASs and BFRs including several metabolites amenable to LC–MS analysis in milk and infant formulas and (ii) to apply a new method for the examination of breast milk and infant formula samples.

2. Materials and method

2.1. Samples collection

The samples of human breast milk were obtained from 50 Czech women living in the Olomouc region (located in the north-east part of the Czech Republic) from April to August 2010 thanks to co-operation with the Gyneacological-maternity Clinic, Faculty Hospital in Olomouc. The age of participating mothers ranged from 20 to 43 years (mean and median age was 30 years). To acquire information that could be relevant to the estimation of contamination pathways, patients completed a questionnaire about their age, body weight, current area of residence (rural/urban), number of children (primapara/multipara), occupation, and dietary habits. Approximately 50 mL of each breast milk sample was collected by hand expressing into a pre-cleaned glass bottle and samples were stored at −20 °C until analysis. The lipid content of the human breast milk samples was determined gravimetrically (results ranging from 0.5–4.9%, mean 2.4%); for this purpose the liquid–liquid extraction (LLE) with hexane and diethyl ether followed by filtration of organic phase through anhydrous sodium sulfate was used [18].

In addition, 6 different types of infant formula from the Czech retail market were examined in this study: (i) one powdered infant and two toddler milk formulas, and (ii) one special formula for babies with lactose intolerance, one formula for premature babies and one soya based formula for babies with non-milk diets.

(i) Milk formulas were supplied in 800 g paper packages and the content of proteins, fats and carbohydrates were in the range of 9.3–10.4, 2.9–3.1 and 55.8–59.1 g in 100 g of formula, respectively.
(ii) Special formulas were obtained in 400 g tin packaging with the composition in proteins, fat and carbohydrates in the range of 10.8–12.8, 10.9–27.3 and 46.1–56 g in 100 g of formula, respectively.

2.2. Standards and chemicals

The individual standards of PFASs and HBCD isomers as well as isotopically labeled internal standards of PFASs and HBCD isomers were purchased from the Wellington Laboratories (Guelph, ON, Canada). PFOS standard supplied by Wellington Laboratories contained 78.8% linear (L-PFOS) and 21.2% branched isomers (Br-PFOS), thus separate quantification of L- and Br-PFOS was possible. Individual standards of OH-PBDEs: 6-hydroxy-2,2′,4,4′-tetrabromodiphenylether (6-OH-BDE-47), 2′-hydroxy-2,3′,4,5′-tetrabromodiphenylether (2′-OH-BDE-68), 4′-hydroxy-2,2′,4,5′-tetrabromodiphenylether (4′-OH-BDE-49), 6-hydroxy-2,2′,4,4′,5-pentabromodiphenylether (6-OH-BDE-99), and brominated phenols: 2,4-dibromophenol (2,4-DBP), 2,4,6-TBP, pentabromophenol (PBP), were purchased from AccuStandard (New Haven, CT, USA). The standard of TBBPA was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). The purity of individual standards was at least 98%. Working standard mixtures of all analytes were prepared in methanol (MeOH) and stored in the refrigerator (5 °C); PFASs and OH-PBDEs were at concentrations 0.25; 0.5; 1; 5; 10; 50 and 100 ng/mL, the concentrations of HBCD isomers, TBBPA and brominated phenols were five times higher. Calibration was prepared by mixing 30 μL of particular working standard mixture with 270 μL of blank matrix extract prepared as described below (without addition of isotopically labeled internal standards) to obtain matrix-matched standards corresponding to the relevant concentration levels: 0.025; 0.05; 0.1; 0.5; 1; 5 and 10 ng/mL for PFASs and OH-PBDEs, and 0.125; 0.25; 0.5; 2.5; 5; 25 and 50 ng/mL for HBCD isomers, TBBPA and brominated phenols.

High performance liquid chromatography (HPLC) grade MeOH was supplied by Merck (Darmstadt, Germany). Acetonitrile (MeCN), anhydrous magnesium sulfate and HPLC grade ammonium acetate (99.99%) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Water purified by a Milli-Q® Integral system (no PFASs containing polymers), supplied by Merck (Darmstadt, Germany), was used throughout the study. Sodium chloride was supplied by Lach-Ner

(Neratovice, Czech Republic). Formic acid (85%) was purchased from Penta (Chrudim, Czech Republic) and Bondesil C18 sorbent (40 μm) was supplied by Varian (Harbor City, CA, USA). Polypropylene (PP) centrifuge tube filters (nylon, pore size 0.22 μm) were supplied by Sigma-Aldrich.

2.3. Extraction and instrumental analysis

An amount of 15 mL of human breast milk sample was transferred into a 50 mL PP centrifuge tube and the isotopically labeled internal standards, 360 μL formic acid, and 15 mL MeCN were added and the mixture was vigorously shaken by hand for 1 min. In the case of infant formula samples, 5 g of powdered sample was weighed and 15 mL of 18 MΩ MilliQ water was added and mixed by shaking for 1 min.

The following steps of sample preparation were identical for both matrices. 6 g anhydrous $MgSO_4$ and 1.5 g NaCl were added and the tube was immediately shaken again for 1 min. The tube was then centrifuged (Hettich, Germany) for 5 min at 10,000 rpm. An amount of 12 mL of the upper organic layer of supernatant was transferred to the new centrifuge tube containing 180 mg C18 sorbent and 1.8 g anhydrous $MgSO_4$. The tube was shaken again for 1 min and centrifuged for 5 min at 10,000 rpm. Subsequently, 8 mL of a purified extract was evaporated near to dryness and the residues were dissolved in 0.5 mL MeOH. The reconstituted extract was filtered through a 0.22 μm nylon centrifuge tube filter and transferred into the vial for the LC–MS/MS analysis.

The UHPLC analyses of PFASs and BFRs were performed using an Acquity Ultra-Performance LC system (Waters, USA) equipped with PEEK tubing and a 10 μL sample loop. Analytes were separated on an Acquity UPLC HSS T3 analytical column (100 mm × 2.1 mm i.d., 1.8 μm particle size, Waters, USA) maintained at 40 °C. The column isolator (50 mm × 2.1 mm i.d., Waters, USA) was inserted between the mixer and the sample valve to separate background contaminates from the sample to be analyzed. The mobile phase consisted of (A) 5 mM ammonium acetate in Milli-Q water and (B) MeOH. The elution gradient conditions for the LC mobile phase were as follows: 10–50% B over 0.5 min, then 50–100% B over 7.5 min followed by an isocratic hold at 100% B for 4 min. The total run time for each injection was 15 min. The flow rate began at 0.3 mL/min and the sample volume injected was 5 μL. The UHPLC system was coupled to a triple quadrupole mass spectrometer Xevo TQ-S (Waters, USA) with negative electrospray ionization (ESI–) and operated in multiple reaction monitoring (MRM) mode. The retention times and quantitative and qualitative MRM transitions of target analytes are listed in the Table S1 in the Supplementary Data.

2.4. Quality assurance/quality control

To demonstrate the applicability of the implemented analytical method, validation experiments on milk and infant formula were performed. Together with each batch of samples, a procedural blank (i.e. sample prepared in a common way, but without the use of test matrix) was prepared to document the absence of background contamination. Recoveries, repeatabilities expressed as relative standard deviations (RSDs), and limits of quantification (LOQs) were established on the data obtained by analyzing 6 spiked blank cow milk (3.5% fat content) and 6 blank powdered milk infant formula samples. For recovery testing, the spike concentrations for milk were 30 pg/mL for PFASs and OH-PBDEs and 300 pg/mL for BFRs (namely HBCDs, TBBPA and brominated phenols). Powdered milk infant formula was spiked at a concentration of 60 pg/g for PFASs and OH-PBDEs, 1000 pg/g for HBCDs, TBBPA and brominated phenols. The LOQs were estimated as the lowest matrix matched calibration standard which provided a signal-to-noise ratio (S/N) higher than 10 and the second MS/MS transition (if available) had to provide a $S/N > 3$. Weighted linear regression ($1/x$) was used and regression coefficient (R^2) was calculated for the calibration curve from the LOQ up to the highest calibration point. Finally, the whole optimized procedure was validated by employing isotopically labeled surrogates.

3. Results and discussion

3.1. Chromatographic separation of PFASs and BFRs

In the first phase of our study, the UHPLC conditions had to be optimized with regard to OH-PBDEs separation. As far as UHPLC system consisted of common C18 column with 5 mM ammonium acetate and MeOH mobile phase, isomers of PBDE metabolites (2′-OH-BDE-68 and 6-OH-BDE-47) were totally co-eluted. Due to the missing characteristic fragments, a spectral resolution was not possible. The separation of these isomers was achieved only when mobile phase selectivity was changed, i.e. MeOH was replaced by MeCN, see Fig. 1 [29]; however, an undesirable decrease in signal sensitivity for other BFRs (TBBPA by 80% and PBP by 90%) was detected. The adverse drop of PFASs signal was also found (FOSAs by 50%, PFCAs and PFSAs by 10%). To obtain as low as possible detection limits for most of major target analytes (see Table 1), 5 mM aqueous ammonium acetate and MeOH was selected as a primary mobile phase, supposing OH-PBDEs signal was detected, sample re-analysis in the second UHPLC system enabling separation of 2′-OH-BDE-68 and 6-OH-BDE-47 might be performed.

3.2. Method for determination of PFASs and BFRs in milk and infant formula

For the development of the new analytical approach for the determination of PFASs and BFRs in milk and infant formula, we took an inspiration from the QuEChERS (“Quick, Easy, Cheap, Effective, Rugged and Safe”) procedure. This method was originally developed for the pesticide residues analysis [30], but has been

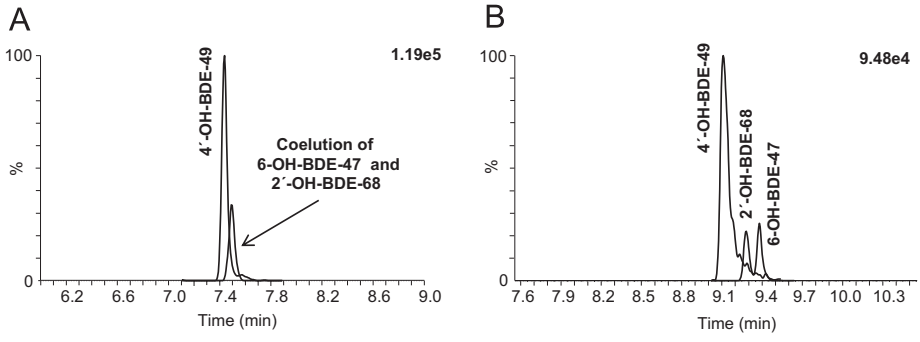


Fig. 1. Chromatograms of OH-PBDEs in matrix matched calibration standard (milk) at 0.5 ng/mL recorded with (A) the mobile phase consisted of 5 mM aqueous ammonium acetate/MeOH and (B) 5 mM aqueous ammonium acetate/MeCN.

Table 1
Recoveries, repeatabilities and LOQs achieved during validation on spiked cow milk (3.5% fat content) and milk infant formula.

Analyte	Milk ($n=6$)				Milk infant formula ($n=6$)			
	Spike PFASs and OH-PBDEs: 30 pg/mL				Spike PFASs and OH-PBDEs: 60 pg/g			
	Spike BFRs[a]: 300 pg/mL				Spike BFRs[a]: 1000 pg/g			
	Recovery (%)	RSD (%)	LOQ[b] (pg/mL)	LOQ[c] (pg/mL)	Recovery (%)	RSD (%)	LOQ[b] (pg/g)	LOQ[c] (pg/g)
PFBA	92	19	20	20	98	23	50	50
PFPeA	91	10	20	20	115	13	20	20
PFHxA	97	4	6	6	107	4	9	9
PFHpA	95	1	6	6	96	3	9	9
PFOA	104	12	6	6	111	4	9	9
PFNA	94	12	6	6	113	3	9	9
PFDA	97	7	6	6	111	4	9	9
PFUdA	99	1	6	6	117	4	9	9
PFDoA	90	3	6	6	110	1	9	9
PFTrDA	101	10	6	6	105	3	9	9
PFTeDA	98	5	6	6	117	3	9	9
PFBS	99	3	6	6	93	4	9	9
PFHxS	101	2	6	6	98	3	5	5
Br-PFOS	110	11	10	10	115	3	20	20
L-PFOS	98	9	5	5	108	3	7	7
PFDS	92	13	6	6	96	2	9	9
PFOSA	101	5	3	10	105	2	5	20
N-EtFOSA	94	5	6	20	104	3	9	20
N-MeFOSA	106	4	6	20	94	5	5	20
6-OH-BDE-47	87	5	n.s.	3	101	9	n.s.	5
4′-OH-BDE-49	94	6	3	3	105	6	5	5
2′-OH-BDE-68	87	5	n.s.	3	95	7	n.s.	5
6′-OH-BDE-99	80	5	3	3	82	10	5	5
2,4-DBP	89	5	200	200	101	6	450	450
2,4,6-TBP	94	4	30	30	99	6	50	50
PBP	82	4	30	100	89	10	50	120
α-HBCD	111	7	30	30	83	11	90	90
β-HBCD	108	5	30	30	81	7	50	50
γ-HBCD	111	4	6	6	84	16	90	90
TBBPA	103	6	60	150	94	7	50	150

[a] Brominated phenols, TBBPA and HBCDs.
[b] The mobile phase consisted of 5 mM ammonium acetate (A) and MeOH (B) was used.
[c] The mobile phase consisted of 5 mM ammonium acetate (A) and MeCN (B) was used n.s. - not specified, because of coelution of 6-OH-BDE-47 and 2′-OH-BDE-68.

already employed also for the analysis of veterinary drugs [31] or mycotoxins [32]. The QuEChERS method is based on a sample extraction with a mixture of MeCN/H_2O and subsequent solvents partition induced by added inorganic salts, NaCl and $MgSO_4$. Crude MeCN extracts can be purified by a dispersive solid-phase extraction (d-SPE) employing e.g. C18 silica, PSA and/or EnviCarb sorbent, depending on the type of co-isolated matrix components. For our purpose C18 sorbent was used for the removal of co-extracted fat and other lipophilic compounds from MeCN extract [33]. To assess the efficiency of this clean-up step, matrix effects (signal suppression/enhancement, SSE) for individual analytes were determined (comparison of a matrix matched calibration slope with the solvent calibration slope in the LOQ range). Matrix effects for most of the PFASs and all brominated phenols were within 30% of ion signal enhancement, except for N-MeFOSA and N-EtFOSA with 10% ion signal suppression. In the case of other BFRs ion signal suppression was observed for OH-PBDEs and TBBPA at 20% and for HBCD isomers at 50%. To overcome these matrix effects, matrix matched calibration was used for the quantification of each analyte.

3.3. Method validation

The validation experiments for milk (Table 1) were performed with spike concentrations of 30 pg/mL for PFASs and 300 pg/mL for BFRs. BFRs were spiked higher, because of their lower instrumental sensitivity compared to PFASs. The recoveries of PFASs were 90–110% with RSD $<19\%$, and for BFRs recoveries ranged from 80–111% with RSD $<7\%$. LOQs of PFASs ranged from 3 to 20 pg/mL, which are slightly lower or comparable with other recent published studies in the range of 5–25 pg/mL for PFOS and PFOA [9,20,21,34–36]. The higher LOQs of short chain PFCAs, PFBA and PFPeA (both 20 pg/mL) were due to frequently observed matrix interferences. LOQs of BFRs were in the range of 3–60 pg/mL, except 2,4-DBP with an LOQ 200 pg/mL. The detection sensitivity of this compound is approximately 10 times lower in comparison to 2,4,6-TBP and PBP (both LOQ 30 pg/mL). In regards to our LOQs of α, β and γ-HBCD (30, 30 and 6 pg/mL; 1, 1 and 0.2 ng/g lw, respectively) and TBBPA (60 pg/mL; 2 ng/g lw), values are also comparable to recent published studies. Shi et al. reported LOQs for α, β, γ-HBCD 50, 30 and 20 pg/g, respectively and 20 pg/g for TBBPA [37]; Kakimoto et al. published LOQs for isomers of HBCD 0.1, 0.1 and 0.2 ng/g lw [38].

The validation experiments of the method for infant formula were performed by at spike concentration of 60 pg/g for PFASs and OH-PBDEs and 1000 pg/g for brominated phenols, HBCDs and TBBPA. Recoveries of PFASs and BFRs were in the range of 93–117% and 81–101%, respectively. RSDs for all target analytes were lower than 23%. The achieved LOQs for PFASs and BFRs were in the range of 5–50 pg/g and 50–450 pg/g, respectively. LOQs for 2,4-DBP (450 pg/g), PFBA (50 pg/g) and PFPeA (20 pg/g) were higher due to the above mentioned reasons. This method was

also verified for special formulas with higher contents of proteins, fats and carbohydrates than milk formula. Recoveries and RSDs were comparable for both matrices. An example chromatogram including all of the target analytes involved in this study is shown in Fig. 2.

Within our experiments, all target analytes fulfill the linearity in the calibration range mentioned above with R^2 higher than 0.99.

3.4. Concentrations of PFASs and BFRs in human breast milk samples

The results obtained by the analysis of 50 breast milk samples are summarized in Table 2. From the 18 target PFASs, only 5 analytes were detected above the LOQ, specifically PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoro-1-hexanesulfonate (PFHxS) and PFOS. The total concentration of PFASs in human breast milk ranged from 38 to 279 pg/mL (mean 115, median 105 pg/mL). The most abundant substances were PFOA and PFOS, which were detected in 100% of the examined samples. Concentrations were in the range of 12–128 pg/mL (mean 50, median 44 pg/mL) and 7–114 pg/mL (mean 33, median 30 pg/mL) for PFOA and L-PFOS, respectively. Other detected analytes were PFNA found in 48% of milk samples in the range of $<$6–15 pg/mL, followed by PFDA and PFHxS found in less than 10% of samples with concentrations ranging from $<$6 to 22 pg/mL. In the case of PFOS, its isomers, L-PFOS and Br-PFOS, were determined separately. Br-PFOS were quantified in 72% of the samples in the concentration range of $<$10–63 pg/mL (mean 20, median 17 pg/mL). The chromatogram of L-PFOS and Br-PFOS in a real milk sample is shown in Fig. 3. Origination of branched isomer occurs during electrochemical fluorination (ECF), process used for PFOS synthesis [2]. The resulting technical mixture typically contains 21–35% of Br-PFOS and 65–79% of L-PFOS [39]. The relative contribution of L-PFOS in the samples positive also for Br-PFOS was in the range of 31–88%. It was slightly wider compared to the technical PFOS mixture. Several studies [40–42] published L/Br-PFOS relative contributions in human serum/plasma ranging from 50% to 70% for L-PFOS, which also did not match the ratio in the technical mixture. The recent studies [43,44] reported isomer specific rates of PFOS-precursor (PFOSA) biotransformation, which may explain the common

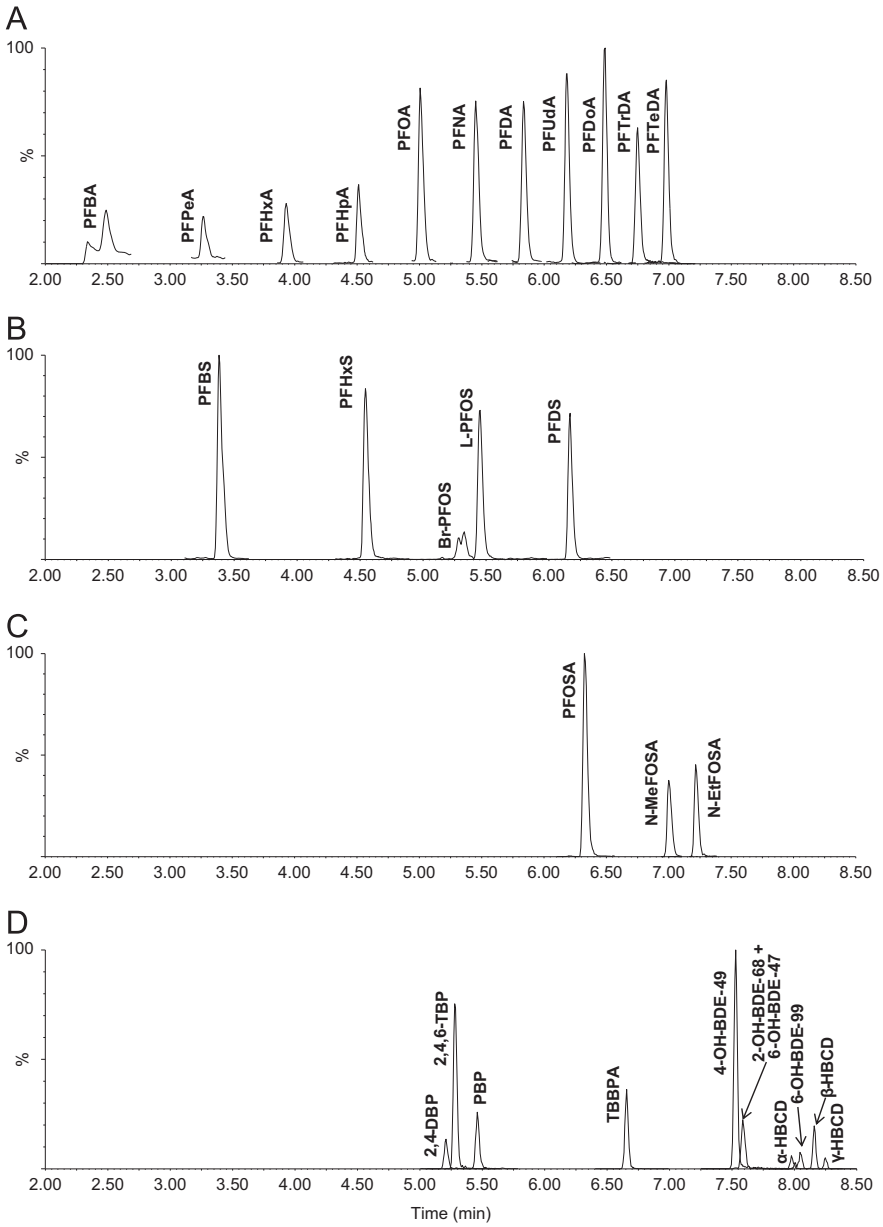


Fig. 2. Chromatograms of (A) PFCAs, (B) PFSAs, (C) non-ionic FOSAs and (D) BFRs in matrix match calibration standard (milk) at concentration 0.5 ng/mL.

Table 2
Concentrations of detected PFASs and BFRs in human breast milk ($n=50$)[a,b].

	Analyte	Samples > LOQ (%)	LOQ	Mean	Median	Minimum	Maximum
pg/mL	PFOA	100	6	50	44	12	128
	L-PFOS	100	5	33	30	7	114
	Br-PFOS	72	10	20	17	< 10	63
	PFNA	48	6			< 6	15
	PFDA	10	6			< 6	12
	PFHxS	8	6			< 6	22
	α-HBCD	28	30			< 30	1660
	TBBPA	30	60			< 60	16,200
ng/g lw	α-HBCD	28	1			< 1	76
	TBBPA	30	2			< 2	688

[a] Mean and median values were calculated when more than 70% samples were positively detected in concentrations above the LOQ.
[b] For results below LOQ one-half the LOQ value was used.

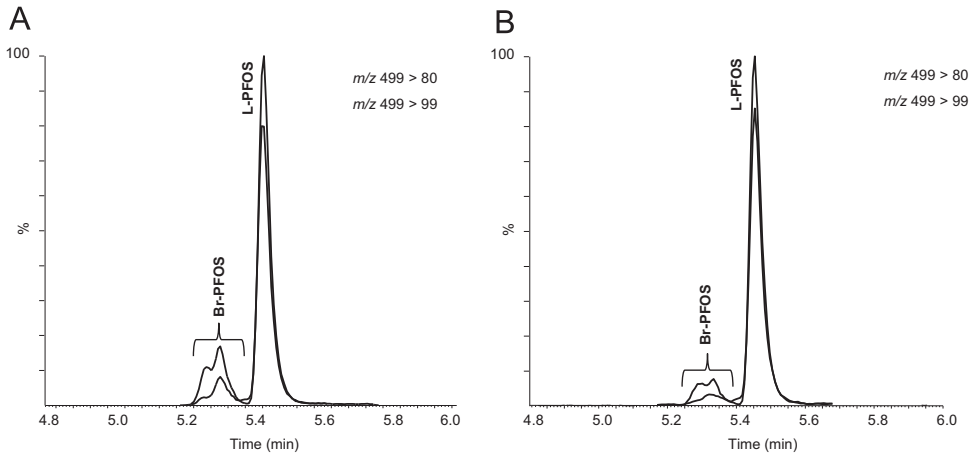


Fig. 3. Chromatograms of (A) Br- and L-PFOS in matrix matched calibration standard ($c=1$ ng/mL) and (B) human breast milk sample. The concentrations of Br- and L-PFOS in sample were 16 pg/mL and 114 pg/mL, respectively.

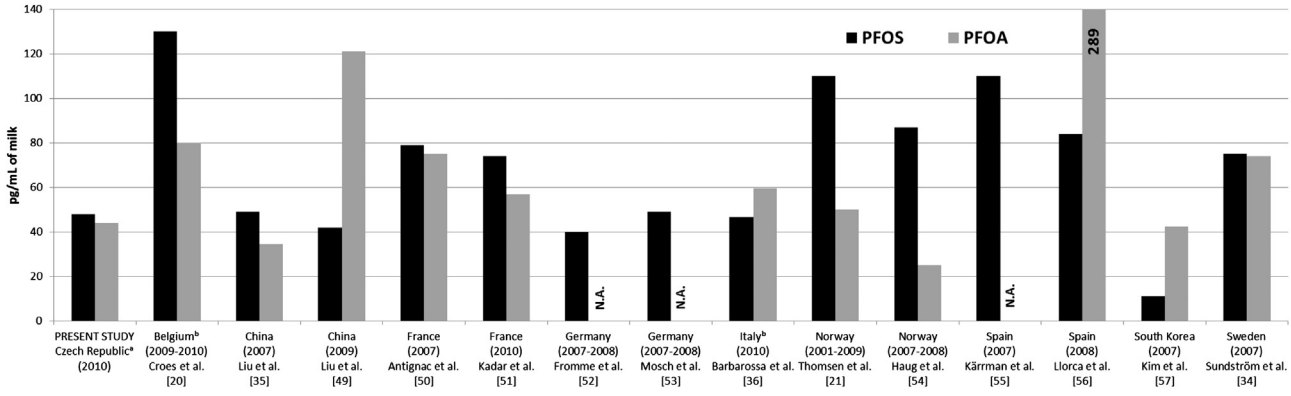


Fig. 4. Median concentrations of PFOS and PFOA (pg/mL) in human breast milk samples from various countries according to years of sampling (in the brackets), N.A. – not available. [a]Median of PFOS is expressed based on the sum of L- and Br-PFOS (pg/mL). [b]Mean of PFOS and PFOA (pg/mL).

observation of enriched PFOS isomers in human sera. However, the PFOSA concentrations in food are often minimal or undetected [45,46]. It is assumed, that the main route of exposure to PFOS isomers is through diet [47] or dust [48]. When we searched data from similar studies concerned with PFASs in other countries, mainly PFOS and PFOA were studied. Fig. 4 gives an overview on PFOS and PFOA concentrations in human milk samples collected since the year 2007. Our data are comparable with other recent reported results [20,21,34–36,49–57].

From 11 target BFRs, only TBBPA and α-HBCD were identified and quantified in the human breast milk samples. TBBPA was found in 30% of samples at a wide concentration range, < 60–16,200 pg/mL (< 2 to 688 ng/g lw). As the number of published studies related to TBBPA concentration levels in human breast milk is extremely limited, a robust comparison of our results with other available data is hardly achievable. Nevertheless, the maximum concentration of TBBPA was approximately 100 times higher compared to those previously published studies [37,58–60].

α-HBCD was detected in 28% of milk samples at concentrations ranging from < 30 to 1660 pg/mL (< 1 to 76 ng/g lw). In the technical mixture γ-HBCD is the most abundant isomer (> 70%), but in the majority of biota samples including our results the

α-HBCD dominated. Some reports have suggested that β- and γ-HBCD can be more extensively metabolized in organisms than α-HBCD [61]. Nevertheless, a few published studies showed the dominance of γ-HBCD in milk samples, which may be related to a recent exposure to a technical mixture [62,63]. In any case, there is a high variability between reported concentrations of the sum of the three isomers (Σ HBCDs) previously published and recently summarized [64]; the mean concentrations are varied from 0.09 to 27 ng/g lw, compared with our results, these data are lower.

Similar to other studies dealing with the occurrence of halogenated pollutants in human milk, the correlation between the age of mother and other factors (e.g. number of children, body weight, occupation and dietary habits) was assessed, but no such trend was found for any of the target compounds. Also the correlation between detected analytes representing both target groups was investigated, nevertheless no relationship was observed.

3.5. PFASs and BFRs in infant formula

No significant contamination was found in examined infant formula samples. In a three samples, the analytes exceeding the LOQ were PFOS, PFOA and PFNA, and the highest contamination in one sample was 19, 10 and 11 pg/g, respectively. BFRs were not detected in investigated samples of infant formula.

Currently, there is no information available regarding the occurrence of BFRs in this type of samples and also limited data exists on PFASs occurrence in infant formula. Although a few positive samples were found in our study, infant formula, under certain conditions, can be also important source of exposure to PFASs and, therefore should be controlled. As documented by a recent study, several long chain PFCAs were detected in milk infant formula made in Japan and China [65]. PFOA, PFNA and PFDA were detected and the mean concentrations ranged between 10 and 28 pg/mL for both countries. In a former study [66] PFOS and PFHxS were found in concentrations also comparable with our study. Within a German study, no PFASs were detected in infant formula samples above the LOQs which ranged from 10 to 50 pg/mL [52]. Finally, in the Spanish study [56], six PFASs were found in milk infant formula and cereals baby food samples, represented by PFOA, perfluoro-7-methyl perfluorooctanoic acid (i,p-PFNA), PFDA, PFOS and perfluorodecanesulfonate (PFDS) in the concentration range of 55–1290 pg/g. The authors assumed that the presence of PFASs in samples could be associated with possible migration/contamination from packaging and production processes. This was supported by the fact that the profile of PFASs present in these products differed from that in most human breast milk analyzed within this Spanish study in which only PFOS, PFOA and i,p-PFNA were detected.

4. Conclusions

Within this study, the new analytical approach for the simultaneous determination of 18 PFASs and 11 BFRs including their metabolites in milk and infant formula has been developed and validated. The sample preparation procedure is based on extraction with acetonitrile and subsequent purification of a crude organic extract (obtained by partition induced by added inorganic salts) with d-SPE using a C18 sorbent. Good performance characteristics were achieved for all target analytes in both matrices, the method recoveries ranged from 80 to 117% with the relative standard deviations lower than 23% for all analytes and the quantification limits were in range from 3–200 pg/mL for milk and 5–450 pg/g for infant formula. The benefits of the method are a rapid and simple sample extraction (ten samples/hour) with a minimal solvent consumption and practically no need of expensive laboratory equipment.

This novel method was successfully applied within the pilot survey for examination of PFASs and BFRs in 50 human breast milks from Olomouc region in the Czech Republic. The results clearly indicated the ubiquitous occurrence of PFASs and some of BFRs in the general Czech population, with PFOS and PFOA being the most abundant contaminants. Subsequently, investigation of six commercial powdered milk formula samples showed no significant contamination, only traces above LOQs were found in few samples.

This is the very first study enabling simultaneous analysis of PFASs and BFRs including several metabolites in human milk and infant formulas, which has never been published before.

Acknowledgments

This study was funded by projects MSMT 6046137305 and 7E08068 supported by the Ministry of Education, Youth and Sports, Czech Republic, and the method was developed within the FP7 EU project PERFOOD (PERFluorinated Organics in Our Diet), no. 227525.

Appendix A. supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.08.040.

References

[1] M. Alaee, P. Arias, A. Sjödin, Å. Bergmann, Environ. Int. 29 (2003) 683–689.
[2] H.J. Lehmler, Chemosphere 58 (2005) 1471–1496.
[3] S.C. Cunha, K. Kalachova, J. Pulkrabova, J.O. Fernandes, M.B.P.P. Oliveira, A. Alves, J. Hajslova, Chemosphere 78 (2010) 1263–1271.
[4] W. D´Hollander, L. Roosens, A. Covaci, C. Cornelis, H. Reynders, K. Van CampenhoutP. de VoogtL. Bervoets, Chemosphere 81 (2010) 478–487.
[5] I. Ericson, R. Marti-Cid, M. Nadal, B. van Bavel, G. Lindström, J.L. Domingo, J. Agric. Food Chem. 56 (2008) 1787–1794.
[6] S.P.J. van Leeuwen, M.J.M. van Velzen, C.P. Swart, I. van der Vee, W.A. Traag, J. de Boer, Environ. Sci. Technol. 43 (2009) 4009–4015.
[7] M. Wilhelm, S. Bergamnn, H.D. Dieter, Int. J. Hyg. Environ. Health 213 (2010) 224–232.
[8] G. Malarvannan, T. Isobe, A. Covaci, M. Prudente, S. Tanabe, Sci. Total Environ. 442 (2013) 366–379.
[9] A. Kärrman, G. Lindström, TrAC Trends Anal. Chem. 46 (2013) 118–128.
[10] G.W. Olsen, J.L. Buttenhoff, L.R. Zobel, Reprod. Toxicol. 27 (2009) 212–230.
[11] European Food Safety Authority Website; ⟨http://www.efsa.europa.eu/en/topics/topic/bfr.htm⟩ (accessed 05.01.13).
[12] EFSA Panel on Contaminants in the Food Chain (CONTAM). Scientific opinion on brominated flame retardants (BFRs) in food: brominated phenols and their derivatives. EFSA J. 10, 2012, p. 2634.
[13] Scientific Opinion of the Panel on Contaminants in the Food Chain. Perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and their salts. EFSA J. 653, 2008, pp. 1–131.
[14] European Food Safety Authority. Perfluoroalkylated substances in food: occurence and dietary exposure. EFSA J. 10, 2012, p. 2743.
[15] R. Schoula, J. Hajslova, V. Bencko, J. Poustka, K. Holadova, V. Vizek, Chemosphere 33 (1993) 1485–1494.
[16] T. Cajka, J. Hajslova, Bull. Environ. Contam. Toxicol. 70 (2003) 913–919.
[17] V. Bencko, M. Cerna, L. Jech, J. Smid, Environ. Toxicol. Pharmacol. 18 (2004) 83–90.
[18] R. Kazda, J. Hajslova, J. Poustka, T. Cajka, Anal. Chim. Acta 520 (2004) 237–243.
[19] L. Roosens, W. D´Hollander, L. Bervoets, H. Reynders, K. Van Campenhout, C. Cornelis, R. Van den Heuvel, G. Koppen, A. Covaci, Environ. Pollut. 158 (2010) 2546–2552.
[20] K. Croes, A. Colles, G. Koppen, E. Govarts, L. Bruckers, E. Van de Mieroop, V. Nelen, A. Covaci, A.C. Dirtu, C. Thomsen, L.S. Haug, G. Becher, M. Mampaey, G. Schoeters, N. Van Larebeke, W. Baeyens, Chemosphere 89 (2012) 988–994.
[21] C. Thomsen, L.S. Haug, H. Stigum, M. Frøshaug, S.L. Broadwell, G. Becher, Environ. Sci. Technol. 44 (2010) 9550–9556.
[22] A. Covaci, S. Voorspoels, J. de Boer, Environ. Int. 29 (2003) 735–756.
[23] S. Morris, P. Bersuder, C.R. Allchin, B. Zegers, J.P. Boon, P.E.G. Leonards, J. de Boer, TrAC Trends Anal. Chem. 25 (2006) 343–349.
[24] A. Covaci, S. Voorspoels, M.A.E. Abdallah, T. Geens, S. Harrad, R.J.Law, J. Chromatogr. A 1216 (2009) 346–363.
[25] S. Lacorte, M.G. Ikonomou, Chemosphere 74 (2009) 412–420.

[26] H. Chang, Y. Wan, J. Naile, X. Zhang, S. Wiseman, M. Hecker, M.H.W. Lam, J. P. Giesy, P.D. Jones, J. Chromatogr. A 1217 (2010) 506–513.
[27] C. Carignan, M.A.E. Abdallah, N. Wu, W. Heiger-Bernays, M.D. McClean, S. Harrad, T.F. Webster, Environ. Sci. Technol. 46 (2012) 12146–12153.
[28] E. Eljarrat, P. Guerra, E. Martínez, M. Farré, J.G. Alvarez, M. López-Teijón, D. Barceló, Environ. Sci. Technol. 43 (2009) 1940–1946.
[29] S. Mas, O. Jáuregui, F. Rubio, A. de Juan, R. Tauler, S. Lacorte, J. Mass Spectrom. 42 (2007) 890–899.
[30] M. Anastassiades, S.J. Lehotay, D. Stajnbaher, F.J. Schenck, J. AOAC Int. 86 (2003) 412–431.
[31] J.L.M. Vidal, A.G. Frenich, M.M. Aguilera-Luiz, R. Romero-González, Anal. Bioanal. Chem. 397 (2010) 2777–2790.
[32] O. Lacina, M. Zachariasova, J. Urbanova, M. Vaclavikova, T. Cajka, J. Hajslova, J. Chromatogr. A 1262 (2012) 8–18.
[33] S.J. Lehotay, K. Mastovska, S.J. Yun, J. AOAC Int. 88 (2005) 630–638.
[34] M. Sundström, D.J. Ehresman, A. Bignert, J.L. Butenhoff, G.W. Olsen, S.C. Chang, Å Bergman, Environ. Int. 37 (2011) 178–183.
[35] J. Liu, J. Li, Y. Zhao, Y. Wang, L. Zhang, Y. Wu, Environ. Int. 36 (2010) 433–438.
[36] A. Barbarossa, R. Masetti, T. Gazzotti, D. Zama, A. Astolfi, B. Veyrand, A. Pession, G. Pagliuca, Environ. Int. 51 (2013) 27–30.
[37] Z. Shi, Y.N. Wu, J.G. Li, Y.F. Zhao, J.F. Feng, Environ. Sci. Technol. 43 (2009) 4314–4319.
[38] K. Kakimoto, K. Akutsu, Y. Konishi, Y. Tanaka, Chemosphere 71 (2008) 1110–1114.
[39] S. Chu, R.J. Letcher, Anal. Chem. 81 (2009) 4256–4262.
[40] A. Kärrman, I. Langlois, B. van Bavel, G. Lindström, M. Oehme, Environ. Int. 33 (2007) 782–788.
[41] N. Riddell, G. Arsenault, J.P. Benskin, B. Chittim, J.W. Martin, A. McAlees, R. McCrindle, Environ. Sci. Technol. 43 (2009) 7902–7908.
[42] L.S. Mosch, C. Thomsen, G. Becher, Environ. Sci. Technol. 43 (2009) 2131–2136.
[43] J.P. Benskin, A. Holt, J.W. Martin, Environ. Sci. Technol. 43 (2009) 8566–8572.
[44] M.S. Ross, C.S. Wong, J.W. Martin, Environ. Sci. Technol. 46 (2012) 3196–3203.
[45] P. Hradkova, J. Poustka, V. Hlouskova, J. Pulkrabova, M. Tomaniova, J. Hajslova, Czech J. Food Sci. 28 (2007) 333–342.
[46] A. Schecter, J. Colacino, D. Haffner, K. Patel, M. Opel, O. Päpke, L. Birnbaum, Environ. Health Perspect. 118 (2010) 796–802.
[47] M. Houde, G. Czub, J.M. Small, S. Backus, X. Wang, M. Alaee, D.C.G. Muir, Environ. Sci. Technol. 42 (2008) 9397–9403.
[48] S. Beesoon, G.M. Webster, M. Shoeib, T. Harner, J.P. Benskin, J.W. Martin, Environ. Health Perspect. 119 (2011) 1659–1664.
[49] J. Liu, J. Li, Y. Liu, H.M. Chan, Y. Zhao, Z. Cai, Y. Wu, Environ. Int. 37 (2011) 1206–1212.
[50] J.P. Antignac, B. Veyrand, H. Kadar, P. Marchand, A. Oleko, B. Le Bizec, S. Vandentorren, Chemosphere 91 (2013) 802–808.
[51] H. Kadar, B. Veyrand, A. Barbarossa, G. Pagliuca, A. Legrand, C. Bosher, C. Y. Boquien, S. Durand, F. Monteau, J.P. Antignac, B. Le Bizec, Chemosphere 85 (2011) 473–480.
[52] H. Fromme, C. Mosch, M. Morovitz, I. Alba-Alejandre, S. Boehmer, M. Kiranoglu, F. Faber, I. Hannibal, O. Genzel-Boroviczény, B. Koletzko, W. Volkel, Environ. Sci. Technol. 44 (2010) 7123–7129.
[53] C. Mosch, M. Kiranoglu, H. Fromme, W. Völkel, J. Chromatogr. B 878 (2010) 2652–2658.
[54] L.S. Haug, S. Huber, G. Becher, C. Thomsen, Environ. Int. 37 (2011) 687–693.
[55] A. Kärrman, J. Domingo, X. Llebaria, M. Nadal, E. Bigas, B. Bavel, G. Lindström, Environ. Sci. Pollut. Res. 17 (2010) 750–758.
[56] M. Llorca, M. Farré, Y. Picó, M.L. Teijón, J.G. Álvarez, D. Barceló, Environ. Int. 36 (2010) 584–592.
[57] S.K. Kim, K.T. Lee, C.S. Kang, L. Tao, K. Kannan, K.R. Kim, C. Kim, J.S. Lee, P. S. Park, Y.W. Yoo, J.Y. Ha, Y.S Shin, J.H. Lee, Environ. Pollut. 159 (2011) 169–174.
[58] Z. Shi, Y. Jiao, Y. Hu, Z. Sun, X. Zhou, J. Feng, J. Li, Y. Wu, Sci. Total Environ. 452–453 (2013) 10–18.
[59] M.A.E. Abdallah, S. Harrad, Environ. Int. 37 (2011) 443–448.
[60] R. Cariou, J.P. Antignac, D. Zalko, A. Berrebi, J.P. Cravedi, D. Maume, P. Marchand, F. Monteau, D.A. Riu, F. Andre, B. Le Bizec, Chemosphere 73 (2008) 1036–1041.
[61] B.N. Zegers, A. Met, R. Van Bommel, C. Minkenberg, T. Hamers, J.H. Kamstra, G. J. Pierce, J.P. Boon, Environ. Sci. Technol. 39 (2005) 2095–2100.
[62] G. Malarvannan, T. Kunisue, T. Isobe, A. Sudaryanto, S. Takahashi, M. Prudente, A. Subramanian, S. Tanabe, Environ. Pollut. 157 (2009) 1924–1932.
[63] E. Eljarrat, P. Guerra, E. Martínez, M. Farré, J.G. Alvarez, M. López-Teijón, D. Barceló, Environ. Sci. Technol. 43 (2009) 1940–1946.
[64] G. Malarvannan, T. Isobe, A. Covaci, M. Prudente, S. Tanabe, Sci. Total Environ. 442 (2013) 366–379.
[65] Y. Fujii, J. Yan, K.H. Harada, T. Hitomi, H. Yang, P. Wang, A. Koizumi, Chemosphere 86 (2012) 315–321.
[66] L. Tao, J. Ma, T. Kunisue, E.L. Libelo, S. Tanabe, K. Kannan, Environ. Sci. Technol. 42 (2008) 8597–8602.

APPENDIX III

Urbancova K., Lankova D., Sram R.J., Hajslova J., Pulkrabova J.

Urinary metabolites of phthalates and di-iso-nonyl cyclohexane-1,2-dicarboxylate (DINCH) – Czech mothers´ and newborns´ exposure biomarkers

Environmental Research (2019) 173: 342–348

doi: 10.1016/j.envres.2019.03.067

Environmental Research 173 (2019) 342–348

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Urinary metabolites of phthalates and di-*iso*-nonyl cyclohexane-1,2-dicarboxylate (DINCH)–Czech mothers' and newborns' exposure biomarkers

Katerina Urbancova[a], Darina Lankova[a], Radim J. Sram[a,b], Jana Hajslova[a], Jana Pulkrabova[a,*]

[a] University of Chemistry and Technology, Faculty of Food and Biochemical Technology, Department of Food Analysis and Nutrition, Technicka 3, Prague 6, 16628, Czech Republic

[b] Institute of Experimental Medicine CAS, Videnska 1083, Prague 4, 14220, Czech Republic

ARTICLE INFO

Keywords:
Biomonitoring
Phthalates
DINCH
Metabolites
UHPLC–MS/MS

ABSTRACT

To assess human exposure to hazardous diesters of phthalic acid and their substitute di-*iso*-nonyl cyclohexane-1,2-dicarboxylate (DINCH), concentrations of their metabolites in urine should be determined. For the purpose of this biomonitoring study, a quick and easy sample preparation procedure for the simultaneous determination of eight phthalate and four DINCH metabolites in urine has been implemented and validated. Following the enzymatic hydrolysis and dilution with methanol, the sample is ready for the analysis by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). The limits of quantification of this method ranged from 0.15 to 0.4 ng/mL urine with recoveries of 60–126% and repeatability in the range of 1–11%. The validated method was subsequently used for the analysis of urine samples collected from mothers and their newborn children living in two localities of the Czech Republic (Karvina and Ceske Budejovice, 2013–2014). Median concentrations of all measured metabolites (Σmetabolites) were slightly lower in the urine samples collected from children (77.7 ng/mL urine) compared to their mothers (115.3 ng/mL urine), but no correlation was found between the concentrations of target compounds in children's and mothers' urine samples. The analyte with the highest concentration was monobutyl phthalate (MBP), with the median concentration of 32.1 ng/mL urine in the urine samples collected from mothers and 17.2 ng/mL urine in the samples collected from their children. This compound was also found in almost all of the measured samples. On the other hand, mono-isononyl-cyclohexane-1,2-dicarboxylate (MINCH) was not found in any urine sample. The most contaminated samples were collected from children living in the Karvina locality (median Σmetabolites 103.2 ng/mL urine), where the mono (2-ethyl-5-carboxypentyl) phthalate (cx-MEHP) compound contributed 43% to the total content of phthalate metabolites in newborns' urine. The results from our study are comparable with concentrations of the target compounds from Norway and Germany and lower compared to the results concluded in Sweden.

1. Introduction

A number of environmental substances have been targeted within various national biomonitoring programmes aimed at an assessment whether and to what extent respective contaminants entered human bodies and how exposure may be changing over time. Diesters of phthalic acid and their substitute di-*iso*-nonyl cyclohexane-1,2-dicarboxylate DINCH represent groups of chemicals involved in the priority list of the current large HBM4EU project (HBM4EU 2017).

Diesters of phthalic acid, also known as phthalates, have been used for a wide range of practical applications. Although most commonly used as PVC plasticisers, they are also added to paints, inks, cosmetics products and coated tablets (Thayer et al., 2015). A compound DINCH is currently the most widely used alternative plasticiser as a substitute for endocrine disrupting and reprotoxic phthalates. It was introduced in 2002 under the Hexamoll® trademark and is structurally similar to di-*iso*-nonyl phthalate (DiNP). DINCH is mostly used as a plasticiser in medical devices, toys and food packaging (Bui et al., 2016; Correia-Sa et al. 2017; Fromme et al., 2016). Phthalates and DINCH belong to a group of additive plasticisers that are not bound to the material by a strong chemical bond. Consequently, these compounds are easily released into the environment via many processes such as evaporation, leaching or mechanical damage (Abb et al., 2009; Marie et al., 2015; Sabaredzovic et al., 2015). Because of their ubiquitous presence in the environment, humans can be exposed to these chemicals through various pathways (ingestion, inhalation or absorption through the skin).

* Corresponding author.
E-mail address: Jana.Pulkrabova@vscht.cz (J. Pulkrabova).

https://doi.org/10.1016/j.envres.2019.03.067
Received 10 January 2019; Received in revised form 26 March 2019; Accepted 26 March 2019
Available online 30 March 2019
0013-9351/

As soon as phthalates and/or DINCH enter the human body, they are rapidly metabolised by intestinal lipases and esterases. Monoesters, the products of a respective parent compound hydrolysis, are then excreted via urine or further oxidised to alcohols, ketones or carboxylic acids. Both monoesters and oxidation products leave the body in either free form or conjugated with glucuronic acid (Johns et al., 2015; Minguez-Alarcon et al., 2016; Schütze et al., 2014).

Phthalate metabolites have adverse effects on human health, such as disruption of the endocrine system. Specifically, their reprotoxicity has been documented in several toxicological studies (Engel et al., 2018; Hartmann et al., 2015; Kim et al., 2017; Perng et al., 2017; Thayer et al., 2015). Because of the health risks, the European Commission recently restricted articles containing four phthalates (di (2-ethylhexyl) phthalate (DEHP), dibutyl phthalate DBP, benzyl butyl phthalate (BBP) and di-*iso*-butyl phthalate (DiBP)) from placement on the market (European Commission 2018). It is assumed that DINCH and its metabolites do not have harmful effects on human health. However, since this compound is relatively new, more data about its behaviour in the human body are needed (Correia-Sa et al. 2017). In order to assess to what extent people are exposed to these compounds, it is important to monitor concentrations of phthalate and DINCH metabolites in biological samples. At present, for these purposes, urine is the most widely used matrix. Its collection is not invasive to the human body and it is available in a sufficient quantity (Esteban and Castaño, 2009).

To date, there is only a limited number of publications focused on the analysis of both groups of these environmental contaminants in human urine within a single method. The analytical procedure applicable to the determination of phthalate and DINCH metabolites in urine requires several steps. Generally, the first step in sample preparation for both groups is an enzymatic hydrolysis of conjugated forms of the target compounds, using β-glucuronidase. For isolation of phthalate metabolites, solid phase extraction (SPE) or online SPE is most commonly applied, with the sorbent typically being silica C_{18} or hydrophilic-lipophilic balanced sorbent (HLB). Polar solvents such as water, methanol, acetonitrile, or their mixtures are then used for elution (Axelsson et al., 2015; Chang et al., 2017; Dewalque et al., 2015; Myridakis et al., 2015; Rocha et al., 2017). Similarly, DINCH metabolites are isolated after the enzymatic hydrolysis, mainly by online SPE with a C_{18} sorbent and acidified acetonitrile (0.1% acetic acid) as an elution solvent (Giovanoulis et al., 2016; Larsson et al., 2017; Wu et al., 2017). Identification and quantification of the target compounds are performed using ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS). The reported limits of quantification (LOQs) range from 0.1 to 1.8 ng/mL urine (Giovanoulis et al., 2016; Heffernan et al., 2016; Machtinger et al., 2018; Gomez Ramos et al., 2016).

There is only very limited information (based on a measurement of biomarkers) on the exposure of the Czech population to phthalates and DINCH. The only human biomonitoring studies in the Czech Republic were performed in 2011 within the COPHES/DEMOCOPHES project, when concentrations of 9 phthalate metabolites in urine samples collected from mothers and their children (age 6–11) were measured (Cerna et al., 2015) and in 2013 where concentration of 15 phthalate metabolites was assessed in urine samples collected from non-obese adults (age 18–65) (Mullerova et al., 2016).

The current study was focused on an application of a new simple sample preparation procedure which after the enzymatic deconjugation requires only dilution of the urine sample with an organic solvent. The key objective was to have available a quick and easy method with sufficiently low LOQs, enabling both phthalate and DINCH metabolites in urine samples to be controlled. And then use the validated procedure for the assessment of the exposure of mothers and their newborn children living in two localities of the Czech Republic (Karvina and Ceske Budejovice, 2013–2014) to phthalates and DINCH.

2. Materials and methods

2.1. Standards

The Standard Reference Material® (SRM) 3673 (Organic Contaminants in Non-Smokers´ Urine) used for the method evaluation and validation was supplied by the US National Institute of Standards and Technology (NIST, USA). This material contains certified concentrations of eight phthalate metabolites (monoethyl phthalate (MEP), mono-*iso*-butyl phthalate (MiBP), mono-*n*-butyl phthalate (MBP), monobenzyl phthalate (MBzP), mono (2-ethylhexyl) phthalate (MEHP), mono (2-ethyl-5-hydroxyhexyl) phthalate (OH-MEHP), mono (2-ethyl-5-oxohexyl) phthalate (oxo-MEHP) and mono (2-ethyl-5-carboxypentyl) phthalate (cx-MEHP)). Certified standards of phthalate metabolites represented by MEP, MiBP, MBP, MBzP and MEHP were purchased from AccuStandard®, Inc. (USA). OH-MEHP, oxo-MEHP, cx-MEHP, isotopically labelled compounds monoethyl phthalate-d_4 (d_4-MEP), mono-*n*-butyl phthalate-d_4 (d_4-MBP) and mono (2-ethylhexyl) phthalate-d_4 (d_4-MEHP), metabolites of compound DINCH, namely 1,2-cyclohexanedicarboxylic acid mono 4-methyloctyl ester (MINCH), 2-(((hydroxy-4-methyloctyl)oxy)carbonyl)cyclohexanecarboxylic acid (OH-MINCH), 2-(((4-methyl-7-oxyooctyl)oxy)carbonyl)cyclohexanecarboxylic acid (mixture of diastereomers) (oxo-MINCH), 1,2-cyclohexanedicarboxylic acid mono 4-methyl-7-carboxy-heptyl ester (cx-MINCH) and isotopically-labelled metabolites 2-(((hydroxy-4-methyloctyl)oxy)carbonyl) cyclohexanecarboxylic-d8 acid (d_8-OH-MINCH) and 2-(((4-Methyl-7-oxyooctyl)oxy)carbonyl) (cyclohexane-d8)carboxylic acid (mixture of diastereomers) (d_8-oxo-MINCH) were purchased from Toronto Research Chemicals, Inc. (Canada).

2.2. Chemicals, reagents and other materials

Enzyme β-glucuronidase (from *E. coli* K 12) and picric acid were supplied by Sigma Aldrich (USA). HPLC gradient methanol was delivered by Merck (Germany). Unsterile polytetrafluoroethylene (PTFE, 5.0 µm, Ø 25 mm) filters were purchased from Rotilabo® (Germany). 96-well microtiter plates were obtained from the Gama Group (Czech Republic). Polypropylene (PP) centrifuge tube filters (nylon, pore size 0.22 µm) were supplied by Sigma-Aldrich (USA).

2.3. Sample collection

The urine samples were collected in the Ceske Budejovice Hospital, Department of Obstetrics and Department of Neonatology, and in the Karvina Hospital, Department of Obstetrics and Department of Neonatology in years 2013–2014. The study was approved by the Ethics Committee of both hospitals and the Institute of Experimental Medicine CAS in Prague. Each mother signed a written consent. All mothers had a pregnancy without complications, their age ranged from 19 to 44 years and their body mass index (BMI) was in the range of 17–38. The average length of the pregnancy was 40 weeks and the birth weight of the children ranged from 2.400 g to 4.900 g. A total of 204 samples (102 samples from mothers and 102 samples from their newborn children) was obtained. The urine samples from both mothers and their newborns were collected 3–4 days after the birth in a hospital as spot samples. The urine samples from the newborns were collected using a special plastic bag called “Adhesive urine bag for children” which is made of foil with a hypoallergenic patch. In the center of the patch is a small hole which can be enlarged as required. This bag was directly attached to the newborn's diaper. Each mother sampled herself, using a plastic 50 mL tube after the childbirth. All mothers were instructed how to collect their samples to avoid contamination. The adhesive bags used for the sampling of newborns and the tubes used for the sampling of mothers were also tested (before sampling) for the presence of target compounds and their precursors and were evaluated as clean (no contaminating compounds were present in the blank samples from the

adhesive bags and tubes). All samples were stored in a freezer (−80 °C) prior to analysis.

2.4. Analytical method

2.4.1. Measurement of urinary creatinine

The creatinine values were used to normalise the urine concentration/dilution in individual samples, in order to ensure data comparability. The creatinine concentration was determined using a Jaffe's spectrophotometric method according to our previous study (Lankova et al., 2016). In brief, a coloured complex of creatinine with alkaline picrate was formed and consequently measured at 505 nm using a Spectrophotometer Epoch (BioTek, USA). The data obtained were evaluated by Gen5.02™ Microplate Data Analysis software.

2.4.2. Determination of phthalate and DINCH metabolites in urine

2.4.2.1. Sample preparation. In this study, we used a fast and simple sample preparation method for the simultaneous determination of eight phthalate and four DINCH metabolites in human urine. After the enzymatic hydrolysis, this procedure requires only dilution of the urine sample with an organic solvent. Sample preparation starts with 300 µL of urine sample, to which 50 µL of ammonium acetate buffer (pH 6.5) are added, together with 10 µL of enzyme β-glucuronidase and 44 µL of internal standard solution (mixture of three isotopically-labelled compounds–d_4-MEP, d_4-MBP, d_4-MEHP, d_8-OH-MINCH and d_8-oxo-MINCH at concentration 100 ng/mL). After 90 min incubation at 37 °C, 20 µL of methanol and 20 µL of formic acid are added and the prepared sample is centrifuged for 30 min at 4 °C and 10 000 rpm.

2.4.2.2. Instrumentation. An ultra-high performance liquid chromatograph (UHPLC) (Acquity UPLC®, Waters, USA) coupled to a tandem mass spectrometer (MS/MS) (Xevo TQ-S, Waters, USA) was used to identify and quantify the target analytes. The mobile phase consisted of A) 0.1% acetic acid in deionised water and B) 0.1% acetic acid in methanol. A gradient of the mobile phase is shown in Table S1 in Supporting Materials. For the separation of the target compounds, an analytical column containing reverse-phase Acquity HSS T3 (100 mm × 2.1 mm × 1.8 µm; Waters, USA) was applied. For the identification and quantification, MS/MS was used with ionisation by electrospray in negative mode (ESI-). Cone voltage was set to −2 kV, source temperature to 150 °C and desolvation temperature to 500 °C. The instrument was operated in a multiple reaction monitoring (MRM) mode. The quantitative/qualitative MRM transitions and retention times of target analytes are listed in Tables S2 and S3 in Supporting Materials.

2.4.2.3. Quality assurance/quality control. The method validation for phthalate metabolites was carried out on the SRM 3673 (urine sample of a non-smoker) in six repetitions (Table 1). Since DINCH metabolites were not certified in this material, the method validation was performed using spike (artificial contamination) of a blank urine sample. The blank urine sample was measured before it was used for the validation on the presence of target compounds and no DINCH metabolites were detected in this sample. The blank urine sample was spiked to two concentration levels (level 1–0.7 ng/mL urine and level 2–7 ng/mL urine) (Table 2). To ensure that there is no external contamination, a procedural blank sample, which contained deionised water instead of urine, was prepared with each set of samples.

The calibration standards were prepared in a solvent solution (methanol) since there was no urine sample available without the presence of all target analytes. The LOQs were determined as the lowest calibration standards where the signal to noise ratio was S/N > 10.

Measured concentrations for phthalate metabolites were in good agreement with those certified in the SRM 3673 (Table 1). Limits of quantification (LOQs) of target analytes ranged between 0.15 and 0.4 ng/mL urine, with recoveries of 70–126%. Performance characteristics obtained for DINCH metabolites (Table 2) were comparable with those reported in the literature (Minguez-Alarcon et al., 2016; Gomez Ramos et al. 2016; Silva et al., 2013). LOQs were 0.15 ng/mL urine for DINCH metabolites with recoveries of 74–100%. The lowest recovery of 60% was observed for MINCH. However, research of the DINCH metabolism indicates that MINCH is only a minor metabolite and is not present in the majority of the tested samples (Koch et al., 2013). Our laboratory also participated in the first round of the Interlaboratory Comparison Investigations (ICI) organized by the HBM4EU project (the European Union's Horizon 2020 research and innovation programme under grant agreement No 733032). The classification of the laboratory performance was based on Z-scores, which were in a satisfactory range of 0.2–1.9 for all measured analytes.

Matrix effects of this method were also evaluated using 6 different non-pooled urine samples. The matrix effects were assessed with and without the correction by the internal standards. The matrix effects non-corrected by the internal standards ranged from 5 to 47% of signal suppression. When we corrected these effects using the internal standards the signal suppression was only 0.5–15%.

3. Results and discussion

The validated method was subsequently used for the analysis of 204 real urine samples collected from mothers and their newborn children, living in two localities of the Czech Republic – Ceske Budejovice and Karvina. The results (ng/mL urine) are summarised in Table 3.

As Table 3 shows, the phthalate metabolite present in all urine samples collected from mothers was MiBP (100% of urine samples), followed by MBP (99%), MEP (98%), cx-MEHP (92%), MBzP (92%), MEHP (90%), OH-MEHP (66%), oxo-MEHP (63%). The DINCH metabolites (OH-MINCH, oxo-MINCH and cx-MINCH) were found in only 7% of all urine samples collected from mothers. Only MINCH was not

Table 1
Performance characteristics of the analytical method (n = 6)–Phthalate metabolites.

Analyte	Internal standard	Certified concentration[a] [ng/mL]	Measured concentration [ng/mL]	LOQ		REC [%]	RSD [%]
				[ng/mL urine]	[µg/g creatinine]		
MEP	d_4-MEP	80.2 ± 2.1	83.5	0.15	0.15	104	3
MiBP	d_4-MBP	5.18 ± 0.26	5.7	0.15	0.15	116	8
MBP	d_4-MBP	11.2 ± 0.7	10.4	0.15	0.15	93	6
MBzP	d_4-MBP	5.69 ± 0.17	6.5	0.15	0.15	113	5
MEHP	d_4-MEHP	4.34 ± 0.16	5.4	0.15	0.15	126	6
OH-MEHP	d_4-MEHP	22.3 ± 0.3	24.7	0.40	0.40	111	7
oxo-MEHP	d_4-MEHP	12.2 ± 0.2	11.8	0.40	0.40	97	8
cx-MEHP	d_4-MEHP	30.1 ± 1.0	21.1	0.40	0.40	70	5

LOQ–limit of quantification, REC–recovery, RSD–relative standard deviation; LOQ [µg/g creatinine] was calculated with the median concentration of creatinine (0.98 mg/mL).

[a] Concentration certified in SRM 3673 (urine of a non-smoker).

Table 2
Performance characteristics of the analytical method (n = 6)–DINCH metabolites.

Analyte	Internal standard	LOQ		Concentration level 1 (0.7 ng/mL urine)		Concentration level 2 (7 ng/mL urine)	
		[ng/mL urine]	[μg/g creatinine]	REC [%]	RSD [%]	REC [%]	RSD [%]
MINCH	d_8-OH-MINCH	0.15	0.15	62	14	60	2
OH-MINCH	d_8-OH-MINCH	0.15	0.15	83	5	74	1
oxo-MINCH	d_8-oxo-MINCH	0.15	0.15	93	9	94	2
cx-MINCH	d_8-oxo-MINCH	0.15	0.15	104	11	100	4

LOQ–limit of quantification, REC–recovery, RSD–relative standard deviation.

detected in any of the tested urine samples. In the case of newborns' urine samples, the percentage of positive samples was very similar compared to their mothers. However, the DINCH metabolites were found in only one newborn's urine sample (1%), again with the exception of MINCH, which was not detected in any of the measured samples.

In terms of concentration, the dominating phthalate metabolite was MBP with the median 32.1 ng/mL urine in samples collected from mothers and 17.2 ng/mL urine in the case of newborns' urine samples. Another compound with high concentration was MiBP and cx-MEHP. For the DINCH metabolites, the highest levels were found for cx-MINCH in both mothers' and newborns' urine samples, followed by OH-MINCH and oxo-MINCH.

The median concentrations of all measured metabolites (Σmetabolites) were slightly lower in urine samples collected from children (77.7 ng/mL urine) compared to their mothers (115.3 ng/mL urine), but no correlation was found between the levels of target compounds in children's and mothers' urine samples (the detailed results are shown in Table S4 in the Supporting Material). This result could be probably caused by the different exposure sources of phthalates. The mothers could use some personal care products containing different phthalates than those products used on the newborn children. And there also could be a different intake of phthalates by the mothers caused by the consumption of food which contains phthalates.

The highest amounts of target compounds were measured in urine samples obtained from children born in Karvina (Fig. 1). Also, a different profile of phthalate metabolites was identified in urine samples obtained from children born in the Karvina region, compared to the urine samples collected from their mothers. Cx-MEHP contributed 43% to the total content of phthalate metabolites in newborns' urine (the median concentration of cx-MEHP was 43.9 ng/mL urine and the median concentration of Σphthalate metabolites was 103.3 ng/mL urine), but only 11% in mothers' urine (the median concentration of cx-MEHP was 11.3 ng/mL urine and the median concentration of Σphthalate metabolites was 108.3 ng/mL urine) (Fig. 1). The urine

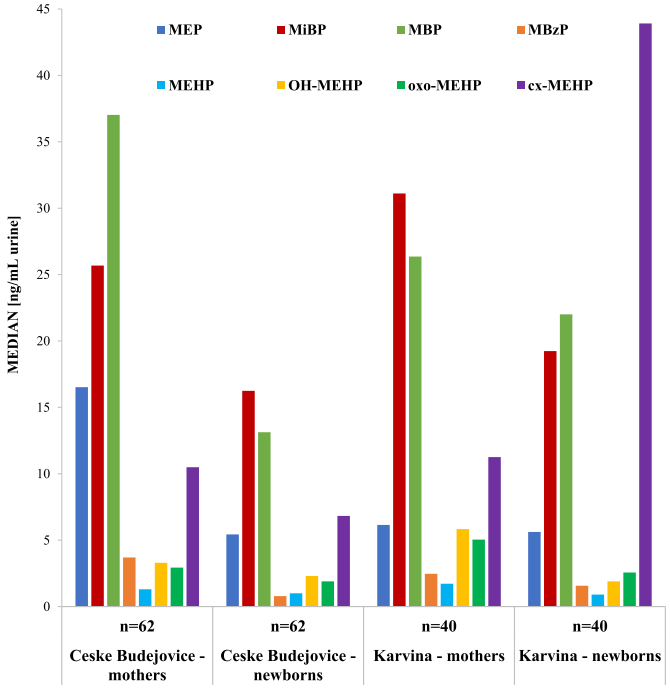


Fig. 1. Concentrations of phthalate metabolites in urine samples of mothers and their newborn children (2013–2014).

Table 3
Concentrations of phthalate and DINCH metabolites in urine samples collected from mothers and their newborn children (n = 204).

Parent compound	Target analyte	LOQ [ng/mL urine]	Mothers (n = 102) [ng/mL urine]						Newborns (n = 102) [ng/mL urine]					
			Mean	Median	Min	Max	% positive samples	95% percentile	Mean	Median	Min	Max	% positive samples	95% percentile
DEP	MEP	0.15	32.4	10.4	0.5	267.2	98	154.8	10.8	5.6	0.3	159.9	100	29.5
DiBP	MiBP	0.15	77.3	26.5	0.3	293.1	100	252.4	22.4	16.6	1.3	110.3	100	62.0
DBP	MBP	0.15	49.0	32.1	3.5	336.5	99	160.5	21.4	17.2	1.1	73.5	100	57.4
DBzP	MBzP	0.15	5.4	2.9	0.2	83.1	92	17.2	1.7	1.2	0.3	13.2	76	6.4
DEHP	MEHP	0.15	3.9	2.2	0.3	25.4	90	12.0	2.1	1.7	0.2	6.3	90	5.4
	OH-MEHP	0.40	7.6	6.7	4.6	116.2	66	19.1	2.9	0.5	2.0	17.7	50	7.7
	oxo-MEHP	0.40	4.4	3.9	2.7	54.3	63	10.9	1.5	0.2	0.5	10.1	42	4.7
	cx-MEHP	0.40	13.8	10.8	2.2	79.6	92	28.2	25.8	12.7	3.0	248.5	91	75.0
Σ phthalate metabolites		-	193.1	108.9	0.9	1060.5	100	463.3	87.3	77.4	3.7	394.2	100	206.2
DINCH	MINCH	0.15	-	-	< 0.15	< 0.15	0	-	-	-	< 0.15	< 0.15	0	-
	OH-MINCH	0.15	1.1	< 0.15	1.1	45.7	7	3.8	0.1	< 0.15	< 0.15	6.3	1	0.05
	oxo-MINCH	0.15	0.7	< 0.15	0.8	20.4	7	3.5	0.1	< 0.15	< 0.15	3.8	1	0.05
	cx-MINCH	0.15	1.4	< 0.15	1.4	48.2	7	5.6	0.2	< 0.15	< 0.15	8.8	1	0.05
Σ DINCH metabolites		-	3.1	< 0.15	3.0	114.3	-	13.1	0.3	< 0.15	< 0.15	19.0	1	0.05

When target analyte was below LOQ for the mean and median calculation 1/2 LOQ value was used.

samples were collected 3–4 days after the birth in a hospital. Based on the premise that phthalate metabolites have very short excretion lifetimes (24–48 h) (Frederiksen et al., 2007), the source of the parent compound (DEHP) of this metabolite is most probably from the hospital. This will be further investigated in a subsequent part of this study. It is also worth to notice that only the concentration of cx-MEHP is higher in urine samples collected from children. The concentration of other DEHP metabolites (OH-MEHP and oxo-MEHP) are higher in urine samples collected from mothers. This result could demonstrate that the metabolism of phthalates is different in adults and children as concluded by Enke et al. (2013) and Silva et al. (2006).

Our results indicate that phthalate metabolites should in future be analysed in studies of the newborn, as they may represent a real health risk to children's development of visual recognition memory (Ipapo et al., 2017) and ADHD (Engel et al., 2018).

3.1. Comparison to results of other studies

The measured concentrations of the target analytes were compared to other published papers dealing with the exposure to phthalates/DINCH in other regions. The comparison of the results has some limitationsbecause the results are differently presented in the papers. In some papers, geometric or arithmetic means or median values are used. For a better comparison, it would be preferable to evaluate the same data (means, medians). However, unified data are often unavailable. The units also vary between papers. For this reason, the results from our paper are presented in Table 4 in ng/mL urine and in ng/g creatinine. As mentioned above, there is only a limited number of studies where metabolites of phthalates and metabolites of the DINCH compound are measured using one method. For this reason, the concentrations of phthalate and DINCH metabolites were assessed separately.

Within the phthalate metabolites, the analyte commonly found at the highest concentration in similar studies is MEP (Table 4). However, in our study, we observed the highest concentrations for the compound MBP in the case of the urine samples collected both from mothers and their newborn children. The same result was also observed by Mullerova et al. (2016), where urinary phthalate metabolites of Czech adults were measured and Enke et al. (2013), where concentrations of phthalate metabolites in urine samples collected from German children were assessed. The overall concentrations of target compounds are comparable with those published in other surveys. Evaluation why the Czech samples have a higher concentration of MBP whereas other studies reported the dominant metabolite MEP is very difficult. For example, there is no evidence that Czech women or adults, in general, use cosmetic differently or buy other brands. Since there is available countless cosmetic products from all over the world it is difficult to say that it has an impact on the exposure of the Czech population. Moreover, in the Czech Republic, we have the European legislation. Legislatively some phthalates are restricted in cosmetic products (Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products). However, the banned phthalates are dibutyl phthalate (DBP), diethylhexyl phthalate (DEHP), bis(2-methoxyethyl) phthalate (DMEP), n-pentyl-isopentylphthalate (PiPP), di-n-pentyl phthalate (DPP), diisopentyl phthalate (DiPP), benzyl butyl phthalate (BBP) and not diethyl phthalate (DEP). The same case can be concluded in children toys, materials that are used in contact with food or medical equipment. However, such a result in the Czech population should be further investigated.

With regard to DINCH metabolites (Table 5), the compound MINCH was not detected in any of the measured samples, as published in this paper. This result is in good agreement with the expected excretion ratios proposed by Koch et al. (2013), where they concluded that less than 1% of the DINCH compound is excreted in urine as MINCH. The concentrations of the other measured metabolites are comparable between studies and on very low levels.

Table 4
Comparison of measured concentrations of phthalate metabolites in the presented study with other papers.

Analyte	Germany Women[a] (Enke et al., 2013)	Germany Children[a] (Enke et al., 2013)	Finland Children[a] (Frederiksen et al., 2014)	Poland Women[a] (Polanska et al., 2014)	Poland Children[a] (Polanska et al., 2014)	Czech Republic Women[b] (Cerna et al., 2015)	Czech Republic Children[b] (Cerna et al., 2015)	Czech Republic Adults[b] (Mullerova et al., 2016)	Australia General population[c] (Gomez Ramos et al., 2016)	USA Women[d] (Wu et al. 2017)	Israel Women[d] (Machtinger et al., 2018)	Sweden Women[d] (Shu et al., 2018)	Czech Republic Women[e] (Presented study)	Czech Republic Newborns[e] (Presented study)
Samples	n = 9	n = 9	n = 58	n = 165	n = 148	n = 117	n = 120	n = 201	n = 24 pooled samples	n = 49	n = 136	n = 1062	n = 102	n = 102
MEP	61.4	11.4	10.7	18.7	9.8	53.3	32.2	16.3	127	43.1	150.5	70.0	10.4 (11.6)	5.6 (10.5)
MiBP	15.3	6.5	21.0	10.3	2.5	n.a.	n.a.	19.5	20.6	5.8	24.9	n.a.	26.5 (26.9)	16.6 (30.2)
MBP	14.3	22.5	15.2	3.6	4.0	n.a.	n.a.	36.4	24.4	6.7	17.4	70.0	32.4 (31.7)	17.2 (31.1)
MBzP	4.0	2.2	24.6	0.03	0.3	4.4	8.5	n.a.	5.2	4.4	1.9	16.4	3.3 (3.5)	1.6 (2.5)
MEHP	3.3	0.9	0.4	0.2	0.02	3.1	3.1	3.0	5.7	8.0	3.8	3.9	2.3 (2.8)	1.8 (2.8)
OH-MEHP	5.6	1.7	5.0	2.0	2.1	18.5	37.7	11.5	25.6	19.7	12.8	17.1	8.9 (12.0)	6.5 (18.6)
oxo-MEHP	4.8	1.3	3.9	1.3	1.2	11.7	25.3	7.4	15.6	13.8	9.9	16.5	5.5 (5.4)	3.2 (6.1)
cx-MEHP	10.4	11.9	10.7	n.a.	n.a.	n.a.	n.a.	42.1	41.6	27.8	20.1	11.6	11.5 (11.6)	14.9 (27.8)

n.a.–not analysed.
[a] Median ng/mL urine.
[b] Geometric mean μg/g creatinine.
[c] Arithmetic mean ng/mL urine.
[d] Geometric mean ng/mL urine.
[e] Median ng/mL urine (μg/g creatinine).

Table 5
Comparison of measured concentrations of DINCH metabolites in the presented study with other published data.

Analyte	USA General population[a] (Silva et al., 2013)	Germany General population[a] (Schütze et al., 2014)	Germany Children[b] (Fromme et al., 2016)	Norway General population[c] (Giovanoulis et al., 2016)	Israel Women[a] (Machtinger et al., 2018)	Czech Republic Women[d] (Presented study)	Czech Republic Newborns[d] (Presented study)
Samples	n = 121	n = 60	n = 208	n = 61	n = 136	n = 102	n = 102
MINCH	n.a.	< 0.1	< 0.1	n.a.	n.a.	< 0.15	< 0.15
OH-MINCH	1.4	2.1	1.7	0.3	1.2	8.8 (4.0)	6.3 (19.2)
oxo-MINCH	1	0.9	1.5	n.a.	n.a.	6.2 (3.1)	3.8 (11.6)
cx-MINCH	2.4	1.8	1.1	0.2	0.6	10.0 (6.8)	8.8 (26.3)

n.a.–not analysed.
[a] Geometric mean ng/mL urine.
[b] Median ng/mL urine.
[c] Geometric mean μg/g creatinine.
[d] Median ng/mL urine (μg/g creatinine).

4. Conclusion

Within this study, the fast and simple method has been validated that requires only the dilution of the hydrolysed urine sample with an organic solvent for the determination of 8 phthalate metabolites and 4 DINCH metabolites. The identification and quantification of the target compounds was performed by UHPLC–MS/MS. The obtained LOQs were comparable with those reported in the literature (0.15–0.4 ng/mL urine), with recoveries of 60–126% and repeatability of 1–11%.

The validated method was subsequently used for the measurement of phthalate and DINCH metabolites in urine samples collected from mothers and their newborn children from two localities in the Czech Republic – Karvina and Ceske Budejovice (2013–2014).

The most contaminated samples were collected from newborn children living in the Karvina industrial region, where the median concentration of phthalate metabolites was approximately two times higher compared to their mothers, and to the levels of these compounds found in the urine samples obtained from mothers and their newborn children living in Ceske Budejovice. Worth noting is the fact that cx-MEHP contributed 43% to the overall contamination in newborns' samples from Karvina. The source of the parent compound (DEHP) will be investigated in a subsequent part of this study.

With regard to the DINCH metabolites, MINCH was not detected in any of the measured samples. The other DINCH metabolites were found in only 7% of the measured samples collected from mothers and in 1% of the samples collected from children. Their median concentrations were very low (0.8–8.8 ng/mL urine), which is similar to those of other published papers.

The pattern of phthalate metabolites rather differed from those reported in similar studies abroad. While, in most of the Czech samples, MBP was found at the highest concentration, in other worldwide studies MEP was typically the dominating metabolite. This result could be caused by the different exposure sources of phthalates in the Czech Republic and other countries and should be further investigated.

Acknowledgments

This work was supported by the “National Programme of Sustainability I” - NPU I (LO1601 - No.: MSMT-43760/2015), a specific university research (MSMT No 21-SVV/2018) and LTAUSA 17182. The authors sincerely appreciate the support of Dr Radek Susil, Vice-Director of Karvina-Raj Hospital, and Dr Bretislav Schon, Director of the Ceske Budejovice Hospital. The study would not have been possible without the close cooperation of all obstetricians, paediatricians, nurses and laboratory workers in both hospitals.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2019.03.067.

References

Abb, M., Heinrich, T., Sorkau, E., Lorenz, W., 2009. Phthalates in house dust. Environ. Int. 35 (6), 965–970.

Axelsson, J., Rylander, L., Rignell-Hydborn, A., Jönsson, B.A.G., Lindh, C.H., Giwercman, A., 2015. Phthalate exposure and reproductive parameters in young men from the general Swedish population. Environ. Int. 85, 54–60.

Bui, T.T., Giovanoulis, G., Palm Cousins, A., Magner, J., Cousins, I.T., de Wit, C.A., 2016. Human exposure, hazard and risk of alternative plasticizers to phthalate esters. Sci. Total Environ. 541, 451–467.

Cerna, M., Maly, M., Rudnai, P., Közepesy, S., Naray, M., Halzlova, K., Jajcaj, M., Grafnetterova, A., Krskova, A., Antosova, D., Forysova, K., 2015. Case study: Possible differences in phthalates exposure among the Czech, Hungarian, and Slovak populations identified based on the DEMOCOPHES pilot study results. Environ. Res. 141, 118–124.

Chang, W.H., Wu, M.H., Pan, H.A., Guo, P.L., Lee, C.C., 2017. Semen quality and insulin-like factor 3: Associations with urinary and seminal levels of phthalate metabolites in adult males. Chemosphere 173, 594–602.

Correia-Sa, L., Schütze, A., Norberto, S., Calhau, C., Domingues, V.F., Koch, H.M., 2017. Exposure of Portuguese children to the novel non-phthalate plasticizer di-(iso-nonyl)-cyclohexane-1,2-dicarboxylate (DINCH). Environ. Int. 102, 79–86.
Dewalque, L., Pirard, C., Vandepaer, S., Charlier, C., 2015. Temporal variability of urinary concentrations of phthalate metabolites, parabens and benzophenone-3 in a Belgian adult population. Environ. Res. 142, 414–423.
Engel, S.M., Villanger, G.D., Nethery, R.C., Thomsen, C., Sakhi, A.K., Drover, S.S.M., Hoppin, J.A., Zeiner, P., Knudsen, G.P., Reichborn/Kjennerud, T., Herring, A.M., Aase, H., 2018. Prenatal phthalates, maternal thyroid function, and risk of attention/deficit hyperactivity disorder in the Norwegian mother and child cohort. Environ. Health Perspect. 126 (5), 057004.
Enke, U., Schleussner, E., Pälmke, C., Seyfarth, L., Koch, H.M., 2013. Phthalate exposure in pregnant women and newborns–The urinary metabolite excretion pattern differs distinctly. Int. J. Hyg Environ. Health 216 (6), 735–742.
Esteban, M., Castaño, A., 2009. Non-invasive matrices in human biomonitoring: A review. Environ. Int. 35, 438–449.
European Commission - Daily News 11/07/, 2018. European Commission - press releases. http://europa.eu/rapid/midday-express-11-07-2018.htm.
Frederiksen, H., Skakkebaek, N.E., Andersson, A.M., 2007. Metabolism of phthalates in humans. Mol. Nutr. Food Res. 51 (7), 899–911.
Frederiksen, H., Kuiri-Hänninen, T., Main, K.M., Dunkel, L., Sankilampi, U., 2014. A longitudinal study of urinary phthalate excretion in 58 full-term and 67 preterm infants from birth through 14 months. Environ. Health Perspect. 122 (9), 998.
Fromme, H., Schütze, A., Lahrz, T., Kraft, M., Fembacher, L., Siewering, S., Burkardt, R., Dietrich, S., Koch, H.M., Völkel, W., 2016. Non-phthalate plasticizers in German daycare centers and human biomonitoring of DINCH metabolites in children attending the centers (LUPE 3). Int. J. Hyg Environ. Health 219, 33–39.
Giovanoulis, G., Alves, A., Papadopoulou, E., Cousins, A.P., Schütze, A., Koch, H.M., Haug, L.S., Covaci, A., Magner, J., Voorspoels, S., 2016. Evaluation of exposure to phthalate esters and DINCH in urine and nails from a Norwegian study population. Environ. Res. 151, 80–90.
Gomez Ramos, M.J., Heffernan, A.L., Toms, L.M., Calafat, A.M., Ye, X., Hobson, P., Broomhall, S., Mueller, J.F., 2016. Concentrations of phthalates and DINCH metabolites in pooled urine from Queensland, Australia. Environ. Int. 88, 179–186.
Hartmann, C., Uhl, M., Weiss, S., Koch, H.M., Scharf, S., König, J., 2015. Human biomonitoring of phthalate exposure in Austrian children and adults and cumulative risk assessment. Int. J. Hyg Environ. Health 218 (5), 489–499.
HBM4EU priority substances, 2017. HBM4EU - Science and Policy for a Healthy Future. https://www.hbm4eu.eu/.
Heffernan, A.L., Thompson, K., Eaglesham, G., Vijayasarathy, S., Mueller, J.F., Sly, P.D., Gomez, M.J., 2016. Rapid, automated online SPE-LC-QTRAP-MS/MS method for the simultaneous analysis of 14 phthalate metabolites and 5 bisphenol analogues in human urine. Talanta 151, 224–233.
Ipapo, K.N., Factor-Livak, P., Whyatt, R.M., Calafat, A.M., Diaz, D., Perera, F., Rauh, V., Herbstman, J.B., 2017. Maternal prenatal urinary phthalate metabolite concentrations and visual recognition memory among infants at 27 weeks. Environ. Res. 155, 7–14.
Johns, L.E., Cooper, G.S., Galizia, A., Meeker, J.D., 2015. Exposure assessment issues in epidemiology studies of phthalates. Environ. Int. 85, 27–39.
Kim, E.H., Jeon, B.H., Kim, J., Kim, Y.M., Han, Y., Ahn, K., Cheong, H.K., 2017. Exposure to phthalates and bisphenol A are associated with atopic dermatitis symptoms in children: a time-series analysis. Environ. Health 16, 1–8.
Koch, H.M., Schütze, A., Pälmke, C., Angerer, J., Brüning, T., 2013. Metabolism of the plasticizer and phthalate substitute diisononyl-cyclohexane-1, 2-dicarboxylate (DINCH®) in humans after single oral doses. Arch. Toxicol. 87 (5), 799–806.
Lankova, D., Urbancova, K., Sram, R.J., Hajslova, J., Pulkrabova, J., 2016. A novel strategy for the determination of polycyclic aromatic hydrocarbon monohydroxylated metabolites in urine using ultra-high-performance liquid chromatography with tandem mass spectrometry. Anal. Bioanal. Chem. 408, 2515–2525.
Larsson, K., Lindh, C.H., Jönsson, B.A.G., Giovanoulis, G., Bibi, M., Bottai, M., Bergström, A., Berglund, M., 2017. Phthalates, non-phthalate plasticizers and bisphenols in Swedish preschool dust in relation to children's exposure. Environ. Int. 102, 114–124.
Machtinger, R., Gaskins, A.J., Racowsky, C., Mansur, A., Adir, M., Baccarelli, A.A., Hauser, R., 2018. Urinary concentrations of biomarkers of phthalates and phthalate alternatives and IVF outcomes. Environ. Int. 111, 23–31.
Marie, C., Vendittelli, F., Sauvant-Rochat, M., 2015. Obstetrical outcomes and biomarkers to assess exposure to phthalates: A review. Environ. Int. 83, 116–136.
Minguez-Alarcon, L., Souter, I., Chiu, Y.H., Williams, P.L., Ford, J.B., Ye, X., Calafat, A.M., Hauser, R., 2016. Urinary concentrations of cyclohexane-1,2-dicarboxylic acid monohydroxy isononyl ester, a metabolite of the non-phthalate plasticizer di(isononyl)cyclohexane-1,2-dicarboxylate (DINCH), and markers of ovarian response among women attending a fertility center. Environ. Res. 151, 595–600.
Mullerova, D., Bouchalova, V., Matejkova, D., Kovarova, K., Svacina, S., Vrbik, K., Pavlouskova, J., Dvorakova, J., Muller, L., 2016. Phthalates exposure indicators determined by urinary phthalate metabolites in healthy non-obese Czech adults: FANTOM study. Food Addit. Contam. Part A 33 (12), 1817–1825.
Myridakis, A., Fthenou, E., Balaska, E., Vakinti, M., Kogevinas, M., Stephanou, E.G., 2015. Phthalate esters, parabens and bisphenol-A exposure among mothers and their children in Greece (Rhea cohort). Environ. Int. 83, 1–10.
Perng, W., Watkins, D.J., Cantoral, A., Mercado-Garcia, A., Meeker, J.D., Téllez-Rojo, M.M., Peterson, K.E., 2017. Exposure to phthalates is associated with lipid profile in peripubertal Mexican youth. Environ. Res. 154, 311–317.
Polanska, K., Ligocka, D., Sobala, W., Hanke, W., 2014. Phthalate exposure and child development: The polish mother and child cohort study. Early Hum. Dev. 90 (9), 477–485.
Rocha, B.A., Asimakopoulos, A.G., Barbosa, F., Kannan, K., 2017. Urinary concentrations of 25 phthalate metabolites in Brazilian children and their association with oxidative DNA damage. Sci. Total Environ. 586, 152–162.
Sabaredzovic, A., Sakhi, A.K., Brantsaeter, A.L., Thomsen, C., 2015. Determination of 12 urinary phthalate metabolites in Norwegian pregnant women by core–shell high performance liquid chromatography with on-line solid-phase extraction, column switching and tandem mass spectrometry. J. Chromatogr. B 1002, 343–352.
Schütze, A., Kolossa-Gehring, M., Apel, P., Brüning, T., Koch, H.M., 2014. Entering markets and bodies: Increasing levels of the novel plasticizer Hexamoll® DINCH® in 24 h urine samples from the German Environmental Specimen Bank. Int. J. Hyg Environ. Health 217, 421–426.
Shu, H., Wikstrom, S., Jönsson, B.A., Lindh, C.H., Svensson, Å., Nånberg, E., Bornehag, C.G., 2018. Prenatal phthalate exposure was associated with croup in Swedish infants. Acta Paediatr. 107 (6), 1011–1019.
Silva, M.J., Reidy, J.A., Preau Jr., J.L., Samandar, E., Needham, L.L., Calafat, A.M., 2006. Measurement of eight urinary metabolites of di (2-ethylhexyl) phthalate as biomarkers for human exposure assessment. Biomarkers 11 (1), 1–3.
Silva, M.J., Jia, T., Samandar, E., Preau Jr., J.L., Calafat, A.M., 2013. Environmental exposure to the plasticizer 1, 2-cyclohexane dicarboxylic acid, diisononyl ester (DINCH) in US adults (2000—2012). Environ. Res. 126, 159–163.
Thayer, K.A., Doerge, D.R., Hunt, D., Schurman, S.H., Twaddle, N.C., Churchwell, M.I., Garantziotis, S., Kissling, G.E., Easterling, M.R., Bucher, J.R., Birnbaum, L.S., 2015. Pharmacokinetics of bisphenol A in humans following a single oral administration. Environ. Int. 83, 107–115.
Wu, H., Olmsted, A., Cantonwine, D.E., Shahsavari, S., Rahil, T., Sites, C., Pilsner, J.R., 2017. Urinary phthalate and phthalate alternative metabolites and isoprostane among couples undergoing fertility treatment. Environ. Res. 153, 1–7.

APPENDIX IV

Lankova D., Urbancova K., Sram R.J., Hajslova J., Pulkrabova J.

A novel strategy for the determination of polycyclic aromatic hydrocarbon monohydroxylated metabolites in urine using ultra-high-performance liquid chromatography with tandem mass spectrometry

Analytical and Bioanalytical Chemistry (2016) 408: 2515–2125

doi: 10.1007/s00216-016-9350-1

Anal Bioanal Chem (2016) 408:2515–2525
DOI 10.1007/s00216-016-9350-1

RESEARCH PAPER

A novel strategy for the determination of polycyclic aromatic hydrocarbon monohydroxylated metabolites in urine using ultra-high-performance liquid chromatography with tandem mass spectrometry

Darina Lankova[1] · Katerina Urbancova[1] · Radim J. Sram[2] · Jana Hajslova[1] · Jana Pulkrabova[1]

Received: 12 November 2015 / Revised: 4 January 2016 / Accepted: 20 January 2016 / Published online: 9 February 2016

Abstract In this study, a novel analytical approach for the determination of 11 monohydroxylated polycyclic aromatic hydrocarbon metabolites (OH-PAHs) in urine was developed and validated. The rapid, simple and high-throughput sample preparation procedure based on ethyl acetate extraction and subsequent purification by dispersive solid-phase extraction (d-SPE) employing a Z-Sep sorbent is used for the first time. For the identification/quantification of target compounds, ultra-high-performance liquid chromatography (U-HPLC) interfaced with tandem mass spectrometry (MS/MS) was applied. The results of validation experiments performed on the Standard Reference Material (SRM) 3673 (organic contaminants in non-smokers' urine) were in accordance with the certified values. The method recoveries ranged from 77 to 114 % with the relative standard deviation lower than 20 % and the quantification limits in the range of 0.010–0.025 ng mL^{-1} (except for benzo[a]pyren-3-ol with 0.9 ng mL^{-1}). Within the pilot study, the new method was used for the analysis of OH-PAHs in 50 urine samples. The concentrations of ΣOH-PAHs were in the range of 0.87–63 ng mL^{-1} (1600–33,000 ng g^{-1} creatinine), with naphthalen-2-ol (2-OH-NAP) and phenanthren-1-ol (1-OH-PHEN) being the most abundant exposure biomarkers detected in all samples.

Keywords Monohydroxylated metabolites of polycyclic aromatic hydrocarbons · SRM 3673 · Tandem mass spectrometry · Ultra-high-performance liquid chromatography · Urine

Introduction

Polycyclic aromatic hydrocarbons (PAHs), produced by incomplete combustion of organic materials, are ubiquitous contaminants present in the environment. The toxicity of some PAHs has been extensively explored due to their carcinogenic, mutagenic and teratogenic potency [1]. The human organism may be exposed to PAHs through various pathways; inhalation of polluted air or cigarette smoke together with dietary intake represents the major ones [2, 3]. As soon as PAHs enter the human body, rapid biotransformation process starts with phase I metabolism in which PAHs are oxidised by the hepatic cytochrome P450 monooxygenases to form reactive epoxide intermediates, followed by reduction or hydrolysis to hydroxylated derivates (monohydroxylated PAH metabolites (OH-PAHs)). In phase II metabolism, the OH-PAHs are conjugated to glucuronic acid or sulphate to increase the water solubility of the metabolite [4]. Depending of the molecular weight, the OH-PAH conjugates (typically glucuronides and/or sulphates) are excreted either into urine (species with two to three benzene rings) or in faeces (greater than or equal to four benzene rings) [5]. These biological processes lead to the formation of multiple metabolites including epoxide, dihydrodiols and monohydroxylated and polyhydroxylated PAHs. The

Electronic supplementary material The online version of this article (doi:10.1007/s00216-016-9350-1) contains supplementary material, which is available to authorized users.

✉ Jana Pulkrabova
jana.pulkrabova@vscht.cz

[1] Faculty of Food and Biochemical Technology, Department of Food Analysis and Nutrition, University of Chemistry and Technology, Prague, Technicka 3, 166 28 Prague 6, Czech Republic

[2] Institute of Experimental Medicine AS CR, Videnska 1083, 142 20 Prague 4, Czech Republic

selection of a reliable biomarker(s) for human PAH exposure is a crucial point since a PAH pattern may fairly vary among the particular exposure sources. Moreover, each PAH is metabolised in the exposed organism to more than one metabolite constituting different positional isomers [6]. Considering various environmental and/or occupational studies [7–11] concerned with monitoring of PAH emissions associated with petrol or diesel combustion, pyren-1-ol (1-OH-PYR), the major monohydroxylated metabolite of pyrene, is one of the most popular exposure biomarkers. However, pyrene itself is not carcinogenic; thus, determination of its biotransformation product is not fully relevant to a health risk assessment process. Bearing in the mind that benzo[a]pyrene is the key representative of carcinogenic PAHs, the monitoring of its major metabolite benzo[a]pyren-3-ol (3-OH-BaP) is a more realistic option. Nevertheless, the measurement of urinary 3-OH-BaP is a rather difficult task. Due to its predominant elimination via biliary excretion in faeces, concentrations of urinary 3-OH-BaP are under common exposure conditions by 3 orders of magnitude lower compared to those of the pyrene metabolite, 1-OH-PYR [12]. With regard to multiple exposures to different PAHs under real-life conditions, measurements of other hydroxylated metabolites originated from naphthalene, phenanthrene, fluorene and chrysene have been realised by many authors in the recent decade [4, 6, 13–19].

The quantitative determination of multiple OH-PAHs and other PAH-related biomarkers in human urine is an analytical challenge due to a large range of their polarities and typically (ultra)-trace levels. Currently, typical sample preparation procedures for isolation of OH-PAHs from urine include enzymatic hydrolysis with β-glucuronidase/sulfatase and a subsequent extraction by applying liquid/liquid extraction (LLE) using pentane [4, 20], pentane/toluene mixture [17] or, alternatively, hexane [6] and, as an extraction/preconcentration alternative, also solid-phase extraction (SPE) [13, 14, 16, 18, 19, 21, 22] or solid-phase microextraction (SPME) [23, 24] were employed. Analytical platforms, which have proved to be sufficiently sensitive for the quantification of urinary OH-PAHs, include high-performance liquid chromatography with fluorescence detection (HPLC–FLD) [12, 18], gas chromatography (GC) coupled with (high-resolution) mass spectrometry ((HR)MS) [6, 13, 19, 20] or tandem mass spectrometry (MS/MS) [25], and liquid chromatography (LC) with MS/MS, either with [22, 26] or without prior derivatisation [13–17, 27]. When selecting the most relevant instrumental set-up, both advantages and disadvantages should have to be considered. For example, GC–MS (/MS) methods for OH-PAHs require derivatisation of the analytes but, on the other hand, thanks to their high separation power, differentiation of the isomeric metabolites is enabled. Moreover, applications of isotope dilution techniques for quantification offer a high-precision analysis. HPLC–FLD, representing a common equipment of many laboratories, benefits from the high sensitivity of fluorescence detection to analytes with PAH structures. Contrary to GC, LC separation suffers of lower separation efficiency which may lead to, together with limited detection, misidentification of analytes; also, the impossibility to compensate matrix effects by isotopically labelled surrogates may complicate accurate quantification [28, 29]. Owing to these facts, a LC–MS/MS technique seems to be, nowadays, a *gold standard* in this field because of its applicability for highly sensitive quantification of a wide range of substances including multiple PAH biomarkers in a complex biological matrix such as urine.

The main aims of this study were to (i) develop and validate a simple, rapid and high-throughput sample preparation procedure for the simultaneous isolation of most often monitored OH-PAHs (due to their toxicity or potential negative effect on human health) represented by metabolites of various PAH classes such as naphthalene, fluorene, phenanthrene, chrysene, pyrene and benzo[a]pyrene using ultra-HPLC (U-HPLC)–MS/MS and to (ii) apply this method for the examination of 50 urine samples collected from the Czech women within the project ‘Impact of air pollution to genome of newborns’ (No. 13-13458S).

Materials and methods

Standards

Certified standards of OH-PAHs represented by naphthalen-1-ol (1-OH-NAP, 1000 μg mL^{-1} of methanol, purity 99 %) and naphthalen-2-ol (2-OH-NAP, 1000 μg mL^{-1} of methanol) were obtained from Absolute Standards, Inc. (USA). Fluoren-2-ol (2-OH-FLUO), phenanthren-1-ol (1-OH-PHEN), phenanthren-2-ol (2-OH-PHEN) and phenanthren-4-ol (4-OH-PHEN) were supplied by Toronto Research Chemicals, Inc. (USA). Phenanthren-3-ol (3-OH-PHEN), phenanthren-9-ol (9-OH-PHEN), 1-OH-PYR and 3-OH-BaP were purchased from Neochema (Germany). Chrysen-6-ol (6-OH-CHR) was obtained from AccuStandard® (USA). Isotopically labelled analogues, specifically $[^2H]_7$-naphthalen-1-ol (d_7-1-OH-NAP), $[^2H]_7$-naphthalen-2-ol (d_7-2-OH-NAP), $[^2H]_9$-fluoren-2-ol (d_9-2-OH-FLUO), $[^2H]_9$-phenanthren-1-ol (d_9-1-OH-PHEN), $[^2H]_9$-phenanthren-2-ol (d_9-2-OH-PHEN), $[^2H]_9$-phenanthren-3-ol (d_9-3-OH-PHEN), $[^2H]_8$-phenanthren-9-ol (d_8-9-OH-PHEN), $[^2H]_9$-pyren-1-ol (d_9-1-OH-PYR) and $[^2H]_{11}$-benzo[a]pyren-3-ol (d_{11}-3-OH-BaP), were supplied by Toronto Research Chemicals, Inc. Creatinine was delivered by Sigma-Aldrich (USA). The purity of all standards and their isotopically labelled analogues was at least 98 %.

Individual OH-PAHs delivered as solids were dissolved with respect to the manufacturers’

recommendations. Mixtures of OH-PAHs and their isotopically labelled analogues (*d*-OH-PAHs) were prepared in methanol at concentrations of 10, 100 and 1000 ng mL^{-1}. Each calibration solution of OH-PAHs corresponding to the calibration curve at levels of 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 ng mL^{-1} in methanol contained an internal standard at 10 ng mL^{-1} except for d_{11}-3-OH-BaP, for which the concentration was 10 times higher, 100 ng mL^{-1}, because of its lower instrumental sensitivity compared to other *d*-OH-PAHs. All solutions were stored at −20 °C in the freezer.

The Standard Reference Material® 3673 (organic contaminants in non-smokers' urine) used for the method evaluation and validation experiments was supplied by the US National Institute of Standards and Technology (NIST, Gaithersburg, Maryland, USA).

Chemicals, reagents and other materials

Analytical grade ethyl acetate for pesticide residue analysis, SupraSolv® *n*-hexane for gas chromatography ECD and FID (purity ≥ 98 %), picric acid, enzyme β-glucuronidase (type HP-2, glucuronidase activity ≥ 100, 000 units mL^{-1}, sulfatase activity ≤ 7500 units mL^{-1}) and sorbents, specifically Supel™ QuE Z-Sep, Supel™ QuE Z-Sep + and Supelclean™ ENVI-Carb™, were supplied by Sigma-Aldrich. Acetone and sodium hydroxide were purchased from Lach-Ner (Czech Republic). LC–MS CHROMASOLV® methanol (purity ≥ 99.9 %) was delivered by Merck (Germany). Sorbents C18 silica gel and primary–secondary amine (PSA), both Bondesil (40 μm), were obtained from Agilent Technologies (USA). Sep-Pak® Vac 3 cc (500 mg) SPE cartridges were supplied by Waters (USA). Unsterile polytetrafluoroethylene (PTFE, 5.0 μm, Ø 25 mm) filters were purchased from Rotilabo® (Germany). Ninety-six-well microtiter plates were obtained from Gama Group (Czech Republic). Polypropylene (PP) centrifuge tube filters (nylon, pore size 0.22 μm) were supplied by Sigma-Aldrich.

Sample collection

All 50 urine samples were collected during winter in 2013/2014 from Czech women living in the Karviná (situated in highly polluted North Moravian industrial agglomeration with a black coal mining and coke and steel production) within the project Impact of air pollution to genome of newborns (No. 13-13458S). The urine was stored in the freezer at −20 °C before analysis. Prior to the analysis, thawed samples were filtered through PTFE filters in order to remove solid impurities.

Method description

Determination of creatinine

The creatinine values were used to normalise the urine concentration/dilution in individual samples, thus ensuring improved data comparability. Samples with excessive physiological dilution or concentration outside the range of 0.3 mg mL^{-1} ≤ creatinine ≤ 3.0 mg mL^{-1} were excluded. A spectrophotometric method for the estimation of urinary creatinine was based on Jaffé's reaction [30, 31], where a coloured complex of creatinine with alkaline picrate formed and then measured at 505 nm. The reaction mixture consisted of 75 μL creatinine calibration standards at 8, 16, 24, 32 and 40 μg mL^{-1}, or a 75 μL 100 times diluted urine, together with 75 μL of saturated picric acid solution, 75 μL of 1 M sodium hydroxide and 75 μL of deionised water, was transferred directly into a well of a microtiter plate. Jaffé's reaction was carried for 20 min at laboratory temperature, and then the absorbance was measured using the spectrophotometer Epoch (BioTek, USA). The obtained data were evaluated by Gen5™ Microplate Data Analysis software.

Enzymatic hydrolysis

An amount of 5 mL of urine was transferred into a 50-mL polypropylene centrifuge tube, and 20 μL of β-glucuronidase and 10 mL of acetate buffer (pH 5) were added. After the addition of an internal standard (d_x-OH-PAHs) at 100 ng $mL^{-1,}$ the mixture was incubated overnight (37 °C, 15 h) to release OH-PAHs from conjugated forms.

SPE

The SPE cartridges (sorbent C18) were conditioned with 5 mL of methanol followed by 10 mL of acetate buffer (pH 5). After the loading of 15 mL urine hydrolysate with the addition of an internal standard into the cartridges, washing with 5 mL of deionised water and 5 mL of 25 % (*v*/*v*) aqueous methanol was performed. During these procedures, the flow rate was held lower than 1 mL min^{-1}. The cartridges were dried using a vacuum pump and centrifuged for 2 min at 5000 rpm (Hettich, Germany). Finally, the target analytes were eluted with 10 mL of ethyl acetate, methanol or acetone. Collected eluates were allowed to evaporate (Büchi Rotavapor, Flawil, Switzerland) and the residual solvent was removed under the gentle stream of nitrogen, if needed. The residues were dissolved in 250 μL of methanol and transferred into the vial for the U-HPLC–MS/MS analysis.

LLE with clean-up using dispersive solid phase extraction

In total, 15 mL of ethyl acetate was added to the enzymatic hydrolysate and the tube was vigorously shaken for 1 min. The tube was then centrifuged for 5 min at 10,000 rpm to facilitate phase separation. An amount of 12 mL of the upper organic layer was transferred into the new tube containing 180 mg of Z-Sep, Z-Sep+, C18, PSA or ENVI-Carb sorbent and 1.8 g anhydrous $MgSO_4$. The tube was shaken again for 1 min and centrifuged for 5 min at 10,000 rpm. Subsequently, 8 mL of a purified extract was allowed to evaporate using a rotary vacuum evaporator near to dryness. The residual solvent was removed under a gentle stream of nitrogen. The residues were dissolved in 250 μL of methanol. The reconstituted extract was filtered through a 0.22-μm nylon centrifuge tube filter and transferred into the vial for the U-HPLC–MS/MS analysis.

Quality assurance/quality control

The validation of the final method (LLE followed by clean-up based on dispersive solid-phase extraction (d-SPE)) was evaluated through the analysis of the NIST Standard Reference Material® (SRM) 3673 (organic contaminants in non-smokers' urine) in six replicates. In the case of 6-OH-CHR and 3-OH-BaP, for which the concentrations are not certified, an artificially contaminated urine sample, previously tested for the content of target analytes, was analysed. The recoveries (REC, %) and repeatabilities (expressed as relative standard deviations, RSDs, %) for 6-OH-CHR and 3-OH-BaP were calculated from six analyses at a concentration level of 0.05 and 1 ng mL^{-1} (based on a regular occurrence of these metabolites in urine). In the case of 4-OH-PHEN and 6-OH-CHR, for which their deuterated analogues were not available, d_8-9-OH-PHEN and d_9-1-OH-PYR, respectively, were chosen. To control background contamination by target analytes, the procedural blank (i.e. in the same sample procedure, only instead of urine, the same volume of deionised water was used) was prepared together with each batch of 20 samples (per day). The determined concentration of OH-PAHs in each procedural blank was subtracted from the respective sample. The limits of quantification (LOQs) were determined as the lowest calibration standard at which analytes provided a signal-to-noise ratio (S/N) of >10. For the compensation of the unexpected influence of matrix or losses of targeted analytes, the entire method was validated using isotopically labelled surrogates.

As regards the miniaturised spectrophotometric method based on Jaffe's reactions, which was implemented within our study, the concentration of creatinine was also determined in the SRM 3673 in six replicates.

Instrumental analysis

The U-HPLC analyses of OH-PAHs were performed using an Acquity Ultra-Performance LC system (Waters, USA) equipped with a 10-μL sample loop. Analytes were separated on a pentafluorophenyl (PFP; Kinetex, Phenomenex, USA) column (100 mm × 2.1 mm × 1.7 μm) maintained at 40 °C. Water (A) and methanol (B) were used as mobile phases at a flow rate of 300 μL min^{-1} and with a gradient, specifically 10–40 % B over 0.5 min then 40–100 % B over 11 min followed by an isocratic hold at 100 % B for 2 min. The total run time for each injection was 13 min. The flow rate began at 0.3 mL min^{-1}, and the sample volume injected was 5 μL at 10 °C. The U-HPLC system was coupled to a triple–quadrupole mass spectrometer Xevo TQ-S (Waters, USA) with electrospray ionisation (ESI-) that was operated in the negative ion mode with a capillary voltage of −2000 V, and ionisation and desolvation temperatures were 150 and 400 °C, respectively. The instrument was operated in multiple reaction monitoring (MRM) mode. The retention times and quantitative/qualitative MRM transitions of target analytes are listed in Table S1 in the Electronic Supplementary Material (ESM).

Results and discussion

U-HPLC–MS/MS method development

In the first stage, the instrumental method for the determination of OH-PAHs was optimised. In order to achieve the lowest LOQs required for real-sample analysis, MS/MS and ionisation parameters (capillary voltage, ionisation temperature) were extensively tested. In the next phase, the chromatographic separation of isomeric compounds, mainly OH-PHEN compounds, was performed on the three types of columns.

To optimise mass spectrometric parameters, standard solutions of individual OH-PAHs at 5000 ng mL^{-1} in a mixture of methanol/water (50:50, *v*/*v*) were directly infused into ESI-. The MRM transition $[M–H]^-$ > $[M–H–28]^-$ was the most abundant for most of the analytes, with the exception of 2-OH-FLUO. A neutral loss of 28 Da has been observed by other investigators and has been attributed to a loss of CO from the parent ion. No further fragmentation of the $[M–H–28]^-$ ion occurred which indicated a high stability of this daughter ion under the experimental conditions. In the case of 2-OH-FLU, two fragment ions $[M–H–28]^-$ and $[M–H–1]^-$ were observed, the latter probably due to five membered rings present in its structure. The sensitivity of the MRM transition $[M–H]^-$ > $[M–H–1]^-$, i.e. *m*/*z* 181 > 180, was 20 times higher compared to that of the reaction $[M–H]^-$ > $[M–H–28]^-$, i.e. 181 > 154. Regarding with the capillary voltage, three different values, −2000, −3000 and −4000 V, were tested. The

highest sensitivity was obtained when −2000 V was set. Compared to −3000 and −4000 V, the signals for all compounds were significantly higher, by 20 and 90 %, respectively. Also, the influence of mobile phase composition was recognised as an important factor affecting sensitivity. The best results under the above-mentioned mobile phase gradient were achieved when aqueous methanol was used (system 1). For example, the addition of ammonium acetate in water (system 2) caused the decrease in signal intensity by 50 % for most analytes compared to water and methanol (system 1). When acetonitrile was used instead of methanol (system 3), similar signal intensities were obtained for OH-NAP, 2-OH-FLUO and OH-PHEN compounds, contrary to later eluted compounds, 1-OH-PYR, 6-OH-CHR and 3-OH-BaP, when signal decreased by 50 % (Fig. 1).

Because of identical MRM transitions of OH-PHEN and OH-NAP isomers, their individual chromatographic separation had to be optimised. Besides the different compositions of the mobile phase, gradient and total time of analysis, various types of columns were tested: Kinetex (i) PFP (100 × 2.1 mm; 1.7 μm), (ii) BEH (C18) (100 × 2.1 mm; 1.7 μm) and (iii) HSS T3 (C18) (100 × 2.1 mm; 1.8 μm). The most critical isomers were OH-PHEN compounds, when the total co-elution of pairs 2-OH-PHEN/3-OH-PHEN and 1-OH-PHEN/9-OH-PHEN using a column with C18 stationary phase under tested conditions was observed. Better selectivity for these aromatic isomers was achieved using PFP stationary phase, when individual OH-PHEN compounds were partially separated (Fig. 2). Due to the unique properties (high electronegativity, low polarizability and strong lipo- and hydrophobicity) of organofluorines, the fluorinated phases offered many possibilities that could not be accomplished by conventional C8 and C18 reverse phases. The PFP column offers dispersive, dipole–dipole, π–π, charge transfer and ion exchange interactions to enable the retention of different types of compounds including aromatics and polycyclic aromatic hydrocarbons [32]. From these reasons, Kinetex PFP (100 × 2.1 mm; 1.7 μm) column was used for the U-HPLC–MS/MS analysis.

Extraction method development

Two different analytical approaches were tested for the isolation of OH-PAHs from urine. With regard to unsatisfactory results obtained by common SPE with C18 sorbent, mainly low method recovery and poor purification of extract, we decided to isolate the target compounds by LLE using ethyl acetate as a medium to which they are to be transferred. Due to a limited selectivity of this extraction step, purification was needed. To have the analytical procedure as simple as possible, d-SPE was chosen in this particular case. In paragraphs below, the development/modification steps are described in detail. The problem we had to overcome was the practical unavailability of a truly blank matrix. Given that the human body is exposed to PAHs practically throughout life (inhalation of polluted air or cigarette smoke, ingestion of contaminated food, dermal absorption), traces of OH-PAHs and their glucuronides/sulphates are unavoidably present in any urine sample. The only way to assess the performance characteristics of both methods during optimisation was to spike urine with a mixture of isotopically labelled analogues (d_x-OH-PAHs) prior to the hydrolysis. For the most effective enzymatic deconjugation step, 10, 20 or 50 μL of β-glucuronidase was added to the naturally contaminated urine sample (pH 5, 37 °C, 15 h). The comparable amounts of OH-PAHs were released (20 and 50 μL) for both sample preparation procedures discussed below. From this reason, for the final validation experiments and examination of real samples, 20 μL of β-glucuronidase was used.

SPE

Considering the relatively non-polar nature of OH-PAHs compared to other substances occurring in urine, C18 silica-based SPE was selected for isolation/preconcentration of these compounds. Within the

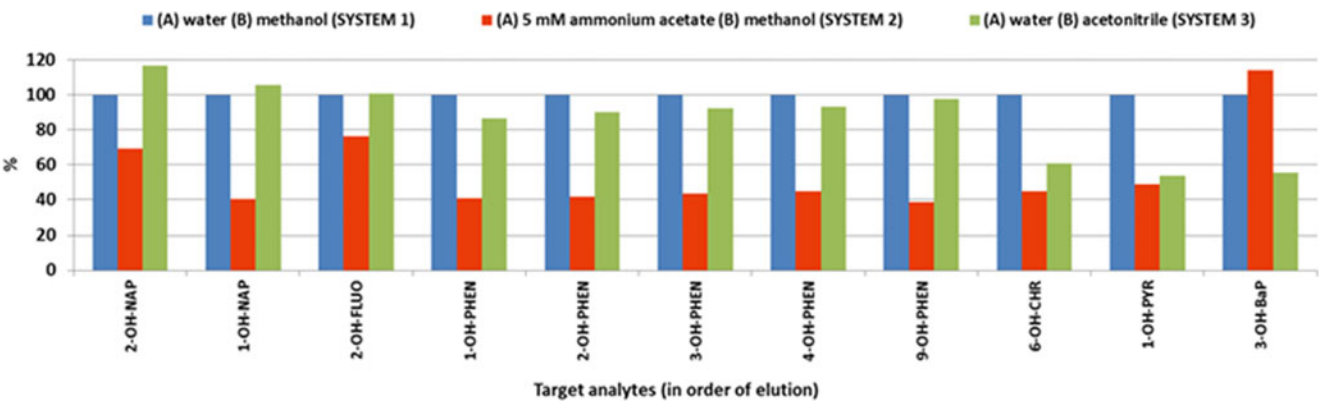


Fig. 1 The influence of mobile phase composition on the signal intensity of OH-PAHs (responses are normalised to the mobile phase composition of water (A) and methanol (B) of 100 %)

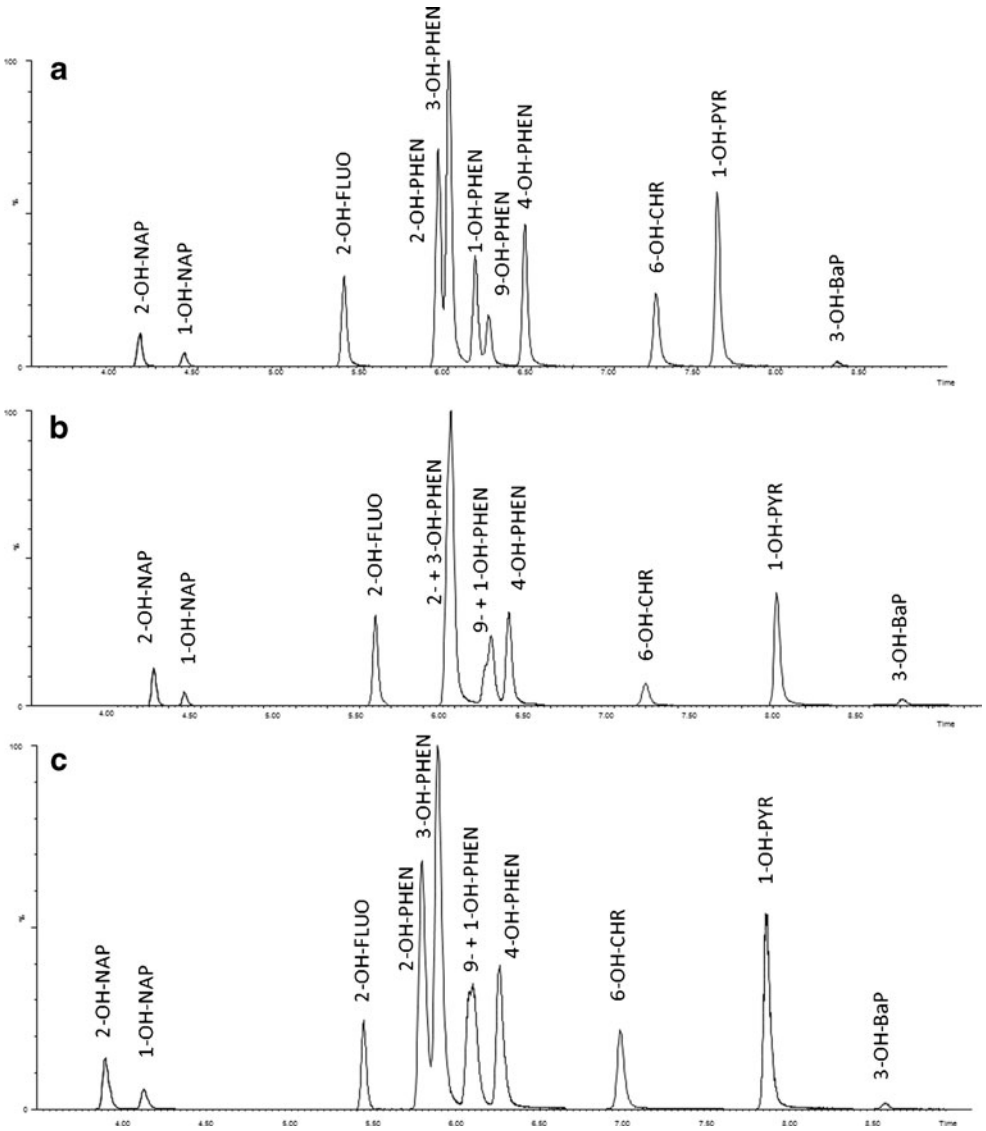


Fig. 2 Chromatographic separation of OH-PAHs on various columns ($c = 10$ ng mL^{-1}): *A* Kinetex PFP (100 × 2.1 mm; 1.7 μm), *B* BEH (C18) (100 × 2.1 mm; 1.7 μm) and *C* HSS T3 (C18) (100 × 2.1 mm; 1.8 μm)

optimisation, various solvent mixtures were tested for both washing (starting with water and followed by 10, 25 or 50 % aqueous MeOH) and elution (methanol, acetone or ethyl acetate). Regarding with the washing step, the best set-up avoiding *d*-OH-PAH losses and, at the same time, enabling removing of some interfering substances was the combination of water and 25 % (*v*/*v*) aqueous MeOH. From tested elution solvents, the highest recoveries in the range of 50–70 % were achieved when ethyl acetate was used. On the other hand, we experienced a similar retention behaviour of yellow/amber urine pigments and other matrix components that eluted from the SPE cartridge together with analytes. These residues of matrix co-extracts had a negative impact on the analyte ionisation, which resulted in the signal suppression by up 90 %. For the compensation of these effects, matrix calibration can be used. However, for its preparation, it is necessary to have sufficient quantities of the blank matrix, which is complicated as described above. Other difficulties were associated with residues of water (100–300 μL) after evaporation of the collected eluate. Considering that the extract was reconstituted in 250 μL of methanol, the final volume was increased and resulted in a dilution of matrix followed by higher LOQs. Finally, after the enzymatic deconjugation, the consistency of hydrolysate compared to non-hydrolysed urine was changed. After the loading of the hydrolysed sample onto the column, the precipitate accumulated on top of the sorbent, which extended the time required for the elution. The clogging of cartridge, which resulted in the lost of samples, has been identified as problematic in a similar study [20]. From these reasons, the new analytical method had to be implemented. The major requirements were as follows: (i) LOQs below 0.05 ng mL^{-1}, (ii) effective removing of matrix co-

extracts and (iii) simple and fast extraction with regard to the consistency of the hydrolysate.

LLE with clean-up using d-SPE

For this purpose, a simple LLE extraction using ethyl acetate (for which the highest recoveries within the SPE method evaluation were observed) including d-SPE was implemented. Various sorbents, specifically C18, PSA, Z-Sep, Z-Sep$^+$ and ENVI-Carb, were tested in the purification step. The best results were obtained when Z-Sep was used. Compared to the other tested sorbents, the response of analytes in the purified extract increased by 30–80 %. The removing of matrix interferences was significant especially for d_9-OH-PYR and d_{11}-3-OH-BaP. As can be expected, ENVI-Carb adsorbs planar compounds, which resulted in the elimination of most OH-PAHs from the extract. Similar results were achieved using C18, PSA and Z-Sep$^+$; nevertheless, the signals in the purified extract decreased by approximately 50 % compared to Z-Sep. The effectivity of the clean-up step was documented by the assessment of the matrix effects. The comparison of matrix effects in both extracts obtained by SPE and LLE with and without d-SPE documented (Fig. 3) that the cleaning of the urine extract with d-SPE led to the reduction of these effects by approximately 50 %, with the exception of d_{11}-3-OH-BaP, when only 20 % reduction was observed.

Method validation

Recoveries and repeatabilities (expressed as RSD, %) of the final method (LLE with clean-up using d-SPE) for OH-NAP isomers, 2-OH-FLUO, OH-PHEN isomers and 1-OH-PYR were calculated from six replicate analyses of the SRM 3673 (Table 1). The results were in good agreement with the certified values except for 1-OH-NAP, the measured concentration of which was slightly lower, 162 ± 11 ng mL^{-1}, compared to the certified value (211 ± 34 ng mL^{-1}). A similar trend as regards the concentrations of 1-OH-NAP determined in the SRM 3673 was observed also in other studies, for instance 151 ± 11 ng mL^{-1} by Motorykin et al. [19] and 197 ± 3 ng mL^{-1} by Li et al. [25]. Recoveries of nine OH-PAHs were in the range of 77–113 %, and repeatabilities were less than 20 %. LOQs for OH-PAHs were in the range of 0.01–0.025 ng mL^{-1}. For the 6-OH-CHR and 3-OH-BaP, which were not certified in the SRM 3673, the performance parameters were determined by the analysis of the artificially contaminated urine blank sample. The validation level was 0.05 ng mL^{-1} for 6-OH-CHR and 1 ng mL^{-1} for 3-OH-BaP (the concentration was higher because of its lower instrumental sensitivity). The recoveries for 6-OH-CHR and 3-OH-BaP were 95 % (RSD 13 %, LOQ 0.01 ng mL^{-1}) and 97 % (RSD 16 %, LOQ 0.9 ng mL^{-1}), respectively. As overviewed in Table 2, present LOQs (0.01–0.025 ng mL^{-1}) are comparable with the other reported values within the similar multi-analyte determination of OH-PAHs using LC–MS/MS [13–17, 34, 35]. The only exception is for 3-OH-BaP, when higher LOQ 0.9 ng mL^{-1} was achieved as compared to Xu et al. [13] (0.005 ng mL^{-1}) or Barbeau et al. [12], who use the LC–FLD detection for the single determination of this metabolite and achieved 0.05 ng L^{-1}.

Finally, the determined concentration of creatinine (508 ± 4 mg mL^{-1}, RSD 0.8 %, LOQ 0.1 ng mL^{-1}) was in accordance with the reference value in the certificate (505 ± 2 mg mL^{-1}). Regarding with the control of background contamination, procedural blanks were prepared. From all target OH-PAHs, traces of 2-OH-NAP, 2-OH-PHEN and 3-OH-PHEN were detected in the concentration range of 0.01–0.09 ng mL^{-1}.

Method applicability

Following the successful method validation, 50 samples of urine (collected within the project Impact of air pollution to genome of newborns, No. 13-13458S) were analysed. The

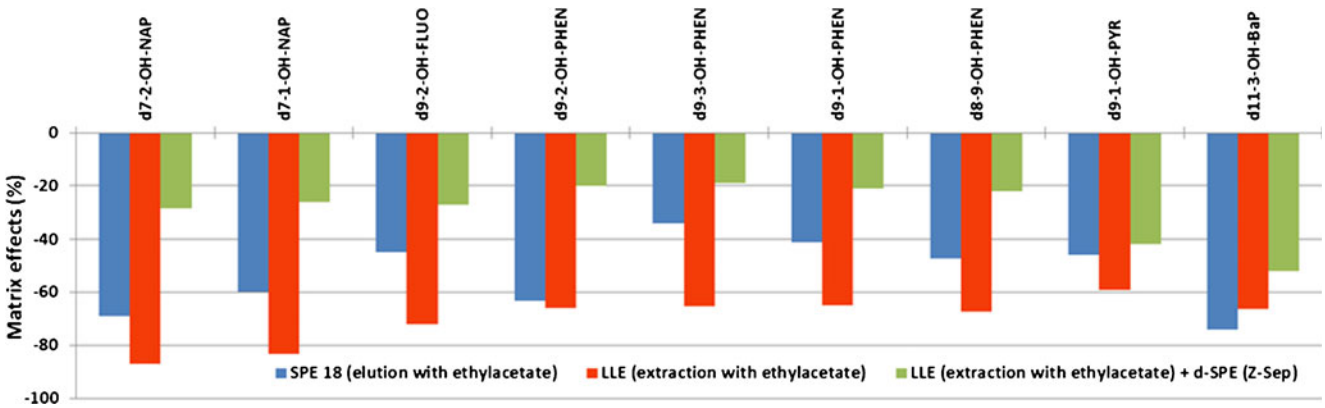


Fig. 3 Comparison of matrix effects (ME, %) for tested extraction techniques. ME (%) = [peak area of matrix-matched standards / peak area of solution standards − 1] × 100 (%). ME = 0 %, no matrix effects; ME < 0 %, ionisation suppression; ME > 0 %, ionisation enhancement [33]

Table 1 Mean values (ng mL^{-1}), standard deviations (SDs), recoveries (REC) and repeatabilities (RSD) of selected OH-PAHs analysed in the SRM 3673

OH-PAHs	ISTD	LOQ (ng mL^{-1})	Measured concentrations ($n = 6$)		Certified concentrations		REC (%)	RSD (%)
			Mean	SD	Mean	SD		
1-OH-NAP	d_7-1-OH-NAP	0.025	162	11	211	34	77	7
2-OH-NAP	d_7-2-OH-NAP	0.025	1.36	0.10	1.35	0.03	100	7
2-OH-FLUO	d_9-2-OH-FLUO	0.025	0.121	0.003	0.107	0.007	113	3
1-OH-PHEN	d_9-1-OH-PHEN	0.010	0.0449	0.0036	0.0488	0.0075	92	8
2-OH-PHEN	d_9-2-OH-PHEN	0.010	0.0215	0.0044	0.0247	0.0043	87	20
3-OH-PHEN	d_9-3-OH-PHEN	0.010	0.0296	0.0031	0.0276	0.0014	107	11
4-OH-PHEN	d_8-9-OH-PHEN	0.010	0.0114	0.0009	0.0104	0.001	109	8
9-OH-PHEN	d_8-9-OH-PHEN	0.010	0.0119	0.0014	0.0116	0.0009	103	12
1-OH-PYR	d_9-1-OH-PYR	0.025	0.0348	0.0028	0.0305	0.0018	114	8

Mass fraction concentrations (μg kg^{-1}) were converted to urinary concentrations (ng mL^{-1}) using a urine density value of 1.019 g mL^{-1}, as specified in the certificate of analysis

samples were obtained from Czech women living in the industrial city Karviná, which represents a locality with high PAH air pollution, especially during winter season due to emissions from the residential heating [36, 37]. The results, expressed both in nanograms per milliliter and nanograms per gram creatinine, are summarised in Table 3. The total concentration of OH-PAHs (ΣOH-PAHs) was in the range of 0.87–63 ng mL^{-1} (1600–33,000 ng g^{-1} creatinine). The most abundant metabolites were 2-OH-NAP and 1-OH-PHEN, which were detected in all examined urine samples. Their concentrations were in the range of 0.51–53 ng mL^{-1} (720–25,000 ng g^{-1} creatinine) and 0.07–3.2 ng mL^{-1} (90–2400 ng g^{-1} creatinine), respectively. The predominance of 2-OH-NAP in the urine of general population (children, non-smokers) was published within recent studies in the range of median concentrations 1.1–5.3 ng mL^{-1} [38–41] and 2600–7400 ng g^{-1} creatinine [35, 42, 43]. The other detected OH-PAHs, namely 1-OH-NAP, 2-OH-FLUO, 2-OH-PHEN, 3-OH-PHEN, 4-OH-PHEN, 9-OH-PHEN and 1-OH-PYR, were found in more than 72 % of samples. 6-OH-CHR and 3-OH-BaP were not detected in any tested urine, and their occurrence was reported only in highly exposed subjects [44], since they are mainly excreted in faeces [5]. The example of U-HPLC–MS/MS chromatogram of urine sample is documented in Fig. S1 (see ESM).

Conclusions

Within our study, the novel analytical approach for the multiple determination of OH-PAHs including the metabolites of carcinogenic PAHs in urine has been developed and validated. The sample preparation procedure based on extraction with ethyl acetate and further purification of a crude urine extract with d-SPE using the sorbent Z-Sep is demonstrated for the first time in the analysis of PAH metabolites in the biological matrices such as urine and substitutes the demanding and time-consuming SPE on columns (with the risk of clogging of cartridge resulting in the loss of the sample or analytes) and thus significantly streamlines the analysis of urine in human biomonitoring studies. Great performance characteristics were obtained during the validation experiments on the SRM 3673, and the method recoveries ranged from 77 to 114 % with the relative standard deviation lower than 20 % and the quantification limits in the range of 0.010–0.025 ng mL^{-1} (except for 3-OH-BaP with 0.9 ng mL^{-1}). With regard to the optimisation of the U-HPLC–MS/MS method, the chromatographic separation of OH-PHEN isomers was achieved using the PFP stationary phase.

The final method was successfully used for the analysis of 50 urine samples obtained from Czech women who are residents of Karviná region, which represent the highly industrialised locality with relatively high PAH exposure. The concentrations of ΣOH-PAHs were in the range of 0.87–63 ng mL^{-1} (1600–33,000 ng g^{-1} creatinine), with 2-OH-NAP and 1-OH-PHEN being the most abundant contaminants. Their concentrations were in the range of 0.51–53 ng mL^{-1} (720–25,000 ng g^{-1} creatinine) and 0.07–3.2 ng mL^{-1} (90–2400 ng g^{-1} creatinine), respectively. These results were comparable to those reported in similar studies since 2014.

This is the very first study which reported the use of ethyl acetate for the isolation of OH-PAHs from urine, followed by the clean-up step of extraction by d-SPE with the sorbent Z-Sep. We recommend that it became a routine method to measure urinary OH-PAHs, which was documented by the investigation of OH-PAH occurrence in the urine of Czech general population.

Table 2 The overview of LC–MS/MS-based method for the analysis of OH-PAHs in urine

OH-PAHs	Sample volume (mL)	Extraction procedure[a]	Instrumental analysis	LOQs	Reference
1-OH-NAP, 2-OH-NAP, 2-OH-FLUO, 3-PHEN, 9-PHEN, 1-OH-PYR, 3-OH-FAT, 1-OH-BaAN, 2-OH-BcPHEN, 3-OH-CHR, 6-OH-CHR, 3-OH-BaP, 9-OH-BaP	10	Enzymatic deconjugation SPE (Sep-Pak C18) Elution MeOH	LC–(ESI-)–MS/MS Nova-Pak C18 column (150 mm × 3.9 mm)	LOD[b] = 0.005–0.25 ng mL^{-1}	[13]
1-OH-NAP, 2-OH-NAP, 2-OH-FLUO, 1-OH-PHEN, 2-OH-PHEN, 3-OH-PHEN, 4-OH-PHEN, 9-OH-PHEN, 1-OH-PYR, 1-OH-BaAN, 2-OH-BaAN, 3-OH-CHR, 6-OH-CHR, 3-OH-BaP, 7-OH-BaP, 9-OH-BaP	3	Enzymatic deconjugation SPE (EnvirElut PAH) Elution MeOH	LC–(ESI-)–MS/MS ThermaGold C18 column (150 mm × 2.1 mm × 3 μm)	0.002–0.010 ng mL^{-1} 0.002 ng mL^{-1} (2 + 3)-OH-PHEN[c] 0.002 ng mL^{-1} 2-OH-BaAN + 6-OH-CHR[c]	[14]
1-OH-NAP, 2-OH-NAP, 2-OH-FLUO, 1-OH-PYR, 1-OH-PHEN, 2-OH-PHEN, 3-OH-PHEN, 4-OH-PHEN, 9-OH-PHEN	5	Enzymatic deconjugation SPE (C18 Bond Elut) MeOH /MeOH (100:0.7)	LC–(ESI-)–MS/MS Kinetex C18 (100 mm × 10 mm × 2.6 μm)	0.001 ng mL^{-1} (4-OH-PHEN) 0.02 ng mL^{-1} (1 + 9)-OH-PHEN[c], 0.01–0.5 ng mL^{-1}	[15]
8-Hydroxy-2′-deoxyguanosine, 1-OH-NAP, 2-OH-NAP, 2-OH-FLUO, 3-OH-FLUO, 1-OH-PHEN, 2-OH-PHEN, 3-OH-PHEN, 4-OH-PHEN, 9-OH-PHEN, 1-OH-PYR	2	Enzymatic deconjugation SPE (C18 Bond Elut) Elution MeCN	LC–(ESI-)–MS/MS Zorbax RRHD Eclipse Plus C18 (100 mm × 2.1 mm × 1.8 μm)	MDL = 0.023–0.625 ng mL^{-1} 0.038 ng mL^{-1} (1 + 9)-OH-PHEN[c]	[16]
1-OH-NAP, 2-OH-NAP, 2-OH-FLUO, 3-OH-FLUO, 1-OH-PHEN, 2-OH-PHEN, 3-OH-PHEN, 4-OH-PHEN, 9-OH-PHEN, 1-OH-PYR	2	Enzymatic deconjugation LLE (80 % pentane/20 % toluene) Purification ($AgNO_3$)	LC–(ESI-)–MS/MS Zorbax RRHD Eclipse Plus C18 (100 mm × 2.1 mm × 1.8 μm)	0.006–0.06 ng mL^{-1} 0.012 ng mL^{-1} (1 + 9)-OH-PHEN[c]	[17]
1-OH-NAP, 2-OH-NAP, 2-FLUO, 2-OH-PHEN, 3-OH-PHEN, 4-OH-PHEN, 9-OH-PHEN, 1-OH-PYR, 1-OH-CHR, 6-OH-CHR, 3-OH-BcPHEN, 1-OH-BaAN	0.5–2	Enzymatic deconjugation LLE (80 % pentane/20 % toluene) Purification ($AgNO_3$)	LC–(ESI-)–MS/MS Eclipse Plus C18 (100 mm × 4.6 mm × 3.5 μm)	0.040 ng mL^{-1} (OH-NAPs), 0.010 ng mL^{-1}	[34]
1-OH-NAP, 2-OH-NAP, 2-OH-FLUO, 1-OH-PHEN, 2-OH-PHEN, 3-OH-PHEN, 4-OH-PHEN, 1-OH-PYR, 6-OH-CHR	10	Enzymatic deconjugation SPE (C18 Bond Elut) Elution MeCN with 0.05 % ammonia	LC–(ESI-)–MS/MS Zorbax SB-C18 (150 × 3.0 mm × 1.8 μm)	0.004–0.015 ng mL^{-1}	[35]

3-OH-FAT 3-hydroxyfluoranthene, *1-OH-BaAN* 1-hydroxybenzo[a]anthracene, *2-OH-BcPHEN* 2-hydroxy-benzo[c]phenanthrene, *MDL* method detection limit

[a] Enzymatic deconjugation using β-glucuronidase/aryl sulfatase

[b] LOQ not specified for 3-OH-BaP

[c] Isomers could not be chromatographically separated and were quantified together

Table 3 Concentrations of detected OH-PAHs in urine samples ($n = 50$)

Analytes	Samples > LOQ (%)	ng mL^{-1}					ng g^{-1} creatinine[a]			
		LOQ	Mean	Median	Minimum	Maximum	Mean	Median	Minimum	Maximum
1-OH-NAP	92	0.03	0.75	0.62	0.09	3.0	810	700	190	2400
2-OH-NAP	100	0.03	8.9	4.6	0.50	53	6900	5400	720	25,000
2-OH-FLUO	86	0.03	0.82	0.63	0.12	2.7	620	570	100	1200
1-OH-PHEN	100	0.01	0.77	0.60	0.07	3.2	780	690	90	2400
2-OH-PHEN	90	0.01	0.43	0.34	0.06	1.5	390	310	55	1600
3-OH-PHEN	86	0.01	0.19	0.15	0.05	0.64	170	150	70	380
4-OH-PHEN	74	0.01	0.22	0.13	0.05	1.0	170	120	40	770
9-OH-PHEN	72	0.01	0.95	0.38	0.08	19	800	390	110	10,000
1-OH-PYR	84	0.03	0.32	0.22	0.05	1.3	280	240	60	960
ΣOH-PAHs			13	8.3	0.87	63	10,000	9000	1600	33,000

[a] The mean, median, minimum and maximum of creatinine were 1.1, 0.92, 0.30 and 2.9 mg mL^{-1} of urine, respectively

Acknowledgments This study was funded by the Czech Science Foundation Project No. 13-13458S. The authors would like to thank the gynaecological and maternity ward at the Hospital and Polyclinic Karviná for organising the logistic and collection of samples. The 'Operational Program Prague – Competitiveness' (CZ.2.16/3.1.00/22197) and 'National Program of Sustainability' (NPU I (LO) MSMT - 34870/2013) are also gratefully acknowledged.

Compliance with ethical standards The study has been approved by the appropriate ethics committee and has been performed in accordance with the ethical standards.

Conflict of interest The authors declare that they have no competing interests.

Informed consent Informed consent was obtained from all participants for being included in the study.

References

1. Scientific opinion of the panel on contaminants in the food chain on a request from the European Commission on Polycyclic Aromatic Hydrocarbons in Food. EFSA J. 2008;724:1–114.
2. Ma Y, Harrad S. Spatiotemporal analysis and human exposure assessment on polycyclic aromatic hydrocarbons in indoor air, settled house dust, and diet: a review. Environ Int. 2015;84:7–16.
3. Moustafa GA, Xanthopoulou E, Riza E, Linos A. Skin disease after occupational dermal exposure to coal tar: a review of the scientific literature. Int J Dermatol. 2015;54:868–79.
4. Li Z, Romanoff LC, Trinidad DA, Hussain N, Jones RS, Porter EN, et al. Measurement of urinary monohydroxy polycyclic aromatic hydrocarbons using automated liquid-liquid extraction and gas chromatography/isotope dilution high-resolution mass spectrometry. Anal Chem. 2006;78:5744–51.
5. Ramesh A, Walker SA, Hood DB, Guillen MD, Schneider K, Weyand EH. Bioavailability and risk assessment of orally ingested polycyclic aromatic hydrocarbons. Int J Toxicol. 2004;23:301–33.
6. Campo L, Rossella F, Fustinoni S. Development of a gas chromatography/mass spectrometry method to quantify several urinary monohydroxy metabolites of polycyclic aromatic hydrocarbons in occupationally exposed subjects. J Chromatogr B. 2008;875:531–40.
7. Jongeneelen FJ. Benchmark guideline for urinary 1-hydroxypyrene as biomarker of occupational exposure to polycyclic aromatic hydrocarbons. Ann Occup Hyg. 2001;45:3–13.
8. Jacob J, Seidel A. Biomonitoring of polycyclic aromatic hydrocarbons in human urine. J Chromatogr B. 2002;778:31–47.
9. Zhang W, Xu D, Zhuang G, Ding C, Wang G, Chang J, et al. A pilot study on using urinary 1-hydroxypyrene biomarker for exposure to PAHs in Beijing. Environ Monit Assess. 2007;131:387–94.
10. Freire C, Abril A, Fernández MF, Ramos R, Estarlich M, Manrique A, et al. Urinary 1-hydroxypyrene and PAH exposure in 4-year-old Spanish children. Sci Total Environ. 2009;407:1562–9.
11. Yamano Y, Hara K, Ichiba M, Hanaoka T, Pan G, Nakadate T. Urinary 1-hydroxypyrene as a comprehensive carcinogenic biomarker of exposure to polycyclic aromatic hydrocarbons: a cross-sectional study of coke oven workers in China. Int Arch Occup Environ Health. 2014;87:705–13.
12. Barbeau D, Maître A, Marques M. Highly sensitive routine method for urinary 3-hydroxybenzo [a] pyrene quantitation using liquid chromatography-fluorescence detection and automated off-line solid phase extraction. Analyst. 2011;136:1183–91.
13. Xu X, Zhang J, Zhang L, Liu W, Weisel CP. Selective detection of monohydroxy metabolites of polycyclic aromatic hydrocarbons in urine using liquid chromatography/triple quadrupole tandem mass spectrometry. Rapid Commun Mass Spectrom. 2004;18:2299–308.
14. Onyemauwa F, Rappaport SM, Sobus JR, Gajdošová D, Wu RA, Waidyanatha S. Using liquid chromatography–tandem mass spectrometry to quantify monohydroxylated metabolites of polycyclic aromatic hydrocarbons in urine. J Chromatogr B. 2009;877:1117–25.
15. Ramsauer B, Sterz K, Hagedorn HW, Engl J, Scherer G, McEwan M, et al. A liquid chromatography/tandem mass spectrometry (LC-MS/MS) method for the determination of phenolic polycyclic aromatic hydrocarbons (OH-PAH) in urine of non-smokers and smokers. Anal Bioanal Chem. 2011;399:877–89.
16. Fan R, Wang D, Ramage R, She J. Fast and simultaneous determination of urinary 8-hydroxy-2′-deoxyguanosine and ten monohydroxylated polycyclic aromatic hydrocarbons by liquid chromatography/tandem mass spectrometry. Chem Res Toxicol. 2012;25:491–9.
17. Fan R, Ramage R, Wang D, Zhou J, She J. Determination of ten monohydroxylated polycyclic aromatic hydrocarbons by liquid–

liquid extraction and liquid chromatography/tandem mass spectrometry. Talanta. 2012;93:383–91.
18. Chetiyanukornkul T, Toriba A, Kameda T, Tang N, Hayakawa K. Simultaneous determination of urinary hydroxylated metabolites of naphthalene, fluorene, phenanthrene, fluoranthene and pyrene as multiple biomarkers of exposure to polycyclic aromatic hydrocarbons. Anal Bioanal Chem. 2006;386:712–8.
19. Motorykin O, Schrlau J, Jia Y, Harper B, Harris S, Harding A, et al. Determination of parent and hydroxy PAHs in personal PM 2.5 and urine samples collected during native American fish smoking activities. Sci Total Environ. 2015;505:694–703.
20. Romanoff LC, Li Z, Young KJ, Blakely NC, Patterson DG, Sandau CD. Automated solid-phase extraction method for measuring urinary polycyclic aromatic hydrocarbon metabolites in human biomonitoring using isotope-dilution gas chromatography high-resolution mass spectrometry. J Chromatogr B. 2006;835:47–54.
21. Li Y, Li X, Zhou Z. A novel facile method using polyetheretherketone as a solid phase extraction material for fast quantification of urinary monohydroxylated metabolites of polycyclic aromatic hydrocarbons. RSC Adv. 2014;4:39192–6.
22. Luo K, Gao Q, Hu J. Derivatization method for sensitive determination of 3-hydroxybenzo [a] pyrene in human urine by liquid chromatography–electrospray tandem mass spectrometry. J Chromatogr A. 2015;1379:51–5.
23. Smith CJ, Walcott CJ, Huang W, Maggio V, Grainger J, Patterson DG. Determination of selected monohydroxy metabolites of 2-, 3- and 4-ring polycyclic aromatic hydrocarbons in urine by solid-phase microextraction and isotope dilution gas chromatography–mass spectrometry. J Chromatogr B. 2002;778:157–64.
24. Luan T, Fang S, Zhong Y, Lin L, Chan SM, Lan C, et al. Determination of hydroxy metabolites of polycyclic aromatic hydrocarbons by fully automated solid-phase microextraction derivatization and gas chromatography–mass spectrometry. J Chromatogr A. 2007;1173:37–43.
25. Li Z, Romanoff LC, Trinidad DA, Pittman EN, Hilton D, Hubbard K, et al. Quantification of 21 metabolites of methylnaphthalenes and polycyclic aromatic hydrocarbons in human urine. Anal Bioanal Chem. 2014;406:3119–29.
26. Jacob P, Wilson M, Benowitz NL. Determination of phenolic metabolites of polycyclic aromatic hydrocarbons in human urine as their pentafluorobenzyl ether derivatives using liquid chromatography–tandem mass spectrometry. Anal Chem. 2007;79:587–98.
27. Li X, Zenobi R. Use of polyetheretherketone as a material for solid phase extraction of hydroxylated metabolites of polycyclic aromatic hydrocarbons in human urine. Anal Chem. 2013;85:3526–31.
28. Hagedorn HW, Scherer G, Engl J, Riedel K, Cheung F, Errington G, et al. Urinary excretion of phenolic polycyclic aromatic hydrocarbons (OH-PAH) in nonsmokers and in smokers of cigarettes with different ISO tar yields. J Anal Toxicol. 2009;33:301–9.
29. Zhang X, Hou H, Xiong W, Hu Q. Development of a method to detect three monohydroxylated polycyclic aromatic hydrocarbons in human urine by liquid chromatographic tandem mass spectrometry. J Anal Methods Chem. 2015;2015:1–8.
30. Butler AR. The Jaffè reaction: identification of the colored species. Clin Chim Acta. 1976;59:227–32.
31. Bonsnes RW, Taussky HH. On the colorimetric determination of creatinine by the Jaffe reaction. J Biol Chem. 1954;158(3):581–91.
32. Euerby MR, McKeown AP, Petersson P. Chromatographic classification and comparison of commercially available perfluorinated stationary phases for reversed-phase liquid chromatography using principal component analysis. J Sep Sci. 2003;26:295–306.
33. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem. 2003;75:3019–30.
34. Guo Y, Senthilkumar K, Alomirah H, Moon HB, Minh TB, Mohd MA, et al. Concentrations and profiles of urinary polycyclic aromatic hydrocarbon metabolites (OH-PAHs) in several Asian countries. Environ Sci Technol. 2013;47:2932–8.
35. Wang Z, Zheng Y, Zhao B, Zhang Y, Liu Z, Xu J, et al. Human metabolic responses to chronic environmental polycyclic aromatic hydrocarbon exposure by a metabolomic approach. J Proteome Res. 2015;14:2583–93.
36. Svecova V, Topinka J, Solansky I, Rossner P, Sram RJ. Personal exposure to carcinogenic polycyclic aromatic hydrocarbons in the Czech Republic. J Expo Anal Environ Epidemiol. 2013;23:350–5.
37. Sykorova B, Kucbel M, Raclavska H, Drozdova J, Raclavsky K. Seasonal variations of polycyclic aromatic hydrocarbons (PAHs) in the air of Moravian-Silesian region, Czech Republic. In: Sung WP, Kao J, editors. Environment, energy and applied technology. London: Taylor & Francis Group; 2015. p. 367–72.
38. Thai PK, Li Z, Sjödin A, Fox A, Diep NB, Binh TT, et al. Biomonitoring of polycyclic aromatic hydrocarbons exposure in small groups of residents in Brisbane, Australia and Hanoi, Vietnam, and those travelling between the two cities. Chemosphere. 2015;139:358–64.
39. Jung KH, Lovinsky-Desir S, Perzanowski M, Liu X, Maher C, Gil E, et al. Repeatedly high polycyclic aromatic hydrocarbon exposure and cockroach sensitization among inner-city children. Environ Res. 2015;140:649–56.
40. Li J, Fan R, Lu S, Zhang D, Zhou Y, Lv Y. Exposure to polycyclic aromatic hydrocarbons could cause their oxidative DNA damage: a case study for college students in Guangzhou, China. Environ Sci Pollut Res. 2015;22:1770–7.
41. Kim HW, Kam S, Lee DH. Synergistic interaction between polycyclic aromatic hydrocarbons and environmental tobacco smoke on the risk of obesity in children and adolescents: the US National Health and Nutrition Examination Survey 2003–2008. Environ Res. 2014;135:354–60.
42. Li J, Lu S, Liu G, Zhou Y, Lv Y, She J, et al. Co-exposure to polycyclic aromatic hydrocarbons, benzene and toluene and their dose–effects on oxidative stress damage in kindergarten-aged children in Guangzhou, China. Sci Total Environ. 2015;524:74–80.
43. Fan R, Li J, Chen L, Xu Z, He D, Zhou Y, et al. Biomass fuels and coke plants are important sources of human exposure to polycyclic aromatic hydrocarbons, benzene and toluene. Environ Res. 2014;135:1–8.
44. Förster K, Preuss R, Roßbach B, Brüning T, Angerer J, Simon P. 3-Hydroxybenzo[a] pyrene in the urine of workers with occupational exposure to polycyclic aromatic hydrocarbons in different industries. J Occup Environ Med. 2008;65:224–9.

APPENDIX V

López M.A., Göen T., Mol H., Nübler S., Haji-Abbas-Zarrabi K., Koch H.M., Kasper-Sonnenberg M., Dvorakova D., Hajslova J., Antignac J.P., Vaccher V., Elbers I., Thomsen C., Vorkamp K., Pedraza – Díaz S., Kolossa-Gehring M., Castaño A.

The European human biomonitoring platform - Design and implementation of a laboratory quality assurance/quality control (QA/QC) programme for selected priority chemicals

International Journal of Hygiene and Environmental Health (2021) 234: 113740

doi: 10.1016/j.ijheh.2021.113740

International Journal of Hygiene and Environmental Health 234 (2021) 113740

Contents lists available at ScienceDirect

International Journal of Hygiene and Environmental Health

journal homepage: www.elsevier.com/locate/ijheh

The European human biomonitoring platform - Design and implementation of a laboratory quality assurance/quality control (QA/QC) programme for selected priority chemicals

Marta Esteban López [a,*], Thomas Göen [b], Hans Mol [c], Stefanie Nübler [b], Karin Haji-Abbas-Zarrabi [b], Holger M. Koch [d], Monika Kasper-Sonnenberg [d], Darina Dvorakova [e], Jana Hajslova [e], Jean-Philippe Antignac [f], Vincent Vaccher [f], Ingrid Elbers [c], Cathrine Thomsen [g], Katrin Vorkamp [h], Susana Pedraza – Díaz [a], Marike Kolossa-Gehring [i], Argelia Castaño [a,**]

[a] National Centre for Environmental Health, Instituto de Salud Carlos III (ISCIII), Majadahonda, Spain
[b] Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine, Friedrich-Alexander Universität Erlangen-Nürnberg (IPASUM), Erlangen, Germany
[c] Wageningen Food Safety Research, Part of Wageningen University & Research, Wageningen, Netherlands
[d] Institute for Prevention and Occupational Medicine of the German Social Accident Insurance, Institute of the Ruhr-University-Bochum (IPA), Bochum, Germany
[e] Department of Food Analysis and Nutrition, Faculty of Food and Biochemical Technology, University of Chemistry and Technology (UCT), Prague, Czech Republic
[f] Oniris, INRAE, LABERCA, Nantes, France
[g] Department of Environmental Health, Norwegian Institute of Public Health (NIPH), Oslo, Norway
[h] Department of Environmental Science, Aarhus University (AU), Roskilde, Denmark
[i] German Environment Agency (UBA), Berlin, Germany

ARTICLE INFO

Keywords:
Human biomonitoring (HBM)
Interlaboratory comparison investigation (ICI)
External
Quality assurance scheme (EQUAS)
HBM4EU

ABSTRACT

A fundamental objective of the human biomonitoring for Europe initiative (HBM4EU) is to progress toward comparable and robust exposure data for a wide variety of prioritized chemicals in human samples. A programme for Quality Assurance/Quality Control (QA/QC) was designed in HBM4EU with the purpose of creating a network of European laboratories providing comparable analytical data of high quality. Two approaches were chosen for two sets of prioritized chemicals with different timelines: (i) Scheme 1, where interested candidate laboratories participated in multiple rounds of proficiency tests (ii) Scheme 2, where selected expert laboratories participated in three rounds of interlaboratory comparison investigations. In both cases, the results were used to identify laboratories capable of generating consistent and comparable results for sample analysis in the frame of HBM4EU. In total, 84 laboratories from 26 countries were invited to participate in Scheme 1 that covered up to 73 biomarkers from Hexamoll® DINCH, phthalates, bisphenols, per- and polyfluoroalkyl substances, halogenated flame retardants (HFRs), organophosporous flame retardants (OPFRs), polycyclic aromatic hydrocarbons (PAH), cadmium, chromium and aromatic amines. 74 of the participants were successful for at least one biomarker in Scheme 1. Scheme 2 involved 22 biomarkers and successful results were obtained by 2 expert laboratories for arsenic, 5 for acrylamide, 4 for mycotoxins, 2 for pesticides and 2 for UV-filters in skin care products. The QA/QC programme allowed the identification of major difficulties and needs in HBM analysis as well of gaining insight in the analytical capacities of European laboratories. Furthermore, it is the first step towards the establishment of a sustainable European network of HBM laboratories.

1. Introduction

Human biomonitoring (HBM) is the gold standard for assessing the actual, overall exposure to chemicals in individuals or populations, irrespective of the detailed knowledge of contributing exposure sources or pathways. Differences in body burdens are mainly reflective of diet,

* Corresponding author. National Centre for Environmental Health, Instituto de Salud Carlos III (ISCIII), Ctra. Majadahonda – Pozuelo, Km 2.2, 28220, Majadahonda, Madrid, Spain.
** Corresponding author. National Center for Environmental Health, Instituto de Salud Carlos III (ISCIII) Majadahonda, Spain
E-mail addresses: m.esteban@isciii.es (M. Esteban López), castano@isciii.es (A. Castaño).

https://doi.org/10.1016/j.ijheh.2021.113740
Received 12 November 2020; Received in revised form 25 February 2021; Accepted 10 March 2021
Available online 26 March 2021

consumer goods and lifestyles as the main exposure determinants for many environmental or product-use related chemicals (Scherer, 2005; Ginsberg and Balk, 2016; Pacyga et al., 2019). These differences are generally small, whereas occupational exposure to chemicals could result in relatively high body burdens. The chemical analysis of HBM samples is facing a number of challenges, related to the environmental exposure at low levels, to the complexity of mixtures of different chemicals (ubiquitous in some cases) and the complex biological matrices. Being a relatively young discipline, with a rapidly growing number of exposure biomarkers in various human tissues and an increasing number of laboratories venturing into the field of HBM, there is a growing demand for establishing a common platform for HBM laboratories, facilitating knowledge exchange, cross-validations, and an international standardization/harmonization of appropriate biomarkers and analytical methods. In this context, it is crucial to have the confidence that the observed differences in multicentre HBM studies are due to variations in exposure and not due to variability or artefacts in the analytical or pre-analytical phase.

In the majority of the EU countries, HBM has been applied as a tool in research projects, often focusing on specific populations, with the exception of some countries such as Germany, Belgium or France, which have established full-scale HBM programmes (Kolossa-Gehring et al., 2012; Schoeters et al., 2012; Dereumeaux et al., 2017). Although significant HBM data have been generated in European countries in the last few decades, the available information appears somewhat fragmented and not always fully comparable. The differences range from the study design (e.g. target population, selection of biological matrices, etc.) to the pre – analytical phase (e.g. sampling procedures, type of samples, etc.) and to the applied analytical methods. With regard to the latter, unlike in some other fields, no standard reference methods do exist for HBM surveillance purposes yet, as there is currently no structure/network of European and/or National Reference Laboratories as it exists in other fields, such chemical food safety (von Holst et al., 2016; Parvaneh et al., 2017; Broothaerts et al., 2020). In addition, sustainable procedures and schemes for proficiency testing applied to human matrices have not yet been extensively developed and there are only few suppliers of proficiency tests for HBM biomarkers (e.g. G-EQUAS, QMEQAS, OSEQAS), offering a limited (though increasing) range of biomarker/matrix combinations and relevant environmental and product-use related exposure levels (Göen et al., 2012).

This lack of harmonization was already addressed during the preparation of the EU Environment and Health Action Plan 2004–2010 (COM 416, 2004) and as a consequence, efforts were made to harmonize HBM in Europe. The first steps were implemented by ESBIO (Expert Team to Support Biomonitoring in Europe), followed by COPHES (Consortium to Perform Human Biomonitoring on a European Scale) (Becker et al., 2014; Schindler et al., 2014; Esteban López et al., 2015) and DEMOCOPHES (DEMOnstration of a study to COordinate and Perform Human biomonitoring on a European Scale) (Den Hond et al., 2015) and most recently, by the Human Biomonitoring for Europe Initiative (HBM4EU, www.hbm4eu.eu).

HBM4EU is an EU Joint Programme that has developed its research programme for priority substances as defined by EU services and partner countries' policy makers to answer open policy relevant questions. HBM4EU aims to harmonize and use HBM to understand human exposure to chemicals, in occupational settings, through the use of consumer products or behavioural choices and the related health risks to improve the chemical risk management and to support policy-making (Ganzleben et al., 2017). Based on policy-related research needs regarding chemical exposure and potential health effects, two sets of priority chemicals were selected in HBM4EU. First set, including phthalates and their substitute 1,2-cyclohexane dicarboxylic acid diisononyl ester (Hexamoll® DINCH), bisphenols, per- and polyfluoroalkyl substances (PFAS), halogenated flame retardants (HFRs), organophosphorus flame retardants (OPFRs), polycyclic aromatic hydrocarbons (PAHs), cadmium, chromium and aromatic amines and the second, acrylamide, aprotic solvents, arsenic, diisocyanates, lead, mercury, mycotoxins, pesticides and UV-filters in skin care products.

Because the chemical analyses in HBM4EU and its predecessors have been organized in a decentralized manner, involving multiple laboratories in several countries the need to ensure data comparability has been a central aspect of the project early on. First steps in this direction were undertaken in COPHES/DEMOCOPHES where a programme consisting of Interlaboratory Comparison Investigations (ICIs) and External Quality Assessment Schemes (EQUAS) was implemented, supporting the generation of comparable HBM data in 17 EU countries (Schindler et al., 2014; Esteban López et al., 2015). In HBM4EU the challenge has been even greater since the number of laboratories, countries and chemicals are significantly higher. Also, as the conclusions on exposure differences in HBM4EU will have important public health consequences at policy-making level, the quality and comparability of the analytical results has to be guaranteed by strictest Quality Assurance and Quality Control (QA/QC) measures. Based on the two list of priority substances established along the project and the time frame, two different approaches were applied to ensure the full comparability of the analytical results. The first one comprised 4 rounds of proficiency tests with a high number of participating laboratories while the second approach was an intensive interlaboratory comparison investigation with a reduced number of expert participants.

This paper presents the main results and compares the two approaches developed in HBM4EU to obtain high quality and comparable analytical results in multicentre HBM studies for a variety of chemicals and laboratories with different degrees of expertise.

2. Material & methods

2.1. Quality Assurance Unit and QA/QC programme

A Quality Assurance Unit (QAU) was established to discuss and decide all issues related to the QA/QC of the chemical analyses in HBM4EU, including the design of the QA/QC programme. The QAU was formed by experts in the field of HBM and analytical chemistry and included the leaders of the COPHES/DEMOCOPHES QAU, to ensure the continuation of previous successful approaches.

The prime objective of the HBM4EU QA/QC programme was to identify (and in the end certify) analytical laboratories that could analyse the HBM4EU samples accurately, precisely and the most important in a comparable way. For that, the QAU designed two different schemes for each set of prioritized compounds mainly to address the time constraints (Fig. 1). Scheme 1 covered the substances on the 1st priority list and involved four rounds of proficiency tests. Participants were free to decide for which biomarkers they participated. The exercises were organised and evaluated as ICIs or EQUAS, depending on the needs and situation for each substance group. In both cases, the exercises involved the assessment of the comparability of analytical results for the same control material analysed in parallel by multiple laboratories, with their own analytical method. As measure of proficiency, Z-scores were calculated using an assigned value, and a preset target standard deviation (e.g. fit-for-purpose standard deviation). In case of ICIs, the assigned value was derived from the participants' results, in case of EQUAS, the assigned value was the mean concentration as established from data generated by designated expert laboratories (ELs).

Scheme 2 addressed a reduced list of chemicals, compared with the original 2nd list of prioritization, to match the studies planned in HBM4EU: acrylamide, arsenic, mycotoxins, pesticides and UV-filters. Scheme 2 included three rounds of ICIs and laboratories should participate for all the biomarkers within a substance group.

For both schemes, two control materials were sent to the participants in each round. The target concentrations of the biomarkers in the control materials was in the range commonly observed in the general population (between P25–P90 percentile in available national reference values of

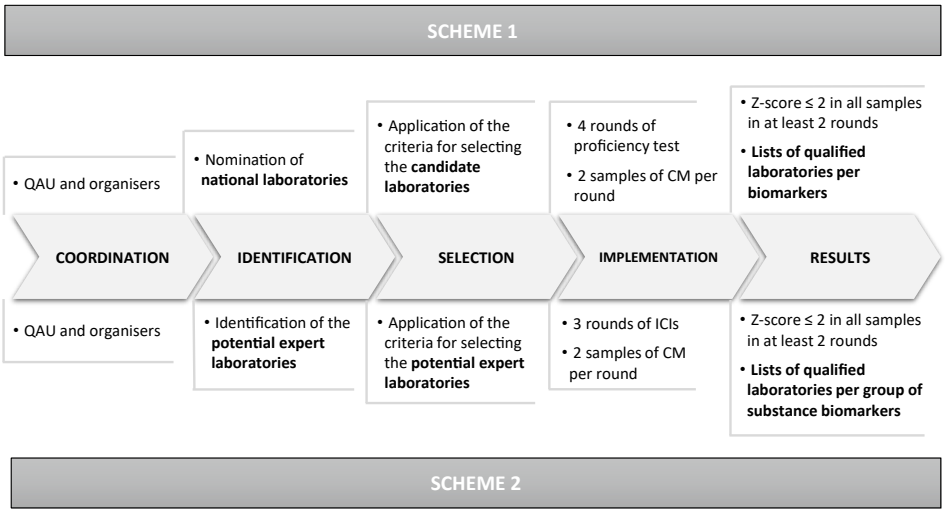


Fig. 1. Steps followed in the two schemes of the HBM4EU QA/QC programme. CM: control materials. ICI: interlaboratory comparison investigation.

EU countries (Den Hond et al., 2015), occupational exposure in the case of Cr) (table S7).

The rounds were spread out over time in such a way that laboratories received feedback on their performance well before the next round, allowing them to perform corrective actions, if needed, before participation in the next round.

To achieve satisfactory results in the schemes and take part in the analysis of the samples in HBM4EU, participants had to obtain successful results in at least two rounds.

2.2. Identification of the supporting and participating laboratories

The objective of this part of the study was to identify laboratories that could support the QA/QC programme by organising the proficiency tests for a specific group of substances, as well as those laboratories that like to analyse samples in HBM4EU.

The potential candidate laboratories were identified by the HBM4EU National Hub Contact Points (i.e. the contact point for each participating country), who provided information on laboratories performing chemical analyses of the prioritized compounds in human matrices and announcing the activity at country level. Additionally, an announcement was launched on the websites of HBM4EU (www.hbm4eu.eu) and the European Environment Agency (EEA) and in different scientific societies and fora, requesting interested laboratories to sign up as potential candidate laboratories in HBM4EU.

Questionnaires were sent to all nominated laboratories to collect information on their experience in the analysis of the target chemicals in human matrices as well as in organising proficiency tests. The responses were evaluated according to the criteria previously defined by the QAU, having as first criterion the experience in the chemical analysis of the target compound group in human samples (Tables S2 and S3). This process resulted in a list of candidate laboratories who were invited to participate in Scheme 1 or to support the QA/QC programme. Fig. 1 summarises the process.

In Scheme 2, it was agreed to select a reduced number of expert laboratories according to technical and practical criteria defined by the QAU (Table S4). The ELs were invited to join the ICIs and alerted about the tight time frame of these ICIs. All the HBM4EU analyses of the 2nd set of priority substances would be performed only in the ELs obtaining satisfactory results (Fig. 1).

The QA/QC programme was coordinated by the QAU and the supporting selected laboratories were responsible for preparing and sending the control materials to the participants, establishing the communication with the participants, evaluating the results and preparing the reports.

2.3. Selection of the biomarker/matrix combinations in the QA/QC programme

Specific exposure biomarkers and most suitable matrices to be included in the QA/QC programme were selected for the first group of prioritized chemicals as described in Vorkamp et al. (2021). Briefly, compound-independent criteria were developed for the selection of most suitable biomarkers and matrices for HBM, for example considering the specificity, biological sensitivity and stability of a certain biomarker/matrix combination. These criteria were then applied to review the scientific literature of the last ten years approx., with a view to identify the most suitable biomarkers and matrices for each of the prioritized chemical. This evaluation resulted in a first list of pairs of biomarker/matrix (typically serum or urine) for each compound group (Vorkamp et al., 2021). In the next step, the list of exposure biomarkers was further reduced based on technical feasibility, expected body burdens and policy-related research needs, as evaluated by the QAU. This shortlist was used in the QA/QC programme.

The same procedure was applied to define the biomarkers for the 2nd list of priority substances addressed in Scheme 2.

2.4. Organization of the proficiency tests

In order to provide a harmonised approach for the organization and evaluation of the different ICI/EQUAS exercises, protocols were drafted and described in standard operating procedures (SOPs). These SOPs were based on existing protocols originated from ISO17043 accredited organisations, and included detailed instructions for all aspects of the QA/QC programme, such as the description of the roles and responsibilities of the organisers, timeline of the exercises, definitions of different terms or templates for communication with the participants and reporting of the results. Additional SOPs were drafted for the preparation and characterisation of control materials and for the evaluation of participants' results. Details for the preparation of the various control materials are available in the online library of the HBM4EU website (www.hbm4eu.eu). The characterization of the control materials included homogeneity and stability testing.

Homogeneity testing was based on ISO13528:2015 and Fearn and Thompson (2001). This involved duplicate analysis of 10 randomly selected test samples of a control material. The control material was considered sufficiently homogeneous if the between-sample standard deviation did not exceed a critical value (0.3 x target standard

deviation).

The stability of the control materials during the period from shipment to the deadline for submission of the participants' results was assessed in line with ISO 13528:2015 and the international harmonised protocol for the proficiency testing of analytical laboratories (Thompson et al., 2006). For this, the organiser stored test samples under the conditions recommended to the participants (typically freezer < -18 °C), and optionally an additional set at −80 °C. Stability was assessed by comparison of the mean of six stored samples (−18 °C, t = after receiving all participants' results), with the mean of a reference set of six samples. The reference was either the mean as obtained at/before shipment of the samples, or the mean obtained for samples stored at −80 °C (assumed stable) analysed concurrently with the stored samples.

2.5. Evaluation of laboratory performance

The laboratory performance was assessed by calculation of z-scores for each biomarker in the test samples according to the following formula:

$$Z = \frac{x - A}{\sigma_T} \quad (1)$$

with Z = z-score
x = participant's result
A = assigned value
σ_T = standard deviation for proficiency, with $\sigma_T = 0.25*A$

A z-score of $|Z| \leq 2$ was interpreted as satisfactory, $2<|Z|<3$ as questionable, and $|Z|\geq 3$ as unsatisfactory performance. The assigned value was either the consensus value derived from the participants (used in ICI) or a value derived from analysis by selected expert laboratories (used in EQUAS). The parameters from equation (1) are briefly explained l below.

- Standard deviation for proficiency, or target standard deviation (σ_T), determines the performance boundaries of the ICI/EQUAS. The performance boundaries should be fit-for-purpose and take into account the interlaboratory variability (reproducibility relative standard deviation, RSD_R) currently considered achievable in HBM analysis. The available data on the latter is scarce and variable. Schindler et al. (2014) reported RSD_Rs ranging from 6% to 32% for cadmium in urine, and 31%–45% (even higher in some cases) for phthalate biomarkers in urine. For selected highly experienced reference laboratories, Göen et al. (2012) reported RSD_Rs in the range 7%–19% for cadmium in urine. Outside the HBM domain, a generic relationship between expected RSD_R and concentration was originally proposed by Horwitz et al. (1980) and later modified by Thompson (2000), and has often been used as a fitness-for-purpose criterion in proficiency testing. The modified Horwitz equation suggests a constant RSD_R of 22% for concentrations below 120 μg/kg, and a decrease of RSD_R for higher concentrations. The validity of the modified Horwitz function has been a matter of debate (Linsinger and Josephs 2006). A constant RSD_R of 25% over a range of 1 μg/kg to 10 mg/kg has been suggested as more appropriate (Alder et al., 2001). Based on the literature it appears that, as long as concentrations are (well) above the method limit of detection (LOD), there is no consistent relationship between concentration and RSD_R. It may depend on the analyte and technique, but at this stage, in lack of exhaustive data for achievable RSD_Rs in HBM analysis and based on the data and discussions from the literature, it was decided to apply a fixed RSD_R of 25% as fit-for-purpose criterion to be used as target relative standard deviation in the ICI/EQUAS programme.

- Assigned value = consensus value (ICI). For determination of the consensus value, robust statistics was performed in accordance with Thompson et al. (2006), the guidelines from (Analytical Methods Committee, 1989a&b), and ISO 13528. The robust mean was taken as consensus value when the following requirements were met: the number of results submitted for a biomarkers had to be at least seven, the uncertainty (u) of the consensus value should be negligible (not exceed $0.3*\sigma_T$), with u being 1.25 times the standard deviation of the participants' results, divided by the square root of the number of participants. When the uncertainty of the consensus was not negligible, but not exceeding $0.7*\sigma_T$, the consensus value was still used for calculation of z-scores, but the uncertainty of the consensus value was taken into account for calculation of the z-scores using the following formula:

$$Z' = \frac{x - A}{\sqrt{\sigma_T{}^2 + u^2}} \quad (2)$$

with Z' = z-score ($0.3*\sigma_T < u \leq 0.7*\sigma_T$)
u = uncertainty of consensus value

In case the uncertainty of the consensus exceeded $0.7*\sigma_T$, the variability of results was considered too high to derive a meaningful consensus, and, consequently, also z-scores using such consensus value were considered unfit for evaluating individual participants' performance.

- Assigned value = Expert value (EQUAS). Establishment of the expert value to be used as assigned value involved the analysis of six replicates of the control material by at least three selected laboratories with a high level of expertise in the determination of the biomarker. For each expert laboratory, the mean value was calculated. Based on these means, the mean of the expert laboratories was calculated, the RSD, and the relative uncertainty (RSD divided by the square root of the number of expert laboratories). The expert value was considered suitable for use as assigned value as long as the uncertainty did not exceed $0.7*\sigma_T$ (i.e. 17.5%). For EQUAS, equation (1) was used for calculation of z-scores.

2.6. Programme coordination and follow – up

Monthly web conferences were organised among the QAU, the programme coordinator and the organisers of the different exercises to discuss the problems encountered and to exchange experiences. A help desk was available for the participating laboratories during the whole duration of the scheme and web conferences were offered to the participants when necessary in order to solve the main analytical problems faced during the exercise. Furthermore, participants also received recommendations after each round in the result reports. Nevertheless, some groups (i.e. Hexamoll® DINCH and phthalates) required a more intense and continuous support and a specific training school was organised.

3. Results

3.1. Details of the QA/QC programme

Both approaches were designed under common premises, but there were some important differences, for example in the number of biomarkers and matrices included (Fig. 2). Scheme 1 covered 73 biomarkers in 3 different human matrices while Scheme 2 involved 22 biomarkers in urine. The duration of Scheme 1 was 18–20 months depending on the substance group. The time for implementing Scheme 2 was shorter, from 3 up to 7 months depending on the group of substances.

3.2. Identification of the supporting and participating laboratories

For Scheme 1, a total of 183 questionnaires were sent to identify candidate laboratories for supporting the QA/QC programme and for

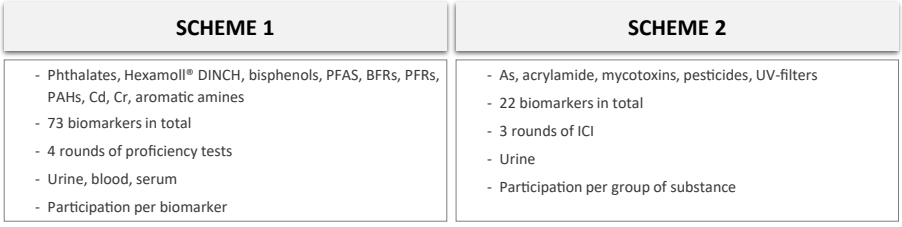


Fig. 2. Main characteristics of the two approaches followed in the HBM4EU QA/QC programme.

participating in the proficiency tests. 115 replies were received (63% response rate), some of them from the same laboratory to participate for different substance groups. Fig. 3 presents the number of candidate laboratories for Scheme 1 per group of substance. Approximately one year later, and after the 1st round of the proficiency test, the list of candidate laboratories was updated (Fig. 3). Questionnaires were sent to 229 potentially interested laboratories, including those already registered as candidate laboratories. The response rate was lower (37%) since some of the laboratories already included on the list of candidate laboratories did not update their data. In order to increase the participation, the selection process was simplified and the only criteria applied for participating in Scheme 1 was the exclusive criterion in table S2 ("have experience in analysing human samples for the given chemical").

With regard to the origin of the candidate laboratories, almost half of them (48%) were from universities or university hospitals, followed by governmental laboratories with 39% of the total number. Private laboratories accounted for 9%.

Of the laboratories completing the questionnaire for supporting the HBM4EU QA/QC programme, 19% reported experience in organising proficiency tests and 18 laboratories were selected in the first call, based on the criteria in Table S3. In the update, 11 participants out of 48 (23%) provided a positive answer and only three new laboratories were added to the list of potential supporting laboratories. Finally, five laboratories were involved as organisers (Table 1). In case of Scheme 2, the organisers were selected from those supporting the previous scheme based on their proven expertise (Table 1). The proficiency test for aromatic amines required a different organization as so, it will not be addressed in this publication.

Table 2 shows the biomarkers covered in the programme (except those for aromatic amines) as a result of the selection process, grouped by substances classes. While Scheme 1 offered a broad range of biomarkers and the participants decided for which they reported results, Scheme 2 offered a more limited number of biomarkers and the ELs had to participate for all biomarkers included in the programme. However, while the biomarkers were pre-defined, the laboratories were free to choose their own analytical method.

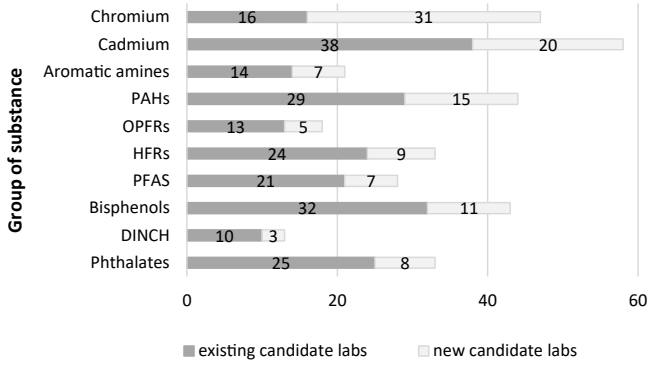


Fig. 3. Number of candidate laboratories in the two calls.

Table 1
Laboratories supporting the HBM4EU QA/QC programme. (Acronyms are defined in Table S1).

Substance group	Organiser	Laboratory preparing & testing control material
1st list of prioritization – Scheme 1		
Phthalates	RIKILT	RIKILT, IPA
Hexamoll® DINCH	RIKILT	RIKILT, IPA
Bisphenols	INRAE	INRAE
PFAS	IPASUM	IPASUM
HFRs	UCT	UCT
OPFRs	UCT	IPASUM
PAHs	IPASUM	IPASUM, UCT[a], ABF[b]
Cadmium	IPASUM	IPASUM
Chromium	IPASUM, JSI[a]	IPASUM, JSI[a]
2nt list of prioritization – Scheme 2		
Acrylamide	IPASUM	IPASUM
Arsenic	IPASUM	IPASUM
Mycotoxins	RIKILT	RIKILT
Pesticides	RIKILT	RIKILT, IPA
UV-filters	IPASUM	IPA, Region H

RIKILT, current name: Wageningen Food Safety Research, part of Wageningen University & Research, The Netherlands.
IPA, Institute for Prevention and Occupational Medicine of the German Social Accident Insurance - Institute of the Ruhr-University Bochum, Germany.
INRAE, Laboratoire d'Etude des Résidus et Contaminants dans les Aliments, LABERCA, Oniris-INRAE, France.
IPASUM, Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine, Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany.
UCT, University of Chemistry and Technology, Czech Republic.
ABF, ABF GmbH Analytisch-Biologisches Labor, Planegg, Germany.
JSI, Jozef Stefan Institute, Slovenia.
Region H, Dep. of Growth and Reproduction, Rigshospitalet, University of Copenhagen.
Selection of the parameters in the programme.
[a] Only the 1st round.
[b] Only 3-BaP in the 1st and 2nd round.

3.3. Participation

A total of 84 laboratories from 26 countries were invited to participate in Scheme 1 but not all of them confirmed their participation. The percentage of invited laboratories that registered in the course of the complete scheme per group of substance varied from 85% for Hexamoll® DINCH to 35% for OPFRs (considering the highest number of registrations in each group). In the case of Hexamoll® DINCH the registration was constant in the four rounds while for the rest of substances, in general, the number of registered laboratories increased after the first two rounds, up to twice or more in case of cadmium and chromium, and decreased after the 3rd round. In total 9 laboratories from Canada, Japan and United States collaborated as reference laboratories in the rounds that were organised as EQUAS (from the 2nd to the 4th round, except for Cr in which all rounds were ICIs) (Table S5).

Looking at the participation of the laboratories in Scheme 1 for different groups of chemicals, more than the half (61%) of the

Table 2
Biomarkers covered in the HBM4EU QA/QC programme.

Substance group	Matrix	Biomarkers
1st list of prioritization – Scheme 1		
Phthalates	urine	MEP, MBzP, MiBP, MnBP, MCHP, MnPeP, MEHP, 5OH-MEHP, 5oxo-MEHP, 5cx-MEPP, MnOP, OH-MiNP, cx-MiNP, OH-MiDP, cx-MiDP
Hexamoll® DINCH	urine	OH-MINCH, cx-MINCH
Bisphenols	urine	BPA, BPF, BPS
PFAS	serum	PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFBS, PFHxS, PFHpS, PFOS (sum of all isomers)
HFRs	serum	BDE-47, BDE-153, BDE-209, α-HBCD, γ-HBCD, TBBPA, Syn-DP, Anti-DP, DBDPE, 2,4,6-TBP
OPFRs	urine	DPHP, BDCIPP, BCEP, BCIPP
PAHs	urine	1-naphthol, 2-naphthol, 1,2-DHN[a], 2-FLUO, 3-FLUO, 9-FLUO, 1-PHEN, 2-PHEN, 3-PHEN, 4-PHEN, 9-PHEN, 1-PYR, 3-BaP[a]
Cadmium	urine blood	Cd
Chromium	urine blood serum	Cr
2nt list of prioritization – Scheme 2		
Acrylamide	urine	AAMA, GAMA
Arsenic	urine	As total, As (III), As (V), MMA, DMA, AsB
Mycotoxins	urine	DON (total)
Pesticides	urine	TCPy, glyphosate, AMPA, cis-DBCA, cis-DCCA, trans-DCCA, 3-PBA, 4-F-3-PBA, ClF3CA
UV-filters	urine	BP1, BP2[b], BP3, BP7[b]

[a] Only in the 1st and 2nd round.
[b] Only in the 1st round.

laboratories participated in the proficiency test for one or two substance groups (36% and 25% respectively) while a very limited number of laboratories (3%) participated for six or more substance groups.

The registration of the laboratories in the biomarkers offered in Scheme 1 varied considerably and showed a high intra-group variation for some substances. The low number of participants sometimes led to insufficient data for results evaluation and a detailed revision was required, including different statistical evaluations in order to obtain reliable results and conclusions. The specific difficulties encountered in each group of substances and the solutions applied will be addressed elsewhere. Table S6 shows the number of laboratories that registered in the four rounds as well as those reporting results and those consistently achieving satisfactory performance in Scheme 1.

Considering the global results, 74 participants reported successful results for at least one biomarker. The maximum number of biomarkers for which a laboratory reported successful results was 47. The average and P90 were 11 and 30 biomarkers, respectively.

Regarding participation in Scheme 2, five expert laboratories participated for acrylamides, three for arsenic, six for mycotoxins, four for pesticides and three for UV-filters, however, this was reduced to two in the second and third round (Table 3). At least two thirds of the participating laboratories returned satisfactory results (Table 3). All participants in the acrylamide exercise obtained satisfactory results.

4. Discussion

Although QA/QC is an essential component in any analytical laboratory, robust results that are comparable between laboratories can still be a challenge, in particular in the context of human biomonitoring of the general population, including low concentrations and the co-occurrence of a multitude of chemicals. For the first time, two different QA/QC approaches were implemented to ensure the quality and comparability of the analytical results in a multicentre EU-wide HBM project.

The design of the HBM4EU QA/QC programme had to be adapted to certain predefined characteristics of the project, mainly the time constraints and the support of capacity building in the participating countries. Scheme 1 offered the possibility of including a high number of laboratories (including less HBM experienced laboratories) and improving their analytical performance while Scheme 2, had to be done in a shorter time period and focused on assessing comparability of results for a small pre-selected group of expert laboratories. Thus, the two approaches were designed according to different priorities.

This work has allowed the identification of a high number of EU laboratories with experience in human biomonitoring and created the first HBM laboratory network in Europe. Nevertheless, despite the two calls and different communication channels employed to reach the laboratories, the authors are aware that a number of analytical laboratories from the different participating countries were not involved in the programme. This could be due to the information not reaching the laboratories or to a lack of interest in participating in the programme (e.g. not aligned with the laboratory interests or because it was a non-funded activity). However, the number of participants allowed to achieve the objectives of HBM4EU, i.e. obtain high quality and comparable HBM results and to provide the capacities for a Europe wide HBM study.

The process of identifying the candidate laboratories revealed interesting information. Significant differences were observed in the number of candidate laboratories for the different groups of substances, primarily reflecting expertise in the analysis of the substances involved. For example, the analysis of cadmium in human samples has been established for years, and validated analytical methods are available. As a consequence, the highest number of candidate laboratories to participate in the QA/QC programme was found for cadmium analysis. However, for biomarkers related to chemicals of more emerging concern the number of laboratories with experience is lower and therefore the number of candidate laboratories was reduced e.g. for Hexamoll® DINCH or OPFRs. A kind of specialization or interest in certain substances was observed for the majority of the laboratories since in general the participation was restricted to 1–3 groups of substances while a reduced number of them covered a wider spectrum. Nevertheless, this could be influenced by other factors such as individual interests and therefore not reflect the real situation in terms of expertise and capacities of the laboratories. Independently of that, there was a clear difference between the participation and results of the inorganic and organic chemicals selected in the project.

A great challenge during the first stages of the programme was the identification of the laboratories to support the QA/QC programme (proficiency tests organisers) since only a limited number of laboratories

Table 3
Participants and results in Scheme 2.

	ROUND 1	ROUND 2	ROUND 3	no.reporting satisfactory results [a] (%)
	no. registered/no.reporting	no. registered/no.reporting	no. registered/no.reporting	
Arsenic and compounds	3/3	3/3	3/3	2 (67%)
Acrylamide	5/5	5/5	5/5	5 (100%)
Mycotoxins	6/6	6/5	5/5	4 (67%)
Pesticides	4/4	4/4	4/4	2 (50%)
UV-filters	3/3	2/2	2/2	2 (100%)

[a] Achieving satisfactory z-scores for the biomarker in both control materials from a round, in at least two rounds from the QA/QC programme.

meet the HBM4EU criteria to support the QA/QC programme. In addition the short timeframe did not help neither since proficiency test had to be organized in parallel for 73 biomarkers in 3 matrices for more than 80 laboratories across 30 countries. In some cases, the laboratories did not have experience in organising proficiency tests for the target compounds. In others, the laboratories had wide experience even in the target chemicals but in other research areas, including in non-human matrices. In addition, although some laboratories had experience in organising these exercises, they could not prepare and test the homogeneity and stability of the control materials employed in the programme and, for some substances (phthalates, Hexamoll® DINCH, OPFRs and PAHs in Scheme 1 and for pesticides and UV – filters in Scheme 2), it was necessary to involve both an organiser and an expert laboratory able to prepare and test an adequate and reliable control material to use in the QA/QC programme. This was indeed another great challenge since, due to the lack of reference materials (i.e. target matrix and biomarkers in the concentrations expected in the general population) the preparation (and test the homogeneity and stability) of the control material for all the exercises increased the time period of the programme. This process was done under strict QA/QC measures and precisely described in the corresponding SOP, to ensure that organisation and evaluation by the different parties involved were done in a harmonised way.

In general, the adherence to the programme was good, although in the first rounds of phthalates, HFRs, OPFRs and PAHs the percentage of registered laboratories reporting results was low for certain biomarkers. In case of phthalates, this occurred for OH-MiNP, OH-MiDP and cx-MiDP, with reporting percentages below 90%. This could probably be explained by initial difficulties in the laboratories that were solved after the first round. The same tendency was observed for BPS and PFPeA. The HFRs also showed an increase in the reporting percentage after the first round except for TBBPA, DBDPE and 2,4,6-TBP. For these compounds, the potential analytical problems were not solved as the number of laboratories with satisfactory results remained low. The situation was similar for OPFRs with a low number of participants reporting results (and high variability among them) for the four biomarkers, making the evaluation of the results difficult. The highest variability in the participation per biomarker and the percentage of registered laboratories reporting results was found in the PAHs group, not only due to the technical difficulties but also due to the specialization of the participants in specific biomarkers.

To achieve satisfactory results in the programme and take part in the analysis of samples in HBM4EU, participants had to obtain successful results in at least two rounds of the proficiency tests and this could explain the general decrease in the number of participants in the 4th round, especially for Cd and Cr. However, this reduction was not so clear in the PAHs group.

Looking at the laboratories that obtained satisfactory results in the programme, the overall goal of analysing the samples in HBM4EU in a comparable way was achieved for all the target chemicals with a high improvement in the number of biomarkers with satisfactory results per laboratory (Figs. 4 and 5). For Hexamoll® DINCH, around 70% of participating laboratories obtained successful results for the two biomarkers (OH-MINCH and cx-MINCH). The phthalates group had higher variability with 31–95% of the participants with satisfactory results depending on the biomarker. There was a set of phthalate metabolites (MEP, MBzP, MnBP, 5OH-MEHP, 5oxo-MEHP, 5cx-MEPP and cx-MiDP) with a satisfactory percentage above 75% while a second set had fewer participants and poorer results (MCHP, MnPeP, MnOP, OH-MiNP, cx-MiNP and OH-MiDP). Issues encountered for the phthalates group included the diversity in coverage of biomarkers (ranging from 3 to all prioritized 15) and limits of quantification (LOQs) (0.02–3.5 ng/ml), as well as background contamination for some of the biomarkers. For biomarkers of the long-chain phthalates (OH-MiNP, cx-MiNP, OH-MiDP, cx-MiDP) and Hexamoll® DINCH (OH-MINCH, cx-MINCH) initially a very high variability of results was observed. The reason for this was that

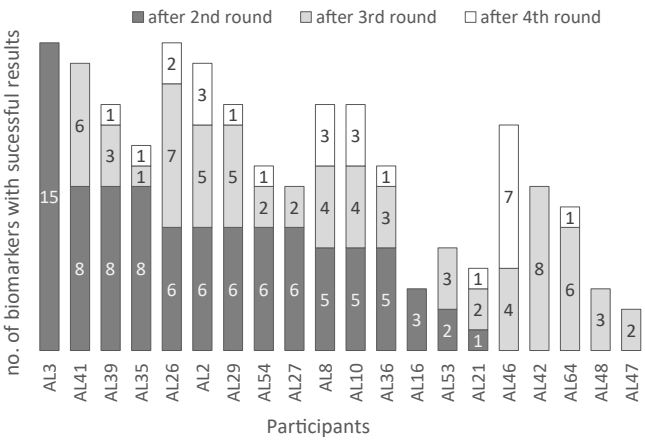


Fig. 4. Progress in the satisfactory results of phthalate biomarkers.

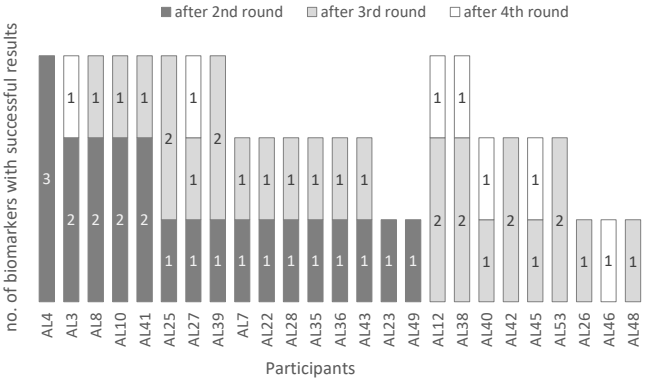


Fig. 5. Progress in the satisfactory results of bisphenols.

the parent compounds are mixtures of isomers resulting in multiple and/or broad peaks in real samples, and because the transition used for quantification in LC-MS/MS analysis affected the results. Standardizing to prescribed mass-transitions for quantification and recommendations regarding the acquisition window to ensure all relevant isomer peaks were included in the measurement reduced the variability. The inter-laboratory variability (RSD_R) derived from the participants' results improved during the programme. Details will be presented in a future paper.

For bisphenols while no major differences were found in terms of participation, the laboratories with satisfactory results varied with the highest number for BPA (83%) and lowest for BPF (50%). Some issues were encountered for this group, especially during the 1st round. Firstly, BPA results appeared overestimated for some participants, probably impacted by an external contamination source. Secondly, BPS and especially BPF were more rarely included and reported by participants, leading to a non-achievable performance assessment especially for BPF during the 1st round, together with a high variability of the results reported by this limited number of laboratories. However, a significant improvement of the results for both BPA, BPS and especially BPF was observed between the 1st and 4th round, demonstrating a good capacity building and methodological consolidation after considering lessons learnt from each round (detailed results and discussion will be presented in a future paper). Globally, the whole exercise for bisphenols finally permitted to attest the existence of a core network of competent HBM laboratories for BPA, BPS and BPF.

For the PFAS group, in general, laboratories showed a high reporting rate for all the biomarkers and the percentage of successful results was above 70% (except for PFHxA) and up to 100% for PFHpA, PFOA, PFNA, PFDA, PFHxs and PFOS. While laboratory performance in the 1st round

(ICI) varied considerably for the individual PFAS biomarkers (PFPeA: 54%; PFOA and PFHxS: 94% each, of laboratories with successful results), the switch to EQUAS from the 2nd round on led to an overall improvement and better homogeneity of results. However, despite this general improvement, especially the analysis of PFHxA at low levels and PFDoDA at high levels proved to be challenging for some laboratories. Detailed results and discussion will be presented in a future paper.

For HFRs, BDE-47, BDE-153 and BDE-209 showed the highest registration in all rounds, thus the calculation of consensus value in the 1st round was possible unlike the other HFR biomarkers. From the 2nd round, organized as EQUAS, an expert assigned value was established for BDE-47, BDE-153, BDE-209, anti-DP and syn-DP. For others (α-HBCD, γ-HBCD, DBDPE, TBBPA and 2,4,6-TBP) the calculation of an assigned value was not possible because of the limited scope of reported results by experts or too high uncertainty of the assigned value. In this case, the calculation of the consensus value from the results submitted by experts and candidates was successful only for α-HBCD and γ-HBCD. Due to the low number of candidates and expert results the calculation of consensus or assigned value was not possible for DBDPE, TBBPA and 2,4,6-TBP (the laboratory in table S6 correspond to the laboratory preparing and testing the CM for this exercise). The highest number of satisfactory results was achieved for BDE-47 and BDE-153. For BDE-209 the success rate was not as high, because of a higher number of results assessed as questionable or unsatisfactory.

The group that presented most difficulties due to the low number of participants and high variability of the results was the OPFRs. The calculation of consensus or assigned values according to standardized ICI/EQUAS approach was not possible at all (BDClPP, BClPP and BCEP) or only to a limited extent (DPHP) in the first three rounds. It was necessary to apply a more flexible approach, in order to draw conclusions. It is worth noting, that following discussions of main analytical difficulties after the 1st and 3rd round, the 4th round was very successful. The calculation of assigned values and the evaluation of results using the ICI/EQUAS approach was realized for BDClPP, BClPP and DPHP. Finally, from a total of six laboratories, which participated in any or the four rounds, five laboratories were successful for DPHP and BDClPP and four for BClPP. Details of the flame retardants results will be presented in a future paper.

The PAHs group had the highest variability in the number of participants per biomarker and also in the laboratories reporting satisfactory results. The main difficulties with PAHs metabolites was that no evaluation was possible for some biomarkers (1,2-DHN, 3-FLUO, 9-FLUO, 9-PHEN, 3-BaP) as the number of participating laboratories was too small (<7). Even after switching to EQUAS from the 2nd round on, no z-scores could be obtained for 9-FLUO, 9-PHEN and 3-BaP, while the only laboratory to analyse 1,2-DHN could not provide quantitative results. From the 3rd round on, the initial scope of 13 biomarkers were reduced to 11 (1,2-DHN and 3-BaP were omitted), but still no z-scores could be provided for 9-FLUO and 9-PHEN in the remaining two rounds. A general improvement in results from the 2nd to the 4th round is not discernible, in fact for some biomarkers even the opposite development is noticeable (especially for 2-FLUO). Details will be presented in a future paper.

The groups involving inorganic biomarkers were those with the highest rate of participation probably due to a more well-established and robust methodology for the analysis of these metals, although the percentage of satisfactory results were in line with those observed in other groups (e.g. PFAS, HFRs). The proficiency tests on cadmium (in blood and urine) were characterized by a large number of participants and satisfactory results from the 1st round on. Thus, there was no noticeable improvement from one round to the next. The main problem encountered was that some laboratories had a too high LOQ for their analytical method and thus failed at low analyte concentrations (Nübler et al., 2021). Unlike the other substance groups, no EQUAS was performed for chromium from the 2nd round onwards, but an ICI was performed in all four rounds. The ICI exercises on chromium (in blood, urine and serum) were characterized by satisfactory results throughout, with too high LOQs being the only problem encountered for some laboratories. Detailed results and discussion will be presented in a future paper.

Globally, the participation in Scheme 1 improved the capacities of the laboratories since the number of laboratories obtaining satisfactory results and the number of biomarkers with successful results per laboratory increased from the first to the last round.

For Scheme 2, the main difficulty was the selection of a reduced number of laboratories with enough expertise because in addition to the technical capability, practical aspects had to be considered in order to ensure the availability of the analytical results by the deadline defined within HBM4EU. The reduced time for implementing Scheme 2 was challenging for both the organisers and the participating ELs. The unexpected shutdown due to the first wave of covid-19 sanitary crisis put further strain on the scheme. The first round was implemented as planned from January to February 2020. The second round started in February–March, but the shutdown of laboratories and restrictions in the shipment of samples because of the covid-19 caused a significant delay in the ICI for acrylamide, UV-filters, pesticides and mycotoxins (samples for As had been sent before the shutdown). Furthermore, some laboratories withdrew their participation in the ICI as a result of the difficulties derived from this situation. Despite these delays, Scheme 2 was implemented between 3 months (arsenic) and 7 months (mycotoxins), so the objective of reducing the time for identifying laboratories with comparable results that could analyse the HBM4EU samples was achieved (Scheme 1 lasted at least one year and a half). In general, Scheme 2 presented less difficulty related to technical aspects since the laboratories involved had wide experience in the analysis of the target biomarkers and a baseline for their selection was defined (e.g. limit of quantification). Nevertheless, some interesting observations were made, for example the differences in total DON levels reported depending on the enzyme used for deconjugation. In several control materials, significantly lower concentrations of total DON were obtained when using β-glucuronidase/sulfatase from Helix Pomatia than when using β-glucuronidase from *E. Coli*. As expected, the pesticide group was the one with more difficulties, with consequences for the results evaluation. While for glyphosate and AMPA results were comparable in general, for chlorpyrifos and pyrethroid biomarkers the relative uncertainty of the mean was too high in several cases for a straightforward statistical evaluation of the reported results.

5. Conclusions

The QA/QC programme designed and implemented in the frame of the HBM4EU initiative can be termed a success and, as in previous studies, the need and utility of this kind of activities in HBM studies was evident. As long as there are no commercial proficiency tests offering a wide of biomarkers of interest in HBM at the concentrations in the range observed in the general population, the proposed approach appears as the best tool to investigate and improve results comparability. However, its implementation in the framework of such research project is complex and sustainability beyond the project is an issue regardless of the approach applied. Apart from the time constraints, questions such as the experience and capacities of the partners for supporting these activities should be considered, as well as the issues related to the funding. The organization of and participation in proficiency tests require a large amount of resources that cannot always be justified within a research project, possibly limiting the participation and thereby, the results achieved.

The main challenges of Scheme 1 approach were the time required for completing the scheme and, for some biomarkers, the rather low number of valid results, which hampered the evaluation of the ICI/EQUAS. Scheme 2 approach permitted to reduce the time required for having a set of laboratories with comparable results and, since it is based on the participation of laboratories fulfilling specific technical criteria (e.g. having a minimum LOQ for all the biomarkers in a group), the

potential problems related to the low experience were avoided. However, there was no opportunity for capacity building and supporting the national hubs in HBM4EU. Therefore, the design of the QA/QC programme has to consider the specific requirements or objectives for each situation.

Although the development and implementation of Scheme 1 required more efforts and time, it is the preferred one in the HBM4EU context since it allows the participation of more laboratories, and provides an opportunity to improve their analytical skills. This approach therefore boosts capacity building in EU laboratories, which in the end contributes to the sustainability of human biomonitoring in Europe. Major milestones and challenges for the future in this field are the definition of standard analytical methods and the establishment of a sustainable and periodical HBM QA/QC programme in Europe to support research activities and analytical laboratories. In line with this, the creation of an institution/network to prepare and provide certified control material in different human matrices would be very helpful for analytical laboratories working in human biomonitoring.

The HBM4EU QA/QC programme has revealed the utility and need in establishing a European network of analytical laboratories for human biomonitoring. This network would support the increasing human biomonitoring and risk assessment studies providing expertise for new method development and high quality analytical results. The network of laboratories created in HBM4EU can be considered as the project's legacy for future human biomonitoring actions in Europe.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgements

This study was part of the HBM4EU project receiving funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 733032. The authors acknowledge all the participating and expert laboratories (Centre de Toxicologie du Québec/Institut National de Santé Publique du Québec; IDEA Consultants, Inc.; Otsuka Pharmaceutical Co., Ltd.; Otsuka Pharmaceutical Co., Ltd.; Arizona Department of Health Services Laboratory; Centers for Disease Control and Prevention (CDC); New York State Department of Health Wadsworth Center; Wisconsin State Laboratory of Hygiene), that made the HBM4EU QA/QC programme possible, as well as the Management and Advisory Boards of HBM4EU.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2021.113740.

References

Alder, L., Korth, W., Patey, A., et al., 2001. Estimation of measurement uncertainty in pesticide residue analysis. J. AOAC Int. 84, 1569–1578. https://doi.org/10.1093/jaoac/84.5.1569.

Analytical Methods Committee, 1989. Robust statistics - how not to reject outliers Part 1. Basic concepts. Analyst 114, 1693–1697. https://doi.org/10.1039/AN9891401693.

Analytical Methods Committee, 1989. Robust statistics - how not to reject outliers Part 2. Interlaboratory trials. Analyst 114, 1699–1702. https://doi.org/10.1039/AN9891401699.

Becker, K., Seiwert, M., Casteleyn, L., et al., 2014. A systematic approach for designing a HBM Pilot Study for Europe. Int J Hyg Environ 214, 321–322. https://doi.org/10.1016/j.ijheh.2013.07.004.

Broothaerts, W., Cordeiro, F., Robouch, P., Emons, H., 2020. Ten years of proficiency testing reveals an improvement in the analytical performance of EU National Reference Laboratories for genetically modified food and feed. Food Contr. 114 https://doi.org/10.1016/j.foodcont.2020.107237.

COM, 2004. Communication from the Commission to the Council, the European Parliament, the European Economic and Social Committee -. The European Environment & Health Action Plan 2004-2010" {SEC(2004) 729}, p. 416.

Den Hond, E., Govarts, E., Willems, H., et al., 2015. The First steps towards harmonised human biomonitoring in Europe. Environ Health Perspect 123, 255–263. https://doi.org/10.1289/ehp.

Dereumeaux, C., Fillol, C., Charles, M.A., et al., 2017. The French human biomonitoring program: first lessons from the perinatal component and future needs. Int J Hyg Environ 220, 64–70. https://doi.org/10.1016/j.ijheh.2016.11.005.

Esteban López, M., Schindler, B.K., Jimenez, J.A., et al., 2015. Mercury analysis in hair: Comparability and quality assessment within the transnaational COPHES/DEMOCOPHES project. Environ Res 141, 24–30. https://doi.org/10.1016/j.envres.2014.11.014.

Fearn, T., Thompson, M., 2001. A new test for 'sufficient homogeneity'. Analyst 126, 1414–1417. https://doi.org/10.1039/b103812p.

Ganzleben, C., Antignac, J.P., Barouki, R., et al., 2017. Human biomonitoring as a tool to support chemicals regulation in the European Union. Int J Hyg Environ Health 220, 94–97. https://doi.org/10.1016/j.ijheh.2017.01.007.

Ginsberg, G.L., Balk, S.J., 2016. Consumer products as sources of chemical exposure to children: case study to triclosan. Curr Opin Pediatr 28, 235–242. https://doi.org/10.1097/MOP.0000000000000329.

Göen, T., Schaller, K.H., Drexler, H., 2012. External quality assessment of human biomonitoring in the range of environmental exposure levels. Int J Hyg Environ Health 215, 229–232. https://doi.org/10.1016/j.ijheh.2011.08.012.

Horwitz, W., Kamps, L.R., Boyer, K.W., 1980. Quality assurance in the analysis of foods and trace constituents. JAOAC Int. 63, 1344. https://doi.org/10.1093/jaoac/63.6.1344.

ISO 13528, 2015. Statistical Methods for Use in Proficiency Testing by Interlaboratory Comparison.

Kolossa-Gehring, M., Becker, K., Conrad, A., et al., 2012. Environmental surveys, specimen bank and health related environmental monitoring in Germany. Int J Hyg Environ Health 215, 120–126. https://doi.org/10.1016/j.ijheh.2011.10.013.

Linsinger, ThPJ., Josephs, R.D., 2006. Limitations of the application of the Horwitz equation. Trends Anal. Chem. 25, 1125–1130. https://doi.org/10.1016/j.trac.2006.11.002.

Nübler, S, Esteban López, M, Mol, H, et al., 2021. Interlaboratory comparison investigations (ICI) and external quality assurance schemes (EQUAS) for cadmium in urine and blood: results from the HBM4EU project. Int. J. Hyg Environ. Health 234, 113711. https://doi.org/10.1016/j.ijheh.2021.113711.

Pacyga, D., Sathyanarayana, S., Strakovsky, R.S., 2019. Dietary predictors of phthalate and bisphenol exposures in pregnant women. Adv Nutr 10, 803–815. https://doi.org/10.1093/advances/nmz029.

Parvaneh, H., Herrmann, S.S., Poulsen, M.E., 2017. Role of sample processing strategies at the European union national reference laboratories (NRLs) concerning the analysis of pesticide residues. J Agric Food Chem 65, 5759–5767. https://doi.org/10.1021/acs.jafc.7b00728.

Scherer, G., 2005. Biomonitoring of inhaled complex mixtures–ambient air, diesel exhaust and cigarette smoke. Exp Toxicol Pathol 57 (Suppl. 1), 75–110. https://doi.org/10.1016/j.etp.2005.05.007.

Schindler, B.K., Esteban, M., Koch, H.M., et al., 2014. The European COPHES/DEMOCOPHES project: towards transnational comparability and reliability of human biomonitoring results. Int J Hyg Environ Health 217, 653–661. https://doi.org/10.1016/j.ijheh.2013.12.002.

Schoeter, G., Den Hond, E., Collesa, A., et al., 2012. Concept of the Flemish human biomonitoring programme. Int J Hyg Environ Health 215, 102–108. https://doi.org/10.1016/j.ijheh.2011.11.006.

Thompson, M., 2000. Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations in relation to fitness for purpose criteria in proficiency testing. Analyst 125, 385–386. https://doi.org/10.1039/b000282h.

Thompson, M., Ellison, R., Wood, R., 2006. The international harmonized protocol for the proficiency testing of analytical chemistry laboratories. Pure Appl Chem 78, 145–196. https://doi.org/10.1351/pac200678010145.

Von Holst, C., Robouch, P., Bellorini, S., González de la Huebra, M.J., Ezerkis, Z., 2016. The work of the European Union Reference Laboratory for Food Additives (EURL) and its support for the authorisation process of feed additives in the European Union: a review. Food Addit Contam 33, 66–77. https://doi.org/10.1080/19440049.2015.1116127.

Vorkamp, K., Castaño, A., Antignac, J.-P., Boada, L.D., Cequier, E., Covaci, A., Esteban López, M., Haug, L.S., Kasper-Sonnenberg, M., Koch, H.M., Luzardo, O.P., Osīte, A., Rambaud, L., Pinorini, M.-T., Sabbioni, G., Thomsen, C., 2021. Biomarkers, matrices and analytical methods targeting human exposure to chemicals selected for a European human biomonitoring initiative. Environ Int 146, 106082. https://doi.org/10.1016/j.envint.2020.106082.

APPENDIX VI

Dvorakova D., Pulkrabova J., Nübler S., Haji-Abbas-Zarrabi K., Göen T., Mol H., Koch H. M., Vaccher V., Antignac J.P., Haug L. S., Vorkamp K., López M.E., Castaño A., Hajslova J.

Interlaboratory Comparison Investigations (ICIs) and External Quality Assurance Schemes (EQUASs) for Flame Retardants Analysis in Biological Matrices: Results from the HBM4EU Project

Environmental Research (2021)

doi: 10.1016/j.envres.2021.111705

Environmental Research 202 (2021) 111705

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

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

ARTICLE INFO

Keywordsabr:
Human biomonitoring (HBM)
Interlaboratory comparison investigation (ICI)
External quality assurance scheme (EQUAS)
Halogenated flame retardants (HFRs)
Organophosphorus flame retardants (OPFRs)
HBM4EU

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.

☆ This work was funded by the European Union's Horizon 2020 research and innovation programme under the grant agreement No. 733032.

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

https://doi.org/10.1016/j.envres.2021.111705
Received 23 April 2021; Received in revised form 13 July 2021; Accepted 13 July 2021
Available online 20 July 2021

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 σ_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 (σ_{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: σ_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: σ_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 σ_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

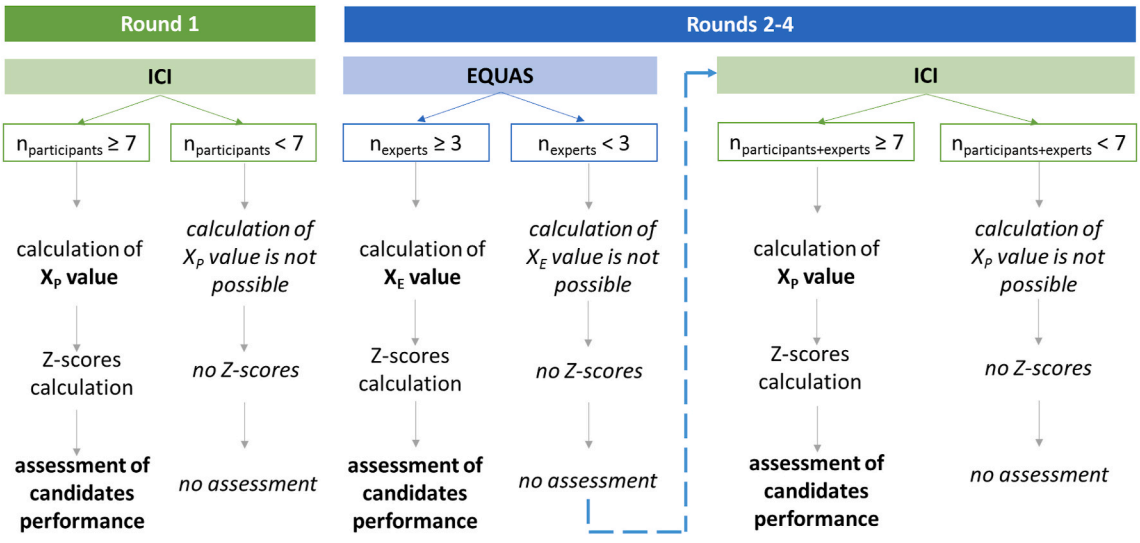


Fig. 1. ICI/EQUAS evaluation scheme for HFRs in serum

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_Rs 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_Rs (expressed as a mean of study RSD_Rs 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_Rs 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_Rs, 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

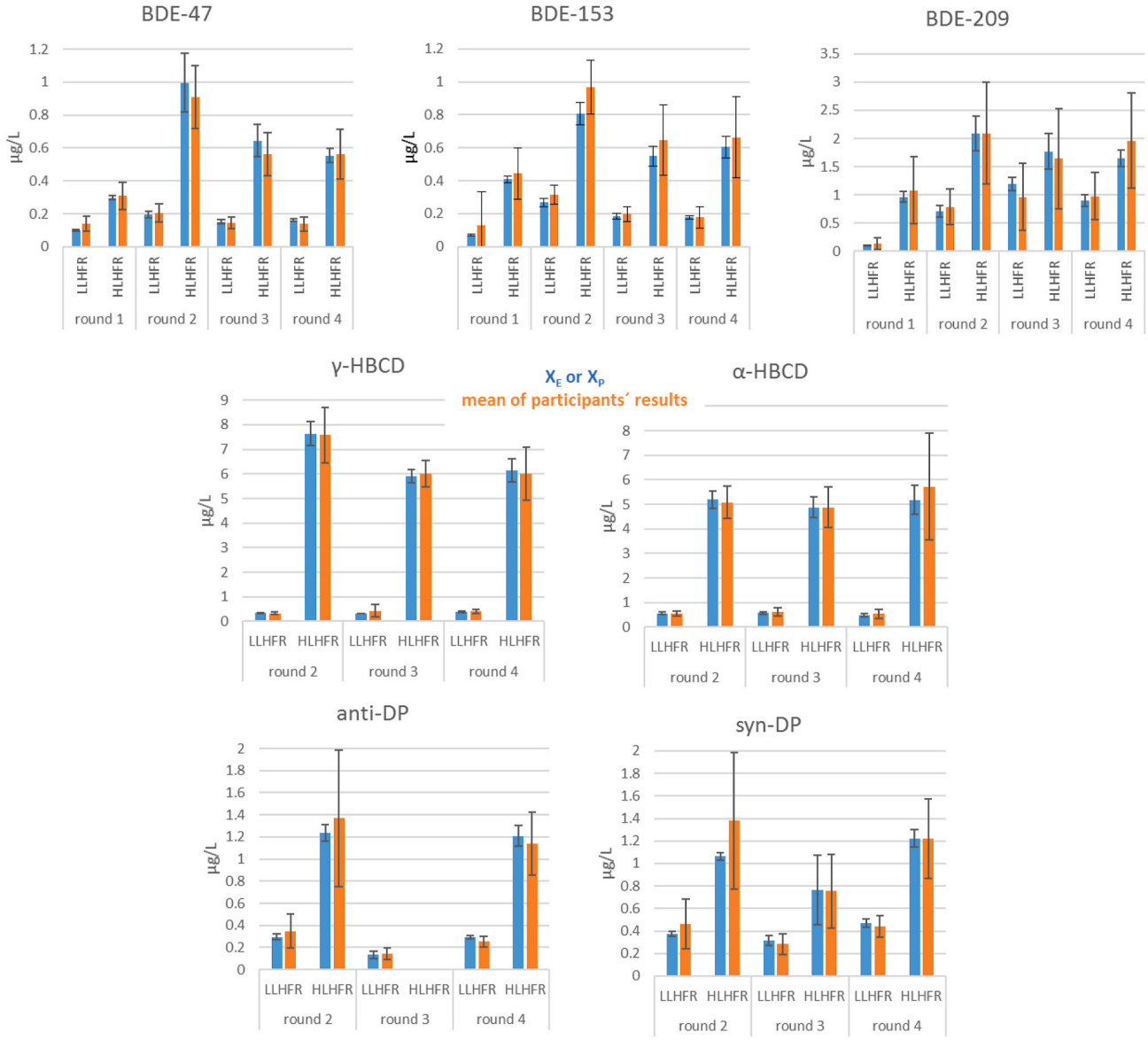


Fig. 2. The comparison of mean of participants results and relevant X_E or X_P for BDEs, HBCDs and DPs (X_E – BDEs in rounds 2-4, DPs in rounds 2 and 4, X_P – BDEs in round 1, DPs in round 3 and HBCDs in rounds 2-4; error bars indicate uncertainty for X_E/X_P and RSD_R for mean of participants results)

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.

Acknowledgements

We gratefully acknowledge funding by the European Union's Horizon 2020 research and innovation programme under the grant agreement No. 733032. The authors would like to thank the HBM4EU Secretariat at the German Environment Agency for administrative support. Furthermore, we thank the Management and Advisory Boards of HBM4EU and all supporting members of the HBM4EU Consortium. We would like to express special thanks to University of Chemistry and Technology Prague, Department of Food Analysis and Nutrition (Czech Republic); Research Centre for Toxic Compounds in the Environment (RECETOX), Trace Analytical Laboratory (Czech Republic); Norwegian Institute of Public Health, Environmental Exposure and Epidemiology (Norway); University of Antwerp, Department of Pharmaceutical Sciences (Belgium); Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine IPASUM (Germany); Centre de Toxicologie du Québec (CTQ)/INSPQ (Canada) and Wisconsin State Laboratory of Hygiene (USA) for their significant support as expert laboratories. We would also like to thank all participating laboratories for their efforts that made the HBM4EU QA/QC programme possible.

Appendix A. Supplementary data

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

References

Abdallah, M.A.E., Harrad, S., 2011. Tetrabromobisphenol-A, hexabromocyclododecane and its degradation products in UK human milk: relationship to external exposure. Environ. Int. 37 (2), 443–448. https://doi.org/10.1016/j.envint.2010.11.008.

Analytical Methods Committee, 1989a. Robust statistics–how not to reject outliers. Part 1. Basic concepts. Analyst 114 (12), 1693–1697. https://doi.org/10.1039/AN9891401693.

Analytical Methods Committee, 1989b. Robust statistics–how not to reject outliers. Part 2. Inter-laboratory trials. Analyst 114 (12), 1699–1702. https://doi.org/10.1039/AN9891401699.

Bastiaensen, M., Van den Eede, N., Su, G., Letcher, R.J., Stapleton, H.M., Covaci, A., 2019. Towards establishing indicative values for metabolites of organophosphate ester contaminants in human urine. Chemosphere 236, 124348. https://doi.org/10.1016/j.chemosphere.2019.124348.

Belli, M., Ellison, S.L., Fajgelj, A., Kuselman, I., Sansone, U., Wegscheider, W., 2007. Implementation of proficiency testing schemes for a limited number of participants. Accred Qual. Assur. 12 (8), 391–398. https://doi.org/10.1007/s00769-006-0247-0.

Bjermo, H., Aune, M., Cantillana, T., Glynn, A., Lind, P.M., Ridefelt, P., Darnerud, P.O., 2017. Serum levels of brominated flame retardants (BFRs: PBDE, HBCD) and influence of dietary factors in a population-based study on Swedish adults. Chemosphere 167, 485–491. https://doi.org/10.1016/j.chemosphere.2016.10.008.

Blum, A., Behl, M., Birnbaum, L.S., Diamond, M.L., Phillips, A., Singla, V., Sipes, N.S., Stapleton, H.M., Venier, M., 2019. Organophosphate ester flame retardants: are they a regrettable substitution for polybrominated diphenyl ethers? Environ. Sci. Technol. Lett. 6 (11), 638–649. https://doi.org/10.1021/acs.estlett.9b00582.

Cequier, E., Marcé, R.M., Becher, G., Thomsen, C., 2013. Determination of emerging halogenated flame retardants and polybrominated diphenyl ethers in serum by gas chromatography mass spectrometry. J. Chromatogr. A 1310, 126–132. https://doi.org/10.1016/j.chroma.2013.08.067.

Cequier, E., Marcé, R.M., Becher, G., Thomsen, C., 2015a. Comparing human exposure to emerging and legacy flame retardants from the indoor environment and diet with concentrations measured in serum. Environ. Int. 74, 54–59. https://doi.org/10.1016/j.envint.2014.10.003.

Cequier, E., Sakhi, A.K., Marcé, R.M., Becher, G., Thomsen, C., 2015b. Human exposure pathways to organophosphate triesters-A biomonitoring study of mother–child pairs. Environ. Int. 75, 159–165. https://doi.org/10.1016/j.envint.2014.11.009.

Darnerud, P.O., Lignell, S., Aune, M., Isaksson, M., Cantillana, T., Redeby, J., Glynn, A., 2015. Time trends of polybrominated diphenylether (PBDE) congeners in serum of Swedish mothers and comparisons to breast milk data. Environ. Res. 138, 352–360. https://doi.org/10.1016/j.envres.2015.02.031.

de Boer, J., Cofino, W.P., 2002. First world-wide interlaboratory study on polybrominated diphenylethers (PBDEs). Chemosphere 46 (5), 625–633. https://doi.org/10.1016/S0045-6535(01)00226-0.

de Boer, J., Wells, D.E., 2006. Pitfalls in the analysis of brominated flame retardants in environmental, human and food samples–including results of three international interlaboratory studies. Trac. Trends Anal. Chem. 25 (4), 364–372. https://doi.org/10.1016/j.trac.2006.01.008.

De Wit, C.A., 2002. An overview of brominated flame retardants in the environment. Chemosphere 46 (5), 583–624. https://doi.org/10.1016/S0045-6535(01)00225-9.

Dereumeaux, C., Saoudi, A., Pecheux, M., Berat, B., de Crouy-Chanel, P., Zaros, C., Brunel, S., Delamaire, C., le Tertre, A., Lefranc, A., Vandentorren, S., Guldner, L., 2016. Biomarkers of exposure to environmental contaminants in French pregnant women from the Elfe cohort in 2011. Environ. Int. 97, 56–67. https://doi.org/10.1016/j.envint.2016.10.013.

Duffek, A., Leonards, P., Lepom, P., 2008. Interlaboratory study on deca-BDE analysis in environmental samples involving routine laboratories. Organohalogen Compd. 70, 002025–002028.

Dufour, P., Pirard, C., Charlier, C., 2017. Determination of phenolic organohalogens in human serum from a Belgian population and assessment of parameters affecting the human contamination. Sci. Total Environ. 599, 1856–1866. https://doi.org/10.1016/j.scitotenv.2017.05.157.

Eljarrat, E., Guerra, P., Martinez, E., Farre, M., Alvarez, J.G., Lopez-Teijon, M., Barcelo, D., 2009. Hexabromocyclododecane in human breast milk: levels and enantiomeric patterns. Environ. Sci. Technol. 43 (6), 1940–1946. https://doi.org/10.1021/es802919e.

Esteban López, M., Göen, T., Mol, H., Nübler, S., Haji-Abbas-Zarrabi, K., Koch, H.M., Kasper-Sonnenberg, M., Dvorakova, D., Hajslova, J., Antignac, J.-P., Vaccher, V., Elbers, I., Thomsen, C., Vorkamp, K., Pedraza-Díaz, S., Kolossa-Gehring, M., Castaño, A., 2021. The European human biomonitoring platform-Design and implementation of a laboratory quality assurance/quality control (QA/QC) programme for selected priority chemicals. Int. J. Hyg Environ. Health 234, 113740. https://doi.org/10.1016/j.ijheh.2021.113740.

European Commission Commission, 2014. Directive 2014/79/EU of 20 june 2014 amending appendix C of annex II to directive 2009/48/EC of the European parliament and of the council on the safety of toys, as regards TCEP, TCPP and TDCP. Off. J. Eur. Union. L182/49– 51.

European Commission, 2008. European Union risk assessment report tris[2-chloro-1-(chloromethyl)ethyl]phosphate (TDCP). https://echa.europa.eu/documents/10162/13630/trd_rar_ireland_tdcp_en.pdf.

European Commission, 2009. European Union Risk Assessment Report. Tris (2-chloroethyl) Phosphate. TCEP. https://echa.europa.eu/documents/10162/2663989d-1795-44a1-8f50-153a81133258.

Fängström, B., Athanassiadis, I., Odsjö, T., Norén, K., Bergman, Å., 2008. Temporal trends of polybrominated diphenyl ethers and hexabromocyclododecane in milk from Stockholm mothers, 1980–2004. Mol. Nutr. Food Res. 52 (2), 187–193. https://doi.org/10.1002/mnfr.200700182.

Fearn, T., Thompson, M., 2001. A new test for 'sufficient homogeneity'. Analyst 126 (8), 1414–1417. https://doi.org/10.1039/B103812P.

Frederiksen, M., Thomsen, C., Frøshaug, M., Vorkamp, K., Thomsen, M., Becher, G., Knudsen, L.E., 2010. Polybrominated diphenyl ethers in paired samples of maternal and umbilical cord blood plasma and associations with house dust in a Danish cohort. Int. J. Hyg Environ. Health 213 (4), 233–242. https://doi.org/10.1016/j.ijheh.2010.04.008.

Fromme, H., Cequier, E., Kim, J.T., Hanssen, L., Hilger, B., Thomsen, C., Chang, Y.-S.-, Völkel, W., 2015. Persistent and emerging pollutants in the blood of German adults: occurrence of dechloranes, polychlorinated naphthalenes, and siloxanes. Environ. Int. 85, 292–298. https://doi.org/10.1016/j.envint.2015.09.002.

Fromme, H., Hilger, B., Albrecht, M., Gries, W., Leng, G., Völkel, W., 2016. Occurrence of chlorinated and brominated dioxins/furans, PCBs, and brominated flame retardants in blood of German adults. Int. J. Hyg Environ. Health 219 (4–5), 380–388. https://doi.org/10.1016/j.ijheh.2016.03.003.

Fromme, H., Lahrz, T., Kraft, M., Fembacher, L., Mach, C., Dietrich, S., Burkardt, R., Völkel, W., Göen, T., 2014. Organophosphate flame retardants and plasticizers in the air and dust in German daycare centers and human biomonitoring in visiting children (LUPE 3). Environ. Int. 71, 158–163. https://doi.org/10.1016/j.envint.2014.06.016.

Ganzleben, C., Antignac, J.P., Barouki, R., Castaño, A., Fiddicke, U., Klánová, J., Lebret, E., Olea, N., Sarigiannis, D., Schoeters, G.R., Sepai, O., Tolonen, H., Kolossa-Gehring, M., 2017. Human biomonitoring as a tool to support chemicals regulation in the European Union. Int. J. Hyg Environ. Health 220 (2 Pt A), 94–97. https://doi.org/10.1016/j.ijheh.2017.01.007.

Geyer, H.J., Schramm, K.W., Feicht, E.A., Fried, K.W., Henkelmann, B., Lenoir, D., Darneud, P., Aune, M., Schmid, P., McDonald, T.A., 2004. Terminal elimination half-lives of the brominated flame retardants TBBPA, HBCD, and lower brominated PBDEs in humans. Organohalogen Compd. 66, 3867–3872.

Hoffman, K., Daniels, J.L., Stapleton, H.M., 2014. Urinary metabolites of organophosphate flame retardants and their variability in pregnant women. Environ. Int. 63, 169–172. https://doi.org/10.1016/j.envint.2013.11.013.

ISO 13528, 2015. Statistical methods for use in proficiency testing by interlaboratory comparison. https://www.iso.org/standard/56125.html.

Jansen, A., Polder, A., Müller, M.H., Skjerve, E., Aaseth, J., Lyche, J.L., 2018. Increased levels of persistent organic pollutants in serum one year after a great weight loss in humans: are the levels exceeding health based guideline values? Sci. Total Environ. 622, 1317–1326. https://doi.org/10.1016/j.scitotenv.2017.11.241.

Kalantzi, O.I., Geens, T., Covaci, A., Siskos, P.A., 2011. Distribution of polybrominated diphenyl ethers (PBDEs) and other persistent organic pollutants in human serum from Greece. Environ. Int. 37 (2), 349–353. https://doi.org/10.1016/j.envint.2010.10.005.

Kierkegaard, A., Björklund, J., Fridén, U., 2004. Identification of the flame retardant decabromodiphenyl ethane in the environment. Environ. Sci. Technol. 38 (12), 3247–3253. https://doi.org/10.1021/es049867d.

Kuselman, I., Fajgelj, A., 2010. IUPAC/CITAC Guide: selection and use of proficiency testing schemes for a limited number of participants—chemical analytical laboratories (IUPAC Technical Report). Pure Appl. Chem. 82 (5), 1099–1135. https://doi.org/10.1351/PAC-REP-09-08-15.

Louro, H., Heinälä, M., Bessems, J., Buekers, J., Vermeire, T., Woutersen, M., van Engelen, J., Borges, T., Rousselle, C., Ougier, E., Alvito, P., Martins, C., Assunção, R., Silva, M.J., Pronk, A., Schaddelee-Scholten, B., Gonzalez, M.D.C., de Alba, M., Castaño, A., Viegas, S., Humar-Juric, T., Kononenko, L., Lampen, A., Vinggaard, A. M., Schoeters, G., Kolossa-Gehring, M., Santonen, T., 2019. Human biomonitoring in health risk assessment in Europe: current practices and recommendations for the future. Int. J. Hyg Environ. Health 222 (5), 727–737. https://doi.org/10.1016/j.ijheh.2019.05.009.

Lyche, J.L., Rosseland, C., Berge, G., Polder, A., 2015. Human health risk associated with brominated flame-retardants (BFRs). Environ. Int. 74, 170–180. https://doi.org/10.1016/j.envint.2014.09.006.

Ma, Y., Stubbings, W.A., Cline-Cole, R., Harrad, S., 2020. Human exposure to halogenated and organophosphate flame retardants through informal e-waste handling activities-a critical review. Environ. Pollut. 115727 https://doi.org/10.1016/j.envpol.2020.115727.

Melymuk, L., Goosey, E., Riddell, N., Diamond, M.L., 2015. Interlaboratory study of novel halogenated flame retardants: INTERFLAB. Anal. Bioanal. Chem. 407 (22), 6759–6769. https://doi.org/10.1007/s00216-015-8843-7.

Nübler, S., López, M.E., Castaño, A., Mol, H., Schäfer, M., Haji-Abbas-Zarrabi, K., Koch, H.M., Vaccher, V., Antignac, J.-P., Dvorakova, D., Hajslova, J., Thomsen, C., Vorkamp, K., Göen, T., 2021. Interlaboratory comparison investigations (ICI) and external quality assurance schemes (EQUAS) for cadmium in urine and blood: results from the HBM4EU project. Int. J. Hyg Environ. Health 234, 113711. https://doi.org/10.1016/j.ijheh.2021.113711.

Proposal to list Dechlorane Plus, 2019. (CAS No: 13560-89-9) and its syn-isomer(CAS No: 135821-03-3) and anti-isomer (CAS No:135821-74-8) in Annexes A, B and/or C to the Stockholm Convention on Persistent Organic Pollutants. Persistent Organic Pollutants Review Committee. UNEP/POPS/POPRC.15/3.

Reemtsma, T., Lingott, J., Roegler, S., 2011. Determination of 14 monoalkyl phosphates, dialkyl phosphates and dialkyl thiophosphates by LC-MS/MS in human urinary samples. Sci. Total Environ. 409 (10), 1990–1993. https://doi.org/10.1016/j.scitotenv.2011.01.032.

Ricci, M., Shegunova, P., Vorkamp, K., 2020. State of the art in the analysis of brominated flame retardants in biota and sediment: insights from the characterisation of two new certified reference materials. Environ. Sci. Pollut. Control Ser. 1–14. https://doi.org/10.1007/s11356-020-08950-7.

Roosens, L., Abdallah, M.A.E., Harrad, S., Neels, H., Covaci, A., 2009. Exposure to hexabromocyclododecanes (HBCDs) via dust ingestion, but not diet, correlates with concentrations in human serum: preliminary results. Environ. Health Perspect. 117 (11), 1707–1712. https://doi.org/10.1289/ehp.0900869.

Rousseeuw, P.J., Verboven, S., 2002. Robust estimation in very small samples. Comput. Stat. Data Anal. 40 (4), 741–758. https://doi.org/10.1016/S0167-9473(02)00078-6.

Roze, E., Meijer, L., Bakker, A., Van Braeckel, K.N., Sauer, P.J., Bos, A.F., 2009. Prenatal exposure to organohalogens, including brominated flame retardants, influences motor, cognitive, and behavioral performance at school age. Environ. Health Perspect. 117 (12), 1953–1958. https://doi.org/10.1289/ehp.0901015.

Sahlström, L.M., Sellström, U., de Wit, C.A., Lignell, S., Darnerud, P.O., 2014. Brominated flame retardants in matched serum samples from Swedish first-time mothers and their toddlers. Environ. Sci. Technol. 48 (13), 7584–7592. https://doi.org/10.1021/es501139d.

Sharkey, M., Harrad, S., Abdallah, M.A.E., Drage, D.S., Berresheim, H., 2020. Phasing-out of legacy brominated flame retardants: the UNEP Stockholm Convention and other legislative action worldwide. Environ. Int. 144, 106041. https://doi.org/10.1016/j.envint.2020.106041.

Shaw, S.D., Harris, J.H., Berger, M.L., Subedi, B., Kannan, K., 2014. Brominated flame retardants and their replacements in food packaging and household products: uses, human exposure, and health effects. In: Toxicants in Food Packaging and Household Plastics. Springer, London, pp. 61–93.

Schindler, B.K., Weiss, T., Schütze, A., Koslitz, S., Broding, H.C., Bünger, J., Brüning, T., 2013. Occupational exposure of air crews to tricresyl phosphate isomers and organophosphate flame retardants after fume events. Arch. Toxicol. 87 (4), 645–648. https://doi.org/10.1007/s00204-012-0978-0.

Sochorová, L., Hanzlíková, L., Černá, M., Drgáčová, A., Fialová, A., Švarcová, A., Gramblička, T., Pulkrabová, J., 2017. Perfluorinated alkylated substances and brominated flame retardants in serum of the Czech adult population. Int. J. Hyg Environ. Health 220 (2), 235–243. https://doi.org/10.1016/j.ijheh.2016.09.003.

Svarcova, A., Lankova, D., Gramblicka, T., Stupak, M., Hajslova, J., Pulkrabova, J., 2019. Integration of five groups of POPs into one multi-analyte method for human blood serum analysis: an innovative approach within biomonitoring studies. Sci. Total Environ. 667, 701–709. https://doi.org/10.1016/j.scitotenv.2019.02.336.

Thompson, M., 2000. Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations in relation to fitness for purpose criteria in proficiency testing. Analyst 125 (3), 385–386. https://doi.org/10.1039/B000282H.

Thompson, M., Ellison, S.L., Wood, R., 2006. The International Harmonized Protocol for the proficiency testing of analytical chemistry laboratories (IUPAC Technical Report). Pure Appl. Chem. 78 (1), 145–196.

Thomsen, C., Stigum, H., Frøshaug, M., Broadwell, S.L., Becher, G., Eggesbø, M., 2010. Determinants of brominated flame retardants in breast milk from a large scale Norwegian study. Environ. Int. 36 (1), 68–74. https://doi.org/10.1016/j.envint.2009.10.002.

Van den Eede, N., Neels, H., Jorens, P.G., Covaci, A., 2013. Analysis of organophosphate flame retardant diester metabolites in human urine by liquid chromatography electrospray ionisation tandem mass spectrometry. J. Chromatogr. A 1303, 48–53. https://doi.org/10.1016/j.chroma.2013.06.042.

Van der Veen, I., de Boer, J., 2012. Phosphorus flame retardants: properties, production, environmental occurrence, toxicity and analysis. Chemosphere 88 (10), 1119–1153. https://doi.org/10.1016/j.chemosphere.2012.03.067.

Varshavsky, J.R., Robinson, J.F., Zhou, Y., Puckett, K.A., Kwan, E., Buarpung, S., Aburajab, R., Gaw, S.L., Sen, S., Gao, S., Smith, S.C., Park, J.-S., Zakharevich, I., Gerona, R.R., Fisher, S.J., Woodruff, T.J., 2021. Organophosphate flame retardants, highly fluorinated chemicals, and biomarkers of placental development and disease during mid-gestation. Toxicol. Sci. https://doi.org/10.1093/toxsci/kfab028.

Vorkamp, K., Castaño, A., Antignac, J.P., Boada, L.D., Cequier, E., Covaci, A., Esteban, M. L., Haug, L.S., Kasper-Sonnenberg, M., Koch, H.M., Luzardo, O.P., Osīte, A., Rambaud, L., Pinorini, M.-T., Sabbioni, G., Thomsen, C., 2021. Biomarkers, matrices and analytical methods targeting human exposure to chemicals selected for a European human biomonitoring initiative. Environ. Int. 146, 106082. https://doi.org/10.1016/j.envint.2020.106082.

Wang, P., Zhang, Q., Zhang, H., Wang, T., Sun, H., Zheng, S., Li, Y., Liang, Y., Jiang, G., 2016. Sources and environmental behaviors of Dechlorane Plus and related compounds—a review. Environ. Int. 88, 206–220. https://doi.org/10.1016/j.envint.2015.12.026.

APPENDIX VII

Polachova A., Gramblicka T., Bechynska K., Parizek O., Parizkova D., Dvorakova D., Honkova K., Rossnerova A., Rossner P., Sram R. J., Topinka J., Pulkrabova J.

Biomonitoring of 89 POPs in blood serum samples of Czech city policemen

Environmental Pollution (2021)

doi: 10.1016/j.envpol.2021.118140

Environmental Pollution 291 (2021) 118140

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Biomonitoring of 89 POPs in blood serum samples of Czech city policemen

Andrea Polachova [a], Tomas Gramblicka [a], Kamila Bechynska [a], Ondrej Parizek [a], Denisa Parizkova [a], Darina Dvorakova [a], Katerina Honkova [b], Andrea Rossnerova [b], Pavel Rossner [c], Radim J. Sram [b], Jan Topinka [b], Jana Pulkrabova [a,*]

[a] University of Chemistry and Technology, Prague, Faculty of Food and Biochemical Technology, Department of Food Analysis and Nutrition, Technicka 3, 166 28, Prague 6, Czech Republic

[b] Institute of Experimental Medicine of the Czech Academy of Sciences, Department of Genetic Toxicology and Epigenetics, Videnska 1083, 142 20, Prague 4, Czech Republic

[c] Institute of Experimental Medicine of the Czech Academy of Sciences, Department of Nanotoxicology and Molecular Epidemiology Videnska 1083, 142 20, Prague 4, Czech Republic

ARTICLE INFO

Keywords:
POPs
Serum
City policemen
Human biomonitoring
Czech Republic

ABSTRACT

In this biomonitoring study, we evaluated the concentrations of 8 polychlorinated biphenyls (PCBs), 11 organochlorinated pesticides (OCPs), 33 brominated flame retardants (BFRs), 7 novel brominated and chlorinated flame retardants (novel FRs) and 30 per- and polyfluoroalkylated substances (PFAS) in human serum samples (n = 274). A total of 89 persistent organic pollutants (POPs) were measured in blood serum samples of city policemen living in three large cities and their adjacent areas (Ostrava, Prague, and Ceske Budejovice) in the Czech Republic. All samples were collected during the year 2019 in two sampling periods (spring and autumn). The identification/quantification of PCBs, OCPs, BFRs, novel FRs and PFAS was performed by means of gas chromatography coupled to (tandem) mass spectrometry (GC–MS/(MS)) and ultra-high performance liquid chromatography coupled to triple quadrupole tandem mass spectrometry (UHPLC–MS/MS). The most frequently detected pollutants were perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluorooctanesulfonate (PFOS), perfluorohexanesulfonate (PFHxS), 2,2′,3,4,4′,5′-hexachlorobiphenyl (CB 138), 2,2′,4,4′,5,5′-hexachlorobiphenyl (CB 153), 2,2′,3,3′,4,4′,5-heptachlorobiphenyl (CB 170), 2,2′,3,4,4′,5,5′-heptachlorobiphenyl (CB 180), hexachlorobenzene (HCB), and *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE) quantified in 100% of serum samples. In the serum samples, the concentrations of determined POPs were in the range of 0.108–900 ng g^{-1} lipid weight (lw) for PCBs, 0.106–1016 ng g^{-1} lw for OCPs, <0.1–618 ng g^{-1} lw for FRs and <0.01–18.3 ng mL^{-1} for PFAS, respectively. Locality, sampling season, and age were significantly associated with several POP concentrations. One of the important conclusions was that within the spring sampling period, statistically significant higher concentrations of CB 170 and CB 180 were observed in the samples from Ostrava (industrial area) compared to Prague and Ceske Budejovice. Older policemen had higher concentrations of five PCBs and two OCPs in blood serum.

1. Introduction

Blood (including plasma, serum and specific blood components – e.g., lymphocytes) represents one of the most important and commonly used biological material for determining the levels of various biomarkers including the persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), organochlorinated pesticides (OCPs), brominated flame retardants (BFRs), novel brominated and chlorinated flame retardants (novel FRs), and per- and polyfluoroalkylated substances (PFAS) (Alves et al., 2014; Angerer et al., 2007; Luque et al., 2012). Biomonitoring studies require accurate and sensitive quantitative measurements of POPs in blood in order to study the relationship between an individual's exposure to these pollutants and their effects on health (Hao et al., 2020).

POPs are listed in the Stockholm Convention for their persistence, potential toxic properties, bioaccumulation, and long-range atmospheric transport ability ("Listing of POPs in the Stockholm Convention," n.d.). However, the main problem is their tendency to accumulate in

* Corresponding author.
E-mail address: jana.pulkrabova@vscht.cz (J. Pulkrabova).

https://doi.org/10.1016/j.envpol.2021.118140
Received 21 May 2021; Received in revised form 20 August 2021; Accepted 6 September 2021
Available online 10 September 2021

the environment for a long time, and despite the ban or restriction on their use, they are still detected in various environmental compartments including living organisms (Alves et al., 2014). Overall, POPs may be hazardous to human health; as an increasing number of epidemiological studies have demonstrated, POP exposure is associated with a number of negative effects such as neurotoxicity, disruption of thyroid function and male reproductive hormones, and declining sperm quality (in terms of concentration, viability, morphology and motility) (Chen et al., 2011; Foster Warren G. et al., 2010; Y. Yu et al., 2020; Zheng et al., 2017). Contrary to the classic POPs, PFAS do not tend to accumulate in lipids, but rather create a strong bond to protein fractions in blood (Jian et al., 2018; Sochorová et al., 2017). Nowadays, PFAS are a constantly emerging issue, mainly because of their bioaccumulation, potential to expand into almost all parts in the ecosystem and the possible negative effects on human health (e.g., allergic disease, endocrine dysfunction, hepatotoxicity, cardiovascular and kidney diseases, low birth weight of newborns and decreased enzyme activity) (Geiger et al., 2014; Lee et al., 2018; Motas Guzmàn et al., 2016; Verner Marc-André et al., 2015). A number of human epidemiological studies provides strong support for the causal associations between exposure to perfluorooctanesulfonate (PFOS)/perfluorooctanoic acid (PFOA) and increased serum cholesterol in adults (Knutsen et al., 2018). In general, human exposure to PFAS is primarily through food intake (fish, seafood, crops, and food packaging), environmental sources, and drinking water. PFAS can easily accumulate in the human body, which is caused by a slow elimination rate and biodegradability of long-chained PFAS (Cui et al., 2020).

Human biomonitoring focuses on the analysis and distribution of pollutants and their metabolites in the human body and related diseases. Regarding human biological samples, studies have monitored a number of POPs in maternal serum, umbilical cord serum, urine, amniotic fluid, seminal fluid, cerebrospinal fluid, and breast milk (Cabrera-Rodríguez et al., 2019; Cui et al., 2020; Kubwabo et al., 2013; Luzardo et al., 2009; McComb et al., 2019; Raymer et al., 2012; Zhang et al., 2018). In addition, POP concentrations in serum may represent an indicator of their circulation through all organs in the human body, and samples can be collected from the entire population, unlike, e.g. breast milk (only from pregnant women). It needs to be emphasized that serum collection is limited by the required volume, especially if infants are studied. Moreover, classic POPs are detected at trace levels in serum compared to adipose tissue. Therefore, a high throughput extraction method using a small volume of serum sample is needed in a combination with a method of instrumental analysis that detects trace amounts of POPs with high accuracy (Artacho-Cordón et al., 2015b; Lee et al., 2020a; Ploteau et al., 2016).

The presented research was conducted within the frame of a project "Healthy Aging in Industrial Environment" (HAIE), which evaluates the effects of selected environmental and lifestyle risk factors on the health and aging of the population in the industrial region. Currently, there is no published study in Europe which has focused on the investigation of such a wide range of POP in blood serum samples obtained from an not yet described population group of city policemen. The main objectives of this study were to evaluate the concentrations of 89 POPs in the serum samples collected in 2019 from 142 city policemen residing in three cities of the Czech Republic (Prague, Ostrava, and Ceske Budejovice) and to evaluate how the POP concentrations were related to locality and age.

2. Materials and methods

2.1. Sample collection

A total of 274 human serum samples were provided within the HAIE research programme (2018–2022). The samples were collected from the same city policemen in two rounds in 2019, spring (February/March; n = 142) and autumn (September/October; n = 132; 10 serum donors did not participate in the second period), living in three cities and their adjacent areas in the Czech Republic (Prague, Ostrava and Ceske Budejovice). The collection of human samples was approved by the Ethics Committee of the Institute of Experimental Medicine of the Czech Academy of Sciences before the start of the HAIE project. All participants of these surveys signed an informed consent.

Table 1
Characteristics of the city policemen in the study.

Variable/Sampling region	Ostrava	Ceske Budejovice	Prague
Number of samples (spring/autumn period)	56/53	18/17	68/62
Personal information			
Age (years) Mean (min-max)	40 (21–61)	38 (22–48)	40 (23–63)
Weight (kg) Mean (min-max)	93 (59–145)	94 (75–130)	93 (67–121)
Height (cm) Mean (min-max)	181 (170–195)	183 (176–190)	181 (168–195)
Body mass index BMI (kg/m^2) Mean (min-max)	28.5 (20.4–44.8)	28.1 (23.1–41.0)	28.5 (19.4–39.1)
Previous smoking (yes/no)	15/41	5/13	20/48
Education level			
Secondary	49	15	52
University	7	3	16

Prague is the capital and the largest city of the Czech Republic and is therefore considered the most densely populated area (1 335 000 inhabitants) in this country, with a high level of traffic. Ostrava, with its 290 000 residents, is the third largest city of the Czech Republic. Thanks to its historical consequences in heavy industry and coal mining (the last coal mining ended in 1994), it is also known as the most industrial area. At present, Nova Hut (known as Liberty Ostrava), which deals with metallurgy and mechanical engineering, is the largest industrial contributor to environmental pollution, but it has undergone a major modernization. The main causes of environmental pollution in this region can be transport, and rather than industrial character, it is a geographical influence, where pollutants may penetrate from neighbouring Poland most often in inverse weather. Ceske Budejovice is an urban residential area with agriculture and 94 000 inhabitants. The industrial burden in this city is the lowest in comparison with Prague and Ostrava.

All participants (age: 21–63) had been residents in the selected localities for at least five years. Each participant filled a detailed questionnaire describing the exposure and lifestyle. More details about the main characteristics of the city policemen are summarized in Table 1.

2.2. Standards, chemicals and other materials

Methanol, dichloromethane, diethylether, *n*-hexane, isooctane, and sulphuric acid (98%) were supplied by Merck (Germany). Acetonitrile and anhydrous magnesium sulphate (98%) were obtained from Sigma-Aldrich (USA); acetone and sodium chloride (99.9%) from Penta (Czech Republic). Polypropylene centrifuge tube filters (nylon, pore size 0.22 μm) were purchased from Sigma-Aldrich. Florisil® for residual analysis (0.15–0.25 mm) supplied by Merck was activated by heating at 600 °C for 4 h, then at 130 °C for 5 h followed by storage in a desiccator.

The characteristics of all target analytes (full name of the compound, abbreviation, concentration, CAS number and other information) and the preparation of calibration/working standards and solutions are described in Tables S1 and S2, respectively, in the Supplementary material. The individual standards of 30 PFAS were obtained from Wellington Laboratories (Guelph, Ontario, Canada). Standards of BFRs, including 16 PBDE congeners, 4 hydroxylated PBDEs (OH-PBDEs), TBBPA, HBCD isomers (α-, β- and γ-), and other BFRs (PBT, PBEB, HBB, BTBPE, OBIND, and DBDPE), were supplied by Wellington Laboratories. The brominated phenols together with seven novel FRs (DBE-DBCH, *syn*-

Table 2
Concentrations of PCBs, OCPs, BFRs, and PFAS in blood serum samples of city policemen (n = 274).[a,b,c]

	1. Round – spring 2019 (n = 142)						2. Round – autumn 2019 (n = 132)					LOQ
	Analyte	Samples > LOQ (%)	Mean	Median	Min-max	5–95% Percentile	Samples > LOQ (%)	Mean	Median	Min-max	5–95% Percentile	
ng mL^{-1}	**PFBA**	89	0.020	0.020	<0.01–0.332	0.005–0.058	88	0.023	0.023	<0.01–0.475	0.005–0.084	0.01
	PFHpA	81	0.017	0.016	<0.01–0.119	0.005–0.061	77	0.016	0.015	<0.01–0.197	0.005–0.058	0.01
	PFOA	100	0.810	0.934	0.081–2.60	0.166–1.93	100	0.871	1.00	0.075–2.90	0.160–2.10	0.01
	PFNA	100	0.323	0.342	0.034–2.07	0.082–0.810	100	0.361	0.383	0.036–3.00	0.079–0.944	0.01
	PFDA	100	0.150	0.162	0.025–0.554	0.054–0.365	100	0.177	0.178	0.031–0.719	0.056–0.525	0.01
	PFUdA	99	0.059	0.064	<0.01–0.184	0.023–0.148	100	0.060	0.057	0.014–0.322	0.021–0.179	0.01
	PFDoA	62	0.012	0.012	<0.01–0.093	0.005–0.047	55	0.011	0.011	<0.01–0.256	0.005–0.049	0.01
	PFTrDA	34			<0.01–0.062	0.005–0.027	31			<0.01–0.094	0.005–0.026	0.01
	PFHxS	100	0.379	0.436	0.043–1.73	0.077–0.915	100	0.398	0.459	0.044–1.79	0.085–0.908	0.01
	PFHpS	94	0.074	0.089	<0.01–0.565	0.005–0.218	92	0.060	0.076	<0.01–0.380	0.005–0.177	0.01
	PFOS	100	2.59	2.66	0.259–16.6	0.551–7.27	100	2.66	2.94	0.237–18.3	0.591–7.06	0.01
ng g^{-1} lw	**CB 28**	58	0.406	0.668	<0.1–7.28	0.050–5.37	61	0.272	0.399	<0.1–7.00	0.050–2.26	0.1
	CB 52	26			<0.1–0.931	0.050–0.508	31			<0.1–3.31	0.050–0.830	0.1
	CB 101	54	0.229	0.331	<0.1–4.09	0.050–1.58	20			<0.1–3.26	0.050–1.27	0.1
	CB 118	93	2.75	3.45	<0.1–18.7	0.050–8.38	97	3.46	3.85	<0.1–17.5	1.31–10.7	0.1
	CB 138	100	28.7	29.9	5.16–219	8.88–99.2	100	45.5	45.9	4.58–280	10.8–144	0.1
	CB 153	100	65.8	64.8	10.1–548	18.0–226	100	77.8	79.3	10.2–567	19.8–257	0.1
	CB 170	100	38.8	37.5	5.60–387	11.1–141	100	30.3	30.8	4.10–227	8.33–94.7	0.5
	CB 180	100	92.2	94.0	10.4–900	28.3–322	100	86.3	91.3	9.29–717	22.9–309	0.5
	HCB	100	17.4	18.1	2.74–63.4	7.45–44.9	100	15.7	15.5	4.54–68.7	6.28–43.4	0.1
	***α*-HCH**	32			<0.1–7.66	0.050–3.28	1			<0.1–0.573	0.050–0.050	0.1
	***β*-HCH**	75	1.57	3.68	<0.1–19.3	0.050–13.5	44			<0.1–31.3	0.050–19.7	0.1
	***γ*-HCH**	32			<0.1–3.74	0.050–1.33	11			<0.1–29.2	0.050–3.81	0.1
	***p,p′*-DDE**	100	103	101	20.7–1016	35.8–326	100	129	123	22.7–997	41.2–416	0.1
	***p,p′*-DDD**	82	0.691	1.01	<0.1–11.9	0.050–3.83	92	1.09	1.34	<0.1–25.9	0.050–4.28	0.1
	***p,p′*-DDT**	51	1.38	2.97	<0.5–90.5	0.250–12.9	27			<0.5–101	0.250–11.4	0.5
	BDE 47	83	1.38	2.09	<0.1–29.8	0.050–18.9	99	1.65	1.67	<0.1–14.1	0.914–3.08	0.1
	BDE 209	44			<1.5–459	0.750–14.3	21			<1.5–618	0.750–230	1.5

[a] Median and geometric mean values were calculated when in >50% of samples analyte was positively detected in a concentration above LOQ.
[b] For results below LOQ, one-half the LOQ value was used.
[c] PFAS: FOSA, N-MeFOSA, N-EtFOSA, PFDS, PFPeA, PFHxA, PFHxDA, PFODA, PFPrS, PFNS, PFDoS, HFPO-DA, NaDONA, 11Cl-PF3OUdS and PFTeDA, PFBS, PFPeS, 9Cl-PF3ONS were quantified in 0–2 and 1–19% of samples, respectively; OCPs: *o,p′*-DDD, *o,p′*-DDT and *δ*-HCH were quantified in 3–8% and in 0% of samples, respectively; BFRs and novel FRs: BDE 49, BDE 66, BDE 85, BDE 100, BDE 154, BDE 183, BDE 196, BDE 197, BDE 203, BDE 207, DBDPE, EH-TBB, syn-DP and BDE 99, BDE 153, anti-DP were detected in 0–7% and 5–33% of samples, respectively; BDE 28, BDE 206, BTBPE, *α*-HBCD, *β*-HBCD, *γ*-HBCD, TBBPA, HBB, OBIND, PBEB, PBT, DPTE, HCDBCO, TBCO, DBE-DBCH, 2,4-DBP, 2,4,6-TBP, PBP, OH-PBDEs were not detected in any sample.

DP, *anti*-DP, DPTE, EH-TBB, HCDBCO and TBCO) were obtained from AccuStandard (New Haven, CT, USA). The individual standards of PCBs (No. 65, 166, 118, and 170, a standard mixture of PCB-MIX 1 with 6 PCB congeners (No. 28, 52, 101, 138, 153, and 180), and 11 OCP standards were supplied by Dr. Ehrenstorfer (Augsburg, Germany). Internal standards of BFRs and isotopically labelled internal standards of PFAS were purchased from Wellington Laboratories. The purity of all standards was at least 97%.

The human blood serum used for the validation experiments was obtained from Sigma-Aldrich. Standard reference material of fortified human blood serum SRM 1958 was supplied by the National Institute of Standards and Technology (NIST, Gaithersburg, Maryland, USA).

2.3. Sample preparation procedure

The sample preparation procedure used for serum analysis was fully described in detail in our earlier study reported by Svarcova et al. (2019) and summarized in the Supplementary material. Briefly, non-polar compounds (PCBs, OCPs, PBDEs, and novel FRs) were isolated from a human blood serum (3 g) using a triple extraction into a mixture of *n*-hexane:diethylether (9:1; *v/v*), followed by purification using solid-phase extraction on a hand-made Florisil® column. For the isolation of more polar and lipophobic (PFASs, OH-PBDEs, HBCDs, TBBPA and brominated phenols) analytes, the remaining fraction was extracted into the acetonitrile with the support of added inorganic salts (sodium chloride and magnesium sulphate).

2.4. Instrumental analysis

The determination of OCPs, PCBs, novel FRs, and BFRs (PBDEs, BTBPE, DBDPE, DBE-DBCH, *anti*-DP, *syn*-DP, DPTE, EH-TBB, HBB, HCDBCO, OBIND, PBEB, PBT, and TBCO) was performed using an Agilent 7890A GC gas chromatograph (Agilent Technologies, USA), coupled to an Agilent 7000C MS triple quadrupole mass spectrometer (Agilent Technologies, USA), operated in the electron ionisation (EI) mode for OCPs and PCBs and negative chemical ionisation (NCI) mode for PBDEs and novel FRs. For the OCPs and PCBs separation, a capillary column DB-5MS (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Agilent Technologies, USA) was used. In the case of BFR and novel FR analysis, a DB-XLB capillary column (15 m × 0.25 mm i.d. × 0.1 μm film thickness; Agilent Technologies, USA) was applied. For more information about the other parameters of GC-MS analysis, see the studies published by Kalachova et al. (2013a), (2013b) and Svarcova et al. (2019).

The ultra-high performance liquid chromatography coupled to tandem MS (UHPLC-MS/MS) was used for the analysis of PFAS, OH-PBDEs, HBCDs, TBBPA, and brominated phenols. An Agilent 1290 Infinity II LC system interfaced with a mass spectrometer Agilent Triple Quadrupole G6495A (Agilent Technologies, USA), operated in the multiple reaction

Table 3
Overview of current studies (2016–2020) dealing with PFAS, PCB and OCP median concentrations in human serum samples collected worldwide.

A) PFAS median concentrations (ng mL-1) in human blood serum.[a,b,c]

			PFAS (ng mL^{-1})				
Country	Year of sampling	Number of samples	PFOS	PFOA	PFNA	PFHxS	References
Faroe Islands	2007–2008	51	5.59	1.00	0.76	0.50	Hu et al. (2018)
Denmark	2008–2013	1533	8.28	2.02	0.76	0.48	Bjerregaard-Olesen et al. (2016)
Sweden	2007–2010	1616	5.30	1.60	0.52	1.20	Shu et al. (2018)
	2007–2010	1533	5.38	1.61	0.53	1.23	Wikström et al. (2020)
	2011–2014	603	9.4[a]	2.8	1.1	7.5	Stubleski et al. (2016)
Belgium	2008–2009	201	12.6	3.50	0.87	1.61	Colles et al. (2020)
Norway	2007–2008	99	5.52	1.06	0.28	0.25	Papadopoulou et al. (2016)
	2010–2011	495	6.58	1.87	0.47	0.59	Averina et al. (2018)
	2013–2014	61	5.24	1.90	0.94	0.78	Poothong et al. (2020)
Czech Republic	2015	300	2.43	0.756	0.325	0.184	Sochorová et al. (2017)
	2019 (spring)	142	2.66	0.934	0.342	0.436	Presented study
USA	2009–2016	450	3.20	1.07	0.50	0.30	Kim et al. (2020)
	2011–2015	1257	7.07	2.47	0.909	1.58	Hurley et al. (2018)
	2013–2014	458	3.75	1.94	0.700	0.810	Ye et al. (2018)
	2014–2016	123	3.72	1.81	0.61	0.67	Trasande et al. (2017)
	2016	45	23.4[b]	11.7[b]	0.8[b]	7.7[b]	Worley et al. (2017)
	2016–2018	1030	3.04	2.01	0.78	1.22	Yu et al. (2020a)
China	2009	60	16.0	3.00	1.1	2.5	Liu et al. (2020)
	2014	45	20.1	17.0	2.04	0.45	Wu et al. (2017)
	2015	39	9.24	1.96	0.76	0.50	Wang et al. (2018)
	2015–2016	132	4.07	2.21	0.57	0.24	Gao et al. (2019)
	2015–2018	424	4.32	0.99	0.42	0.16	Cai et al. (2020)
	2017	252	14.2	14.8	3.17	0.33	Duan et al. (2020)
	2017	15	13.9[c]	3.59[c]	0.45[c]	10.7[c]	Gao et al. (2018)
	2018	85	4.90	2.80	1.30	0.50	Jin et al. (2020)
South Korea	2009–2010	1874	10.2[b]	2.85[b]	N/A	N/A	Lee et al. (2017)
Japan	2009–2010	339	4.50	2.10	1.80	0.46	Nakayama et al. (2020)
Saudi Arabia	2017–2018	108	5.30	1.03	0.50	1.48	Banjabi et al. (2020)
Australia	2002–2013	29	4.40[c]	2.90[c]	0.55[c]	2.50[c]	Eriksson et al. (2017)
	2016–2017	2400	5.71[c]	1.84[c]	0.47[c]	2.11[c]	Toms et al. (2019)
New Zealand	2011–2013	63	3.40[b]	2.40[b]	0.66[b]	1.0[b]	Coakley et al. (2018)

B) PCB and OCP median concentrations (ng g^{-1} lw) in human blood serum.[a,b,c]

			PCBs (ng g^{-1} lw)			
Country	Year of sampling	Number of samples	CB 138	CB 153	CB 180	References
Spain	2006	322	48.3	122	63.4	Zubero et al. (2017)
	2013	127	4.07	10.7	13.0	Zubero et al. (2017)
Italy (highly polluted area)	2013–2014	816	53.5	93.5	118	Zani et al. (2019)
Belgium	2015	252	ND	53.8	41.1	Pirard et al. (2018)
Czech Republic	2015	38	71	137	158	Svarcova et al. (2019)
	2019 (spring)	142	29.9	64.8	94.0	Presented study
USA	2016–2018	1000	7.45[a]	10.7[b]	7.68[b]	Du et al. (2020)
China	2016–2017	High/low exposure group (n = 38/38)	22.7/6.81	22.5/8.08	19.2/3.92	Yu et al. (2020b)
Korea	1994–2013	151	5.00	10.2	6.95	Moon et al. (2017)
	2011	148	4.67	9.80	ND	Choi et al. (2018)
	2019	25	2.87[c]	4.18[c]	2.72[c]	Lee et al. (2020a, 2020b)
Lebanon	2013–2015	316	8.20	16.4	24.1	Helou et al. (2019)
Iran	2016–2017	300	69.4	159	108	Karimi et al. (2020)

OCPs (ng/g lipid weight)						
Country	**Year of sampling**	**Number of samples**	**HCB**	***p,p′*-DDE**	***p,p′*-DDT**	**References**
Czech Republic	2015	38	29.6	207	13.5	Svarcova et al. (2019)
	2019 (spring)	142	18.1	101	2.97	Presented study
Korea	1994–2013	151	–	101	7.73	Moon et al. (2017)
	2011	147	4.13	63.4	5.31	Choi et al. (2018)
	2019	25	6.10[c]	36.3[c]	3.65[c]	Lee et al. (2020a, 2020b)
China	2014	1923	190	444	11.4	Wang et al. (2017)
Lebanon	2013–2015	316	5.80	15.1	15.6	Helou et al. (2019)
Iran	2016–2017	300	ND	22.4	18.6	Karimi et al. (2020)
Australia	2012–2013	2400	5.60	129	4.30	Thomas et al. (2017)
Russia	2012–2018	152	150	275	–	Abou Ghayda et al. (2020)

NOTES: [a] L-PFOS; [b] Geometric mean; [c] Mean.
NOTES: Not mentioned; ND not detected; [a] CB 138 + CB 158; [b] Geometric mean; [c] Mean.

monitoring mode (MRM) using electrospray ionisation in a negative mode (ESI) was employed. Target analytes were separated on an Acquity UPLC BEH C18 analytical column (100 mm × 2.1 mm i.d., 1.7 μm particle size, Waters, USA). The mobile phase consisted of A) 5 mM ammonium acetate in 20% acetonitrile in deionised water and B) 20% acetonitrile in methanol. Measurement conditions are described in detail in the study (Lankova et al., 2015) and in Table S3 in the Supplementary material.

2.4.1. Quality control and validation

To monitor background contamination, a procedural blank (deionised water was used instead of serum) was prepared together with each batch of 20 samples. When POP concentrations were determined in the procedural blank (the values centred around the limits of quantitation (LOQs)), the procedural blank was subtracted from the detected concentrations in the respective sample batch. LOQs were estimated as the lowest calibration standard with the signal to noise ratio (S/N) > 10 for the quantitative transition (ion), and S/N > 3 for at least one confirmation transition (ion). To compensate the unexpected matrix effects mainly in the GC injector, the standards of PCBs (CB 65 and CB 166) and BFRs (BDE 37, 77 and $^{13}C_{12}$-BDE 209) were used. To monitor the correction of possible matrix effects in the ion source of LC-MS system and the extraction efficiency (recovery of analytes), isotopically labelled surrogates ($^{13}C_{12}$-PFAS, $^{13}C_{12}$-TBBPA, and $^{13}C_{12}$-HBCDs) were applied.

The validation of the analytical method for the analysis of 78 organohalogenated contaminants and newly added 11 PFAS (PFHxDA, PFODA, PFPrS, PFPeS, PFHpS, PFNS, PFDoS, HFPO-DA, NaDONA, 9Cl-PF3ONS, and 11Cl-PF3OUdS) in human blood serum is described in detail in our previous study (Svarcova et al., 2019) and in the Supplementary material.

2.5. Lipid content determination

The resulting concentrations of non-polar POPs (OCPs, PCBs, novel FRs, PBDEs, and other BFRs) are commonly expressed on a lipid weight (lw) basis. Due to the very low lipid content in blood serum, it is not possible to determine its amount gravimetrically, and it is necessary to use a more accurate enzymatic method. The determination of the total lipid content is performed by the measurement of individual lipid classes, such as triacylglycerols and cholesterol in mmol L^{-1}, which are then converted to g l^{-1} according to the equations mentioned in the studies reported by Covaci et al. (2006) and Rugge et al. (2011).

2.6. Statistical analyses

All statistical analyses were performed in Metaboanalyst (metboanalyst.ca) or using custom built R-scripts. Only analytes with concentrations above LOQs in 50% and more samples were used for further statistical evaluation. Analysis of Variance (ANOVA) followed by Tukey's post hoc tests and paired t-tests were used to evaluate different levels of contaminants between localities and sampling time, respectively. For significantly differing analytes (p-value < 0.05), fold changes were calculated using the group's median concentrations. Additionally, Spearman's rank correlation coefficient was calculated to confirm a trend of increasing intensity between age groups. Logarithmic transformation was used prior any univariate statistical test to ensure normal distribution of the data.

3. Results and discussion

3.1. POPs in blood serum samples

The results of 89 POP concentrations (geometric mean, median, minimum and maximum, 5–95% percentile, and the number of positive samples) measured in the serum samples from both sampling periods and three residential areas are summarized in Table 2. The lipid content reached 0.27–1.47% (*w/w*) with a mean value of 0.58%. The most abundant contaminants were PFOA, PFNA, PFDA, PFOS, PFHxS, CB 138, CB 153, CB 170, CB 180, HCB, and *p,p′*-DDE quantified in all samples, followed by PFUdA, PFHpS, PFBA, PFHpA, PFDoA, CB 28, CB 101, CB 118, *β*-HCH, *p,p′*-DDD, and *p,p′*-DDT found in >50% of serum samples. As the values of POP concentrations in both periods are very similar, only the spring period with all participants will be further discussed.

Selected PFAS, PCB, and OCP concentrations in serum samples from the most recent papers (2016–2020) are summarized in Table 3.

PFAS belonged to the dominant group of more polar contaminants in human blood serum. The total concentrations of all detected ∑PFAS ranged from 0.516 to 22.2 ng mL^{-1} (mean 5.54 ng mL^{-1}, median 4.93 ng mL^{-1}). The highest concentrations were determined for PFOS (median 2.66 ng mL^{-1}), followed by PFOA (median 0.934 ng mL^{-1}), PFHxS (median 0.436 ng mL^{-1}) and PFNA (median 0.342 ng mL^{-1}). The medians of PFOA, PFNA, PFHxS, and PFOS from our study are comparable with the only data obtained within the similar Czech study (Sochorová et al., 2017). Moreover PFOA concentration in serum samples obtained from men were lower compared to women (Sochorová et al., 2017). Higher concentrations of PFOA in serum were observed in the elderly population (50–65 years of age) and in the population with higher education, however this trend has not been seen in our study (p-value > 0.05). A contributor to higher levels of PFOA may also be the higher intake of dairy products and milk (Sochorová et al., 2017).

In general, as documented in Table 3, the concentrations of PFOA, PFNA, PFHxS, and PFOS in serum of Czech population are the lowest in comparison with the other countries in Europe. The reason may be that these compounds have never been produced in the Czech Republic. Compared to studies from China (Duan et al., 2020; Wu et al., 2017) and USA (Worley et al., 2017), our results are approximately seven times lower for PFOS and sixteen times lower for PFOA. These levels, which are among the highest (median PFOS: 20.1 ng mL^{-1} and PFOA: 17.0 ng mL^{-1}), were detected in serum samples from Shanghai (sampling of 2014), which is one of the most populous and largest cities in China, where these substances are still produced. A polluted environment also contributes to these high levels (Wu et al., 2017). Higher serum PFOS (3–5 times) and PFOA (3–17 times) levels were found within the recent studies from Belgium (Colles et al., 2020), China (Duan et al., 2020; Gao et al., 2018; Liu et al., 2020) and South Korea (Lee et al., 2017). The serum concentration values of PFOS and PFOA detected in the Belgian study were four times higher compared to our results. Nevertheless, within five years Belgium has announced a decrease in PFOS concentrations (median: 7.58 ng mL^{-1}) by about a half, but PFOA levels (median: 2.94 ng mL^{-1}) are almost unchanged (Colles et al., 2020). In the USA, where these substances were produced on a large scale until about 2002 by 3M Company, a declining trend like in Europe is evident. If we compare the values in the studies from 2011 to 2015 and 2016–2018, the decrease in PFOS amounts is approximately two and a half times. This declining trend can be explained by the overall decline in PFOS concentrations in the general U.S. population (Kato et al., 2011) and blood donors (Olsen et al., 2017) due to the termination of PFOS production in the USA since 2000 (Hurley et al., 2018; C. H. Yu et al., 2020). A study reported by Post et al. (2017) indicated, that ongoing exposures to even relatively low drinking water concentrations of long-chain perfluoroalkyl acids (PFAAs) including PFOA, PFOS, PFNA and PFHxS substantially increase human body burdens, which remain elevated for many years after elimination of possible exposure sources.

The concentrations of non-polar POPs found in previous studies performed in the Czech Republic reached higher concentrations in comparison with other EU countries, thus one of the aim of present study was to confirm that the exposure to these contaminants continues to decline gradually. The obtained results confirmed the predominance of highly chlorinated PCB congeners (No. 138, 153, 170, and 180), which were also detected at the highest levels (median CB 138: 29.9 ng g^{-1} lw; CB 153: 64.8 ng g^{-1} lw; CB 170: 37.5 ng g^{-1} lw, and CB 180: 94.0 ng g^{-1} lw). The total concentrations of targeted ∑8PCBs reached the values of 34.1–2075 ng g^{-1} lw (mean 310 ng g^{-1} lw, median 226 ng g^{-1} lw). In general the indicator PCBs (No. 138, 153, and 180) are commonly found in serum at the highest levels worldwide, therefore they were selected as the main PCB representatives and their concentrations are further compared. As shown in Table 3, the current results of selected PCB congeners are twice as low compared to the previous study (Svarcova et al., 2019). It can be claimed that PCB concentrations in the serum of Czech population are rather high compared to other countries. This means that our values of CB 138, CB 153 and CB 180 detected in serum

samples were approximately 2–13 times higher compared to all other studies from Europe (Zubero et al., 2017); (Esposito et al., 2014); (Kalantzi et al., 2011), USA (Du et al., 2020), China (Moon et al., 2017; Y. Yu et al., 2020b), Korea (Choi et al., 2018; Moon et al., 2017), Lebanon (Helou et al., 2019) and Tunisia (Hassine et al., 2014). The lowest findings of these PCB congeners were detected in one of the most recent studies conducted in Korea (Lee et al., 2020a). In contrast, the highest median concentration, especially for CB 153, was determined within the Iranian study (Karimi et al., 2020) – the measured concentration was about 2 times higher compared to our study. It can be concluded that higher levels of selected PCB congeners in blood serum samples from the Czech Republic and Iran reflect the previous production of technical mixtures in these areas and probably inefficient or insufficient disposal of materials containing these substances.

Subsequently, a total of ten substances from the OCP group were detected in at least one sample, and three analytes (HCB, *p,p′*-DDE, *p,p′*-DDD) were quantified in more than 50% of the samples in both rounds (spring and autumn). Only one analyte (*δ*-HCH) was not detected in any sample. The total ∑DDTs (sum of *p,p′*-DDE; *o,p′*-DDE; *p,p′*-DDD; *o,p′*-DDD; *p,p′*-DDT, and *o,p′*-DDT) content in serum varied between 22.7 and 1047 ng g^{-1} lw (mean 143 ng g^{-1} lw, median 103 ng g^{-1} lw). The highest concentrations were determined for *p,p′*-DDE (median 101 ng g^{-1} lw). The most abundant compounds were HCB and *p,p'*-DDE and therefore are further compared with the published data in Table 3. Within the comparison in the Czech Republic, the median values of HCB and *p,p'*-DDE in current study are twice as low compared to the previous study (Svarcova et al., 2019). The targeted *p,p'*-DDE reached the highest levels in all countries and the current median concentration is in a good agreement with Italian, Tunisian, Korean, and Australian studies (Amodio et al., 2012; Hassine et al., 2014; Moon et al., 2017; Thomas et al., 2017). Two times higher median concentrations were observed for *p,p'*-DDE in serum in South Korea (Kang et al., 2008), Greece (Kalantzi et al., 2011) and Russia (Abou Ghayda et al., 2020). Medians of *p,p'*-DDE are 2–7 times lower in Turkey (Ulutaş et al., 2015), Korea (Choi et al., 2018; Lee et al., 2020a), Lebanon (Helou et al., 2019) and Iran (Karimi et al., 2020) compared to results from the Czech Republic. In the case of HCB, higher median concentrations (2–8 times) were published in the Russian (150 ng g^{-1} lw), Spanish (109 ng g^{-1} lw) and Tunisian studies (39.3 ng g^{-1} lw) (Abou Ghayda et al., 2020; Hassine et al., 2014; Porta et al., 2012). Comparable HCB concentrations were found in other studies from Europe (Kalantzi et al., 2011) (Amodio et al., 2012) and South Korea (Kang et al., 2008). The lowest concentrations of HCB were detected in the study from South Korea (Choi et al., 2018). The highest *p,p'*-DDE and HCB levels were found in blood serum samples from China in a study reported by Wang et al. (2017).

In the past, within the history of organochlorinated substances in Czechoslovakia, the occurrence of PCBs and OCPs was high everywhere in the environment, e.g. PCBs were produced under the name of Delor mixture and their production was stopped in 1984. Moreover, DDTs and HCB, among the other compounds, were produced in the Spolana Neratovice chemical plant. All production of DDTs was terminated between 1978 and 1983. HCB was also formed as an industrial by-product, banned as a pesticide in 1977, the production of which was terminated altogether in 1968. Therefore, these substances still persist in the environment and it is necessary to re-evaluate the exposure of the Czech population after some time. When comparing median concentrations of the most abundant organochlorine analytes (CB 153, HCB, and ∑DDTs) determined by the National Institute of Public Health between 2005 and 2015 in blood serum samples of Czech adults, supplemented by the values from this study (2019), a declining trend can be observed (medians CB 153/∑DDTs/HCB; year 2005: 438/519/97; year 2007: 310/339/63; year 2009: 195/282/48, and year 2019: 64.8/103/18.1 ng g^{-1} lw). Compared to 2005, the quantified levels of selected substances in our study (2019) are approximately 5–6 times lower. The reason is the aforementioned ban on the production and usage of these substances which leads to no further contamination of the environment. Moreover, these compounds are gradually eliminated from the environment, which leads to a reduction in the exposure of the Czech population to these contaminants (Černá et al., 2012, 2008, 2007, The National Institute of Public Health).

Table 4
Fold change of significantly differing (based on ANOVA, p-value < 0.05 followed by Tukey's Post-hoc tests) contaminants between localities (Ostrava, Ceske Budejovice, and Prague).[a]

Season	Group of POPs	Analyte	Fold change (MEDIAN)		
			Ostrava – Ceske Budejovice	Prague – Ceske Budejovice	Prague – Ostrava
Spring (2019)	**PCBs**	CB 28	0.03†	0.65	18.67
		CB 118	1.02	0.84	0.83†
		CB 138	1.29	0.96	0.74#
		CB 153	1.45	0.97	0.67†
		CB 170	1.74†	1.02	0.59‡
		CB 180	1.78†	1.01	0.57‡
	OCPs	*p,p'*-DDD	0.77#	1.03	1.33‡
	BFRs	BDE 47	2.78	0.71	0.26‡
	PFAS	PFHxS	1.44†	1.15	0.80
		PFHpA	1.14	1.36#	1.19
		PFDA	0.55†	0.76	1.36
Autumn (2019)	**PCBs**	CB 118	1.09#	0.93#	0.85
		CB 180	1.55	1.08	0.70#
	PFAS	PFHxS	1.37†	1.12	0.81#
		PFDA	0.41‡	0.68#	1.66‡
		PFUdA	0.46†	0.46†	0.99

[a] Fold changes were calculated using group's median values (#p < 0.05; † p-value < 0.01; ‡ p-value < 0.001 from Tukey's Post-hoc test).

BFRs and novel FRs belong to the minor target analytes in serum. The most frequently detected compounds, present in 83–99% and 21–44% of serum samples, were BDE 47 and BDE 209, respectively. From the novel FRs, anti-DP was quantified in 22–33% of samples. Other FRs, that were quantified in 5–28% of samples, were BDE 99 and BDE 153. Total of 24 of 40 monitored FRs were found below the LOQs in tested samples. In comparison with the former study from the Czech Republic (Sochorová et al., 2017), the detection frequencies of the most common PBDEs (BDE 47, 99, 153, and 209) were much lower than in the presented study. However, the maximum concentration for BDE 209 was higher (<1.5–2693 ng g^{-1} lw) in serum samples collected from 2015 than in samples from 2019 (1.50–459 ng g^{-1} lw). The contribution of individual PBDE congeners is quite variable across different studies and the possible explanation of this phenomenon could be the very different production and consumption rates of BFRs across the countries, e.g. BFRs have been produced in the U.S. and therefore the higher amounts could be expected in human blood serum from the American studies compared to the results from the Czech Republic where those compounds have never been produced. In a recent study from Sweden (Bjermo et al., 2017), congeners BDE 153, BDE 209 and BDE 47 were most commonly found in serum, with a detection frequency (DF) reaching 100%, 74% and 24%, respectively. In similar study, the most abundant PBDEs in blood serum of the Danish population were BDE 47, BDE 99, BDE 153 and BDE 209 reaching DF of 84–98% and 56% (Vorkamp et al., 2014). In an American study BDE 47, BDE 100 and BDE 153 were the ones most frequently found in blood serum, with DF ranging from 78 to 80% (Hurley et al., 2017). Considering the present results, BDE 47 median value is in a good agreement with European, Chinese and New Zealand studies (Antignac et al., 2009; Coakley et al., 2018; Vorkamp et al., 2014; Y. Yu et al., 2020). However, the concentration of this contaminant was approximately seven times lower compared to studies from the USA (Butt et al., 2016; Hurley et al., 2017; Makey et al., 2014). These results support the hypothesis that elevated PBDE concentrations are in human serum samples from country where the technical mixtures of PBDEs have been produced and widely used. The median of BDE 209 measured in the present study was lower compared

Table 5
Fold change of significantly differing (based on paired *t*-test, *p*-value < 0.05) contaminants between serum samples from spring and autumn 2019 (significance in at least one city).[a]

Group of POPs	Analyte	Fold change (MEDIAN) Ostrava (spring-autumn)	Ceske Budejovice (spring-autumn)	Prague (spring-autumn)
PCBs	CB 28	0.08	4.46‡	4.17#
	CB 118	0.91	0.97	0.88†
	CB 138	0.57#	0.61†	0.63‡
	CB 170	1.35†	1.11	1.11
OCPs	*p,p'*-DDE	0.89	0.82	0.68#
	p,p'-DDD	0.60‡	0.64	0.97
BFRs	BDE 47	3.19†	0.89	0.67‡
PFAS	PFBA	0.74#	1.07	1.07
	PFDA	0.98	0.73	0.81†

[a] Fold changes were calculated using group's median values (# p-value <0.05; † p-value < 0.01; ‡ p-value < 0.001 from *t*-test).

to the world, namely nine times lower than in Europe (Antignac et al., 2009; Vorkamp et al., 2014) and thirteen times lower than in serum from China (Qiao et al., 2018; Y. Yu et al., 2020). Nevertheless, compared to blood serum from Sweden (Bjermo et al., 2017; Darnerud et al., 2015), current BDE 47 concentrations were five times higher and for BDE 209 the levels were comparable.

3.2. Differences between sampling localities (Prague, Ostrava and Ceske Budejovice)

As shown in Table 4, within the spring 2019, the median concentrations indicated statistically significant associations between localities for 11 POPs, namely CB 28, CB 118, CB 138, CB 153, CB 170, CB 180, *p,p'*-DDD, BDE 47, PFHxS, PFHpA, and PFDA (ANOVA followed by Tukey's Post-hoc tests). The measured concentrations of five PCBs (CB 118, CB 138, CB 153, CB 170, and CB 180) and BDE 47 were significantly higher (1.2–4 times) in serum samples from the city of Ostrava (an industrial area with a long-term polluted environment) compared to Prague (highly populated area). The highest PCB levels (highly chlorinated PCBs), were also found for the Ostrava region in an earlier Czech study especially in serum samples from men (Černá et al., 2008). However, PFDA and *p,p'*-DDD showed an opposite trend, when their concentrations decreased significantly in serum samples from Ostrava in comparison to Ceske Budejovice. The city of Ceske Budejovice is more focused on agriculture than the other monitored cities, and in the past DDTs were used there, so it can be a historically older burden of the environment. In the case of serum samples collected in autumn 2019 with focus on variability between residential areas, a statistically significant trends were observed for three PFAS (PFDA, PFUdA, and PFHxS) and two PCBs (CB 118 and CB 180). Higher levels of CB 180 and PFHxS in this period were determined again in the city of Ostrava. On the other hand, PFUdA and PFDA concentrations increased significantly in serum samples obtained in Ceske Budejovice, see Table 4 and Fig. S1. In summary, higher findings of PCBs in Ostrava may be caused by the industrial character associated mainly with the past of this city and also by the fact that the only incinerator plant in the Czech Republic with a permit to burn waste containing PCBs is located here. Although, PFAS have never been produced in the Czech Republic, it is possible that some intermediates with PFAS content were processed in Ostrava and therefore, higher amounts of some PFAS in the serum samples from Ostrava were observed. Therefore, more studies with a larger set of samples from different areas of the Czech Republic would be needed.

3.3. Differences between seasons (spring and autumn 2019)

According to the paired *t*-test, statistically significant season-dependent differences in at least one city were noted for CB 28, CB 118, CB 138, CB 170, *p,p'*-DDE, *p,p'*-DDD, BDE 47, PFBA, and PFDA (Table 5). The serum concentrations of CB 170 and BDE 47 in Ostrava showed higher levels (up to three times higher) in spring, while results of CB 138 and *p,p'*-DDD were lower during this period. The observed trends were very diverse: the concentrations of CB 28 in serum collected in Ceske Budejovice during the spring had an increasing trend (up to four times) and, conversely, slightly decreased for CB 138. The last monitored city was Prague, where higher values of CB 138, *p,p'*-DDE, and PFDA in the blood serum from the autumn period were mostly observed. The differences were very variable and could probably be related, for example, to a change of daily diet during spring and autumn, as well as to the origin of the food within each period. Although, the minor differences for less abundant contaminants were observed, they were not consistent (no systematic changes has been documented) as well as without trends and therefore could be assumed that within such short period no significant changes in POP concentrations in blood serum occur.

3.4. Differences between age groups

As summarized in Table 6, all these analytes met the criterium of ANOVA (p-value <0.05), that confirmed significantly different concentrations of the targeted POPs between age groups (21–30; 31–40; 41–50, and 51–60 years of age; for Ceske Budejovice only the first three groups were used). As all significant POPs showed a trend of increasing concentration with increasing age, Spearman rank correlation coefficient was calculated to confirm the positive correlation. For all five PCBs (CB 118, CB 153, CB 170, and CB 180), three OCPs (HCB, *p,p'*-DDE, and

Table 6
The correlation between a selected statisticaly significant POP serum levels (ANOVA, *p*-value < 0.05 and Spearman's rank correlation coefficient) with various age groups (21–30, 31–40, 41–50, and 51–63) in Ostrava, Ceske Budejovice and Prague.

Group of POPs	Analyte	Ostrava ANOVA p-value	Ostrava Spearman's rank correlation coefficient	Ceske Budejovice ANOVA p-value	Ceske Budejovice Spearman's rank correlation coefficient	Prague ANOVA p-value	Prague Spearman's rank correlation coefficient
PCBs	CB 118	6.42E-05	0.496	n.s.	–	7.91E-05	0.467
	CB 138	4.56E-08	0.575	n.s.	–	1.22E-15	0.619
	CB 153	2.88E-10	0.622	3.07E-03	0.498	1.10E-18	0.666
	CB 170	4.15E-14	0.679	1.43E-05	0.633	7.86E-22	0.721
	CB 180	1.22E-15	0.700	1.79E-06	0.647	2.36E-21	0.708
OCPs	HCB	1.25E-04	0.442	n.s.	–	1.89E-06	0.398
	p,p'-DDD	n.s.	–	n.s.	–	2.23E-02	0.273
	p,p'-DDE	3.02E-04	0.405	n.s.	–	1.92E-09	0.464
PFAS	PFOS	n.s.	–	n.s.	–	1.76E-02	0.242
	PFHpS	n.s.	–	n.s.	–	2.22E-02	0.292

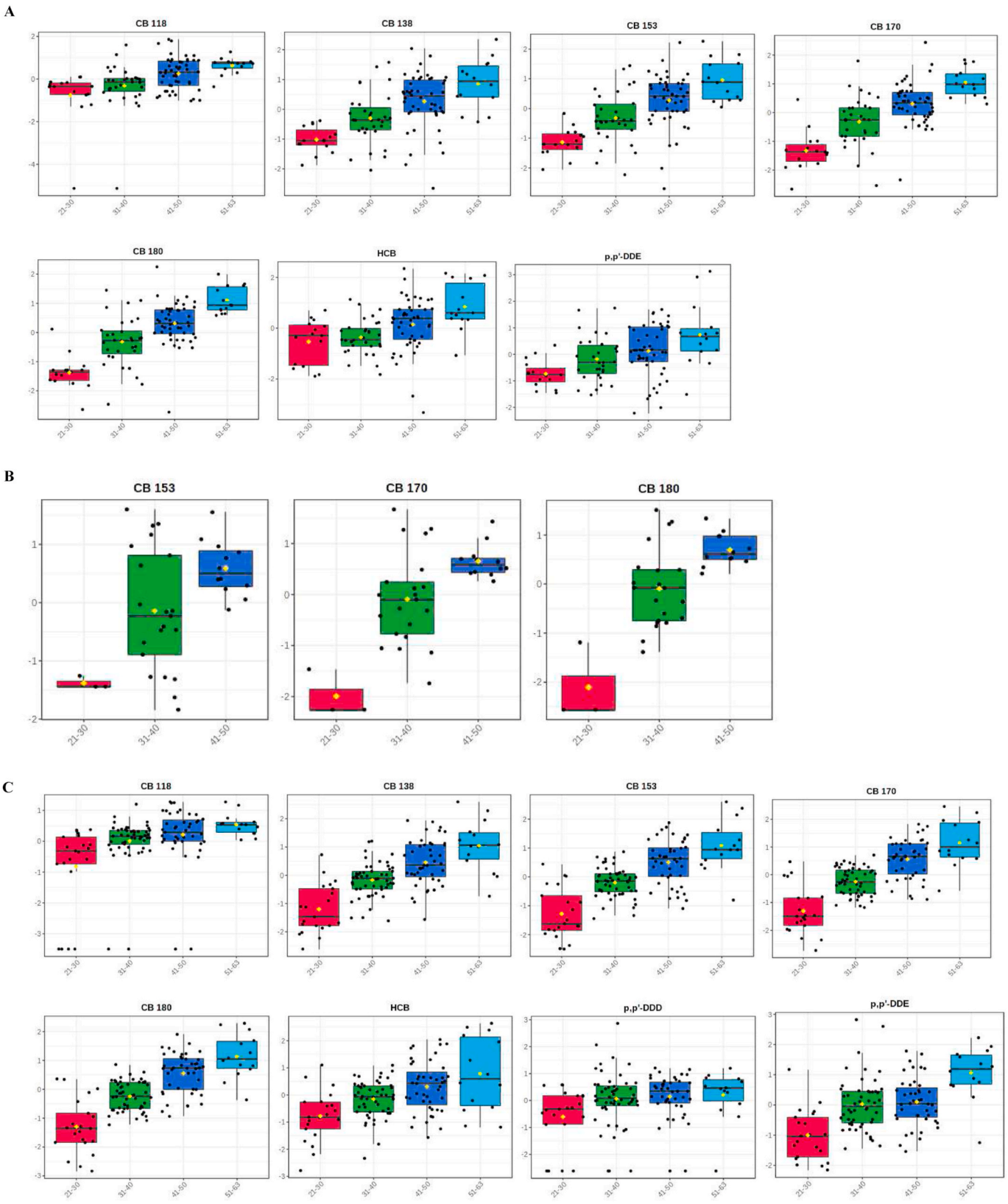


Fig. 1. A Box-plots of human serum concentrations of PCBs and OCPs in Ostrava, divided on the basis of different age groups (21–30, 31–40, 41–50, and 51–63).**B:** Box-plots of human serum concentrations of PCBs in Ceske Budejovice, divided on the basis of different age groups (21–30, 31–40, 41–50).**C:** Box-plots of human serum concentrations of PCBs, OCPs and PFAS in Prague, divided on the basis of different age groups (21–30, 31–40, 41–50, and 51–63).

p,p'-DDD) and two PFAS (PFOS and PFHpS) the correlation coefficients were significant, nevertheless the strongest correlation was observed in case of CB 180 and CB 170 ($r > 0.632$ in all three localities). The obtained results and the associated serum concentrations of selected PCBs, OCPs and PFAS in the various age groups of participants for all three localities are summarized in Fig. 1A–C. The increase in the concentrations of the above-mentioned contaminants is probably caused by the longer exposure to these pollutants during the subjects' lifetimes. In addition, their slow elimination from the body leads to increasing levels of these pollutants in the body as the policemen's age increases as well. Due to the lipophilic properties of these substances, it could be assumed that higher levels of these analytes could be detected in the blood serum of policemen with a higher subcutaneous fat ratio. Other important factor affecting the differences in POP concentrations between the age groups is the period the respective compound has been banned from the usage and subsequent reducing of environmental burden and human exposure (Nøst et al., 2013). The previous human biomonitoring studies also confirmed that higher age was associated with higher PCB and OCP levels (Artacho-Cordón et al., 2015a; Černá et al., 2008; Foster et al., 2012; Ibarluzea et al., 2011; Wittsiepe et al., 2008). Conversely, in the present study, this trend was not confirmed for PFAS, BFRs and novel FRs.

Other aspects such as the correlation between serum samples with lipidomics were assessed. The effect of BMI, cholesterol, education, and former smoking habits on POP levels in serum was further investigated. However, no statistically significant relationships between these examined factors and serum POP concentrations were discovered.

4. Conclusions

The uniqueness of this study lies in the analysis of a wide range of POPs with different physico-chemical properties in a large set of blood serum samples, mapping a sample group of urban policemen. For this population their total body burden to POPs does not depend only on the diet, age, various localities but also the nature of their profession (they move around the city and spend a lot of time outdoors). This is the first study to analyse such a large set of serum pollutants (8 PCBs, 11 OCPs, 33 BFRs, 7 novel FRs, and 30 PFAS) in the unique population group. The total concentrations of ∑PFAS, ∑PCB,s and ∑DDTs in serum samples were in the range of 0.516–22.2 ng mL^{-1}, 34.1–2075 ng g^{-1} lw, and 22.7–1047 ng g^{-1} lw, respectively. In general, the contaminants occurring the most frequently in all serum samples were represented by PFAS (PFOA, PFNA, PFDA, PFOS, and PFHxS); PCBs (No. 138, 153, 170, and 180) and OCPs (HCB and *p,p'*-DDE).

The observed individual groups of serum contaminants can be summarized as follows:

The newly monitored substances (PFHxDA, PFODA, PFPrS, PFPeS, PFHpS, PFNS, PFDoS, HFPO-DA, NaDONA, 9Cl-PF3ONS, 11Cl-PF3OUdS) are included within the monitoring of PFAS in serum. Major PFOS and PFOA serum concentrations are among the lowest compared to population in Europe, China and the USA. Statistically significant differences between localities and seasons were found for PFHxS, PFDA, PFUdA, and PFDA, respectively. However, no significantly different PFAS concentrations associated with various age groups were observed.

Regarding PCBs, the predominance of highly chlorinated PCB congeners (No. 138, 153, 170, and 180) was observed. Higher PCB levels in Czech serum samples were confirmed by the other relevant studies.

The main representatives of OCPs were HCB and *p,p'*-DDE, which were found in all samples with the highest medians (16.3 ng g^{-1} lw and 114 ng g^{-1} lw, respectively). These results were comparable with those concentrations in serum samples from Europe, Australia, Tunisia and Korea (Amodio et al., 2012; Hassine et al., 2014; Kalantzi et al., 2011; Kang et al., 2008; Moon et al., 2017; Thomas et al., 2017).

In general, a declining trend for PCB and OCP content in human blood serum was observed. Based on further statistical evaluation, significantly different concentrations of contaminants between localities and age groups were observed. Specifically, in blood serum samples from spring sampling time, the occurrence of CB 170 and CB 180 was 70% higher in Ostrava compared to Prague and Ceske Budejovice, which could be due to the highly polluted environment of the Ostrava region. When comparing the results from the spring and the autumn sampling times, the minor differences of less abundant contaminants were observed, however no systematic changes as well as trends have been seen. Regarding the age groups, five PCBs (No. 118, 153, 170, and 180), three OCPs (HCB, *p,p'*-DDE, and *p,p'*-DDD) and two PFAS (PFOS and PFHpS) increasing with the increasing age of the policemen was observed. The highest Spearman rank correlation coefficients were noted for CB 170 and CB 180.

Within the minor BFRs and novel FRs in serum, the most abundant representatives were BDE 47, BDE 209, and anti-DP quantified in 83–99%, 21–44% and 22–33% of serum samples, respectively. The congener BDE 209 was commonly detected in highest levels in serum samples, but our levels were lower compared to the European and Chinese studies (Antignac et al., 2009; Qiao et al., 2018; Vorkamp et al., 2014; Y. Yu et al., 2020). In summary, the body burden of these pollutants is very similar to elsewhere in Europe, but there are differences in the contribution of individual PBDEs. Statistically significant associations between concentrations and locality and sampling season were observed only in case of the most abundant BDE 47.

Finally, the obtained results within the project HAIE will partly clarify how long-term stay in differently polluted areas affect the individuals health and also current total body burden of various POPs in population from various localities will be known. The obtained data will be processed within the upcoming epidemiological studies under this project.

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.

Acknowledgements

This work was supported by the European Regional Development Fund under Grant Healthy Aging in Industrial Environment HAIE (CZ.02.1.01/0.0/0.0/16_019/0000798). This research has also received funding from the project No.: MSMT-LTAUSA17182 and the Specific University Research grant No.: A1_FPBT_2020_002.

We would also like to thank the Institute of Experimental Medicine of the Czech Academy of Sciences (Prague, Czech Republic) for providing human serum samples.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.118140.

Credit statement

Andrea Polachova: Writing – original draft preparation, Methodology, Validation, Data Curation. **Tomas Gramblicka:** Methodology, Data Curation. **Kamila Bechynska:** Formal analysis, Software. **Ondrej Parizek:** Investigation, Visualization. **Denisa Turnerova:** Investigation, Visualization. **Darina Dvorakova:** Writing – Reviewing and Editing, Validation. **Katerina Honkova:** Resources. **Andrea Rossnerova:** Data Curation, Conceptualization. **Pavel Rossner, Jr.:** Data Curation, Conceptualization. **Radim J. Sram:** Supervision, Funding acquisition, Project administration. **Jan Topinka:** Supervision, Funding acquisition, Project administration. **Jana Pulkrabova:** Supervision, Conceptualization, Writing – Reviewing and Editing, Funding acquisition.

References

Abou Ghayda, R., Sergeyev, O., Burns, J.S., Williams, P.L., Lee, M.M., Korrick, S.A., Smigulina, L., Dikov, Y., Hauser, R., Mínguez-Alarcón, L., 2020. Peripubertal serum concentrations of organochlorine pesticides and semen parameters in Russian young men. Environ. Int. 144, 106085. https://doi.org/10.1016/j.envint.2020.106085.

Alves, A., Kucharska, A., Erratico, C., Xu, F., Den Hond, E., Koppen, G., Vanermen, G., Covaci, A., Voorspoels, S., 2014. Human biomonitoring of emerging pollutants through non-invasive matrices: state of the art and future potential. Anal. Bioanal. Chem. 406, 4063–4088. https://doi.org/10.1007/s00216-014-7748-1.

Amodio, E., Turci, R., Massenti, M.F., Di Gaudio, F., Minoia, C., Vitale, F., Firenze, A., Calamusa, G., 2012. Serum concentrations of persistent organic pollutants (POPs) in the inhabitants of a Sicilian city. Chemosphere 89, 970–974. https://doi.org/10.1016/j.chemosphere.2012.06.054.

Angerer, J., Ewers, U., Wilhelm, M., 2007. Human biomonitoring: state of the art. Int. J. Hyg Environ. Health 210, 201–228. https://doi.org/10.1016/j.ijheh.2007.01.024.

Antignac, J.-P., Cariou, R., Zalko, D., Berrebi, A., Cravedi, J.-P., Maume, D., Marchand, P., Monteau, F., Riu, A., Andre, F., Le Bizec, B., 2009. Exposure assessment of French women and their newborn to brominated flame retardants: determination of tri- to deca- polybromodiphenylethers (PBDE) in maternal adipose tissue, serum, breast milk and cord serum. Environ. Pollut. 157, 164–173. https://doi.org/10.1016/j.envpol.2008.07.008.

H., B., A., H. Artacho-Cordón, F., Belhassen, H., Arrebola, J.P., Ghali, R., Amira, D., Jiménez-Díaz, I., Pérez-Lobato, R., Olea, N., 2015a. Serum levels of persistent organic pollutants and predictors of exposure in Tunisian women Sci. Total Environ. 511, 530–534. https://doi.org/10.1016/j.scitotenv.2014.12.093.

Artacho-Cordón, F., Fernández-Rodríguez, M., Garde, C., Salamanca, E., Iribarne-Durán, L.M., Torné, P., Expósito, J., Papay-Ramírez, L., Fernández, M.F., Olea, N., Arrebola, J.P., 2015b. Serum and adipose tissue as matrices for assessment of exposure to persistent organic pollutants in breast cancer patients. Environ. Res. 142, 633–643. https://doi.org/10.1016/j.envres.2015.08.020.

Averina, M., Brox, J., Huber, S., Furberg, A.-S., 2018. Perfluoroalkyl substances in adolescents in northern Norway: lifestyle and dietary predictors. The Tromsø study, Fit Futures 1. Environ. Int. 114, 123–130. https://doi.org/10.1016/j.envint.2018.02.031.

Banjabi, A.A., Li, A.J., Kumosani, T.A., Yousef, J.M., Kannan, K., 2020. Serum concentrations of perfluoroalkyl substances and their association with osteoporosis in a population in Jeddah, Saudi Arabia. Environ. Res. 187, 109676. https://doi.org/10.1016/j.envres.2020.109676.

Bjermo, H., Aune, M., Cantillana, T., Glynn, A., Lind, P.M., Ridefelt, P., Darnerud, P.O., 2017. Serum levels of brominated flame retardants (BFRs: PBDE, HBCD) and influence of dietary factors in a population-based study on Swedish adults. Chemosphere 167, 485–491. https://doi.org/10.1016/j.chemosphere.2016.10.008.

Bjerregaard-Olesen, C., Bach, C.C., Long, M., Ghisari, M., Bossi, R., Bech, B.H., Nohr, E. A., Henriksen, T.B., Olsen, J., Bonefeld-Jørgensen, E.C., 2016. Time trends of perfluorinated alkyl acids in serum from Danish pregnant women 2008–2013. Environ. Int. 91, 14–21. https://doi.org/10.1016/j.envint.2016.02.010.

Butt, C.M., Miranda, M.L., Stapleton, H.M., 2016. Development of an analytical method to quantify PBDEs, OH-BDEs, HBCDs, 2,4,6-TBP, EH-TBB, and BEH-TEBP in human serum. Anal. Bioanal. Chem. 408, 2449–2459. https://doi.org/10.1007/s00216-016-9340-3.

Cabrera-Rodríguez, R., Luzardo, O.P., Almeida-González, M., Boada, L.D., Zumbado, M., Acosta-Dacal, A., Rial-Berriel, C., Henríquez-Hernández, L.A., 2019. Association between prenatal exposure to multiple persistent organic pollutants (POPs) and growth indicators in newborns. Environ. Res. 171, 285–292. https://doi.org/10.1016/j.envres.2018.12.064.

Cai, D., Li, Q.-Q., Chu, C., Wang, S.-Z., Tang, Y.-T., Appleton, A.A., Qiu, R.-L., Yang, B.-Y., Hu, L.-W., Dong, G.-H., Zeng, X.-W., 2020. High trans-placental transfer of perfluoroalkyl substances alternatives in the matched maternal-cord blood serum: evidence from a birth cohort study. Sci. Total Environ. 705, 135885. https://doi.org/10.1016/j.scitotenv.2019.135885.

Černá, M., Spěváčková, V., Batáriová, A., Šmíd, J., Čejchanová, M., Očadlíková, D., Bavorová, H., Beneš, B., Kubínová, R., 2007. Human biomonitoring system in the Czech Republic. Int. J. Hyg Environ. Health 210, 495–499. https://doi.org/10.1016/j.ijheh.2007.01.005.

Černá, M., Malý, M., Grabic, R., Batáriová, A., Šmíd, J., Beneš, B., 2008. Serum concentrations of indicator PCB congeners in the Czech adult population. Chemosphere 72, 1124–1131. https://doi.org/10.1016/j.chemosphere.2008.04.019.

Černá, M., Krsková, A., Čejchanová, M., Spěváčková, V., 2012. Human biomonitoring in the Czech Republic: an overview. Int. J. Hyg. Environ. Health, Special Issue: Berlin International Conference on Human Biomonitoring 215, 109–119. https://doi.org/10.1016/j.ijheh.2011.09.007.

Chen, Aimin, Dietrich Kim, N., Xia, Huo, Shuk-mei, Ho, 2011. Developmental neurotoxicants in E-waste: an emerging health concern. Environ. Health Perspect. 119, 431–438. https://doi.org/10.1289/ehp.1002452.

Choi, S., Kim, H., Kim, Sunmi, Choi, G., Kim, Sungjoo, Park, J., Shim, S., Lee, I., Kim, Sungkyoon, Moon, H.-B., Choi, K., Lee, J.J., Kim, S.Y., 2018. Current status of organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) exposure among mothers and their babies of Korea-CHECK cohort study. Sci. Total Environ. 618, 674–681. https://doi.org/10.1016/j.scitotenv.2017.07.232.

Coakley, J., Bridgen, P., Mueller, J., Douwes, J., 't Mannetje, A., 2018. Polybrominated diphenyl ethers and perfluorinated alkyl substances in blood serum of New Zealand adults, 2011–2013. Chemosphere 208, 382–389. https://doi.org/10.1016/j.chemosphere.2018.05.191.

Colles, A., Bruckers, L., Den Hond, E., Govarts, E., Morrens, B., Schettgen, T., Buekers, J., Coertjens, D., Nawrot, T., Loots, I., Nelen, V., De Henauw, S., Schoeters, G., Baeyens, W., van Larebeke, N., 2020. Perfluorinated substances in the Flemish population (Belgium): levels and determinants of variability in exposure. Chemosphere 242, 125250. https://doi.org/10.1016/j.chemosphere.2019.125250.

Covaci, A., Voorspoels, S., Thomsen, C., van Bavel, B., Neels, H., 2006. Evaluation of total lipids using enzymatic methods for the normalization of persistent organic pollutant levels in serum. Sci. Total Environ. 366, 361–366. https://doi.org/10.1016/j.scitotenv.2006.03.006.

Cui, Q., Pan, Y., Wang, J., Liu, H., Yao, B., Dai, J., 2020. Exposure to per- and polyfluoroalkyl substances (PFASs) in serum versus semen and their association with male reproductive hormones. Environ. Pollut. 266, 115330. https://doi.org/10.1016/j.envpol.2020.115330.

Darnerud, P.O., Lignell, S., Aune, M., Isaksson, M., Cantillana, T., Redeby, J., Glynn, A., 2015. Time trends of polybrominated diphenylether (PBDE) congeners in serum of Swedish mothers and comparisons to breast milk data. Environ. Res. 138, 352–360. https://doi.org/10.1016/j.envres.2015.02.031.

Du, S., Rodenburg, L., Patterson, N., Chu, C., Riker, C.D., Yu, C.H., Fan, Z., Tina), 2020. Concentration of polychlorinated biphenyls in serum from New Jersey biomonitoring study: 2016–2018. Chemosphere 261, 127730. https://doi.org/10.1016/j.chemosphere.2020.127730.

Duan, Y., Sun, H., Yao, Y., Meng, Y., Li, Y., 2020. Distribution of novel and legacy per-/polyfluoroalkyl substances in serum and its associations with two glycemic biomarkers among Chinese adult men and women with normal blood glucose levels. Environ. Int. 134, 105295. https://doi.org/10.1016/j.envint.2019.105295.

Eriksson, U., Mueller, J.F., Toms, L.-M.L., Hobson, P., Kärrman, A., 2017. Temporal trends of PFSAs, PFCAs and selected precursors in Australian serum from 2002 to 2013. Environ. Pollut. 220, 168–177. https://doi.org/10.1016/j.envpol.2016.09.036.

Esposito, M., Serpe, F.P., Diletti, G., Messina, G., Scortichini, G., La Rocca, C., Baldi, L., Amorena, M., Monda, M., 2014. Serum levels of polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans and polychlorinated biphenyls in a population living in the Naples area. southern Italy. Chemosphere 94, 62–69. https://doi.org/10.1016/j.chemosphere.2013.09.013.

Foster, W.G., Cheung, A.P., Davis, K., Graves, G., Jarrell, J., Leblanc, A., Liang, C.L., Leech, T., Walker, M., Weber, J.P., Van Oostdam, J., 2012. Circulating metals and persistent organic pollutant concentrations in Canadian and non-Canadian born primiparous women from five Canadian centres: results of a pilot biomonitoring study. Sci. Total Environ. 326–336. https://doi.org/10.1016/j.scitotenv.2012.06.070, 435–436.

Foster Warren, G., Maharaj-Briceño Serena, Cyr Daniel, G., 2010. Dioxin-induced changes in epididymal sperm count and spermatogenesis. Environ. Health Perspect. 118, 458–464. https://doi.org/10.1289/ehp.0901084.

Gao, K., Fu, J., Xue, Q., Li, Y., Liang, Y., Pan, Y., Zhang, A., Jiang, G., 2018. An integrated method for simultaneously determining 10 classes of per- and polyfluoroalkyl substances in one drop of human serum. Anal. Chim. Acta 999, 76–86. https://doi.org/10.1016/j.aca.2017.10.038.

Gao, K., Zhuang, T., Liu, X., Fu, Jianjie, Zhang, J., Fu, Jie, Wang, L., Zhang, A., Liang, Y., Song, M., Jiang, G., 2019. Prenatal exposure to per- and polyfluoroalkyl substances (PFASs) and association between the placental transfer efficiencies and dissociation constant of serum proteins–PFAS complexes. Environ. Sci. Technol. 53, 6529–6538. https://doi.org/10.1021/acs.est.9b00715.

Geiger, S.D., Xiao, J., Ducatman, A., Frisbee, S., Innes, K., Shankar, A., 2014. The association between PFOA, PFOS and serum lipid levels in adolescents. Chemosphere 98, 78–83. https://doi.org/10.1016/j.chemosphere.2013.10.005.

Hao, W., Kingston, H.M., Dillard, A., Macherone, A., Stuff, J., Pamuku, M., 2020. Quantification of persistent organic pollutants in human whole blood samples using stir bar sorptive extraction coupled with GC/MS/MS and isotope dilution mass spectrometry. Microchem. J. 153, 104279. https://doi.org/10.1016/j.microc.2019.104279. Skip,.

Hassine, B.S., Hammami, B., Ben Ameur, W., El Megdiche, Y., Barhoumi, B., El Abidi, R., Driss, M.R., 2014. Concentrations of organochlorine pesticides and polychlorinated biphenyls in human serum and their relation with age, gender, and BMI for the general population of Bizerte, Tunisia. Environ. Sci. Pollut. Res. 21, 6303–6313. https://doi.org/10.1007/s11356-013-1480-9.

Helou, K., Harmouche-Karaki, M., Karake, S., Narbonne, J.-F., 2019. A review of organochlorine pesticides and polychlorinated biphenyls in Lebanon: environmental and human contaminants. Chemosphere 231, 357–368. https://doi.org/10.1016/j.chemosphere.2019.05.109.

Hu, X.C., Dassuncao, C., Zhang, X., Grandjean, P., Weihe, P., Webster, G.M., Nielsen, F., Sunderland, E.M., 2018. Can profiles of poly- and Perfluoroalkyl substances (PFASs) in human serum provide information on major exposure sources? Environ. Health 17, 11. https://doi.org/10.1186/s12940-018-0355-4.

Hurley, S., Goldberg, D., Nelson, D.O., Guo, W., Wang, Y., Baek, H.-G., Park, J.-S., Petreas, M., Bernstein, L., Anton-Culver, H., Reynolds, P., 2017. Temporal evaluation of polybrominated diphenyl ether (PBDE) serum levels in middle-aged and older California women, 2011–2015. Environ. Sci. Technol. 51, 4697–4704. https://doi.org/10.1021/acs.est.7b00565.

Hurley, S., Goldberg, D., Wang, M., Park, J.-S., Petreas, M., Bernstein, L., Anton-Culver, H., Nelson, D.O., Reynolds, P., 2018. Time trends in per- and polyfluoroalkyl substances (PFASs) in California women: declining serum levels, 2011–2015. Environ. Sci. Technol. 52, 277–287. https://doi.org/10.1021/acs.est.7b04650.

Ibarluzea, J., Alvarez-Pedrerol, M., Guxens, M., Marina, L.S., Basterrechea, M., Lertxundi, A., Etxeandia, A., Goñi, F., Vioque, J., Ballester, F., Sunyer, J., 2011. Sociodemographic, reproductive and dietary predictors of organochlorine

compounds levels in pregnant women in Spain. Chemosphere 82, 114–120. https://doi.org/10.1016/j.chemosphere.2010.09.051.
Jian, J.-M., Chen, D., Han, F.-J., Guo, Y., Zeng, L., Lu, X., Wang, F., 2018. A short review on human exposure to and tissue distribution of per- and polyfluoroalkyl substances (PFASs). Sci. Total Environ. 636, 1058–1069. https://doi.org/10.1016/j.scitotenv.2018.04.380.
Jin, H., Lin, S., Dai, W., Feng, L., Li, T., Lou, J., Zhang, Q., 2020. Exposure sources of perfluoroalkyl acids and influence of age and gender on concentrations of chlorinated polyfluorinated ether sulfonates in human serum from China. Environ. Int. 138, 105651. https://doi.org/10.1016/j.envint.2020.105651.
Kalachova, K., Cajka, T., Sandy, C., Hajslova, J., Pulkrabova, J., 2013a. High throughput sample preparation in combination with gas chromatography coupled to triple quadrupole tandem mass spectrometry (GC–MS/MS): a smart procedure for (ultra) trace analysis of brominated flame retardants in fish. Talanta 105, 109–116. https://doi.org/10.1016/j.talanta.2012.11.073.
Kalachova, K., Pulkrabova, J., Cajka, T., Drabova, L., Stupak, M., Hajslova, J., 2013b. Gas chromatography–triple quadrupole tandem mass spectrometry: a powerful tool for the (ultra)trace analysis of multiclass environmental contaminants in fish and fish feed. Anal. Bioanal. Chem. 405, 7803–7815. https://doi.org/10.1007/s00216-013-7000-4.
Kalantzi, O.I., Geens, T., Covaci, A., Siskos, P.A., 2011. Distribution of polybrominated diphenyl ethers (PBDEs) and other persistent organic pollutants in human serum from Greece. Environ. Int. 37, 349–353. https://doi.org/10.1016/j.envint.2010.10.005.
Kang, J.-H., Park, H., Chang, Y.-S., Choi, J.-W., 2008. Distribution of organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) in human serum from urban areas in Korea. Chemosphere 73, 1625–1631. https://doi.org/10.1016/j.chemosphere.2008.07.087.
Karimi, B., Nabizadeh Nodehi, R., Yunesian, M., 2020. Serum level of PCBs and OCPs and leukocyte telomere length among adults in Tehran, Iran. Chemosphere 248, 126092. https://doi.org/10.1016/j.chemosphere.2020.126092.
Kato, K., Wong, L.-Y., Jia, L.T., Kuklenyik, Z., Calafat, A.M., 2011. Trends in exposure to polyfluoroalkyl chemicals in the U.S. Population: 1999−2008. Environ. Sci. Technol. 45, 8037–8045. https://doi.org/10.1021/es1043613.
Kim, K., Bennett, D.H., Calafat, A.M., Hertz-Picciotto, I., Shin, H.-M., 2020. Temporal trends and determinants of serum concentrations of per- and polyfluoroalkyl substances among Northern California mothers with a young child, 2009–2016. Environ. Res. 186, 109491. https://doi.org/10.1016/j.envres.2020.109491.
Knutsen, H.K., Alexander, J., Barregård, L., Bignami, M., Brüschweiler, B., Ceccatelli, S., Cottrill, B., Dinovi, M., Edler, L., Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L., Nebbia, C.S., Oswald, I.P, Petersen, A., Rose, M., Roudot, A.-C., Vleminckx, C., Vollmer, G., Wallace, H., Bodin, L., Cravedi, J.P., Halldorsson, T.I., Haug, L.S., Johansson, N., Loveren, van H., Gergelova, P., Mackay, K., Levorato, S., Manen, van M., Schwerdtle, T., EFSA Panel on Contaminants in the Food Chain (CONTAM), 2018. Risk to human health related to the presence of perfluorooctane sulfonic acid and perfluorooctanoic acid in food. EFSA J. 16 (12), 1–284. https://doi.org/10.2903/j.efsa.2018.5194.
Kubwabo, C., Kosarac, I., Lalonde, K., 2013. Determination of selected perfluorinated compounds and polyfluoroalkyl phosphate surfactants in human milk. Chemosphere 91, 771–777. https://doi.org/10.1016/j.chemosphere.2013.02.011.
Lankova, D., Svarcova, A., Kalachova, K., Lacina, O., Pulkrabova, J., Hajslova, J., 2015. Multi-analyte method for the analysis of various organohalogen compounds in house dust. Anal. Chim. Acta 854, 61–69. https://doi.org/10.1016/j.aca.2014.11.007.
Lee, J.H., Lee, C.K., Suh, C.-H., Kang, H.-S., Hong, C.-P., Choi, S.-N., 2017. Serum concentrations of per- and poly-fluoroalkyl substances and factors associated with exposure in the general adult population in South Korea. Int. J. Hyg Environ. Health 220, 1046–1054. https://doi.org/10.1016/j.ijheh.2017.06.005.
Lee, S., Kim, Sunmi, Park, J., Kim, H.-J., Choi, G., Choi, S., Kim, Sungjoo, Kim, S.Y., Kim, Sungkyoon, Choi, K., Moon, H.-B., 2018. Perfluoroalkyl substances (PFASs) in breast milk from Korea: time-course trends, influencing factors, and infant exposure. Sci. Total Environ. 612, 286–292. https://doi.org/10.1016/j.scitotenv.2017.08.094.
Lee, J.E., Oh, H.B., Im, H., Han, S.B., Kim, K.H., 2020a. Multiresidue analysis of 85 persistent organic pollutants in small human serum samples by modified QuEChERS preparation with different ionization sources in mass spectrometry. J. Chromatogr. A 1623, 461170. https://doi.org/10.1016/j.chroma.2020.461170.
Lee, J.E., Oh, H.B., Im, H., Han, S.B., Kim, K.H., 2020b. Multiresidue analysis of 85 persistent organic pollutants in small human serum samples by modified QuEChERS preparation with different ionization sources in mass spectrometry. J. Chromatogr. A 1623, 461170. https://doi.org/10.1016/j.chroma.2020.461170.
Listing of POPs in the Stockholm Convention. accessed 11.2.20. http://chm.pops.int/TheConvention/ThePOPs/ListingofPOPs/tabid/2509/Default.aspx.
Liu, B., Zhang, R., Zhang, H., Yu, Y., Yao, D., Yin, S., 2020. Levels of perfluoroalkyl acids (PFAAs) in human serum, hair and nails in guangdong province, China: implications for exploring the ideal bio-indicator. Arch. Environ. Contam. Toxicol. 79, 184–194. https://doi.org/10.1007/s00244-020-00743-w.
Luque, N., Ballesteros-Gómez, A., van Leeuwen, S., Rubio, S., 2012. A simple and rapid extraction method for sensitive determination of perfluoroalkyl substances in blood serum suitable for exposure evaluation. J. Chromatogr. A 1235, 84–91. https://doi.org/10.1016/j.chroma.2012.02.055.
Luzardo, O.P., Mahtani, V., Troyano, J.M., Álvarez de la Rosa, M., Padilla-Pérez, A.I., Zumbado, M., Almeida, M., Burillo-Putze, G., Boada, C., Boada, L.D., 2009. Determinants of organochlorine levels detectable in the amniotic fluid of women from Tenerife Island (Canary Islands, Spain). Environ. Res. 109, 607–613. https://doi.org/10.1016/j.envres.2009.03.008.
Makey, C.M., McClean, M.D., Sjödin, A., Weinberg, J., Carignan, C.C., Webster, T.F., 2014. Temporal variability of polybrominated diphenyl ether (PBDE) serum concentrations over one year. Environ. Sci. Technol. 48, 14642–14649. https://doi.org/10.1021/es5026118.
Marc-André, Verner, Loccisano Anne, E., Nils-Halvdan, Morken, Yoon, Miyoung, Wu, Huali, Robin, McDougall, Mildred, Maisonet, Marcus, Michele, Reiko, Kishi, Chihiro, Miyashita, Mei-Huei, Chen, Wu-Shiun, Hsieh, Andersen Melvin, E., Clewell Harvey, J., Longnecker Matthew, P., 2015. Associations of perfluoroalkyl substances (PFAS) with lower birth weight: an evaluation of potential confounding by glomerular filtration rate using a physiologically based pharmacokinetic model (PBPK). Environ. Health Perspect. 123, 1317–1324. https://doi.org/10.1289/ehp.1408837.
McComb, J., Mills, I.G., Muller, M., Berntsen, H.F., Zimmer, K.E., Ropstad, E., Verhaegen, S., Connolly, L., 2019. Human blood-based exposure levels of persistent organic pollutant (POP) mixtures antagonise androgen receptor transactivation and translocation. Environ. Int. 132, 105083. https://doi.org/10.1016/j.envint.2019.105083.
Moon, H.J., Lim, J., Jee, S.H., 2017. Association between serum concentrations of persistent organic pollutants and smoking in Koreans: a cross-sectional study. J. Epidemiol. 27, 63–68. https://doi.org/10.1016/j.je.2016.09.006.
Motas Guzmàn, M., Clementini, C., Pérez-Cárceles, M.D., Jiménez Rejón, S., Cascone, A., Martellini, T., Guerranti, C., Cincinelli, A., 2016. Perfluorinated carboxylic acids in human breast milk from Spain and estimation of infant's daily intake. Sci. Total Environ. 544, 595–600. https://doi.org/10.1016/j.scitotenv.2015.11.059.
Nakayama, S.F., Isobe, T., Iwai-Shimada, M., Kobayashi, Y., Nishihama, Y., Taniguchi, Y., Sekiyama, M., Michikawa, T., Yamazaki, S., Nitta, H., Oda, M., Mitsubuchi, H., Sanefuji, M., Ohga, S., Mise, N., Ikegami, A., Suga, R., Shimono, M., 2020. Poly- and perfluoroalkyl substances in maternal serum: method development and application in Pilot Study of the Japan Environment and Children's Study. J. Chromatogr. A 460933. https://doi.org/10.1016/j.chroma.2020.460933.
Nøst, T.H., Breivik, K., Fuskevåg, O.-M., Nieboer, E., Odland, J.Ø., Sandanger, T.M., 2013. Persistent organic pollutants in Norwegian men from 1979 to 2007: intraindividual changes, age–period–cohort effects, and model predictions. Environ. Health Perspect. 121, 1292–1298. https://doi.org/10.1289/ehp.1206317.
Olsen, G.W., Mair, D.C., Lange, C.C., Harrington, L.M., Church, T.R., Goldberg, C.L., Herron, R.M., Hanna, H., Nobiletti, J.B., Rios, J.A., Reagen, W.K., Ley, C.A., 2017. Per- and polyfluoroalkyl substances (PFAS) in American Red Cross adult blood donors, 2000–2015. Environ. Res. 157, 87–95. https://doi.org/10.1016/j.envres.2017.05.013.
Papadopoulou, E., Sabaredzovic, A., Namork, E., Nygaard, U.C., Granum, B., Haug, L.S., 2016. Exposure of Norwegian toddlers to perfluoroalkyl substances (PFAS): the association with breastfeeding and maternal PFAS concentrations. Environ. Int. 94, 687–694. https://doi.org/10.1016/j.envint.2016.07.006.
Pirard, C., Compere, S., Firquet, K., Charlier, C., 2018. The current environmental levels of endocrine disruptors (mercury, cadmium, organochlorine pesticides and PCBs) in a Belgian adult population and their predictors of exposure. Int. J. Hyg Environ. Health 221, 211–222. https://doi.org/10.1016/j.ijheh.2017.10.010.
Ploteau, S., Antignac, J.-P., Volteau, C., Marchand, P., Vénisseau, A., Vacher, V., Le Bizec, B., 2016. Distribution of persistent organic pollutants in serum, omental, and parietal adipose tissue of French women with deep infiltrating endometriosis and circulating versus stored ratio as new marker of exposure. Environ. Int. 97, 125–136. https://doi.org/10.1016/j.envint.2016.08.011.
Poothong, S., Papadopoulou, E., Padilla-Sánchez, J.A., Thomsen, C., Haug, L.S., 2020. Multiple pathways of human exposure to poly- and perfluoroalkyl substances (PFASs): from external exposure to human blood. Environ. Int. 134, 105244. https://doi.org/10.1016/j.envint.2019.105244.
Porta, M., López, T., Gasull, M., Rodríguez-Sanz, M., Garí, M., Pumarega, J., Borrell, C., Grimalt, J.O., 2012. Distribution of blood concentrations of persistent organic pollutants in a representative sample of the population of Barcelona in 2006, and comparison with levels in 2002. Sci. Total Environ. 423, 151–161. https://doi.org/10.1016/j.scitotenv.2012.02.001.
Post, G.B., Gleason, J.A., Cooper, K.R., 2017. Key scientific issues in developing drinking water guidelines for perfluoroalkyl acids: contaminants of emerging concern. PLoS Biol. 15, e2002855 https://doi.org/10.1371/journal.pbio.2002855.
Qiao, L., Zheng, X.-B., Yan, X., Wang, M.-H., Zheng, J., Chen, S.-J., Yang, Z.-Y., Mai, B.-X., 2018. Brominated flame retardant (BFRs) and Dechlorane Plus (DP) in paired human serum and segmented hair. Ecotoxicol. Environ. Saf. 147, 803–808. https://doi.org/10.1016/j.ecoenv.2017.09.047.
Raymer, J.H., Michael, L.C., Studabaker, W.B., Olsen, G.W., Sloan, C.S., Wilcosky, T., Walmer, D.K., 2012. Concentrations of perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) and their associations with human semen quality measurements. Reprod. Toxicol. Elmsford N 33, 419–427. https://doi.org/10.1016/j.reprotox.2011.05.024.
Rugge, B., Balshem, H., Sehgal, R., Relevo, R., Gorman, P., Helfand, M., 2011. Screening and Treatment of Subclinical Hypothyroidism or Hyperthyroidism, AHRQ Comparative Effectiveness Reviews. Agency for Healthcare Research and Quality (US), Rockville (MD).
Shu, H., Lindh, C.H., Wikström, S., Bornehag, C.-G., 2018. Temporal trends and predictors of perfluoroalkyl substances serum levels in Swedish pregnant women in the SELMA study. PloS One 13. https://doi.org/10.1371/journal.pone.0209255.
Sochorová, L., Hanzlíková, L., Černá, M., Drgáčová, A., Fialová, A., Švarcová, A., Gramblička, T., Pulkrabová, J., 2017. Perfluorinated alkylated substances and brominated flame retardants in serum of the Czech adult population. Int. J. Hyg. Environ. Health, Special Issue: Human Biomonitoring 220, 235–243. https://doi.org/10.1016/j.ijheh.2016.09.003, 2016.
Stubleski, J., Salihovic, S., Lind, L., Lind, P.M., van Bavel, B., Kärrman, A., 2016. Changes in serum levels of perfluoroalkyl substances during a 10-year follow-up period in a

large population-based cohort. Environ. Int. 95, 86–92. https://doi.org/10.1016/j.envint.2016.08.002.

Svarcova, A., Lankova, D., Gramblicka, T., Stupak, M., Hajslova, J., Pulkrabova, J., 2019. Integration of five groups of POPs into one multi-analyte method for human blood serum analysis: an innovative approach within biomonitoring studies. Sci. Total Environ. 667, 701–709. https://doi.org/10.1016/j.scitotenv.2019.02.336.

Thomas, A., Toms, L.-M.L., Harden, F.A., Hobson, P., White, N.M., Mengersen, K.L., Mueller, J.F., 2017. Concentrations of organochlorine pesticides in pooled human serum by age and gender. Environ. Res. 154, 10–18. https://doi.org/10.1016/j.envres.2016.12.009.

Toms, L.M.L., Bräunig, J., Vijayasarathy, S., Phillips, S., Hobson, P., Aylward, L.L., Kirk, M.D., Mueller, J.F., 2019. Per- and polyfluoroalkyl substances (PFAS) in Australia: current levels and estimated population reference values for selected compounds. Int. J. Hyg Environ. Health 222, 387–394. https://doi.org/10.1016/j.ijheh.2019.03.004.

Trasande, L., Koshy, T.T., Gilbert, J., Burdine, L.K., Attina, T.M., Ghassabian, A., Honda, M., Marmor, M., Chu, D.B., Han, X., Shao, Y., Kannan, K., 2017. Serum perfluoroalkyl substances in children exposed to the world trade center disaster. Environ. Res. 154, 212–221. https://doi.org/10.1016/j.envres.2017.01.008.

Ulutaş, O.K., Çok, I., Darendeliler, F., Aydin, B., Çoban, A., Henkelmann, B., Schramm, K.-W., 2015. Blood levels of polychlorinated biphenlys and organochlorinated pesticides in women from Istanbul, Turkey. Environ. Monit. Assess. 187 https://doi.org/10.1007/s10661-015-4358-0.

Vorkamp, K., Nielsen, F., Kyhl, H.B., Husby, S., Nielsen, L.B., Barington, T., Andersson, A.-M., Jensen, T.K., 2014. Polybrominated diphenyl ethers and perfluoroalkyl substances in serum of pregnant women: levels, correlations, and potential health implications. Arch. Environ. Contam. Toxicol. 67, 9–20. https://doi.org/10.1007/s00244-013-9988-z.

Wang, Q., Yuan, H., Jin, J., Li, P., Ma, Y., Wang, Y., 2017. Organochlorine pesticide concentrations in pooled serum of people in different age groups from five Chinese cities. Sci. Total Environ. 586, 1012–1019. https://doi.org/10.1016/j.scitotenv.2017.02.082.

Wang, Y., Zhong, Y., Li, J., Zhang, J., Lyu, B., Zhao, Y., Wu, Y., 2018. Occurrence of perfluoroalkyl substances in matched human serum, urine, hair and nail. J. Environ. Sci. 67, 191–197. https://doi.org/10.1016/j.jes.2017.08.017.

Wikström, S., Lin, P.-I., Lindh, C.H., Shu, H., Bornehag, C.-G., 2020. Maternal serum levels of perfluoroalkyl substances in early pregnancy and offspring birth weight. Pediatr. Res. 87, 1093–1099. https://doi.org/10.1038/s41390-019-0720-1.

Wittsiepe, J., Schrey, P., Lemm, F., Eberwein, G., Wilhelm, M., 2008. Polychlorinated dibenzo-p-dioxins/polychlorinated dibenzofurans (PCDD/fs), polychlorinated biphenyls (PCBs), and organochlorine pesticides in human blood of pregnant women from Germany. J. Toxicol. Environ. Health 71, 703–709. https://doi.org/10.1080/15287390801985034.

Worley, R.R., Moore, S.M., Tierney, B.C., Ye, X., Calafat, A.M., Campbell, S., Woudneh, M.B., Fisher, J., 2017. Per- and polyfluoroalkyl substances in human serum and urine samples from a residentially exposed community. Environ. Int. 106, 135–143. https://doi.org/10.1016/j.envint.2017.06.007.

Wu, M., Sun, R., Wang, M., Liang, H., Ma, S., Han, T., Xia, X., Ma, J., Tang, L., Sun, Y., Xu, G., 2017. Analysis of perfluorinated compounds in human serum from the general population in Shanghai by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Chemosphere 168, 100–105. https://doi.org/10.1016/j.chemosphere.2016.09.161.

Ye, X., Kato, K., Wong, L.-Y., Jia, T., Kalathil, A., Latremouille, J., Calafat, A.M., 2018. Per- and polyfluoroalkyl substances in sera from children 3 to 11 years of age participating in the National Health and Nutrition Examination Survey 2013–2014. Int. J. Hyg Environ. Health 221, 9–16. https://doi.org/10.1016/j.ijheh.2017.09.011.

Yu, Y., Lin, B., Qiao, J., Chen, Xi-chao, Chen, W., Li, L., Chen, Xiao-yan, Yang, L., Yang, P., Zhang, G., Zhou, X., Chen, C., 2020a. Levels and congener profiles of halogenated persistent organic pollutants in human serum and semen at an e-waste area in South China. Environ. Int. 138, 105666. https://doi.org/10.1016/j.envint.2020.105666.

Yu, C.H., Riker, C.D., Lu, S., Fan, Z., Tina), 2020b. Biomonitoring of emerging contaminants, perfluoroalkyl and polyfluoroalkyl substances (PFAS), in New Jersey adults in 2016–2018. Int. J. Hyg Environ. Health 223, 34–44. https://doi.org/10.1016/j.ijheh.2019.10.008.

Zani, C., Magoni, M., Speziani, F., Leonardi, L., Orizio, G., Scarcella, C., Gaia, A., Donato, F., 2019. Polychlorinated biphenyl serum levels, thyroid hormones and endocrine and metabolic diseases in people living in a highly polluted area in North Italy: a population-based study. Heliyon 5, e01870. https://doi.org/10.1016/j.heliyon.2019.e01870.

Zhang, Xiaolan, Wu, X., Lei, B., Jing, Y., Jiang, Z., Zhang, Xinyu, Fang, X., Yu, Y., 2018. Transplacental transfer characteristics of organochlorine pesticides in paired maternal and cord sera, and placentas and possible influencing factors. Environ. Pollut. 233, 446–454. https://doi.org/10.1016/j.envpol.2017.10.075.

Zheng, J., He, C.-T., Chen, S.-J., Yan, X., Guo, M.-N., Wang, M.-H., Yu, Y.-J., Yang, Z.-Y., Mai, B.-X., 2017. Disruption of thyroid hormone (TH) levels and TH-regulated gene expression by polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs), and hydroxylated PCBs in e-waste recycling workers. Environ. Int. 102, 138–144. https://doi.org/10.1016/j.envint.2017.02.009.

Zubero, M.B., Eguiraun, E., Aurrekoetxea, J.J., Lertxundi, A., Abad, E., Parera, J., Goñi-Irigoyen, F., Ibarluzea, J., 2017. Changes in serum dioxin and PCB levels in residents around a municipal waste incinerator in Bilbao, Spain. Environ. Res. 156, 738–746. https://doi.org/10.1016/j.envres.2017.04.039.

APPENDIX VIII

Richterova D., Fabelova L., Patayova H., Pulkrabova J., Lankova D., Rausova K., Sovcikova E., Stencl J., Hajslova J., Trnovec T., Palkovicova Murinova L.

Determinants of prenatal exposure to perfluoroalkyl substances in the Slovak birth cohort

Environment International (2018) 121 (2):1304–1310

doi: 10.1016/j.envint.2018.10.051

Environment International 121 (2018) 1304–1310

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Determinants of prenatal exposure to perfluoroalkyl substances in the Slovak birth cohort

Denisa Richterová[a], Lucia Fábelová[a], Henrieta Patayová[a], Jana Pulkrabová[b], Darina Lanková[b], Katarína Rausová[a], Eva Šovčíková[a], Ján Štencl[c], Jana Hajšlová[b], Tomas Trnovec[a], Ľubica Palkovičová Murínová[a,*]

[a] Slovak Medical University, Faculty of Public Health, Department of Environmental Medicine, Bratislava, Slovakia
[b] University of Chemistry and Technology, Prague, Department of Food Analysis and Nutrition, Prague, Czech Republic
[c] Slovak Medical University, Bratislava, Slovakia

ARTICLE INFO

Handling Editor: Heather Stapleton

Keywords:
Perfluoroalkyl substances
Determinants
Prenatal exposure
Cord blood

ABSTRACT

Background: Perfluoroalkyl substances (PFASs) are man-made fluorinated compounds with endocrine-disrupting properties, detected in 99% of serum samples worldwide and associated with adverse childhood health outcomes. The aim of this study was to describe determinants of prenatal exposure to PFASs in Slovakia.

Methods: This study was based on Slovak multicentric prospective mother-child cohort PRENATAL ($N = 796$). Cord blood samples were collected within 2010–2012 and PFASs were analyzed in a subpopulation of 322 newborns. Concentrations of perfluorooctane sulfonic acid (PFOS), perfluorohexane sulfonic acid (PFHxS), perfluorooctanoic acid (PFOA) and perfluorononanoic acid (PFNA) were measured in the samples of cord blood using an ultrahigh-performance liquid chromatography- mass spectrometry (U-HPLC−MS) method. From questionnaires, we obtained information on medical history of mother, socio-demographic factors, nutrition and environmental factors. Association between maternal characteristics and PFASs exposure was analyzed using multivariable linear regression models.

Results: The highest cord blood concentration (geometric mean ± SD) was observed for PFOA (0.79 ± 2.21 ng/ml) followed by PFOS (0.36 ± 2.56 ng/ml), PFNA (0.20 ± 2.44 ng/ml) and PFHxS (0.07 ± 2.36 ng/ml). Primiparity was associated with higher levels of all four PFAS: PFOS (exp. β = 1.25; 95%CI[1.03; 1.53]), PFOA (exp. β = 1.49; 95%CI[1.18; 1.89]), PFNA (exp. β = 1.30; 95%CI[1.05; 1.60]) and PFHxS (exp. β = 1.49; 95%CI [1.20; 1.86]). In addition, maternal age category 29 years and more was associated with higher PFNA and PFHxS levels (exp. β = 1.27; 95%CI[1.04; 1.55] and exp. β = 1.30; 95%CI[1.06; 1.60], respectively) and higher educational level of mother was associated with higher PFNA levels (exp. β = 1.32; 95%CI[1.04; 1.68]). Higher fish consumption was associated with lower PFNA levels (exp. β = 0.49; 95%CI [0.26; 0.92]).

Conclusions: We observed that PFASs cord blood concentrations were comparable or lower than those measured in western or northern European countries. We identified parity as the main determinant of PFASs exposure in our population and maternal age and education as factors that might be associated with exposure to certain PFASs.

1. Introduction

Perfluoroalkyl substances (PFASs) are man-made fluorinated compounds with high thermal and chemical stability and surface activity. They have been used for over 60 years as surfactants for a wide range of materials and products (e.g. textiles, packaging materials, furniture, cleaning and polishing agents, etc.) (EFSA, 2012). Due to their environmental persistence and capacity to undergo long-range transport, PFASs have been detected in air, dust, surface and ground water, soil and sediment even in remote areas (ATSDR, 2015). The two most studied PFASs and also the final products of degradation of many PFASs, perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) (Quinete et al., 2010), were found in 99% of human serum samples in various studies around the world (Bartolomé et al., 2017; Berg et al., 2014; Calafat et al., 2007; Cariou et al., 2015; Dereumeaux et al., 2016; Kato et al., 2014; Liu et al., 2011; Manzano-Salgado et al.,

* Corresponding author at: Slovak Medical University, Limbová 12, 83303 Bratislava, Slovakia.
E-mail address: lubica.murinova@szu.sk (Ľ. Palkovičová Murínová).

https://doi.org/10.1016/j.envint.2018.10.051
Received 13 June 2018; Received in revised form 1 October 2018; Accepted 25 October 2018
Available online 09 November 2018

2016; Mørck et al., 2015; Ode et al., 2013; Schoeters et al., 2017; Sochorova et al., 2017). Dietary exposure has been suggested as the main route of exposure, however, humans are exposed to PFASs through many pathways. Children can be exposed to PFASs prenatally through placenta, and postnatally via breastmilk and hand-to-mouth activities (ATSDR, 2015; Jusko et al., 2016). As a result of their bioaccumulation and environmental persistence, PFOS, its salts and perfluorooctane sulfonyl fluoride (PFOS-F) were classed as persistent organic pollutants (POPs) by Stockholm Convention (PFOA and perfluorohexane sulfonic acid (PFHxS) currently under review) and production of these substances was restricted (Stockholm Convention, 2009).

Organs and organ systems of child are developing during fetal and early postnatal period, therefore children are particularly vulnerable group, very sensitive to adverse health effects of different environmental stressors. PFASs have endocrine-disrupting properties and recent studies reported associations between maternal PFASs concentrations and health outcomes in children. Maternal PFOS and PFOA concentrations were associated with increased weight, BMI *Z*-score, waist circumference, body fat and overweight status in children (Braun et al., 2016; Høyer et al., 2015; Maisonet et al., 2012). Studies also suggested that cholesterol and insulin levels might be influenced by PFAS concentration in serum (Frisbee et al., 2010; Geiger et al., 2014; Maisonet et al., 2015a; Timmermann et al., 2014; Zeng et al., 2015). PFASs may also affect antibody response to vaccination (Grandjean et al., 2012; Granum et al., 2013; Stein et al., 2015) and positive association was found between PFOS, PFOA and asthma (Dong et al., 2013). There is an evidence for negative effects of PFASs on thyroid function of children (Kim et al., 2011; Kim et al., 2016; Lopez-Espinosa et al., 2012) and sexual hormones in puberty (Kristensen et al., 2013; Lopez-Espinosa et al., 2011; Maisonet et al., 2015b).

In previous studies, maternal PFASs concentrations were associated with age, parity and breastfeeding (Bartolomé et al., 2017; Berg et al., 2014; Cariou et al., 2015; Kato et al., 2014; Manzano-Salgado et al., 2016; Ode et al., 2013), since they bioaccumulate in liver, kidney and bladder through years and both, pregnancy and breastfeeding, represent important routes for PFAS elimination from a body of mother but also for their transition to an infant's body (ATSDR, 2015). Transport through placental barrier and breastmilk was observed and exposure through lactation was higher compared to exposure during gestation (Liu et al., 2011). A country of origin was previously associated with PFAS serum concentrations as their concentrations were higher in samples of women originating from the Nordic countries compared to those of women from the Middle East, North Africa, and sub-Saharan Africa in a Swedish study (Ode et al., 2013) and lower in serum of black women compared to those of white and Hispanic women in a US Multiethnic Cohort study (Kato et al., 2014). In the same study, low income was associated with lower PFASs concentrations (Kato et al., 2014). Considering diet as the main source of exposure, some studies reported high intake of fish and shellfish during pregnancy associated with increased PFOS concentrations (Berg et al., 2014; Manzano-Salgado et al., 2016).

Exposure to PFASs has been studied in many European countries, however, in Slovakia this is the first study to investigate prenatal PFASs levels. Slovakia belongs among countries where fish and seafood consumption is the lowest among EU-28 sub-regions (EUMOFA, 2017). In the present study, we aimed to describe concentrations of PFASs in cord blood and to assess determinants of exposure during pregnancy.

2. Materials and methods

2.1. Population

Within 2010–2012, the Slovak multicentric prospective mother-child cohort PRENATAL was launched in 8 regions covering all parts of Slovakia. Pregnant women were recruited by regional gynecologists after confirmation of pregnancy (8th to 10th week of pregnancy). After signing a written informed consent, participants were interviewed by trained nurses and questionnaires were administered. From the obstetrical record, we collected data on sex of newborns, gestational age (weeks), birth weight (g), maternal age at delivery (years) and parity (primiparous yes/no). We also collected socio-demographic data on mothers from self-reported questionnaires: marital status (2 categories: married = married or living in a couple; single = unmarried, divorced or widowed), education (2 categories: lower = without high school diploma; higher = with high school or university diploma), employment (2 categories: employed (students incl.); unemployed (maternity leave incl.)), pre-pregnancy BMI (lean < 17.99; normal 18–25; overweight or obese > 25.1) and weight gain during pregnancy (kg) as well as smoking before and during pregnancy (yes/no) and alcohol consumption before pregnancy (yes/no). Data on fast food and fish consumption were collected from food frequency questionnaire (FFQ). Weekly intake of fast food in kilograms was calculated based on frequency and amount of four food items consumed: pizza, hamburger, hot dog and kebab. Fish intake was calculated as a sum of sea fish, freshwater fish and canned fish consumption in kilograms per week. 796 pregnant women were followed until birth, when cord blood samples were taken. PFASs were analyzed in a subpopulation of 322 newborns. The study was approved by the Ethical Committee of the Slovak Medical University in Bratislava.

2.2. Sample preparation and standards

An amount of 30 ml cord blood was put in a centrifuge in order to separate the serum. Serum samples (1 ml) were stored at −20 °C until transferred to the laboratory at the Department of Food Analysis and Nutrition, University of Chemistry and Technology, Prague, Czech Republic.

The sample preparation procedure was based on extraction to the acetonitrile. Briefly, an amount of 0.5 ml of the serum was placed into the 15 ml centrifuge tube, then the addition of 50 µl of formic acid and 5 ml of acetonitrile was done. The tube was shaken by hand for 1 min. Subsequently, 1 g of magnesium sulphate was added followed by another shaking for 1 min. After that the tube was centrifuged (5 min, 10,000 rpm) and 3.5 ml of a primary extract was transferred into the flask and evaporated using a rotary vacuum evaporator near to dryness. The residues were dissolved in 250 µl of methanol. The reconstituted extract was filtered through a 0.22-µm nylon centrifuge tube filter and placed into the glass vial for the U-HPLC–MS/MS measurement.

The list of target individual standards including their isotopically labelled analogues is in Supplementary information. The working standards mixtures of PFASs were prepared at concentrations 0.05, 0.1, 0.5, 1, 5 and 10 ng/ml in methanol. Each calibration standards contained internal standards of ^{13}C-PFASs at 1 ng/ml.

2.3. Chemicals, reagents and other materials

Acetonitrile and high performance LC grade methanol were delivered by Merck (Germany). Magnesium sulphate (98%), formic acid (98%), HPLC grade ammonium acetate (99.99%) and polypropylene centrifuge tube filters (pore size 0.22 µm, nylon) were obtained from Sigma-Aldrich (USA). Deionised water was purified by a Milli-Q® Integral system (Merck), which does not include PFASs containing polymers, was used in the study. The human blood serum used for the method development and validation experiments was provided by Sigma-Aldrich (USA).

2.4. Instrumental analysis

The U-HPLC analyses of PFASs were carried out using an Acquity Ultra-Performance LC system (Waters, USA) with a 10-µL sample loop (sample injection was 5 µl). Target compounds were

chromatographically separated on a BEH C18 (100 × 2.1 mm i.d., 1.7 µm, Waters, USA) heated on 40 °C. The column isolator (50 mm × 2.1 mm i.d., Waters, USA) was placed between the sample valve and the mixer of mobile phases in order to separate background contamination from the analyzed serum sample. The flow rate of a mobile phase, which was consisted of (A) 5 mM ammonium acetate in deionised water and B) methanol, started at 0.3 ml/min. The elution gradient conditions were: 10–50% B over 0.5 min, then 50–100% B over 7.5 min followed by an isocratic hold at 100% B for 4 min. The total run time of instrumental analysis for one injection was 15 min.

The LC system was coupled to a triple–quadrupole mass spectrometer Xevo TQ-S (Waters, USA) equipped with electrospray ionisation (ESI) that was set in the negative ion mode with a capillary voltage of −4000 V. The desolvation and ionisation temperatures were 400 and 120 °C, respectively. The MS instrument was operated in a multiple reaction monitoring (MRM) mode. The further information about the LC–MS method is described in detail by Lankova et al. (2013).

2.5. Quality assurance and quality control (QA/QC)

The performance characteristics of the method were assessed through the analysis of the artificially contaminated serum sample (obtained from Sigma-Aldrich), which was previously analyzed for the detection of present concentration of PFASs. The repeatabilities (expressed as relative standard deviations, RSDs, %) and the recoveries (REC, %) for target analytes were calculated from six analyses at a concentration level of 0.1 ng/ml. To control background contamination by PFASs, the procedural blank (i.e. using the same sample procedure, only instead of serum, the 5 ml of deionised water was used) was prepared together with each set of 20 samples (per day). The limits of quantification (LOQs) were determined as the lowest calibration standard at which analyte provided a signal-to-noise ratio (S/N) higher than 10. For the compensation of the matrix effects or losses of PFASs, the whole method was validated using isotopically labelled surrogates.

Regarding to the natural concentration of PFASs in tested human serum used for the validation experiments only PFOS, PFOA and PFNA were present at the concentrations near LOQ (0.01–0.05 ng/ml). Recoveries of PFASs were in the range of 87–120% with the RSD < 20%. The LOQs were determined as 0.05 ng/ml for PFCAs and 0.02 ng/ml for FOSAs and PFSAs.

2.6. Statistical analysis

Statistical analyses were performed using SPSS 20. PFASs levels were measured in a subpopulation of 322 newborns and general characteristics of mothers in subpopulation did not differ from the original PRENATAL cohort ($N = 796$; Table 1). The following PFASs compounds were analyzed: PFOA, PFOS, PFHxS, PFNA, PFBA, PFPeA, PFHxA, PFHpA, PFDA, PFUdA, PFDoA, PFTrDA, PFTeDA, PFBS, PFDS, PFOSA, NMeFOSA and NEtFOSA. Only PFASs with > 80% of samples > LOD were included in further analyses, namely: PFOS, PFHxS, PFOA and PFNA. List of measured PFASs with % of their detection is shown in Supplementary table 1. PFASs concentrations < LOD were imputed using LOD/√2. Since data on PFASs exposure were skewed to the left (range of skewness coefficients 2.4–7.2), concentrations were log-transformed to the base 10 in order to improve normality of the data distribution. We described PFASs concentrations by socio-demographic subgroups within our study population using Kruskal-Wallis and Mann-Whitney tests. Based on literature, we used following covariates – gender, maternal age, gestational age, parity, pre-pregnancy BMI, weight gain during pregnancy, marital status, education, employment, smoking and alcohol consumption before pregnancy, fish consumption and fast food consumption. Correlations among individual PFASs were examined using Spearman's correlation. In the multivariable linear regression models, we included covariates based on data from literature and results from bivariate regression (Supplementary table 2). The final model included: maternal age (2 categories based on median: under 29 years, 29 years and more), parity, educational level of mother and fish consumption. Gender was not included in the final multivariable regression models, based on the results from bivariate analysis, furthermore, sensitivity analysis with gender included in the final model did not significantly change the results (not shown). Employment was strongly associated with parity, maternal age and education, hence it was not included in the final model. Additionally, strong predictors for fast food consumption were both, parity and maternal age, hence, this variable was not included in the final multivariate model. Since our outcome variables were log-transformed, we exponentiated beta coefficients for easier interpretation. Therefore, presented effect estimates are proportional changes in the geometric mean of PFASs concentrations in each category compared to reference group.

Table 1
General characteristics of study population, the whole PRENATAL cohort and general population of women aged 19 to 44 years in Slovakia.

Characteristics	Study population ($n = 322$) % or mean ± std dev	PRENATAL cohort ($n = 796$) % or mean ± std dev	General population ($n = 1,058,977$) % or mean ± std dev
Children gender			
Female	45.9	46.7	–
Male	54.1	53.3	
Gestational age (weeks)	39.4 ± 1.3	39.4 ± 1.4	–
Birthweight (g)	3382 ± 435	3351 ± 474	–
Maternal age at delivery (years)	28.7 ± 4.7	28.9 ± 4.6	–
Primiparous	34.6	38.7	–
Pre-pregnancy BMI (kg/m^2)			
Lean	3.9	3.9	–
Normal	70.4	71.8	
Overweight or obese	25.7	24.4	
Weight gain during pregnancy (kg)	14.0 ± 4.8	13.9 ± 5.1	–
Marital status			
Married	85.1	79.6*	44.8***
Single	14.9	20.4	55.2
Education			
Higher	79.8	82.1	69.3***
Lower	20.2	17.9	30.7
Employment			
Employed	61.5	65.1	66.2
Unemployed	38.5	34.9	33.8
Smoking			
Before pregnancy	27.1	28.8	14.6***
During pregnancy	5.6	6.3	–
Drinking alcohol before pregnancy	45.3	49.3	–
Fish consumption (kg/week)	0.19	0.19	–
Fast food consumption (kg/week)	0.16	0.16	–

* $p < 0.05$.
*** $p < 0.001$.

3. Results

General characteristics of the mother-child birth cohort are summarized in Table 1. Among 322 children, 54.1% were boys. The mean (± SD) gestational age was 39.4 (± 1.3) weeks. Mothers were 28.7 (± 4.7) years old at delivery and for 34.6% it was their first child. 85.1% of mothers were married or living in a couple. Majority of mothers was employed or studied (61.5%) and 79.8% had higher educational level. Pre-pregnancy BMI was lower than normal in 3.9%. On the

Table 2
PFASs cord blood concentrations in the Slovak mother-child cohort (n = 322).

Chemical (ng/ml)	< LOD %	Geometric mean ± std dev	25th percentile	50th percentile	75th percentile
PFOA	0.3	0.79 ± 2.21	0.50	0.88	1.47
PFOS	0	0.36 ± 2.56	0.36	0.64	1.01
PFNA	0.6	0.20 ± 2.44	0.14	0.24	0.35
PFHxS	6.9	0.07 ± 2.36	0.05	0.08	0.12

other hand, 25.7% of mothers were classified as overweight or obese before pregnancy. 27.1% of women were smokers before pregnancy, but only 5.6% reported active smoking during pregnancy. Compared to general population of Slovak women of the same age and the whole PRENATAL cohort, there was lower percentage of employed women in our study population (66%, 65% and 61% respectively). On the other hand, percentage of women with higher education in our population was significantly higher compared to general population but comparable to total PRENATAL cohort (79%, 69% and 82% respectively). We had also significantly higher percentage of married women (85% compared to 45% in general population and 79% in PRENATAL cohort) and 27% of women in our population were smoking before pregnancy (29% in PRENATAL cohort) compared to 15% of daily smokers in general population. There was no significant difference in other characteristics between study population and the whole PRENATAL cohort.

Measurable PFOS concentrations were detected in the whole study population, while 6.9% of PFHxS concentrations in cord blood were < LOD (Table 2). The highest concentration (geometric mean ± SD) in cord blood was observed for PFOA (0.79 ± 2.21 ng/ml), followed by PFOS (0.36 ± 2.56 ng/ml). Low PFHxS levels of 0.07 ± 2.36 ng/ml were measured in cord blood. We observed concentrations of all four PFASs to be highly correlated (Table 3), with the strongest correlation between PFOA and PFNA cord blood levels (r_s = 0.81, $p < 0.001$).

PFOS levels were significantly higher in primiparous compared to multiparous women (0.74 vs. 0.55 ng/ml respectively), women aged 29 years and more compared to women under 29 years (0.44 vs. 0.36 ng/ml respectively) and in those, who were employed compared to unemployed ones (0.73 and 0.53 ng/ml respectively; Table 4). Similar results were observed for PFHxS. PFOA cord blood concentrations were significantly higher in primiparous than multiparous women (1.00 vs. 0.81 ng/ml respectively), in those, who were employed compared to unemployed ones (1.00 vs. 0.62 ng/ml respectively) and those with higher compared to lower level of education (0.91 vs. 0.75 ng/ml respectively). Likewise, PFNA concentrations were significantly higher in primiparous women, older women, employed women and those with higher education. We did not observe significant difference in median concentrations of PFAS by categories of pre-pregnancy BMI, marital status or smoking and drinking alcohol before pregnancy.

In multivariable regression models, the 4 PFASs analyzed in our study were associated with parity (Table 5). Primiparity was associated with higher levels of PFOS (exp. β = 1.25; 95%CI[1.03; 1.53]), PFOA (exp. β = 1.49 95%CI[1.18; 1.89]), PFNA (exp. β = 1.30; 95%CI[1.05; 1.60]) and PFHxS (exp. β = 1.49; 95%CI [1.20; 1.86]). In addition, maternal age category 29 years and more was associated with higher PFNA and PFHxS levels (exp. β = 1.27; 95%CI[1.04; 1.55]and exp. β = 1.30; 95%CI[1.06; 1.60], respectively) and higher educational level of mother was associated with higher PFNA levels (exp. β = 1.32; 95%CI[1.04; 1.68]). Higher fish consumption was associated with lower PFNA levels (exp. β = 0.49; 95%CI[0.26; 0.92]).

Table 3
Correlation between PFASs cord blood concentrations in the Slovak mother-child cohort (n = 322).

Chemical	PFOA	PFOS	PFNA	PFHxS
PFOA	1.00	0.58***	0.81***	0.65***
PFOS		1.00	0.74***	0.78***
PFNA			1.00	0.69***
PFHxS				1.00

*** $p < 0.001$.

Table 4
PFASs median cord blood concentrations in Slovak mother-child cohort by socio-demographic characteristics (n = 322).

Chemical (ng/ml)	PFOA	PFOS	PFNA	PFHxS
Age at delivery				
Under 29 years	0.82	0.36*	0,22*	0.07**
29 years and more	0.97	0.44	0.27	0.09
Parity				
Primiparous	1,00***	0.74*	0.28*	0.10**
Multiparous	0.81	0.55	0.22	0.07
Pre-pregnancy BMI (kg/m^2)				
Lean	0.70	0.47	0.23	0.09
Normal	0.90	0.38	0.23	0.08
Overweight or obese	0.87	0.43	0.26	0.09
Marital status				
Married	0.87	0.40	0.24	0.08
Single	0.86	0.38	0.25	0.08
Education				
Higher	0.91*	0.66	0.25*	0.09
Lower	0.75	0.51	0.20	0.07
Employment				
Employed	1.00***	0.73**	0.28***	0.09***
Unemployed	0.62	0.53	0.019	0.07
Smoking before pregnancy				
No	0.87	0.40	0.25	0.08
Yes	0.87	0.37	0.23	0.08
Drinking alcohol before pregnancy				
No	0.83	0.36	0.22	0.08
Yes	0.93	0.41	0.26	0.08

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 5
Results of multivariable linear regression analysis of the associations between determinants and PFASs in cord blood in Slovak mother-child cohort (n = 322); exponentiated β coefficients with 95%CI.

Chemical (ng/ml)	PFOA	PFOS	PFNA	PFHxS
Age at delivery				
Under 29 years	Ref.	Ref.	Ref.	Ref.
29 years and more	1.21 [0.97; 1.52]	1.18 [0.97; 1.42]	1.27 [1.04; 1.55]*	1.30 [1.06; 1.60]*
Parity				
Multiparous	Ref.	Ref.	Ref.	Ref.
Primiparous	1.49 [1.18; 1.89]**	1.25 [1.03; 1.53]*	1.30 [1.05; 1.60]*	1.49 [1.20; 1.86]**
Education				
Lower	Ref.	Ref.	Ref.	Ref.
Higher	1.27 [0.98; 1.66]	1.19 [0.95; 1.49]	1.32 [1.04; 1.68]*	1.24 [0.97; 1.58]
Fish consumption	0.60 [0.30; 1.20]	0.81 [0.45; 1.46]	0.49 [0.26; 0.92]*	0.53 [0.28; 1.02]

* $p < 0.05$.
** $p < 0.01$.

4. Discussion

We described determinants of PFASs exposure in the Slovak mother-child cohort. The main determinant of PFASs prenatal exposure was parity. Overall, multiparous women had significantly lower cord blood concentrations of all measured PFASs in this population. Furthermore, higher age of mother was associated with higher levels of PFNA and PFHxS, higher educational level was associated with higher PFNA levels

and higher fish consumption with lower PFNA levels.

Prenatal PFASs concentrations vary across Europe (Supplementary table 3). In general, PFASs cord blood concentrations in our study were lower compared to concentrations measured in Sweden (Ode et al., 2013) and Spain (Manzano-Salgado et al., 2015) but comparable with those in Norway (Gützkow et al., 2012) or France (Cariou et al., 2015). A difference in PFOS and PFOA cord blood levels was observed among European countries, with highest levels found in Sweden (median = 6.5 and 1.7 ng/ml, respectively; Ode et al., 2013) and the lowest levels measured in our study (median = 0.6 and 0.9 ng/ml, respectively). The considerable difference in prenatal PFASs exposure may be due to different time period when samples were taken. In Swedish study, samples taken between 1978 and 2001 reflect exposure before implementation of restriction on PFOS production and usage in 2009 (Stockholm Convention, 2009), which could explain higher levels of PFOS compared to our samples. Comparing our results with other European studies performed after 2009, PFOS levels in Slovakia (median = 0.6 ng/ml; geom. mean = 0.4 ng/ml) were lower than those in French (median = 1.1 ng/ml) and Flemish studies (geom. mean = 1.1 ng/ml) (Cariou et al., 2015; Schoeters et al., 2017). In contrast to Swedish, French and Norwegian studies, we observed higher levels of PFOA than PFOS. PFOA levels measured in our study (geom. mean = 0.8 ng/ml; median = 0.9 ng/ml) were lower than those in the Flemish cohort (geom. mean = 1.2 ng/ml) (Schoeters et al., 2017), but comparable to levels in France (median = 0.9 ng/ml) (Cariou et al., 2015). PFOA and its salts are currently under assessment and will be potentially restricted as well (Stockholm Convention, 2009), however, it is still in use and that might be one of the reasons, why we observed slightly higher PFOA compared to PFOS levels, to which restrictions were already applied. PFOA levels were observed to be higher also in cord blood collected within 2007–2009 in Germany (Fromme et al., 2010). One of the sources of PFOA exposure in general population is drinking water (Post et al., 2012). Unfortunately, no information on PFASs levels in drinking water or in other relevant environmental matrices in Slovakia is available.

Median concentration of PFNA in our study (0.2 ng/ml) was similar to other European studies (Sweden = 0.2 ng/ml; Norway = 0.1 ng/ml) (Gützkow et al., 2012; Ode et al., 2013). In Sweden and France, PFNA was detected only in 40 and 74% of samples, respectively (Cariou et al., 2015; Ode et al., 2013), compared to 99% in our study. Comparing our results with other studies conducted in Europe mentioned above, we have to consider that the sample size of some of the studies is relatively small and we have limited information on their representativeness.

Regarding determinants of PFASs exposure, consistent with previous studies (Berg et al., 2014; Cariou et al., 2015; Kato et al., 2014; Manzano-Salgado et al., 2016; Ode et al., 2013), parity appears to be the most important determinant of PFASs concentrations in cord blood in our population. Berg et al. (2014), who investigated levels of 26 PFASs in maternal blood, observed that parity was the strongest predictor, and similarly to our findings, multiparous women had lower concentrations compared to nulliparous ones. *trans*-Placental passage is a significant route of human exposure to PFASs, however maternal concentrations decrease during pregnancy as PFASs cross placental barrier with a certain amount distributed to fetus, making it also important way of PFASs elimination from the body of mother (Cariou et al., 2015).

Higher PFNA levels in cord blood were significantly associated with higher maternal education in our study and the same trend was found for other PFASs in our study as well. Similar in Norway, pregnant women in the highest education category had 45% higher PFNA plasma levels and also higher PFOS and PFHxS levels compared to the lowest education category (Brantsaeter et al., 2013). Higher education was also associated with higher PFASs levels in pregnant women in Denmark (Bjerregaard-Olesen et al., 2016) and higher PFOA concentrations in breast milk in mothers from Czech Republic (SZÚ, 2015). This association may be partly explained by higher income of women with higher education, thus they can afford to buy products containing PFASs, which may be more expensive (e.g. impregnated materials). Another explanation could be that higher education may affect diet preferences, in favor of healthier diet, including e.g. more fish. In our population, despite the low fish consumption in general, we observed the association between higher education of mothers and higher fish consumption. Higher education can open more opportunities for employment as well. In bivariate analysis PFASs levels were higher in women, who were employed or student, which means they probably have less time for eating during day and they possibly prefer fast food. We observed a non-significant trend ($p = 0.09$) for higher fast food consumption among employed women compared to unemployed women in our study population. Zagorsky and Smith (2017) found an association between number of working hours and increased fast food consumption. Fast food is usually packed in materials containing PFASs (Schaider et al., 2017), therefore the levels might be higher in people eating fast food regularly. On the other hand, fast food, being usually cheaper than healthier food, may be preferred by women with lower socioeconomic status (SES). In case we use education level as a proxy marker for SES, we would expect the association in opposite direction than it was found in our study, it means lower education associated with higher PFASs exposure. Nevertheless, we analyzed the association between PFASs levels and consumption of fast food and we did not find any significant association in our study.

One of the main non-occupational sources of PFASs exposure in human population represents fish consumption (EFSA, 2012). The majority, if not all published studies on PFASs determinants in cord blood were performed in populations from coastal countries with fish being the important part of their diet (e.g. Spain, Norway, Sweden, France). Slovak Republic belongs among the Central European countries where fish and seafood consumption is the lowest among EU-28 sub-regions (7.8 kg/per capita per year in Slovakia in 2014; EUMOFA, 2017). In our study, average fish consumption was 188 g/week (9.7 kg/year), which is higher than reported by EUMOFA (2017), but still very low compared to EU-28 average (25.8 kg/year). With respect to PFASs exposure in our study, we found an inverse association between fish consumption and PFNA levels, and the similar non-significant trend was observed for other PFASs. Although unexpected, these results point out that in our population, fish consumption does not seem to be a main determinant of PFASs exposure.

This study has several limitations. First, most of the women in our sample were highly educated (80%), thus findings from this study might not be representative for population with high percentage of women with lower education. During recruitment, women with different SES were approached in regions across the whole country and their involvement was based solely on the signed informed consent. Despite this approach, we have overrepresented women with higher education. Second, we had higher percentage of married women, compared to general Slovak population, which could be explained by women being pregnant in our study thus they are more likely to be married and also high proportion of women from regions, where people stick to traditional family concept more than they do in big cities. Next, smoking in pregnancy was self-reported in the questionnaire, it was not objectified by cotinine measurement in urine, hence, it could result in misclassification of smoking exposure in our study group, since women tend to lower their exposure to tobacco smoke due to its known negative effects on health of child. This issue possibly occurs with alcohol consumption and smoking before pregnancy as well. Compared to general population, we had higher percentage of smoking in our study. This difference is probably caused by the different way of data collection - we did not collected data on daily smokers as it was done on the national level, thus part of women in our population might be smoking occasionally only (we used the question: “Did you smoke during the last year? Yes/No”). And finally, food frequency questionnaire administered at the beginning our study did not focus on fast food consumption specifically and only 4 items (hot-dog, hamburger, pizza and kebab)

were included. This could underestimate fast food consumption of pregnant women in our study.

Regarding strengths of our study, we should mention the sample size that was relatively big, if compared to other European studies on PFASs exposure in cord blood. Furthermore, we recruited pregnant women in all parts of Slovakia and only inclusion criterion was the signed informed consent, therefore, we did not exclude any population group based on the SES or other characteristics (e.g. urban vs. rural regions). Data on mothers were collected prospectively during pregnancy and not only at delivery and that could increase the validity of our questionnaire data.

The current study is the first to investigate prenatal exposure to PFASs and its determinants in eastern part of Europe. Even though the fish consumption in our country is low, PFASs levels that we observed are comparable to those in northern or western countries, pointing out to possible different sources of PFASs exposure in Slovak population.

5. Conclusion

In conclusion, this study points out parity as the main determinant of PFASs exposure in our population. No association between PFASs cord blood levels and BMI, smoking or drinking alcohol before pregnancy was found in our study, however, we observed association between PFNA concentration and educational level of mother. Education and consumption of different types of food as determinants of PFASs exposure are worth of further investigation.

Acknowledgements

This study was supported by Ministry of Health of the Slovak Republic Grants 2007/07-SZU-03, 2012/41-SZU-05, 2012/47-SZU-11, Slovak Research and Development Agency Grants APVV-0444-11, APVV-0571-12, DO7RP-0030-12, 7th FP EU project DENAMIC (no. 282957) and by the project “Center of Excellence of Environmental Health” ITMS No. 26240120033, based on the supporting Operational Research and Development Program financed by the European Regional Development Fund.

Declarations of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2018.10.051.

References

ATSDR, 2015. Toxicological Profile for Perfluoroalkyls. U.S. Department of Health and Human Services, Public Health Service, Atlanta, GA.

Bartolomé, M., Gallego-Picó, A., Cutanda, F., Huetos, O., Esteban, M., Pérez-Gómez, B., Castaño, A., 2017. Perfluorinated alkyl substances in Spanish adults: geographical distribution and determinants of exposure. Sci. Total Environ. 603, 352–360.

Berg, V., Nøst, T.H., Huber, S., Rylander, C., Hansen, S., Veyhe, A.S., Fuskevåg, O.M., Odland, J., Sandanger, T.M., 2014. Maternal serum concentrations of per-and polyfluoroalkyl substances and their predictors in years with reduced production and use. Environ. Int. 69, 58–66.

Bjerregaard-Olesen, C., Bach, C.C., Long, M., Ghisari, M., Bech, B.H., Nohr, E.A., Henriksen, T.B.Ø., Bonefeld-Jørgensen, E.C., 2016. Determinants of serum levels of perfluorinated alkyl acids in Danish pregnant women. Int. J. Hyg. Environ. Health 219 (8), 867–875.

Brantsaeter, A.L., Whitworth, K.W., Ydersbond, T.A., Haug, L.S., Haugen, M., Knutsen, H.K., Thomsen, C., Meltzer, H.M., Becher, G., Sabaredzovic, A., Hoppin, J.A., Eggesbø, M., Longnecker, M.P., 2013. Determinants of plasma concentrations of perfluoroalkyl substances in pregnant Norwegian women. Environ. Int. 54, 74–84.

Braun, J.M., Chen, A., Romano, M.E., Calafat, A.M., Webster, G.M., Yolton, K., Lanphear, B.P., 2016. Prenatal perfluoroalkyl substance exposure and child adiposity at 8 years of age: the HOME study. Obesity 24 (1), 231–237.

Calafat, A.M., Wong, L.Y., Kuklenyik, Z., Reidy, J.A., Needham, L.L., 2007. Polyfluoroalkyl chemicals in the US population: data from the National Health and Nutrition Examination Survey (NHANES) 2003–2004 and comparisons with NHANES 1999–2000. Environ. Health Perspect. 115 (11), 1596.

Cariou, R., Veyrand, B., Yamada, A., Berrebi, A., Zalko, D., Durand, S., Pollono, C., Marchand, P., Leblanc, J.-C., Antignac, J.-P., Le Bizec, B., 2015. Perfluoroalkyl acid (PFAA) levels and profiles in breast milk, maternal and cord serum of French women and their newborns. Environ. Int. 84, 71–81.

Dereumeaux, C., Saoudi, A., Pecheux, M., Berat, B., de Crouy-Chanel, P., Zaros, C., Brunel, S., Delamaire, C., le Tertre, A., Lefranc, A., Vandentorren, S., Guldner, L., 2016. Biomarkers of exposure to environmental contaminants in French pregnant women from the Elfe cohort in 2011. Environ. Int. 97, 56–67.

Dong, G.H., Tung, K.Y., Tsai, C.H., Liu, M.M., Wang, D., Liu, W., Jin, Y.H., Hsieh, W.S., Lee, Y.L., Chen, P.C., 2013. Serum polyfluoroalkyl concentrations, asthma outcomes, and immunological markers in a case–control study of Taiwanese children. Environ. Health Perspect. 121 (4), 507.

EFSA, 2012. Perfluoroalkylated substances in food: occurrence and dietary exposure. EFSA J. 10 (6), 2743. (55 pp.). https://doi.org/10.2903/j.efsa.2012.2743.

EUMOFA, 2017. EU Consumer Habits Regarding Fishery and Aquaculture Products (Final report). European Market Observatory for Fisheries and Aquaculture Products.

Frisbee, S.J., Shankar, A., Knox, S.S., Steenland, K., Savitz, D.A., Fletcher, T., Ducatman, A.M., 2010. Perfluorooctanoic acid, perfluorooctanesulfonate, and serum lipids in children and adolescents: results from the C8 Health Project. Arch. Pediatr. Adolesc. Med. 164 (9), 860–869.

Fromme, H., Mosch, C., Morovitz, M., Alba-Alejandre, I., Boehmer, S., Kiranoglu, M., Faber, F., Hannibal, I., Genzel-Boroviczény, O., Koletzko, B., Völkel, W., 2010. Pre- and postnatal exposure to perfluorinated compounds (PFCs). Environ. Sci. Technol. 44, 7123–7129.

Geiger, S.D., Xiao, J., Ducatman, A., Frisbee, S., Innes, K., Shankar, A., 2014. The association between PFOA, PFOS and serum lipid levels in adolescents. Chemosphere 98, 78–83.

Grandjean, P., Andersen, E.W., Budtz-Jørgensen, E., Nielsen, F., Mølbak, K., Weihe, P., Heilmann, C., 2012. Serum vaccine antibody concentrations in children exposed to perfluorinated compounds. JAMA 307 (4), 391–397.

Granum, B., Haug, L.S., Namork, E., Stølevik, S.B., Thomsen, C., Aaberge, I.S., van Loveren, H., Løvik, M., Nygaard, U.C., 2013. Pre-natal exposure to perfluoroalkyl substances may be associated with altered vaccine antibody levels and immune-related health outcomes in early childhood. J. Immunotoxicol. 10 (4), 373–379.

Gützkow, K.B., Haug, L.S., Thomsen, C., Sabaredzovic, A., Becher, G., Brunborg, G., 2012. Placental transfer of perfluorinated compounds is selective–a Norwegian Mother and Child sub-cohort study. Int. J. Hyg. Environ. Health 215 (2), 216–219.

Høyer, B.B., Ramlau-Hansen, C.H., Vrijheid, M., Valvi, D., Pedersen, H.S., Zviezdai, V., Jönsson, B.A., Lindh, C.H., Bonde, J.P., Toft, G., 2015. Anthropometry in 5-to 9-year-old Greenlandic and Ukrainian children in relation to prenatal exposure to perfluorinated alkyl substances. Environ. Health Perspect. 123 (8), 841.

Jusko, T.A., Oktapodas, M., Palkovičová Murinová, L., Babinská, K., Babjaková, J., Verner, M.A., DeWitt, J.C., Thevenet-Morrison, K., Čonka, K., Drobná, B., Chovancová, J., Thurston, S.W., Lawrence, B.P., Dozier, A.M., Järvinen, K.M., Patayová, H., Trnovec, T., Legler, J., Hertz-Picciotto, I., Lamoree, M.H., 2016. Demographic, reproductive, and dietary determinants of perfluorooctane sulfonic (PFOS) and perfluorooctanoic acid (PFOA) concentrations in human colostrum. Environ. Sci. Technol. 50 (13), 7152–7162.

Kato, K., Wong, L.Y., Chen, A., Dunbar, C., Webster, G.M., Lanphear, B.P., Calafat, A.M., 2014. Changes in serum concentrations of maternal poly-and perfluoroalkyl substances over the course of pregnancy and predictors of exposure in a multiethnic cohort of Cincinnati, Ohio pregnant women during 2003–2006. Environ. Sci. Technol. 48 (16), 9600–9608.

Kim, S., Choi, K., Ji, K., Seo, J., Kho, Y., Park, J., Kim, S., Park, S., Hwang, I., Jeon, J., Yang, H., Giesy, J.P., 2011. Trans-placental transfer of thirteen perfluorinated compounds and relations with fetal thyroid hormones. Environ. Sci. Technol. 45 (17), 7465–7472.

Kim, D.H., Kim, U.J., Kim, H.Y., Choi, S.D., Oh, J.E., 2016. Perfluoroalkyl substances in serum from South Korean infants with congenital hypothyroidism and healthy infants–its relationship with thyroid hormones. Environ. Res. 147, 399–404.

Kristensen, S.L., Ramlau-Hansen, C.H., Ernst, E., Olsen, S.F., Bonde, J.P., Vested, A., Halldorsson, T.I., Becher, G., Haug, L.S., Toft, G., 2013. Long-term effects of prenatal exposure to perfluoroalkyl substances on female reproduction. Hum. Reprod. 28 (12), 3337–3348.

Lankova, D., Lacina, O., Pulkrabova, J., Hajslova, J., 2013. The determination of perfluoroalkyl substances, brominated flame retardants and their metabolites in human breast milk and infant formula. Talanta 117, 318–325. https://doi.org/10.1016/j.talanta.2013.08.040.

Liu, J., Li, J., Liu, Y., Chan, H.M., Zhao, Y., Cai, Z., Wu, Y., 2011. Comparison on gestation and lactation exposure of perfluorinated compounds for newborns. Environ. Int. 37 (7), 1206–1212.

Lopez-Espinosa, M.J., Fletcher, T., Armstrong, B., Genser, B., Dhatariya, K., Mondal, D., Ducatman, A., Leonardi, G., 2011. Association of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) with age of puberty among children living near a chemical plant. Environ. Sci. Technol. 45 (19), 8160–8166.

Lopez-Espinosa, M.J., Mondal, D., Armstrong, B., Bloom, M.S., Fletcher, T., 2012. Thyroid function and perfluoroalkyl acids in children living near a chemical plant. Environ. Health Perspect. 120 (7), 1036.

Maisonet, M., Terrell, M.L., McGeehin, M.A., Christensen, K.Y., Holmes, A., Calafat, A.M., Marcus, M., 2012. Maternal concentrations of polyfluoroalkyl compounds during pregnancy and fetal and postnatal growth in British girls. Environ. Health Perspect. 120 (10), 1432.

Maisonet, M., Näyhä, S., Lawlor, D.A., Marcus, M., 2015a. Prenatal exposures to perfluoroalkyl acids and serum lipids at ages 7 and 15 in females. Environ. Int. 82,

49–60.
Maisonet, M., Calafat, A.M., Marcus, M., Jaakkola, J.J., Lashen, H., 2015b. Prenatal exposure to perfluoroalkyl acids and serum testosterone concentrations at 15 years of age in female ALSPAC study participants. Environ. Health Perspect. 123 (12), 1325.
Manzano-Salgado, C.B., Casas, M., Lopez-Espinosa, M.J., Ballester, F., Basterrechea, M., Grimalt, J.O., Jiménez, A.-M., Kraus, T., Schettgen, T., Sunyer, J., Vrijheid, M., 2015. Transfer of perfluoroalkyl substances from mother to fetus in a Spanish birth cohort. Environ. Res. 142, 471–478.
Manzano-Salgado, C.B., Casas, M., Lopez-Espinosa, M.J., Ballester, F., Martinez, D., Ibarluzea, J., Santa-Marina, L., Schettgen, T., Vioque, J., Sunyer, J., Vrijheid, M., 2016. Variability of perfluoroalkyl substance concentrations in pregnant women by socio-demographic and dietary factors in a Spanish birth cohort. Environ. Int. 92, 357–365.
Mørck, T.A., Nielsen, F., Nielsen, J.K., Siersma, V.D., Grandjean, P., Knudsen, L.E., 2015. PFAS concentrations in plasma samples from Danish school children and their mothers. Chemosphere 129, 203–209.
Ode, A., Rylander, L., Lindh, C.H., Källén, K., Jönsson, B.A., Gustafsson, P., Olofsson, P., Ivarsson, S.A., Rignell-Hydbom, A., 2013. Determinants of maternal and fetal exposure and temporal trends of perfluorinated compounds. Environ. Sci. Pollut. Res. 20 (11), 7970–7978.
Post, G.B., Cohn, P.D., Cooper, K.R., 2012. Perfluorooctanoic acid (PFOA), an emerging drinking water contaminant: a critical review of recent literature. Environ. Res. 116, 93–117.
Quinete, N., Orata, F., Maes, A., Gehron, M., Bauer, K.H., Moreira, I., Wilken, R.D., 2010. Degradation studies of new substitutes for perfluorinated surfactants. Arch. Environ. Contam. Toxicol. 59 (1), 20–30.
Schaider, L.A., Balan, S.A., Blum, A., Andrews, D.Q., Strynar, M.J., Dickinson, M.E., Lunderberg, D.M., Lang, J.R., Peaslee, G.F., 2017. Fluorinated compounds in US fast food packaging. Environ. Sci. Technol. Lett. 4 (3), 105–111.
Schoeters, G., Govarts, E., Bruckers, L., Den Hond, E., Nelen, V., De Henauw, S., Sioen, I., Nawrot, T.S., Plusquin, M., Vriens, A., Covaci, A., Loots, I., Morrens, B., Coertjens, D., Van Larebeke, N., De Craemer, S., Croes, K., Lambrechts, N., Colles, A., Baeyens, W., 2017. Three cycles of human biomonitoring in Flanders – time trends observed in the Flemish Environment and Health Study. Int. J. Hyg. Environ. Health 220 (2), 36–45.
Sochorova, L., Hanzlikova, L., Cerna, M., Drgacova, A., Fialova, A., Svarcova, A., Gramblicka, T., Pulkrabova, J., 2017. Perfluorinated alkylated substances and brominated flame retardants in serum of the Czech adult population. Int. J. Hyg. Environ. Health 220 (2A), 235–243. https://doi.org/10.1016/j.ijheh.2016.09.003.
Stein, C.R., McGovern, K.J., Pajak, A.M., Maglione, P.J., Wolff, M.S., 2015. Perfluoroalkyl and polyfluoroalkyl substances and indicators of immune function in children aged 12–19 y: National Health and Nutrition Examination Survey. Pediatr. Res. 79 (2), 348–357.
Stockholm Convention on persistent organic pollutants (POPs), 2009. Retrieved from December 04, 2017. http://chm.pops.int/Convention/Media/Pressreleases/COP4Geneva9May2009/tabid/542/language/en-US/Default.aspx.
SZÚ, 2015. (In Czech language). Zdravotní důsledky expozice lidského organismu toxickým látkám ze zevního prostředí (biologický monitoring): Odborná zpráva za rok 2014. Státní zdravotní ústav, Praha. http://www.szu.cz/uploads/documents/chzp/biomonitoring/Odborna_biolmon_2014_def.pdf.
Timmermann, C.A.G., Rossing, L.I., Grøntved, A., Ried-Larsen, M., Dalgård, C., Andersen, L.B., Grandjean, P., Nielsen, F., Svendsen, K.D., Scheike, T., Jensen, T.K., 2014. Adiposity and glycemic control in children exposed to perfluorinated compounds. J. Clin. Endocrinol. Metab. 99 (4), E608–E614.
Zagorsky, J.L., Smith, P.K., 2017. The association between socioeconomic status and adult fast-food consumption in the U.S. Econ. Hum. Biol. 27, 12–25.
Zeng, X.W., Qian, Z., Emo, B., Vaughn, M., Bao, J., Qin, X.D., Zhu, Y., Li, J., Lee, Y.L., Dong, G.H., 2015. Association of polyfluoroalkyl chemical exposure with serum lipids in children. Sci. Total Environ. 512, 364–370.

APPENDIX IX

Cerna M., Grafnetterova A.P., Dvorakova D., Pulkrabova J., Maly M., Janos T., Vodrazkova N., Tupa Z., Puklova V.

Biomonitoring of PFOA, PFOS and PFNA in human milk from Czech Republic, time trends and estimation of infant's daily intake

Environmental Research (2020) 188: 109763

doi: 10.1016/j.envres.2020.109763

Environmental Research 188 (2020) 109763

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Biomonitoring of PFOA, PFOS and PFNA in human milk from Czech Republic, time trends and estimation of infant's daily intake☆

Milena Černá[a,b,*], Anna Pinkr Grafnetterová[a], Darina Dvořáková[c], Jana Pulkrabová[c], Marek Malý[a], Tomáš Janoš[a], Nicole Vodrážková[a], Zdeňka Tupá[a], Vladimíra Puklová[a]

[a] National Institute of Public Health, Šrobárova 48, 100 42, Prague, Czech Republic
[b] Charles University, Third Faculty of Medicine, Department of Hygiene, Ruská 87, 100 00, Prague, Czech Republic
[c] University of Chemistry and Technology, Prague, Department of Food Analysis and Nutrition, Prague, Czech Republic

ARTICLE INFO

Keywords:
PFASs
Human milk
Biomonitoring
Daily intake
Risk assessment

ABSTRACT

Background: Perfluoralkylated substances (PFASs) are persistent and bioaccumulative environmental contaminants. They are included on the list of emergent compounds monitored in the frame of HBM4EU project.
Objectives: To analyze PFASs levels in human milk samples collected in the period 2006 through 2017, to follow their time trends, to assess the PFASs exposure in breastfed infants, to calculate the daily intake of PFASs and to compare it with the tolerable daily/weekly) intakes and to quantify risk from exposure using the hazard quotient and hazard index approach.
Material and methods: A broad spectrum of PFASs were analyzed by means of UHPLC-MS/MS in primipara human milk samples collected in four consecutive time periods 2006, 2010/11, 2014, and 2017; N = 46, 183, 164 and 232, respectively. Mothers living in urban and suburban residences were recruited after their delivery at maternity hospitals, and milk samples were taken within 2 and 8 weeks after delivery. The questionnaire was focused on possible sources of exposure, dietary habits and lifestyle.
Results: Only perfluorooctane sulfonate (PFOS) and perfluorooctanoid acid PFOA (in 2017, also perfluorononanoic acid (PFNA)) were quantified in more than 90% of analyzed human milk samples. In all sampling periods, the levels of PFOA were higher than those of PFOS ($p < 0.05$). A significant downward temporal trend ($p < 0.001$) was observed for both PFOA and PFOS levels. The median concentrations in sampling years 2006, 2010/11, 2014, and 2017 were 0.075, 0.059, 0.035, and 0.023 ng/mL for PFOA and 0.045, 0.031, 0.029, and 0.020 ng/mL for PFOS, respectively. In 2017, PFNA was also quantified in 99% of samples with the median concentration of 0.007 ng/mL. The levels of PFASs correlated with maternal sea fish consumption. No maternal age-related relationship was observed. Using the tolerably daily intake (TDI) values for PFOS and PFOA set by the European Food Safety Authority (EFSA) in 2008, the calculated daily intakes from breastfeeding were clearly below these limits. Using the new, more conservative EFSA Provisional Tolerably Weekly Intake (PTWI) values set in 2018, we demonstrated a considerable exceedance of PTWI, with a hazard index above 1.
Conclusion: Significant time-related decreasing trends in the PFOS and PFOA levels in human milk were observed. Nevertheless, the body burden of infants from breastfeeding might pose an enhanced health risk to infants when the current PTWI values are applied. These findings strongly support the present EU efforts to phase out PFOA, its salts and PFOA related compounds. Since PFOS exposure there has still been widely detected despite PFOS usage reduction measures, the major exposure routes should be further monitored and, if possible, eliminated.

1. Introduction

PFASs are a diverse group of man-made chemicals produced for over sixty years and used in multiple industrial and commercial products including paints, packaging materials, and textiles (Lindstrom et al., 2011). The most widely studied and in various matrices mostly

☆ This research was funded by the Ministry of Health of the Czech Republic and in part also by PROGRESS Q16 Environmental Research, Charles University in Prague. The project was approved by the Ethics Committee of the National Institute of Public Health in Prague.
* Corresponding author. National Institute of Public Health, Šrobárova 48, 100 42, Prague, Czech Republic.
E-mail address: milena.cerna@szu.cz (M. Černá).

https://doi.org/10.1016/j.envres.2020.109763
Received 26 February 2020; Received in revised form 5 May 2020; Accepted 29 May 2020
Available online 03 June 2020
0013-9351/

detected representatives are perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA).

Even though PFASs have been produced and used since the late 1940s, they become contaminants of emerging concern only over the last decades, especially due to their widespread application, persistence in the environment, bioaccumulation potential, biomagnification in the food chain (Barbarossa et al., 2013; Fromme et al., 2009; Lau et al., 2007), and adverse effects on animal and human health (Antignac et al., 2013; Landrigan et al., 2002). The half-lives for PFOA and PFOS in humans are estimated to be 3.8 and 5.4 y, respectively (Olsen et al., 2007). The production, use, and disposal of PFASs have resulted in their widespread distribution in the environment and exposure in the general population.

Toxicological and epidemiological studies have reported several adverse health outcomes such as developmental and reproductive toxicity, immunotoxicity resulting in increased frequency of common infection and the reduced antibody response to vaccination in children (Granum et al., 2013), or endocrine disrupting effects (Ballesteros et al., 2017; Christensen et al., 2019; Lee et al., 2018). PFASs exposure has also been found to be associated with a variety of other adverse health outcomes including suspected carcinogenicity (WHO-IARC, 2016), alteration of thyroid hormone levels (Shrestha et al., 2017) and metabolic syndrome (Christensen et al., 2019).

Human exposure may occur through different pathways, but diet seems to be the major route. Important sources are fish and seafood (Domingo, 2012; Domingo and Nadal, 2017; EFSA, 2018). Additional sources of dietary exposure may be drinking water (Ericson et al., 2008) and contamination of food from packaging material. However, humans can be exposed also to PFASs discharged to the environment through inhalation of outdoor/indoor air, house dust ingestion or dermal contact (Winkens et al., 2017; Kim et al., 2019). The exposure occurs mostly due to the PFASs content in consumer products (Bečanová et al., 2016; Winkens et al., 2017). Several studies have concluded that PFASs can cross the placental barrier, reach the fetus, and harm its neurodevelopment (Hanssen et al., 2013; Kim et al., 2011; Zhao et al., 2017).

Concerns about impacts of these chemicals on public health led to efforts to reduce their production and use under the Stockholm Convention. PFOS, its salts, and PFOS-related compounds were added to the Stockholm Convention list in 2010, the inclusion of PFOA, its salts, and PFOA-related compounds is just being finalized, and the proposal for listing perfluorohexane sulfonic acid (PFHxS), its salts, and related compounds is recently under review. Some large manufacturers have been voluntarily phasing out long-chain PFASs since 2002.

Knowledge of population exposure is necessary for health risk assessment and regulation of adverse pollutants in the environment. Human biomonitoring is an ideal instrument, which allows to estimate human exposure to chemicals by measuring either the substances themselves, their metabolites, or markers of subsequent health effects in body fluids or tissues (Angerer et al., 2007; Kolossa-Gehring et al., 2012). Overall, the global biomonitoring of PFASs is mainly focused on human blood or serum/plasma (Hanssen et al., 2013; Fromme et al., 2007; Bartolomé et al., 2017). Likewise, PFASs were found in serum samples from the Czech adult population (Sochorová et al., 2017).

Human milk has been used less often as a matrix for the determination of PFASs. PFASs entering the body are mainly bound to blood proteins (Bischel et al., 2010; Han et al., 2003), but the human milk contains less protein (about 11–12 g/L) than blood plasma or serum (65–85 g/L). This fact along with the difference in protein composition of human milk and blood and reduced transfer of protein-bound chemicals from blood to milk results in lower PFASs levels in human milk than are those in maternal blood (Cariou et al., 2015; Völkel et al., 2008). On the other hand, human milk analysis is an important non-invasive method for assessing the infant body burden since lactation may be a considerable source of their exposure to PFASs (Barbarossa et al., 2013; Wilhelm et al., 2008; Winkens et al., 2017).

Human milk represents the unique food source for newborns at least in the first six months of life, as recommended by the WHO (WHO, 2019). Therefore, the knowledge of PFAS occurrence in milk is crucial for the exposure estimation for breastfed infants (Antignac et al., 2013; Croes et al., 2012; Motas Guzmàn et al., 2016; Raab et al., 2013; Sundström et al., 2011; Völkel et al., 2008).

In the Czech Republic, the pilot data on the presence of PFASs in human milk have already been shown (Lanková et al., 2013). The biomonitoring of PFASs in human milk was thus integrated into the Czech human biomonitoring program (CZ-HBM) which is conducted regularly since 1994 (Černá et al., 2007, 2017).

The main aims of the present study were (a) to assess PFASs concentrations and their temporal trends in human milk samples from Czech breastfeeding primiparas collected within the CZ-HBM project, (b) to assess the potential health risk for breastfed infants using the hazard quotient and hazard index and (c) to compare our results with those obtained in other similar studies.

2. Material and methods

2.1. Survey design

The CZ-HBM is an ongoing survey, initiated in 1994 and designated to measure and monitor population exposure to selected environmental chemicals. It has been carried out as a cross-sectional study of the general urban/suburban population with a rather limited national representatives (Černá et al., 2007). The sampling places were selected to represent both large agglomerations (Prague 1.2 million inhabitants), industrial cities (Ostrava, 0.3 million inhabitants), smaller towns (Liberec 0.1 million. inhabitants) and rather rural municipalities (Žďár n. Sázavou 0.02 million inhabitans, Uherské Hradiště 0.02 million inhabitans) (see Supplementary Material Fig. S1). Generally, toxic chemicals such as heavy metals (Cd, Pb, and Hg) and certain POPs listed in the Stockholm Convention (Van den Berg et al., 2017) have been investigated in blood, urine, and hair of children and adults. Since the very beginning of the CZ-HBM, one of the matrices is also human milk collected from breastfeeding primiparas approximately in three-year intervals.

2.2. Study subjects, recruitment and sampling

The following groups of human milk specimens were analyzed:

a) The residual of samples collected in 2006 and 2010/11 (N = 59 and 183, respectively) for the purpose of preparing pooled samples for the fourth (2005–2007) and fifth (2008–2010) WHO/UNEP global surveys) (Van den Berg et al., 2017) and archived in biobank;
b) Regular samples from 2014 collected within the CZ-HBM survey (N = 164);
c) Regular samples from 2017 collected within the CZ-HBM survey (N = 232).

Women (mothers) were contacted for the first time shortly (1–2 days) after their delivery at the cooperating maternity hospitals in four selected urban/suburban areas (Fig. S1). Signed informed consent was obtained from each donor mother. The participants were provided with the sampling instructions and a pre-washed glass container. They were also asked to complete a self-administered questionnaire focused on general information about themselves and the child, their lifestyle, consumption of selected foods and use of consumer products known to possibly may contain PFASs. The human milk was sampled in the mothers' home; field co-workers transported them to the laboratory on the ice and stored at −80 °C until analysis.

All human milk samples were obtained from healthy primiparas following the Standard Operating Procedure (SOP) taken from the WHO/UNEP global survey of POPs. Similar inclusion criteria were used as well, e.g. normal pregnancy, first born child, delivery of healthy

Table 1
Basic characteristics of study population in different sampling years.

		2006	2010/2011	2014	2017
Number of respondents		59	183	164	232
Location	Prague	18 (31%)	48 (26%)	51 (31%)	56 (24%)
	Liberec	10 (17%)	50 (27%)	41 (25%)	57 (25%)
	Ostrava	13 (22%)	51 (28%)	49 (30%)	65 (28%)
	Uherské Hradiště	5 (8%)	34 (19%)	23 (14%)	–
	Žďár nad Sázavou	–	–	–	54 (23%)
	Kutná Hora	5 (8%)	–	–	–
	Benešov	8 (14%)	–	–	–
Mother's age (mean; min - max)		27 (19–30)	29 (17–38)	30 (20–45)	29 (18–44)
Education	Primary school	NI	8 (4%)	1 (1%)	8 (3%)
	Secondary school	NI	93 (51%)	67 (40%)	91 (40%)
	University	NI	81 (45%)	96 (59%)	133 (57%)
Smoking		0	11 (6%)	6 (4%)	12 (5%)
Chronic diseases	Total	NI	NI	31 (19%)	46 (19%)
	Thyroid disease	NI	NI	12 (7%)	13 (6%)
Fish consumption					
Sea fish	Daily	NI	NI	0	0
	2x - 3x per week	NI	NI	6 (4%)	12 (5%)
	1x per week	NI	NI	25 (15%)	67 (29%)
	1x a month	NI	NI	98 (60%)	101 (44%)
	Never	NI	NI	34 (21%)	51 (22%)
Freshwater fish	Daily	NI	NI	0	0
	2x - 3x per week	NI	NI	5 (3%)	8 (3%)
	1x per week	NI	NI	9 (6%)	33 (14%)
	1x a month	NI	NI	94 (58%)	118 (51%)
	Never	NI	N	54 (33%)	72 (31%)

NI = Not included in questionnaire.

child, exclusive breastfeeding to one child, residence in the Czech Republic and milk collection within the period of 2–8 weeks after delivery. The exception was the age limit of 30 years: it was shifted due to the recent trend towards older aged primiparas.

2.3. Analysis of PFASs and quality assurance

In brief, the sample preparation procedure based on extraction using acetonitrile and subsequent purification by dispersive solid-phase extraction (d-SPE) employing C18 sorbent was used. Ultra-high performance liquid chromatography (UHPLC) interfaced with a tandem mass spectrometry (MS/MS) was employed for the identification/quantification of these compounds. For additional information on analyzed PFASs, respective ISTD and quantification and qualification ions see Supplementary Material Table S1. Because the corresponding certified reference material is not commercially available, the method was evaluated through the analysis of the artificially contaminated cow milk sample. Recoveries were in the range of 80 and 120%, repeatabilites were < 20% and LOQs were in the range of 0.001–0.006 ng/mL. The additional details can be found in Lanková et al. (2013) and in the supplementary material (Additional information S1).

2.4. Daily intake estimation and risk assessment

On the basis of the concentrations of PFOS and PFOA detected in milk samples, the intake of exclusively breastfed infants was estimated. The estimated daily intake of PFASs for the Czech breastfed infants was calculated assuming the consumption of 800 mL/day per infant during the first six months of life and a body mass of 6 kg in accordance with the WHO Infant/Child Growth chart (2009). This approach was used later in Llorca et al. (2010) and Motas Guzmàn et al. (2016) for the calculation of the daily intake from breastfeeding. In our study, two scenarios were considered: a medium intake based on the median values and a high (conservative) intake based on the 95th percentile values.

To quantify health risks from the exposure to PFOS and PFOA, the hazard quotient (HQ) and hazard index (HI) approach was used. The HQs was calculated as a ratio between the daily intake (DI) of the respective PFAS (namely PFOA and PFOS) and the Tolerable Daily Intake (TDI), or Provisional Tolerable Weekly Intake (PTWI) set by the EFSA Panel on Contaminants in the Food Chain (EFSA, 2008; EFSA, 2018). Considering the same most sensitive endpoints, especially adverse effects on the liver, thyroid and development, the HI as a sum of HQs was calculated to assess the risk of the combined exposure.

2.5. Statistical analysis

Only compounds with more than 50% of values above the LOQ were used for statistical analyses. The concentrations of PFOS, PFOA and perfluorononanoic acid (PFNA) (the last one only in 2017) in human milk were summarized using the geometric means and medians together with the minimum and maximum values. This criterion is obligatorily used in the CZ-HBM survey for the definition of population exposure and implementation of statistical analysis. The concentrations below the LOQ were assigned half the value of the LOQ. Differences in PFOA, PFOS (expressed as a sum of linear and branched isomers), and PFNA concentrations between groups and over time were tested by various analyses of variance models applied to logarithmically transformed data. In the case of significant results, Sidak's multiple comparison procedure was used for pairwise comparisons. Spearman's correlation coefficient was used to investigate the possible association between the total PFOS and PFOA milk concentrations. Significance level was set at $p = 0.05$. Data were processed using the Stata statistical software package, release 14.2 (StataCorp LP, College Station, TX, U.S.A.).

3. Results and discussion

3.1. Basic maternal characteristics

The basic characteristics of the breastfeeding women participating in the study in different years are summarized in Table 1.

Table 2
Descriptive statistics of total PFOS, Br-PFOS, L-PFOS, PFOA and PFNA concentrations in human milk and appropriate LOQ (ng/mL).

Compound	2006	2010/2011	2014	2017
Total PFOS				
GM (95% CI)	0.050 (0.041–0.060)	0.031 (0.027–0.035)	0.030 (0.025–0.036)	0.022 (0.013–0.031)
Ranges	0.016–0.162	0.007–0.158	0.003–0.212	0.002–0.169
95th perc.	0.124	0.107	0.111	0.078
Br-PFOS				
LOQ (% > LOQ)	0.01 (89.8)	0.01 (60.1)	0.001 (95)	0.001 (98.3)
GM (95% CI)	0.017 (0.013–0.020)	0.012 (0.009–0.014)	0.011 (0.008–0.014)	0.007 (0.005–0.009)
Ranges	< 0.01–0.068	< 0.01–0.092	< 0.001–0.128	< 0.001–0.099
95th perc.	0.035	0.054	0.059	0.040
L-PFOS				
LOQ (% > LOQ)	0.004 (100)	0.004 (97.3)	0.002 (99)	0.002 (99.6)
GM (95% CI)	0.033 (0.027–0.039)	0.018 (0.016–0.021)	0.018 (0.016–0.021)	0.014 (0.006–0.022)
Ranges	0.011–0.101	0.005–0.108	< 0.002–0.095	< 0.002–0.083
95th perc.	0.088	0.055	0.062	0.041
PFOA				
LOQ (% > LOQ)	0.01 (100)	0.01 (96.7)	0.006 (99)	0.003 (100)
GM (95% CI)	0.078 (0.066–0.090)	0.054 (0.050–0.058)	0.034 (0.031–0.037)	0.024 (0.021–0.026)
Ranges	0.028–0.23	0.016–0.159	< 0.006–0.159	< 0.003–0.16
95th perc.	0.172	0.113	0.083	0.058
PFNA				
LOQ (% > LOQ)	0.01 (1.7)	0.01 (1.6)	0.006 (6.7)	0.003 (98.7)
GM (95% CI)	NC	NC	NC	0.007 (0.006–0.007)
Ranges	NC	NC	NC	< 0.003–0.029
95th perc.	NC	NC	NC	0.012

NC = not calculated.
GM = geometric mean.
(95% CI) = 95% confidence interval calculated from geometric mean.
Total PFOS = branched Br-PFOS + linear L-PFOS isomers (calculated).

3.2. PFASs concentrations in human milk, local differences and time-related trends

The list of the PFASs analyzed in particular sampling years along with the LOQ values and percentages of positive findings is shown in Supplementary Material Table S2. Of a total of 19 (until 2014) or 23 (in 2017) target compounds analyzed in milk samples, only PFOS and PFOA were quantified in more than 90% of samples. In 2017 also PFNA was quantified in more than half (99%) of samples, although, more positive findings of PFNA in the human milk samples from Czech Republic (48%) (< 0.006–0.015 ng/mL) were documented earlier by Lanková et al. (2013). Positive findings were marginally detected also for perfluoroheptanoic acid (PFHpA) in the archived samples and for perfluorohexane sulfonic acid (PFHxS) in the 2014 study (Table S2).

The descriptive statistics with the concentration ranges for PFOS, PFOA and PFNA are shown in Table 2. PFOS was found as a linear (L-PFOS) or branched (Br-PFOS) isomer with proportion of about 34–39% of Br-PFOS in all samples tested (Fig. 1). Thomsen et al. (2010) found the relative amount of the branched isomers in human milk to be about 18%; Haug et al. (2010) reported this proportion to reach about 17%. However, generally, the literature data on the proportions of Br- and L-PFOS in human milk are scarce.

The PFOA levels in human milk were significantly higher than the PFOS levels (calculated as the sum of Br- and L-PFOS isomers) ($p < 0.001$ in 2006 and 2010/2011, $p < 0.05$ in 2014 and 2017), i.e.1.2–1.9-fold higher depending on the sampling year. A similar proportion was observed by Barbarossa et al. (2013), Beser et al. (2019), and Kang at al. (2016), whereas Tao et al. (2008) or Haug et al. (2010) reported an inverse proportion. Other compounds either were not detected or were below the LOQ (Table S2).

A significant downward temporal trend in the geometric mean (GM) levels of both PFOS and PFOA was observed between 2006 and 2017 ($p < 0.001$ for both substances). Each subsequent period represented a significant decrease compared to the previous period with the exception of PFOS levels between 2010/2011 and 2014 (Table 2).

A considerable difference between the samples from different areas was observed (Fig. 2). The PFOS concentrations in milk samples from Liberec in northern Bohemia (see Fig. S1) were significantly higher ($p < 0.001$) than those in other areas during all sampling periods (except for 2006; $p = 0.708$) and remained at roughly the same level between 2006 and 2014 followed by some decrease in 2017. A similar trend was not observed for PFOA in Liberec. Consequently, the association between total PFOS and PFOA was weaker in Liberec compared to other areas (Spearman's correlation coefficient of 0.39 vs. 0.50–0.54).

3.3. Other factors affecting the PFASs concentrations in human milk

3.3.1. Age

In this study, the influence of age was analyzed by comparing two age groups: mothers under 30 years of age and those aged 30 years or older. No statistically significant difference in PFASs levels was found between the both age groups. In other studies, a relationship between maternal age and PFASs levels in milk has not been found either (Llorca et al., 2010; Tao et al., 2008). However, an association of PFASs and age has been confirmed in Lee et al. (2018). It should be taken into account that the maternal age ranges in the study are not wide enough and that the influence of age may be confounded by other factors, especially education.

3.3.2. Education

Maternal education data were available only for the samples from 2014 to 2017. In both years, mothers with tertiary education had significantly higher levels of PFOA in milk compared with those with primary or secondary education ($p = 0.006$), whereas the differences between mothers with primary and secondary education were negligible. A similar shift is seen in total PFOS as well but it does not reach statistical significance ($p = 0.072$). A positive association between the education and serum PFOS and PFOA levels was also observed in Czech adults (Sochorová et al., 2017). The increased PFASs levels in the population group with tertiary education might be related to higher socioeconomic status allowing them to buy high quality consumer

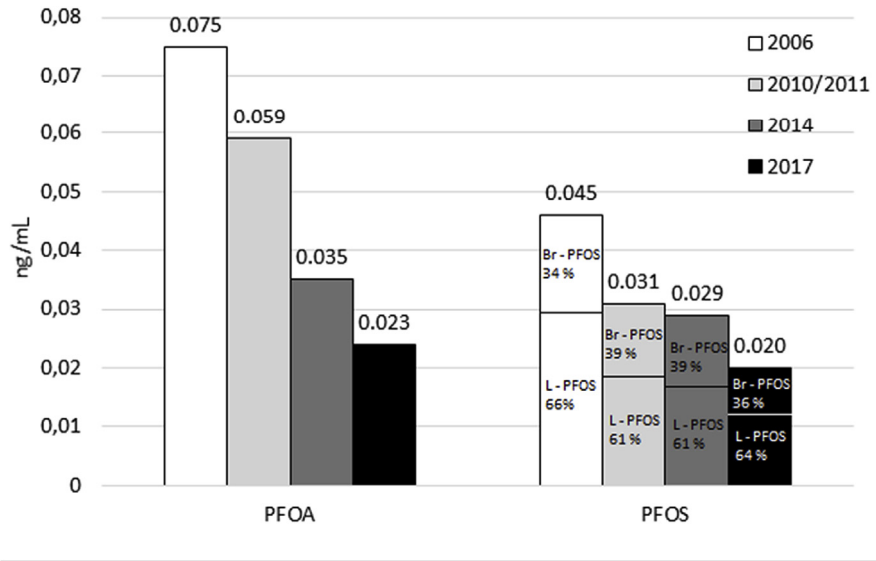


Fig. 1. Median concentrations of PFOA and PFOS (ng/mL) in human milk samples collected in the Czech Republic in the period 2006–2017.

products (waterproof footwear, sportswear, etc.), which may contain more PFASs (Buekers et al., 2018).

3.3.3. Diet

Food has been identified by several studies as the main exposure pathway to PFASs (Domingo et al., 2012; Domingo, Nadal, 2017; Jain, 2014). In our studies from 2014 to 2017, dietary habits were mapped by means of a food frequency questionnaire. The consumption of sea fish, but not of freshwater fish, was positively correlated to the human milk PFOA levels ($p = 0.01$), whereas the consumption of seafood was rather rarely reported. Despite the rising sea fish consumption observed between 2014 and 2017 in the Czech population, mostly as a result of switching from the category "consumption of sea fish meal once a month" to category "once a week" (see Supplementary Material Fig. S2), the PFOS and PFOA human milk levels decreased in 2017 n (Fig. 3). No statistically significant differences were found for milk and dairy products, eggs, meat, offal or frequency of fast-food consumption. An association between the consumption of fish and PFASs level in human body fluids was also described by other authors (Haug et al., 2010; Motas Guzmán at al., 2016; EFSA, 2018).

3.4. Calculation of PFASs intake in breastfed infants and risk assessment

Daily human milk consumption varies with the child's age and its other food intake. In this study we consider an average daily milk consumption rate of 800 mL/day per infant from month 0–6 and exclusive breastfeeding. The median values, 95th percentiles, and ranges of calculated PFOA, PFOS and PFNA daily intakes in infants per sampling year are given in Table 3a. The highest daily intake was observed for PFOA (median values were 10.0, 7.73, 4.69 and 3.09 ng/kg bw/day for the respective sampling periods). Lower daily intakes were observed regularly for PFOS (median values of 6, 4, 4.08 and 2.65 ng/kg bw/day respectively). Based on the PFNA concentrations measured, the daily intake could only be calculated for the last sampling period (median value of 0.89). Unfortunately neither TDI nor TWI for PFNA has been available.

The exposure limits for PFOS and PFOA were set to 150 ng/kg of bw/day and 1500 ng/kg of bw/day respectively in 2008 (EFSA, 2008). Considering the maximum daily intake, none of PFASs values exceeded the recommended TDI of 1500 and 150 ng/kg bw/day for PFOA and PFOS respectively. As shown in Table 3b, the HQs and HIs values are below 1, which represents only negligible health risk. In the past, a number of other authors came to a similar conclusion (Motas Guzmàn et al., 2016; Llorca et al., 2010; Kang et al., 2016; Sundström et al., 2011). Moreover, Kraft et al. (2007) have established a health-risk reference value of 0.540 ng/mL for combined concentrations of PFOS and PFOA in human milk. This threshold limit value was not exceeded in our samples either. The highest individual level found for the sum of PFOS and PFOA was found 0.346 in 2006, e.g. which is 64% of the above mentioned reference value.

The increasing awareness of toxicological effects on humans and the wide spread of PFASs in the environment have led to a re-evaluation of the existing limits. Environmental Protection Agencies from different countries (Swedish EPA, 2012; US EPA, 2014; and Danish EPA, 2015) tried to refine and tighten existing limits. So the European Commission asked the EFSA to assess health risk from the exposure to PFOS and PFOA. The EFSA Panel on Contaminants in the Food Chain established a PTWI of 13 ng/kg bw per week for PFOS and of 6 ng/kg bw per week for PFOA (EFSA, 2018). Their more conservative approach should make the health risk assessment of PFASs more protective. Indeed, when the new PTWI values were applied for the assessment of PFOS and PFOA exposure from human milk in our study, we came to the conclusion that the infant exposure considerably exceeds the PTWI value, with HQ and HI being substantially higher than 1 (Table 3b). It may mean that the PFASs exposure burden of infants from breastfeeding is not negligible and that lactation is a considerable source of exposure for infants.

Given that human milk is a sole food source for infants, breastfeeding could be an important postnatal exposure pathway. It is well known that the exposure to toxic contaminants in early life may impose greater health impacts than later in life (e.g. Landrigan et al., 2002). On the other hand, the TDI/TWI refers to the lifetime intake whereas the breastfeeding period is much shorter. Moreover, breastfeeding is still generally considered to be the optimal form of nutrition for infants (ATSDR, 2019).

4. Strengths and limitations of the study

A strength of the study was the possibility to follow the PFASs levels in human milk over a time period of about ten years. It allowed to demonstrate a significant time-related downward trend. The recruitment and sampling were carried out in accordance with the standard operating procedures used in the whole CZ-HBM system. All samples were analyzed at the same laboratory. On the other hand, sufficient data are not available on external exposure of breastfeeding mothers, their behavior, dietary habits, and socioeconomic status to do a detailed analysis of exposure sources and exposure assessment. The changes

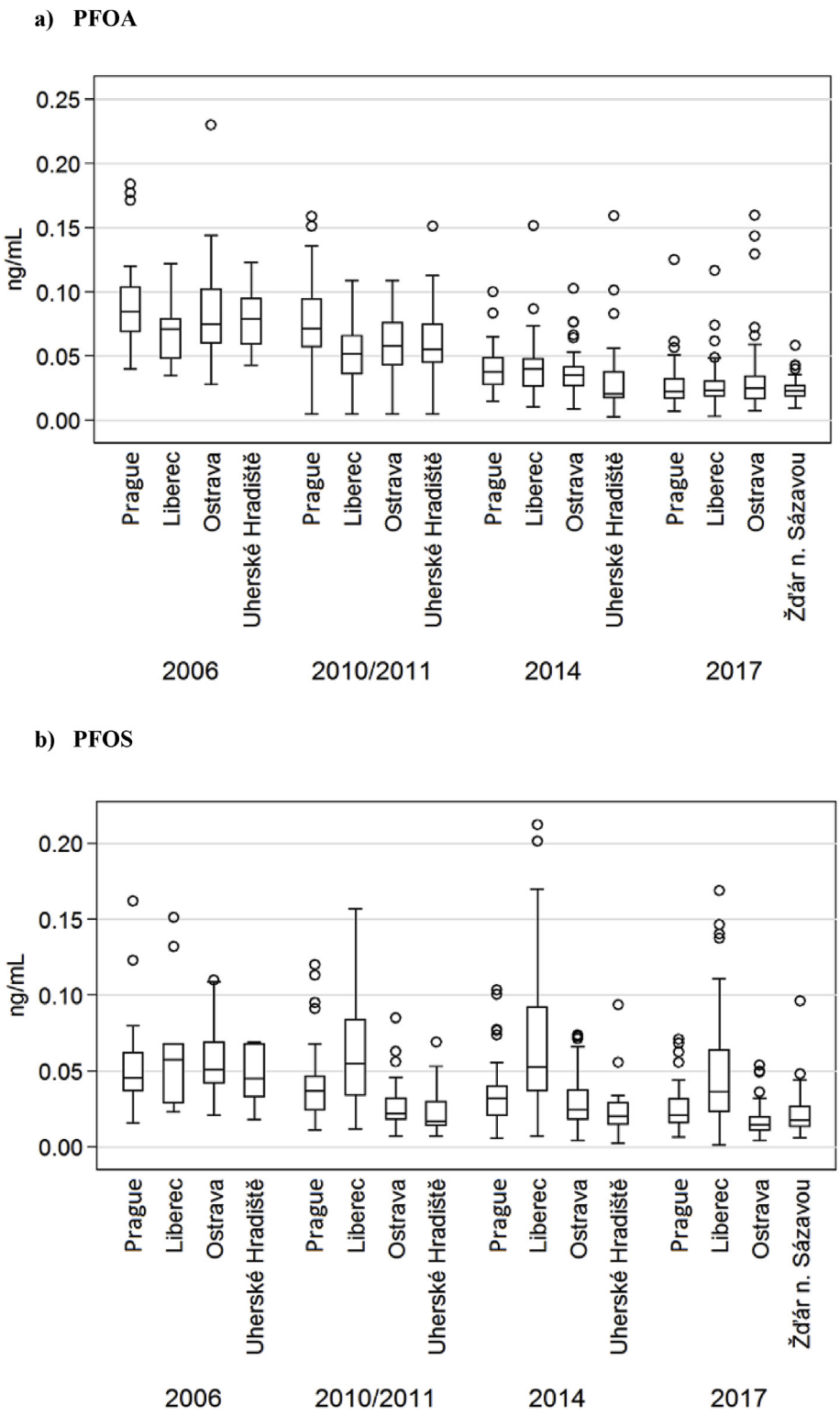


Fig. 2. Concentrations of PFOA (a) and PFOS (b) in the human milk samples from Czech Republic 2006–2017. Boxes represent the interquartile range with the median represented by a horizontal line. Whiskers extend to the farthest data points within 1.5 times the interquartile range, outliers are plotted individually.

made to the structure of the questionnaire in the course of sampling years, in particular, the absence of questions concerning fish consumption in previous years, are the possible limitations of the study. Different number of samples per site is available and thus these data are not generalizable to the Czech general female population or to the lactating women in the selected localities.

5. Conclusion

This study provides significant evidence for decreasing trends in PFOS and PFOA concentrations in human milk from the Czech population between 2006 and 2017. The probable explanation of this declining trends could be gradual and long term regulations of these compounds by authorities worldwide, especially in the frame of

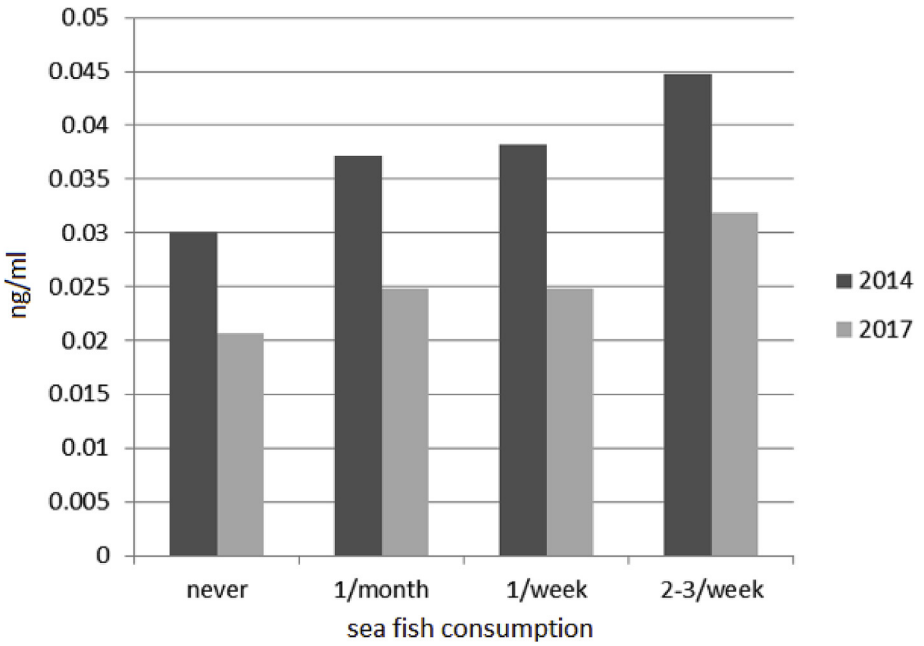


Fig. 3. Geometric mean of PFOA concentrations (ng/mL) in human milk samples related to sea fish consumption.

Table 3a
Daily PFASs intake calculated for breastfed infants (ng/kg bw/day) and the tolerable exposure levels (TDI, PTWI).

		2006 (N = 59)	2010 + 2011 (N = 183)	2014 (N = 164)	2017 (N = 232)	TDI[a]	PTWI[b]
PFOA	Median	10.0	7.73	4.69	3.09	1500	6
	95th perc.	22.9	15.0	10.9	7.76		
	Range	3.73-30.7	0.67-20.1	0.40-21.2	0.46-21.3		
PFOS	Median	6.00	4.00	4.08	2.65	150	13
	95th perc.	16.5	14.2	14.8	10.2		
	Range	2.13–21.6	0.93-20.9	0.33-28.3	0.20–22.5		
PFNA	Median	–	–	–	0.89	–	–
	95th perc.	–	–	–	1.64		
	Range	–	–	–	0.20-3.89		

N - Number of samples.
[a] Source – EFSA (2008).
[b] Source- EFSA (2018).

Table 3b
Hazard Quotients (HQ) and Hazard Index (HI) for PFASs intake in the breastfed infants based on Tolerable Daily Intake (TDI) and Provisional Tolerable Weekly Intake (PTWI).

			2006 (N = 59)	2010 + 2011 (N = 183)	2014 (N = 164)	2017 (N = 232)
Calculation with TDI	HQ PFOA	Median	0.007	0.005	0.003	0.002
		95th perc.	0.015	0.010	0.007	0.005
	HQ PFOS	Median	0.040	0.027	0.027	0.018
		95th perc.	0.110	0.095	0.099	0.068
	HI	Median	0.047	0.032	0.030	0.020
		95th perc.	0.124	0.097	0.103	0.070
Calculation with PTWI	HQ PFOA	Median	11.7	9.02	5.47	3.61
		95th perc.	26.7	17.5	12.8	9.05
	HQ PFOS	Median	3.23	2.15	2.20	1.42
		95th perc.	8.90	7.65	7.98	5.48
	HI	Median	15.2	12.1	8.09	5.33
		95th perc.	32.6	20.4	18.0	13.9

N - Number of samples.

Stockholm convention in 2009. The assessment of health risk to infants from the consumption of human mothers' milk showed that their exposure did not exceed the EFSA 2008 TDI. The exposure levels if TDI established in 2008 appeared to be negligible as daily intake of infant did not exceed the tolerable daily intake. Using PTWI established in 2018 for risk estimation, the exposure levels of breastfed infants were far above upper limit. Further measures to reduce the exposure to PFASs are therefore recommended. However, the risk associated with PFASs exposure via breastfeeding should be balanced against the benefit from breastfeeding.

CRediT authorship contribution statement

Milena Černá: Conceptualization. **Anna Pinkr Grafnetterová:** Methodology. **Darina Dvořáková:** Methodology. **Jana Pulkrabová:** Methodology. **Marek Malý:** Formal analysis, Software. **Tomáš Janoš:** Validation. **Nicole Vodrážková:** Data curation, Writing - original draft. **Zdeňka Tupá:** Data curation, Writing - original draft. **Vladimíra Puklová:** Writing - review & editing.

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.

Acknowledgements

This research was funded by the Ministry of Health of the Czech Republic and in part also by PROGRESS Q16 Environmental Research, Charles University in Prague. The authors want to express their special thanks to all breastfeeding mothers who participated in this study and donated human milk samples. We also thank our colleagues from the maternity hospitals and Regional Public Health Institutes for their hard work with the sample collection and handling.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2020.109763.

References

Angerer, J., Ewers, U., Wilhelm, M., 2007. Human biomonitoring: state of the art. Int. J. Hyg Environ. Health 210, 201–228. https://doi.org/10.1016/j.ijheh.2007.01.024.

Antignac, J.P., Veyrand, B., Kadar, H., Marchand, P., Oleko, A., Bizec, B. Le, Vandentorren, S., 2013. Occurrence of perfluorinated alkylated substances in breast milk of French women and relation with socio-demographical and clinical parameters: results of the ELFE pilot study. Chemosphere 91, 802–808. https://doi.org/10.1016/j.chemosphere.2013.01.088.

ATSDR (Agency for Toxic Substances and Disease Registry), 2019. PFAS, an overview of the science and guidance for clinicians on per- and polyfluoroalkyl substances (PFAS). Available at: https://www.atsdr.cdc.gov/pfas/docs/ATSDR_PFAS_ClinicalGuidance_12202019.pdf, Accessed date: 11 February 2020.

Ballesteros, V., Costa, O., Iñiguez, C., Fletcher, T., Ballester, F., Lopez-Espinosa, M.J., 2017. Exposure to perfluoroalkyl substances and thyroid function in pregnant women and children: a systematic review of epidemiologic studies. Environ. Int. 99, 15–28. https://doi.org/10.1016/j.envint.2016.10.015.

Barbarossa, A., Masetti, R., Gazzotti, T., Zama, D., Astolfi, A., Veyrand, B., Pession, A., Pagliuca, G., 2013. Perfluoroalkyl substances in human milk: a first survey in Italy. Environ. Int. 51, 27–30. https://doi.org/10.1016/j.envint.2012.10.001.

Bartolomé, M., Gallego-Picó, A., Cutanda, F., Huetos, O., Esteban, M., Pérez-Gómez, B., Castaño, A., 2017. Perfluorinated alkyl substances in Spanish adults: geographical distribution and determinants of exposure. Sci. Total Environ. 603–604, 352–360. https://doi.org/10.1016/j.scitotenv.2017.06.031.

Bečanová, J., Melymuk, L., Vojta, Š., Komprdová, K., Klánová, J., 2016. Screening for perfluoroalkyl acids in consumer products, building materials and wastes. Chemosphere 164, 322–329. https://doi.org/10.1016/j.chemosphere.2016.08.112.

Beser, M.I., Pardo, O., Beltrán, J., Yusà, V., 2019. Determination of 21 perfluoroalkyl substances and organophosphorus compounds in breast milk by liquid chromatography coupled to orbitrap high-resolution mass spectrometry. Anal. Chim. Acta 1049, 123–132. https://doi.org/10.1016/j.aca.2018.10.033.

Bischel, H.N., MacManus-Spencer, L.A., Luthy, R.G., 2010. Noncovalent interactions of long-chain perfluoroalkyl acids with serum albumin. Environ. Sci. Technol. 44, 5263–5269. https://doi:10.1021/es101334s.

Buekers, J., Colles, A., Cornelis, C., Morrens, B., Govarts, E., Schoeters, G., 2018. Socio-economic status and health: evaluation of human biomonitored chemical exposure to per-and polyfluorinated substances across status. Int. J. Environ. Res. Publ. Health 15. https://doi.org/10.3390/ijerph15122818.

Cariou, R., Veyrand, B., Yamada, A., Berrebi, A., Zalko, D., Durand, S., Pollono, C., Marchand, P., Leblanc, J.C., Antignac, J.P., Le Bizec, B., 2015. Perfluoroalkyl acid (PFAA) levels and profiles in breast milk, maternal and cord serum of French women and their newborns. Environ. Int. 84, 71–81. https://doi.org/10.1016/j.envint.2015.07.014.

Černá, M., Puklová, V., Hanzlíková, L., Sochorová, L., Kubínová, R., 2017. 25 years of HBM in the Czech republic. Int. J. Hyg Environ. Health 220, 3–5. https://doi.org/10.1016/j.ijheh.2016.08.004.

Černá, M., Spěváčková, V., Batáriová, A., Šmíd, J., Čejchanová, M., Očadlíková, D., Bavorová, H., Beneš, B., Kubínová, R., 2007. Human biomonitoring system in the Czech Republic. Int. J. Hyg Environ. Health 210, 495–499. https://doi.org/10.1016/j.ijheh.2007.01.005.

Christensen, K.Y., Raymond, M., Meiman, J., 2019. Perfluoroalkyl substances and metabolic syndrome. Int. J. Hyg Environ. Health 222, 147–153. https://doi.org/10.1016/j.ijheh.2018.08.014.

Croes, K., Colles, A., Koppen, G., Govarts, E., Bruckers, L., Van de Mieroop, E., Nelen, V., Covaci, A., Dirtu, A.C., Thomsen, C., Haug, L.S., Becher, G., Mampaey, M., Schoeters, G., Van Larebeke, N., Baeyens, W., 2012. Persistent organic pollutants (POPs) in human milk: a biomonitoring study in rural areas of Flanders (Belgium). Chemosphere 89, 988–994. https://doi.org/10.1016/j.chemosphere.2012.06.058.

Domingo, J.L., 2012. Health risks of dietary exposure to perfluorinated compounds. Environ. Int. 40, 187–195. https://doi.org/10.1016/j.envint.2011.08.001.

Domingo, J.L., Nadal, M., 2017. Per-and polyfluoroalkyl substances (PFASs) in food and human dietary intake: a review of the recent scientific literature. J. Agric. Food Chem. 65, 533–543. https://doi.org/10.1021/acs.jafc.6b04683.

EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2008. Opinion of the Scientific Panel on Contaminants in the Food chain on Perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and their salts. The EFSA Journal 653, 1–131 Journal number.

EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2018. Scientific Opinion on the risk to human health related to the presence of perfluorooctanesulfonic acid and perfluorooctanoic acid in food. EFSA Journal 16 (12), 284. https://doi.org/10.2903/j.efsa.2018.5194. 5194.

Ericson, I., Nadal, M., Van Bavel, B., Lindström, G., Domingo, J.L., 2008. Levels of perfluorochemicals in water samples from Catalonia, Spain: is drinking water a significant contribution to human exposure? Environ. Sci. Pollut. Res. 15, 614–619. https://doi.org/10.1007/s11356-008-0040-1.

Fromme, H., Schlummer, M., Möller, A., Gruber, L., Wolz, G., Ungewiss, J., Böhmer, S., Dekant, W., Mayer, R., Liebl, B., Twardella, D., 2007. Exposure of an adult population to perfluorinated substances using duplicate diet portions and biomonitoring data. ES T (Environ. Sci. Technol.) 41, 7928–7933. https://doi: 10.1021/es071244n.

Fromme, H., Tittlemier, S.A., Völkel, W., Wilhelm, M., Twardella, D., 2009. Perfluorinated compounds - exposure assessment for the general population in western countries. Int. J. Hyg Environ. Health 212, 239–270. https://doi.org/10.1016/j.ijheh.2008.04.007.

Granum, B., Haug, L.S., Namork, E., Stølevik, S.B., Thomsen, C., Aaberge, I.S., Van Loveren, H., Løvik, M., Nygaard, U.C., 2013. Pre-natal exposure to perfluoroalkyl substances may be associated with altered vaccine antibody levels and immune-related health outcomes in early childhood. J. Immunot. 10, 373–379. https://doi.org/10.3109/1547691X.2012.755580.

Han, X., Snow, T.A., Kemper, R.A., Jepson, G.W., 2003. Binding of perfluorooctanoic acid to rat and human plasma proteins. Chem. Res. Toxicol. 16, 775–781. https://doi.org/10.1021/tx034005w.

Hanssen, L., Dudarev, A.A., Huber, S., Odland, J.Ø., Nieboer, E., Sandanger, T.M., 2013. Partition of perfluoroalkyl substances (PFASs) in whole blood and plasma, assessed in maternal and umbilical cord samples from inhabitants of arctic Russia and Uzbekistan. Sci. Total Environ. 447, 430–437. https://doi.org/10.1016/j.scitotenv.2013.01.029.

Haug, L.S., Thomsen, C., Brantsæter, A.L., Kvalem, H.E., Haugen, M., Becher, G., Alexander, J., Meltzer, H.M., Knutsen, H.K., 2010. Diet and particularly seafood are major sources of perfluorinated compounds in humans. Environ. Int. 36, 772–778. https://doi.org/10.1016/j.envint.2010.05.016.

Jain, R.B., 2014. Contribution of diet and other factors to the levels of selected polyfluorinated compounds: data from NHANES 2003-2008. Int. J. Hyg Environ. Health 217, 52–61. https://doi.org/10.1016/j.ijheh.2013.03.008.

Kang, H., Choi, K., Lee, H.S., Kim, D.H., Park, N.Y., Kim, S., Kho, Y., 2016. Elevated levels of short carbon-chain PFCAs in breast milk among Korean women: current status and potential challenges. Environ. Res. 148, 351–359. https://doi.org/10.1016/j.envres.2016.04.017.

Kim, D.H., Lee, J.H., Oh, J.E., 2019. Assessment of individual-based perfluoroalkly substances exposure by multiple human exposure sources. J. Hazard Mater. 365, 26–33. https://doi.org/10.1016/j.jhazmat.2018.10.066.

Kim, S.K., Lee, K.T., Kang, C.S., Tao, L., Kannan, K., Kim, K.R., Kim, C.K., Lee, J.S., Park, P.S., Yoo, Y.W., Ha, J.Y., Shin, Y.S., Lee, J.H., 2011. Distribution of perfluorochemicals between sera and milk from the same mothers and implications for prenatal and postnatal exposures. Environ. Pollut. 159, 169–174. https://doi.org/10.1016/j.envpol.2010.09.008.

Kolossa-Gehring, M., Becker, K., Conrad, A., Schröter-Kermani, C., Schulz, C., Seiwert, M., 2012. Environmental surveys, specimen bank and health related environmental monitoring in Germany. Int. J. Hyg Environ. Health 215, 120–126. https://doi.org/10.1016/j.ijheh.2011.10.013.

Kraft, M., Bernsmann, T., Hölzer, J., Just, P., Krüger, C., Quabeck, G., Rauchfluss, K., Wilhelm, M., 2007. Der PFT-Fall im Sauerland aus umweltmedizinischer Sicht – bewertung und Maßnahmen. Umweltmed. Forsch. Prax. 12, 79–86.

Landrigan, P.J., Sonawane, B., Mattison, D., McCally, M., Garg, A., 2002. Chemical contaminants in breast milk and their impacts on children's health: an overview. Environ. Health Perspect. 110, 313–315. https://doi.org/10.1289/ehp.021100313.

Lanková, D., Lacina, O., Pulkrabova, J., Hajslova, J., 2013. The determination of perfluoroalkyl substances, brominated flame retardants and their metabolites in human breast milk and infant formula. Talanta 117, 318–325. https://doi.org/10.1016/j.talanta.2013.08.040.

Lau, C., Anitole, K., Hodes, C., Lai, D., Pfahles-Hutchens, A., Seed, J., 2007. Perfluoroalkyl acids: a review of monitoring and toxicological findings. Toxicol. Sci. 99, 366–394.

https://doi.org/10.1093/toxsci/kfm128.
Lee, S., Kim, Sunmi, Park, J., Kim, H.J., Choi, G., Choi, S., Kim, Sungjoo, Kim, S.Y., Kim, Sungkyoon, Choi, K., Moon, H.B., 2018. Perfluoroalkyl substances (PFASs) in breast milk from Korea: time-course trends, influencing factors, and infant exposure. Sci. Total Environ. 612, 286–292. https://doi.org/10.1016/j.scitotenv.2017.08.094.
Lindstrom, A.B., Strynar, M.J., Libelo, E.L., 2011. Polyfluorinated compounds: past, present, and future. Environ. Sci. Technol. 45, 7954–7961. https://doi.org/10.1021/es2011622.
Llorca, M., Farré, M., Picó, Y., Teijón, M.L., Álvarez, J.G., Barceló, D., 2010. Infant exposure of perfluorinated compounds: levels in breast milk and commercial baby food. Environ. Int. 36, 584–592. https://doi.org/10.1016/j.envint.2010.04.016.
Motas Guzmàn, M., Clementini, C., Pérez-Cárceles, M.D., Jiménez Rejón, S., Cascone, A., Martellini, T., Guerranti, C., Cincinelli, A., 2016. Perfluorinated carboxylic acids in human breast milk from Spain and estimation of infant's daily intake. Sci. Total Environ. 544, 595–600. https://doi.org/10.1016/j.scitotenv.2015.11.059.
Olsen, G.W., Burris, J.M., Ehresman, D.J., Froelich, J.W., Seacat, A.M., Butenhoff, J.L., Zobel, L.R., 2007. Half-life of serum elimination of perfluorooctanesulfonate, perfluorohexanesulfonate, and perfluorooctanoate in retired fluorochemical production workers. Environ. Health Perspect. 115, 1298–1305. https://doi.org/10.1289/ehp.10009.
Raab, U., Albrecht, M., Preiss, U., Völkel, W., Schwegler, U., Fromme, H., 2013. Organochlorine compounds, nitro musks and perfluorinated substances in breast milk - results from Bavarian monitoring of breast milk 2007/8. Chemosphere 93, 461–467. https://doi.org/10.1016/j.chemosphere.2013.06.013.
Shrestha, S., Bloom, M.S., Yucel, R., Seegal, R.F., Rej, R., McCaffrey, R.J., Wu, Q., Kannan, K., Fitzgerald, E.F., 2017. Perfluoroalkyl substances, thyroid hormones, and neuropsychological status in older adults. Int. J. Hyg Environ. Health 220, 679–685. https://doi.org/10.1016/j.ijheh.2016.12.013.
Sochorová, L., Hanzlíková, L., Černá, M., Drgáčová, A., Fialová, A., Švarcová, A., Gramblička, T., Pulkrabová, J., 2017. Perfluorinated alkylated substances and brominated flame retardants in serum of the Czech adult population. Int. J. Hyg Environ. Health 220, 235–243. https://doi.org/10.1016/j.ijheh.2016.09.003.
Sundström, M., Ehresman, D.J., Bignert, A., Butenhoff, J.L., Olsen, G.W., Chang, S.C., Bergman, Å., 2011. A temporal trend study (1972-2008) of perfluorooctanesulfonate, perfluorohexanesulfonate, and perfluorooctanoate in pooled human milk samples from Stockholm, Sweden. Environ. Int. 37, 178–183. https://doi.org/10.1016/j.envint.2010.08.014.
Tao, L., Ma, J., Kunisue, T., Libelo, E.L., Tanabe, S., Kannan, K., 2008. Perfluorinated compounds in human breast milk from several Asian countries, and in infant formula and dairy milk from the United States. Environ. Sci. Technol. 42, 8597–8602. https://doi.org/10.1021/es801875v.
Thomsen, C., Haug, L.S., Stigum, H., Frøshaug, M., Broadwell, S.L., Becher, G., 2010. Changes in concentrations of perfluorinated compounds, polybrominated diphenyl ethers, and polychlorinated biphenyls in Norwegian breast-milk during twelve months of lactation. Environ. Sci. Technol. 44, 9550–9556. https://doi:10.1021/es1021922.
Van den Berg, M., Kypke, K., Kotz, A., Tritscher, A., Lee, S.Y., Magulova, K., Fiedler, H., Malisch, R., 2017. WHO/UNEP global surveys of PCDDs, PCDFs, PCBs and DDTs in human milk and benefit–risk evaluation of breastfeeding. Arch. Toxicol. 91, 83–96. https://doi.org/10.1007/s00204-016-1802-z.
Völkel, W., Genzel-Boroviczény, O., Demmelmair, H., Gebauer, C., Koletzko, B., Twardella, D., Raab, U., Fromme, H., 2008. Perfluorooctane sulphonate (PFOS) and perfluorooctanoic acid (PFOA) in human breast milk: results of a pilot study. Int. J. Hyg Environ. Health 211, 440–446. https://doi.org/10.1016/j.ijheh.2007.07.024.
WHO, 2009. World Health Organization, United Nations Children's, Fund, WHO child growth standards and the identification of severe acute malnutrition in infants and children A Joint Statement. https://www.who.int/nutrition/publications/severemalnutrition/9789241598163/en/.
WHO – IARC, 2016. Agents classified by the IARC monographs, volumes 110 (PFOA).
WHO, 2019. World Health Organization. Exclusive Breastfeeding for Optimal Growth, Development and Health of Infants. 2019. Available at: http://who.int/elena/titles/exclusive_breastfeeding/en, Accessed date: 23 July 2019.
Wilhelm, M., Kraft, M., Rauchfuss, K., Hölzer, J., 2008. Assessment and management of the first German case of a contamination with perfluorinated compounds (PFC) in the region Sauerland, North Rhine-Westphalia. J. Toxicol. Environ. Health Part A Curr. Issues 71, 725–733. https://doi.org/10.1080/15287390801985216.
Winkens, K., Vestergren, R., Berger, U., Cousins, I.T., 2017. Early life exposure to per- and polyfluoroalkyl substances (PFASs): a critical review. Emerg. Contam. 3, 55–68. https://doi.org/10.1016/j.emcon.2017.05.001.
Zhao, Z., Tang, J., Mi, L., Tian, C., Zhong, G., Zhang, G., Wang, S., Li, Q., Ebinghaus, R., Xie, Z., Sun, H., 2017. Perfluoroalkyl and polyfluoroalkyl substances in the lower atmosphere and surface waters of the Chinese Bohai Sea, Yellow Sea, and Yangtze River estuary. Sci. Total Environ. 599–600, 114–123. https://doi.org/10.1016/j.scitotenv.2017.04.147.

APPENDIX X

Urbancova K., Dvorakova D., Gramblicka T., Sram R.J., Hajslova J., Pulkrabova J.

Comparison of polycyclic aromatic hydrocarbon metabolite concentrations in urine of mothers and their newborns

Environmental Research (2020) 188: 109763

doi: 10.1016/j.scitotenv.2020.138116

Science of the Total Environment 723 (2020) 138116

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Comparison of polycyclic aromatic hydrocarbon metabolite concentrations in urine of mothers and their newborns

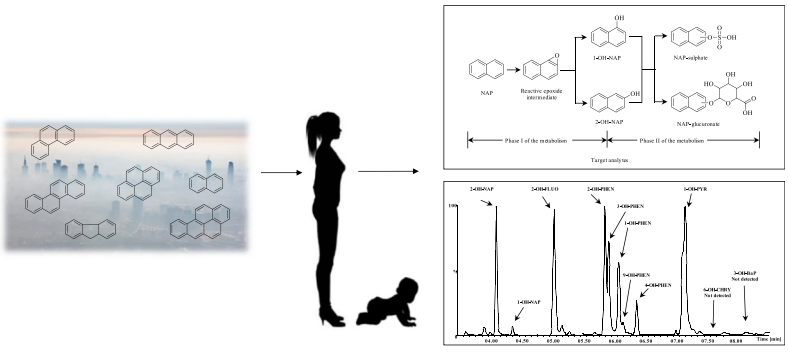
Katerina Urbancova [a], Darina Dvorakova [a], Tomas Gramblicka [a], Radim J. Sram [a,b], Jana Hajslova [a], Jana Pulkrabova [a,*]

[a] University of Chemistry and Technology, Faculty of Food and Biochemical Technology, Department of Food Analysis and Nutrition, Technicka 3, 166 28 Prague, Czech Republic
[b] Institute of Experimental Medicine Medicine CAS, Videnska 1083, 142 20 Prague 4, Czech Republic

HIGHLIGHTS

- The most frequently detected analyte with the highest concentration was 2-OH-NAP.
- 6-OH-CHRY and 3-OH-BaP were not detected in any of the analysed samples.
- ΣOH-PAHs in children's urine samples were1.7× lower compared to their mothers.
- ΣOH-PAHs in urine did not correlate with PAHs measured in the ambient air.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 6 February 2020
Received in revised form 19 March 2020
Accepted 20 March 2020
Available online 21 March 2020

Editor: Damia Barcelo

Keywords:
Environment
Biomonitoring
Biomarkers
OH-PAHs
UHPLC–MS/MS

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are environmental contaminants produced during incomplete combustion of organic matter. Humans can be exposed to them via several pathways (inhalation, digestion, dermal exposure). The aim of this study was to assess the concentration of 11 monohydroxylated metabolites of PAHs (OH-PAHs) in 660 urine samples collected from mothers and their newborns residing in two localities of the Czech Republic — Most and Ceske Budejovice — in 2016 and 2017. After enzymatic hydrolysis, the target analytes were extracted from the urine samples using liquid-liquid extraction, with extraction solvent ethyl acetate and a clean-up step using dispersive solid-phase extraction (d-SPE) with the *Z*-Sep sorbent. For identification and quantification, ultra-high performance liquid chromatography coupled with tandem mass spectrometry was applied. 2-OH-NAP was the compound present in all of the measured samples and it was also the compound at the highest concentration in both mothers' and newborns' urine samples (median concentration 5.15 μg/g creatinine and 3.58 μg/g creatinine). The total concentrations of OH-PAHs in urine samples collected from mothers were 2 times higher compared to their children. The most contaminated samples were collected in Most in the period October 2016–March 2017 from both mothers (12.59 μg/g creatinine) and their newborns (8.29 μg/g creatinine). The concentrations of OH-PAHs in urine samples, which were collected from both mothers and their newborns as presented in this study, are comparable with those found in our previous study between 2013 and 2014. In addition, they are slightly lower or comparable to other studies from Poland, USA, Germany, China, and Australia.

* Corresponding author.
E-mail address: jana.pulkrabova@vscht.cz (J. Pulkrabova).

https://doi.org/10.1016/j.scitotenv.2020.138116

The results might indicate that the population in the previously highly air-polluted mining districts carries some long-term changes (maybe existing changes in genetic information), which also affect the metabolism of PAHs. It could be related to the long-lasting effect, and thus corresponding to the shortened life expectancy.

1. Introduction

PAHs are a group of well-known ubiquitous environmental contaminants that are formed during incomplete combustion or pyrolysis of organic matter (Kim et al., 2013). The most thoroughly investigated substance from this group is benzo[*a*]pyrene (BaP), which is listed as a carcinogen in humans (Group 1) according to a database by the International Agency for Research on Cancer (IARC). Another compound of high concern is chrysene (CHRY), which is listed as possibly carcinogenic to humans (Group 2B) (Li et al., 2016; IARC, 2018). However, PAHs occur in the environment as complex mixtures and thus it is necessary to monitor more than one compound (Yamamoto et al., 2015).

Humans can be exposed to PAH mixtures via various pathways, such as inhalation of contaminated air and/or tobacco smoke, or digestion of contaminated food. For occupationally exposed individuals (e.g., steel, coal or asphalt workers), dermal absorption is the major exposure pathway (Ma and Harrad, 2015; Alicandro et al., 2016). The high molecular PAHs (e.g., BaP or indeno[1,2,3-*cd*]pyren) tend to accumulate to some extent in fat tissue and only a minor part of them is metabolised. The low molecular PAHs (e.g. fluorene or phenanthrene) are metabolised in a larger part. After entering the body, PAHs are metabolised by cytochrome P450 enzymes. Metabolism includes two main phases: In Phase I, OH-PAHs are mainly formed. In Phase II, these compounds are conjugated with glucuronic acid or sulfate to produce compounds that are more water-soluble and are easily excreted from the body via urine or bile (Tombolini et al., 2018).

During their metabolic transformation, PAHs can become harmful to human health. In the first phase of metabolism, reactive species could be formed and interact with proteins or DNA and, as a result, exposure to PAHs can cause a higher incidence of cancer. PAH metabolites (mainly OH-PAHs) can act as endocrine disruptors, and therefore, can negatively affect the endocrine system. Exposure to PAHs during pregnancy can affect foetal development and can lead to intrauterine growth retardation and lower birth weights (Dejmek et al., 2000; Abdel-Shafy and Mansour, 2016). Prenatal PAH exposure can also be associated with the development of attention deficit hyperactivity disorder (ADHD) (Perera et al., 2018). Children from regions with higher PAHs exposure suffer from higher respiratory morbidity (Hertz-Picciotto et al., 2007; Dostal et al., 2013) and increased incidence of asthma bronchiale (Choi et al., 2017; Choi et al., 2019). Scientific research also indicates that PAHs (their metabolites) can affect childhood obesity (Poursafa et al., 2018). The International Programme on Chemical Safety (IPCS), the Scientific Committee on Food (SCF) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) concluded that 15 PAHs show clear evidence of mutagenicity and genotoxicity in somatic cells in experimental animals in vivo (EFSA (European Food Safety Authority), 2008). However, there are no regulations for the restriction of the levels of PAH metabolites in the human body and biological matrices. The only restricted compounds by the legislation are parent PAHs in some foods, and the concentrations of BaP are regulated in the air.

To assess the exposure of the human body to PAHs, it is important to monitor the parent PAHs in the ambient air and food as well as their metabolites in biological samples. Therefore, for this reason, mainly OH-PAHs are measured in the urine.

According to previous researchers, it can be seen that they are more focused on the analysis of OH-PAHs in urine samples (i.e., to evaluate PAH exposure) collected from a broad range of population groups, for example, highly exposed people, such as steel (Onyemauwa et al., 2009) or asphalt workers (Campo et al., 2010), smokers (Ramsauer et al., 2011), or the general population living in contaminated areas (Guo et al., 2013a). The most recent scientific papers are more focused on the population where PAH exposure could be most harmful, such as young children, adolescents (Sochacka-Tatara et al., 2018; Dobraca et al., 2018; Kelishadi et al., 2018), and pregnant women (Nethery et al., 2012; Adetona et al., 2013).

The presented research addresses a specific gap identified for PAHs within the frame of the European HBM4EU project in understanding the relationship between exposure and health. The main aim was to evaluate the concentrations of 11 OH-PAHs in the urine samples collected from July 2016 to August 2017 from mothers and their newborns residing in two localities of the Czech Republic — Ceske Budejovice and Most. The district of Ceske Budejovice in Southern Bohemia was selected as a control locality, according to our previous studies (Sram et al., 2016; Veleminsky Jr. et al., 2016). The district of Most is one of the mining districts in Northern Bohemia, which was characterised by significant air pollution due to power plants and local heating emissions in the '70s and '80s, and use of brown coal with a high content of sulphur. The outcome of this load was seen in the shortening of life expectancy by two years in both males and females. This did not change in the period 1990–2017 (Sram et al., 1996). Our previous study analysed the effect of air pollution by PAHs in a highly polluted locality — the Karvina district in Northern Moravia and the control district of Ceske Budejovice (Urbancova et al., 2017).

2. Materials and methods

Certified standards of OH-PAHs, namely 1-OH-NAP and 2-OH-NAP (both 1000 μg/mL methanol) were purchased from Absolute Standards, Inc. (USA). 3-OH-PHEN, 9-OH-PHEN, 1-OH-PYR and 3-OH-BaP (all 10 μg/mL acetonitrile) were purchased from Neochema (Germany) and 6-OH-CHRY (100 μg/mL acetonitrile) was obtained from AccuStandard® (USA). 2-OH-FLUO, 1-OH-PHEN, 2-OH-PHEN, 4-OH-PHEN and isotopically labelled analogues, specifically $[^2H]_7$-naphtalene-1-ol (d_7-1-OH-NAP), $[^2H]_7$-naphtalene-2-ol (d_7-2-OH-NAP), $[^2H]_9$-fluorene-2-ol (d_9-2-OH-FLUO), $[^2H]_9$-phenanthrene-1-ol (d_9-1-OH-PHEN), $[^2H]_9$-phenanthrene-2-ol (d_9-2-OH-PHEN), $[^2H]_9$-phenanthrene-3-ol (d_9-3-OH-PHEN), $[^2H]_8$-phenanthrene-9-ol (d_8-9-OH-PHEN), $[^2H]_9$-pyrene-1-ol (d_9-1-OH-PYR), $[^2H]_{11}$-benzo[*a*]pyrene-3-ol (d_{11}-3-OH-BaP) were supplied by Toronto Research Chemicals Inc. (Canada) in a solid form. Creatinine was delivered by Sigma-Aldrich (USA). The purity of all standards and their isotopically labelled analogues was at least 98%.

Individual OH-PAHs delivered as solids were dissolved according to the manufacturers' recommendations. Mixtures of OH-PAHs and their isotopically labelled analogues (d_x-OH-PAHs) were prepared in methanol. All solutions were stored at −20 °C in the freezer.

The Standard Reference Material® (SRM) 3673 (Organic Contaminants in Non-Smokers´ Urine) used for the method validation experiments was supplied by the US National Institute of Standards and Technology (NIST, USA).

2.1. Chemicals, reagents and other materials

Ethyl acetate, picric acid, enzyme β-glucuronidase (type HP-2, glucuronidase activity ≥100,000 units/mL, sulfatase activity ≤7500 units/mL), sorbent Supel™ QuE *Z*-Sep and polypropylene centrifuge tube filters (nylon, pore size 0.22 μm) were supplied by Sigma Aldrich (USA). HPLC gradient methanol was delivered by Merck (Germany). Unsterile

polytetrafluoroethylene (5.0 μm, Ø 25 mm) filters were purchased from Rotilabo® (Germany). 96-well microtiter plates were obtained from the Gama Group (Czech Republic).

2.2. Sample collection

The sample collection was carried out within the grant of the Czech Academy of Sciences, Strategy AV21, Qualitas and EU Horizon 2020 HBM4EU. The samples were collected in two localities of the Czech Republic — Ceske Budejovice and Most, namely in the Ceske Budejovice Hospital, Department of Obstetrics and Department of Neonatology and in the Most Hospital, Department of Obstetrics and Department of Neonatology. All the mothers´ urine samples were collected before delivery and newborns´ urine samples were collected on the second day after delivery as spot samples. The study was approved by the Ethics Committee of both hospitals and the Institute of Experimental Medicine of the CAS in Prague. Each mother was required to sign a written consent. A total of 660 samples (330 samples from mothers and 330 samples from their newborns) were collected. The sampling was carried out from July 2016 to August 2017. The urine samples were stored in the freezer at −20 °C before analysis.

Each mother filled in a questionnaire regarding her age, body mass index (BMI), current residency, eating habits and smoking habits. According to the data, all the mothers claimed that they did not smoke during the third trimester of their pregnancy and, therefore, were evaluated as non-smokers. Gestation age, date of child's birth, delivery type, birth weight, and gender were obtained from the questionnaire filled in by medical personnel. The summary of the information is shown in Table 1.

Within the Qualitas project, air samples were also collected for the analysis of PAHs. Air was sampled using Hi-Vol filters, and it was collected every 3 days (10 samples/month). The sample volume was 1627 m^3/24 h in each location in seasons July–September 2016, October 2016–March 2017 and April–May 2017.

2.3. Description of methods

2.3.1. Measurement of urinary creatinine

To normalise the urine concentration/dilution in individual samples for data comparability, the creatinine values were used. The creatinine concentration was measured using a Jaffe's spectrophotometric method according to our previous study (Lankova et al., 2016).

2.3.2. Analysis of OH-PAHs in urine

2.3.2.1. Extraction. The sample preparation procedure based on LLE with the extraction solvent ethyl acetate and a clean-up step, using d-SPE with a *Z*-Sep sorbent, is described in detail in our previous paper (Urbancova et al., 2017).

2.3.2.2. Instrumental analysis. The UHPLC–MS/MS analysis of 11 urinary OH-PAHs was performed using an Acquity Ultra-Performance LC system coupled with a triple quadrupole mass spectrometer Xevo TQ-S (both from Waters, USA) with electrospray ionisation in a negative ion mode (ESI-). Analytes were separated on a PFP (pentafluorophenyl) Kinetex column, Phenomenex (USA) (100 mm × 2.1 mm × 1.7 μm). Measurement conditions are described in more detail in our previously published paper (Lankova et al., 2016).

2.3.2.3. Quality assurance/quality control and validation. The validation of the analytical method for the analysis of 11 urinary OH-PAHs and the spectrophotometric method based on Jaffe's reaction for creatinine determination are described in detail in our previous study [30]. The method accuracy was controlled by using the SRM 3673. Limits of quantification (LOQs) were 0.01–0.025 ng/mL with recovery 77–113% and repeatability (RSD) 3–14%. For 6-OH-CHRY and 3-OH-BaP, which were not certified in the SRM 3673, the performance parameters were measured by the analysis of an artificially contaminated urine blank sample. The recovery for 6-OH-CHRY was 95% (RSD 13%, LOQ 0.01 ng/mL) and for 3-OH-BaP, it was 97% (RSD 16%, LOQ 0.9 ng/mL).

Background contamination by the target analytes was also monitored. A procedural blank sample (deionised water was used instead of urine) was prepared together with each batch of samples. The blank sample contained traces of 1-OH-NAP, 2-OH-NAP, 1-OH-PHEN, 2-OH-PHEN, 3-OH-PHEN and 1-OH-PYR (concentrations below 0.02 ng/mL urine). The concentration of contamination in the blank sample was subtracted from all samples prepared on the same day as the procedural blank sample.

2.3.3. Analysis of PAHs in Hi-Vol filters

The extraction of PAHs from the Hi-Vol filters is described in our other paper (Polachova et al., 2020). Briefly, the target PAHs from the filters were extracted using the mixture hexane:dichloromethane (1:1, *v*/*v*) for 7 h in a Soxhlet apparatus. The primary extract was redissolved after evaporation in hexane and purified from interfering co-extracts by solid-phase extraction (SPE) on a silica column. After another evaporation, the final extract was dissolved in isooctane and analysed for the presence of 24 PAHs, using gas chromatography, combined with high-resolution mass spectrometry (time of flight analyser (TOF)) in electron ionisation mode (GC-HRTOFMS-EI). LOQs of this method ranged from 0.0006 ng/m^3 air to 0.0012 ng/m^3 air with a recovery of 64–118% and repeatability of 4–19%.

3. Results

3.1. Overall urinary concentrations of 11 OH-PAHs in urine samples

A total of 660 urine samples (330 samples collected from mothers, 330 from their newborns) were analysed to detect the presence of 11 OH-PAHs. Table 2 shows the results of OH-PAH concentrations in urine samples collected from mothers and their newborns. In the mothers' urine samples, 2-OH-NAP was the most abundant compound found in 100% of the samples, followed by 1-OH-PHEN (99% of samples), 2-OH-PHEN (99% of samples) and 2-OH-FLUO (99% of samples). Other compounds were present in >89% of the measured samples. In the case of urine samples collected from newborns, the most abundant compound was also 2-OH-NAP (100% of samples), followed by 2-OH-PHEN (88% of samples), 1-OH-PHEN (86% of the samples) and 9-OH-PHEN (82% of samples). The abundance of the other OH-PAHs was lower. Monohydroxylated metabolites of carcinogenic CHRY and BaP, namely 6-OH-CHRY and 3-OH-BaP, were not detected in any of the measured samples collected from mothers or their newborns. This

Table 1
General information about mothers and their newborns — median (min-max).

	Ceske Budejovice			Most		
	Jul-Sep 2016	Oct 2016-Mar 2017	Apr-Aug 2017	Jul-Sep 2016	Oct 2016-Mar 2017	Apr-Aug 2017
Mother's age (years)	32 (18–42)	32 (20–43)	32 (22–41)	30 (19–41)	29 (18–44)	30 (18–43)
BMI	23 (16–44)	22 (16–40)	23 (19–43)	24 (18–41)	22 (16–42)	23 (17–37)
Gestation age (weeks)	39 (36–40)	39 (36–42)	39 (37–40)	40 (35–42)	39 (37–42)	39 (36–40)
Birth weight (g)	3360 (2240–4070)	3445 (2440–4610)	3590 (2140–4340)	3280 (2700–4190)	3425 (1300–4530)	3410 (2090–4360)

Table 2
Urinary concentrations of 11 OH-PAHs measured in 660 samples collected from Czech mothers and their newborns (μg/g creatinine[a]).

Analyte	LOQ[b]	Mothers (n = 330)					Newborns (n = 330)				
		Mean	Median	Min	Max	% positive samples	Mean	Median	Min	Max	% positive samples
1-OH-NAP	0.025	0.77	0.40	0.03	19.66	98	0.24	0.13	0.03	13.31	67
2-OH-NAP	0.025	7.30	5.15	0.56	42.62	100	6.09	3.58	0.46	41.12	100
2-OH-FLUO	0.025	0.33	0.23	0.07	4.15	99	0.17	0.08	0.03	1.06	72
1-OH-PHEN	0.010	0.43	0.26	0.04	13.82	99	0.45	0.13	0.01	4.86	86
2-OH-PHEN	0.010	0.27	0.17	0.03	5.36	99	0.21	0.09	0.01	3.08	88
3-OH-PHEN	0.010	0.10	0.06	0.01	1.99	96	0.05	0.01	0.01	0.57	52
4-OH-PHEN	0.010	0.48	0.17	0.01	16.06	94	0.03	0.01	0.01	0.30	45
9-OH-PHEN	0.010	0.97	0.45	0.05	22.06	89	0.37	0.26	0.01	3.36	82
1-OH-PYR	0.025	0.18	0.12	0.03	2.14	91	0.06	0.01	0.02	0.94	36
6-OH-CHRY	0.010	–	–	<0.01	<0.01	0	–	–	<0.01	<0.01	0
3-OH-BaP	0.900	–	–	<0.900	<0.900	0	–	–	<0.900	<0.900	0
ΣOH-PAH	–	11.12	8.96	1.83	78.76	100	8.48	5.15	0.46	47.62	100

When target analyte was below LOQ for the mean and median calculation 1/2 LOQ value was used.
[a] Mean, median, minimum and maximum concentration of creatinine was 1.1, 0.92, 0.30 and 2.9 mg/mL urine.
[b] LOQ was calculated with the median concentration of creatinine (0.92 mg/mL).

result is in good agreement with other published papers (Onyemauwa et al., 2009; Veleminsky Jr. et al., 2016; Sykorova et al., 2015; Li et al., 2014), and it is probably caused by urine not being the major excretion route for these more lipophilic compounds.

As shown in Table 2, the concentrations of all monitored OH-PAHs (ΣOH-PAHs) was 1.7 times higher in the urine samples collected from mothers (median ΣOH-PAHs 8.96 μg/g creatinine) compared to their children (median ΣOH-PAHs 5.15 μg/g creatinine).

2-OH-NAP was the compound present in all of the measured samples and it was also the compound found at the highest concentration in both mothers' and newborns' urine samples (median concentration 5.15 μg/g creatinine and 3.58 μg/g creatinine). The domination of 2-OH-NAP among other measured OH-PAHs was also found in other similar studies (Tombolini et al., 2018; Sochacka-Tatara et al., 2018; Cathey et al., 2018) and our previous study (Urbancova et al., 2017). It can be assumed that inhalation may be the dominant source of exposure for low molecular PAHs, such as NAP (Guo et al., 2013b). However, this compound was not measured in the Hi-Vol filters analysed in this study due to its high volatility. The median concentrations of approximately one order of magnitude lower were measured for the compound with the second-highest concentration, 9-OH-PHEN (mothers – 0.45 μg/g creatinine, newborns – 0.26 μg/g creatinine). The concentrations of other measured PAHs were about one order of magnitude lower. In addition, their profiles varied in the urine samples compared between mothers and their newborns. No correlation was found in the concentrations of target compounds measured in the urine samples collected from mothers and their newborns. The highest correlation was found for 2-OH-NAP, with the coefficient of determination R^2 0.79 in the urine samples collected in Ceske Budejovice from April to August 2017. However, these coefficients for this compound were lower in urine samples collected in other seasons in Ceske Budejovice and the second sampling locality Most (Table S1 in the Supplementary data). In addition, no relationship was found between concentrations of OH-PAHs found in the urine and mothers' age, and BMI and newborns' birth weight.

3.2. Comparison of OH-PAH concentrations in urine samples collected from mothers and their newborns according to sampling season and locality

The samples were divided into three subgroups based on the sampling periods in relation to the concentrations of BaP in the air, namely from July to September 2016 (BaP concentration in the air <1 ng/m^3), from October 2016 to March 2017 (BaP concentration in the air >1 ng/m^3) and from April to August 2017(BaP concentration in the air <1 ng/m^3).

The concentration data of all PAHs measured in the air are summarised in Table S2 in the Supplementary Data.

To evaluate the differences between groups of samples, a statistical t-test was performed. Detailed results from this test (t-value, t_α and p-value) are summarised in Table S3 in the Supplementary Data.

3.2.1. Urine samples collected from mothers and their newborns

As was earlier mentioned, the median concentration of ΣOH-PAHs in all urine samples was approximately 1.7 times higher in the urine samples collected from mothers (median concentration 8.96 μg/g creatinine) compared to their children (median 5.15 μg/g creatinine) as shown in Fig. 1.

In the samples collected in the period July–September 2016 from mothers living in Ceske Budejovice, the median concentration of ∑OH-PAHs was statistically significantly higher ($\alpha = 0.05$) compared to their newborns (8.88 μg/g creatinine and 3.86 μg/g creatinine). The same trend was observed in Ceske Budejovice in the urine samples collected in the periods of October 2016–March 2017 and April–August 2017. However, in the last two periods, we observed a smaller difference in ∑OH-PAHs concentrations in urine samples collected from mothers compared to their children than in the first sampling period. In October 2016–March 2017, the median concentration of ∑OH-PAHs in mothers' urine was 1.9 times higher compared to their children (6.76 μg/g creatinine and 3.65 μg/g creatinine). In April–August 2017, the median concentration of ∑OH-PAHs in mothers' urine was 1.7 times higher compared to their children (5.82 μg/g creatinine and 3.43 μg/g creatinine) as shown in Fig. 1.

Concerning the second sampling locality, Most, a statistically significant difference ($\alpha = 0.05$) was found between the urine samples collected from mothers and their newborns in the sampling period of July–September 2016. The median concentration of ∑OH-PAHs in mothers' urine from this period was 2.3 times higher compared to their children (11.42 μg/g creatinine and 4.96 μg/g creatinine). In the second sampling season (October 2016–March 2017), the difference between the median concentration of ∑OH-PAHs in mothers' and newborns' urine samples was lower. The median concentration of ∑OH-PAHs in the urine samples collected from mothers was 1.5 times higher compared to their children (12.59 μg/g creatinine and 8.29 μg/g creatinine). No statistical difference ($\alpha = 0.05$) was found in the concentrations of OH-PAHs in samples collected from mothers and their newborns in April–August 2017 in Most (8.65 μg/g creatinine and 8.04 μg/g creatinine) (Fig. 1).

3.2.2. Urine samples collected from mothers living in Ceske Budejovice and Most

The median concentration of ΣOH-PAHs in the urine samples collected from mothers living in Ceske Budejovice in the first sampling round (July–September 2016) was statistically significantly higher ($\alpha = 0.05$) compared to the samples collected from mothers between

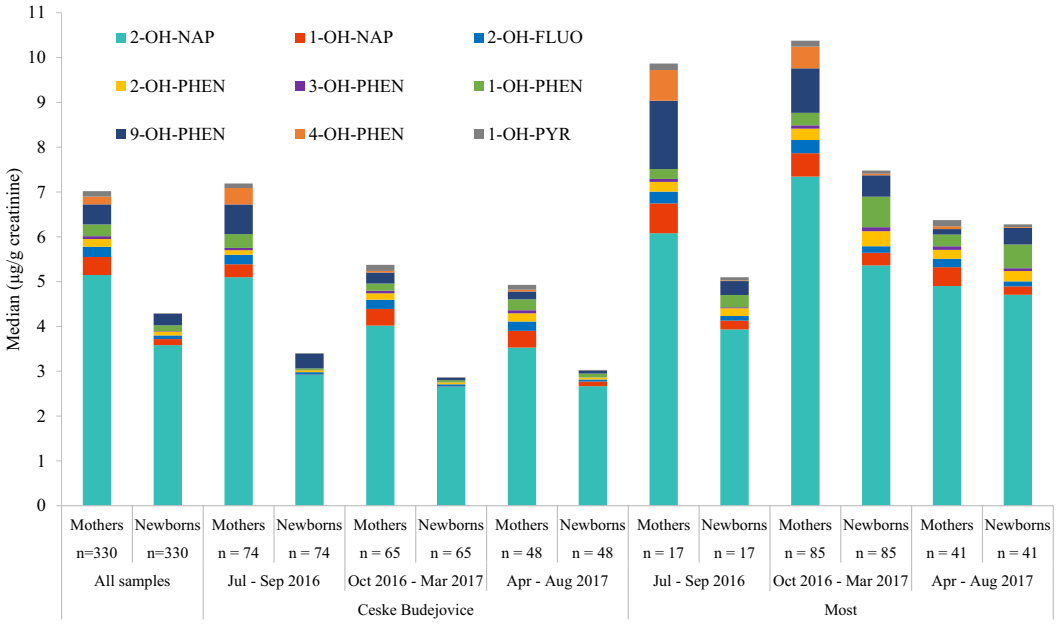


Fig. 1. Concentrations (µg/g creatinine) of detected OH-PAHs in mothers' and newborns' urine samples.

October 2016 to March 2017 in the same locality (8.88 µg/g creatinine and 6.76 µg/g creatinine), and the median concentration of ΣOH-PAHs in urine samples collected in April–August 2017 (5.82 µg/g creatinine) as shown in Fig. 1. However, as Fig. 2 shows, the obtained concentrations of OH-PAHs in urine do not correlate with PAHs measured in the ambient air. We assume that in this case mothers were exposed to PAHs from other sources, such as diet.

In the case of mothers from the Most region, no statistically significant difference ($\alpha = 0.05$) in the ΣOH-PAHs amount was determined between the first two sampling rounds (July–September 2016 (11.42 µg/g creatinine) and October 2016–March 2017 (12.59 µg/g creatinine)). The median concentration of ΣOH-PAHs collected in the third sampling round (April–August 2017) was approximately 1.5 times lower (8.65 µg/g creatinine) compared to the previous sampling rounds (Fig. 1). This outcome is probably caused again by exposure sources other than the inhalation of PAH-contaminated air (Fig. 2).

For a clear demonstration of the correlation between PAHs present in the ambient air and their monohydroxylated metabolites measured in urine, Fig. 2 only shows the results for phenanthrene (PHEN) (measured in the air) and ΣOH-PHEN (sum of 1-, 2-, 3-, 4- and 9-OH-PHEN) measured in mothers' urine samples. The results for other PAHs are very similar. The detailed results of all PAHs in ambient air are documented in **Table S2** in the Supplementary data. Though Fig. 2 shows that the highest concentration of PHEN in the air was measured in February, it was not the month when the highest ΣOH-PHEN in urine was determined. The same trend was observed for all measured PAHs and their monohydroxylated metabolites in urine. The highest concentrations of PAHs were measured in the air samples collected in both Ceske Budejovice and Most in February 2017 (ΣPAHs 28.2 ng/m^3 and 22.0 ng/m^3). However, these results do not correspond with the levels of ΣOH-PAHs measured in the urine. According to the highest concentrations of PAHs in the ambient air in Ceske Budejovice in

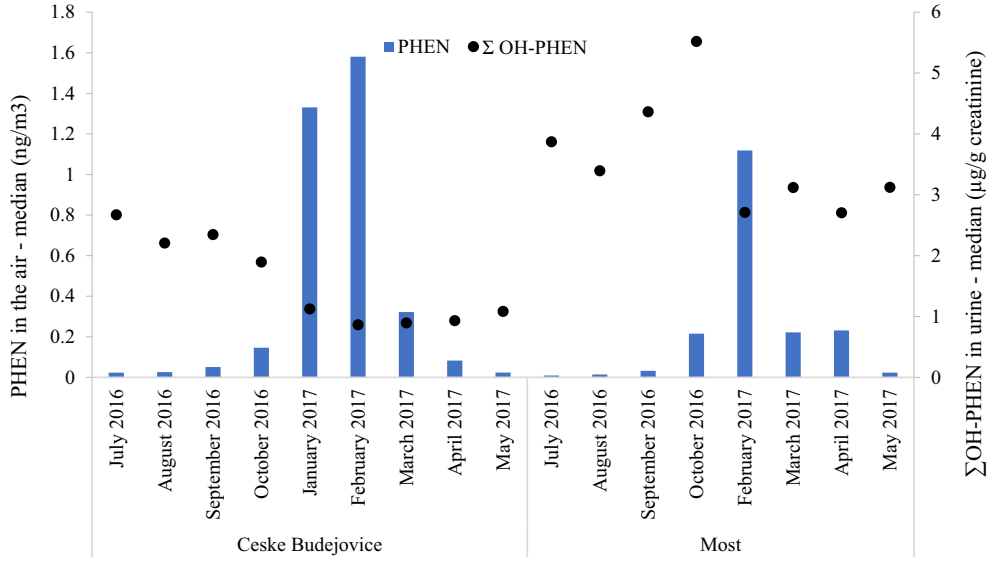


Fig. 2. Comparison of PHEN concentrations in the air and ∑OH-PHEN measured in urine.

February 2017, the concentrations of OH-PAHs in urine should be the highest as well. However, as shown in Fig. 2, OH-PAH concentrations in urine are the lowest in February 2017. The same trend can be observed in Most, the second sampling locality.

3.2.3. Urine samples collected from newborns born in Ceske Budejovice and Most

In the newborns' urine samples, there were comparable median concentration in samples collected in all three sampling rounds in Ceske Budejovice (July–September 2016 (3.86 μg/g creatinine), October 2016–April 2017 (3.65 μg/g creatinine) and May–August 2017 (3.43 μg/g creatinine) as shown in Fig. 1. The amount of ΣOH-PAHs in newborns' urine samples from Most collected in July–September 2016 (4.96 μg/g creatinine) was statistically significantly lower compared to the second and third sampling rounds (October 2016–April 2017 (8.29 μg/g creatinine), May–August 2017 (8.04 μg/g creatinine) as shown in Fig. 1. Then again, these outcomes do not correlate with the concentration of PAHs measured in the ambient air.

Our results indicate that there is possibly another exposure source that has a stronger influence on the concentrations of OH-PAHs in the urine. It is assumed that diet accounts for almost 80% of the total PAH exposure (Li et al., 2016; Ma and Harrad, 2015). The concentrations of PAHs measured in the whole-day diets from mothers participating in this study will be published in our other paper (Polachova et al., 2020). Unfortunately, breast milk samples were not collected within the Qualitas project.

4. Discussion

Our results were compared with other studies and our previous study, where urine samples collected from mothers and their newborns living in two localities of the Czech Republic (control locality Ceske Budejovice and highly air-polluted Karvina) were measured (Urbancova et al., 2017). As shown in Table 3, the analyte with the highest concentration in all of the studies was 2-OH-NAP, which corresponds with our results from this study and our previous study. The profiles of other target analytes are variable between countries. This result indicates that people in different countries are exposed to different mixtures of PAHs present in the air, diet and other sources.

The concentrations of OH-PAHs in urine samples collected from mothers were comparable with those reported in the US study in 2012 (Fan et al., 2012), the study from India in 2012 (Guo et al., 2013a), where samples of the general population were assessed, and our previously published paper (Urbancova et al., 2017). The concentrations of target compounds in urine samples collected from newborn children were comparable with the samples collected from infants living in the USA (Dobraca et al., 2018) and the samples collected from newborns which we analysed in our previous study (Urbancova et al., 2017).

5. Conclusions

A total of 660 samples was measured to detect the presence of 11 OH-PAHs in urine samples collected from mothers and their newborns residing in two localities of the Czech Republic, in order to assess exposure to PAHs in this particular part of the Czech population.

The most abundant compound was 2-OH-NAP, which was found in 100% of the samples. It was also the analyte with the highest concentration, which corresponds with the results from other published papers from Poland, USA, Germany, India, and Iran (Ramsauer et al., 2011; Sochacka-Tatara et al., 2018; Dobraca et al., 2018; Kelishadi et al., 2018; Guo et al., 2013a; Fan et al., 2012) and the results from our previous study (Urbancova et al., 2017).

The median concentration of ΣOH-PAHs in all urine samples was approximately 1.7 times higher in urine samples collected from mothers (median concentration 8.96 μg/g creatinine) compared to their newborns (median 5.15 μg/g creatinine). Moreover, the concentration of these compounds in the urine samples collected in Most were almost 2 times higher compared to the samples from Ceske Budejovice (median concentration of ΣOH-PAHs 9.28 μg/g creatinine and 4.92 μg/g creatinine). The most contaminated samples were collected in Most in the period October 2016–March 2017 from both mothers (12.59 μg/g creatinine) and their newborns (8.29 μg/g creatinine).

The results obtained for newborns in Most seem to be similar to our previous results (Urbancova et al., 2017), where urine samples collected in Karvina and Ceske Budejovice were assessed: BaP level in Karvina in the summer period (August–October 2013) corresponded to Ceske Budejovice in the summer and winter periods (January–April 2014), but the median concentration of ΣOH-PAHs in newborn urine samples in Karvina was approximately 2 times higher in summer and 4 times higher in winter. We may assume that the population in the previously highly air-polluted mining districts carries some long-term changes (maybe existing changes in genetic information), which also affect the metabolism of PAHs. It could be related to the long-lasting effect, corresponding to the shortened life expectancy.

Abbreviations

1-OH-NAP Naphthalene-1-ol
1-OH-PHEN Phenanthrene-1-ol

Table 3
Comparison of measured concentrations of OH-PAHs in urine in the presented study with other papers.

	USA 2006 Girls[a] Dobraca et al., 2018	Poland 2007 Children[b] Sochacka-Tatara et al., 2018	Germany 2010 Smokers[c] Ramsauer et al., 2011	USA 2012 General population[a] Fan et al., 2012	India 2012 General population[a] Guo et al., 2013a	Iran 2016 Children[a] Kelishadi et al., 2018	Czech Republic 2013–2014 Women[d] Urbancova et al., 2017	Czech Republic 2013–2014 Newborns[d] Urbancova et al., 2017	Czech Republic 2016–2017 Women[d] [Presented study]	Czech Republic 2016–2017 Newborns[d] [Presented study]
Samples	$n = 431$	$n = 218$	$n = 100$	$n = 34$	$n = 38$	$n = 150$	$n = 265$	$n = 266$	n = 330	n = 330
1-OH-NAP	1.19	3.30	4.26	1.59	1.10	0.36	0.73 (0.54)	0.23 (0.45)	0.36 (0.41)	0.12 (0.20)
2-OH-NAP	2.16	8.20	8.47	7.65	3.80	0.42	5.98 (5.36)	2.01 (3.10)	4.66 (5.16)	2.28 (3.60)
2-OH-FLUO	0.20	0.91	1.56	0.87	0.35	n.a.	0.41 (0.37)	0.12 (0.18)	0.23 (0.23)	0.07 (0.11)
Σ OH-PHEN	0.26	1.28	0.56	1.76	0.73	0.11	1.19 (0.99)	0.60 (0.88)	1.15 (1.24)	0.41 (0.76)
1-OH-PYR	0.10	0.36	0.16	0.99	0.42	0.10	0.21 (0.21)	0.06 (0.10)	0.12 (0.13)	0.05 (0.10)
6-OH-CHRY	n.a.	n.a.	n.a.	n.a.	< 0.01	n.a.	< 0.01	< 0.01	< 0.01	< 0.01
3-OH-BaP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	< 0.90	< 0.90	< 0.90	< 0.90
Σ OH-PAH	3.92	15.96	15.00	12.86	6.40	0.99	9.55 (8.14)	3.19 (4.99)	7.50 (8.66)	2.97 (4.83)

a – Geometric mean (ng/mL urine).
b – Median (μg/g creatinine).
c – Median (ng/mL urine).
d – Median (ng/mL urine; μg/g creatinine).

1-OH-PYR
Pyrene-1-ol
2-OH-FLUO
Fluorene-2-ol
2-OH-NAP
Naphthalene-2-ol
2-OH-PHEN
Phenanthrene-2-ol
3-OH-BaP Benzo[*a*]pyrene-3-ol
3-OH-PHEN
Phenanthrene-3-ol
4-OH-PHEN
Phenanthrene-4-ol
6-OH-CHRY
Chrysen-6-ol
7-OH-BaP Benzo[*a*]pyrene-7-ol
9-OH-PHEN
Phenanthrene-9-ol
ADHD Attention deficit hyperactivity disorder
BaP Benzo[*a*]pyrene
BMI Body mass index
d-SPE Dispersive solid phase extraction
d_7-1-OH-NAP
$[^2H]_7$-naphtalene-1-ol
d_7-2-OH-NAP
$[^2H]_7$-naphtalene-2-ol
d_8-9-OH-PHEN
$[^2H]_8$-phenanthrene-9-ol
d_9-1-OH-PHEN
$[^2H]_9$-phenanthrene-1-ol
d_9-1-OH-PYR
$[^2H]_9$-pyrene-1-ol
d_9-2-OH-FLUO
$[^2H]_9$-fluorene-2-ol
d_9-2-OH-PHEN
$[^2H]_9$-phenanthrene-2-ol
d_9-3-OH-PHEN
$[^2H]_9$-phenanthrene-3-ol
d_{11}-3-OH-BaP
$[^2H]_{11}$-benzo[*a*]pyrene-3-ol
ESI- Electrospray ionization (negative mode)
HiVol samplers High-Volume air samplers
IARC International Agency for Research on Cancer
IPCS International Programme on Chemical Safety
JECFA Joint FAO/WHO Expert Committee on Food Additives
LLE Liquid-liquid extraction
LOQ Limit of quantification
NIST National Institute of Standards and Technology
OH-PAHs Monohydroxylated polycyclic aromatic hydrocarbons
PAHs Polycyclic aromatic hydrocarbons
PHEN Phenanthrene
PFP Pentafluorophenyl
SCF Scientific Committee on Food
SRM Standard Reference Material
UHPLC–MS/MS
Ultra-high performance liquid chromatography coupled with tandem mass spectrometry

Consent for publication

Author and all co-authors agree with the publication of this article.

Ethical approval and consent to participate

The study was approved by the Ethics Committee of Ceske Budejovice Hospital, Department of Obstetrics and Department of Neonatology, the Most Hospital, Department of Obstetrics and Department of Neonatology and the Institute of Experimental Medicine of the CAS in Prague. Each mother signed a written consent to participate in the study.

Availability of supporting data

Supporting data are available for download as Supplementary information or available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Katerina Urbancova: Investigation, Validation, Writing - original draft.**Darina Dvorakova:**Investigation, Validation.**Tomas Gramblicka:** Investigation, Validation.**Radim J. Sram:**Conceptualization.**Jana Hajslova:**Conceptualization.**Jana Pulkrabova:**Conceptualization, Writing - original draft.

Declaration of competing interest

All authors declare that they have no competing interests.

Acknowledgements

This work was supported by the EU Horizon 2020 Programme grant agreement No 733032 (HBM4EU), the grant of the Czech Academy of Sciences, Strategy AV21, Qualitas, the "National Programme of Sustainability I" - NPU I (LO1601) and the grants LTAUSA17182 and MSMT No 21-SVV/2019. We would like to acknowledge great support from the HBM4EU WP9 (Laboratory analysis and quality assurance unit), in particular Argelia Castaño, Marta Esteban and their colleagues from the Institute of Health Carlos III in Madrid. Finally, this study would not have been possible without the close cooperation of all obstetricians, paediatricians, nurses and laboratory workers in both hospitals, especially Dr. Jiri Bilek in Most and Dr. Milos Veleminsky, Jr. in Ceske Budejovice.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.138116.

References

Abdel-Shafy, J.I., Mansour, M.S.M., 2016. A review on polycyclic aromatic hydrocarbons: source, environmental impact, effect on human health and remediation. Egypt. J. Pet. 25, 107–123.
Adetona, O., Li, Z., Sjödin, A., Romanoff, L.C., Aguilar-Villalobos, M., Needham, L.L., Hall, D.B., Cassidy, B.E., Naeher, L.P., 2013. Biomonitoring of polycyclic aromatic hydrocarbon exposure in pregnant women in Trujillo, Peru—comparison of different fuel types used for cooking. Environ. Int. 53, 1–8.
Alicandro, G., Rota, M., Boffetta, P., La Vecchia, C., 2016. Occupational exposure to polycyclic aromatic hydrocarbons and lymphatic and hematopoietic neoplasms: a systematic review and meta-analysis of cohort studies. Arch. Toxicol. 90, 2643–2656.
Campo, L., Rossella, F., Pavanello, S., Mielzynska, D., Siwinska, E., Kapka, L., Bertazzi, P.A., Fustinoni, S., 2010. Urinary profiles to assess polycyclic aromatic hydrocarbons exposure in coke-oven workers. Toxicol. Lett. 192 (1), 72–78.
Cathey, A., Ferguson, K.K., McElrath, T.F., Cantonwine, D.E., Pace, G., Alshawabkeh, A., Cordero, J.F., Meeker, J.D., 2018. Distribution and predictors of urinary polycyclic aromatic hydrocarbon metabolites in two pregnancy cohort studies. Environ. Pollut. 232, 556–562.
Choi, H., Tabashidze, N., Rossner Jr., P., Dostal, M., Pastorkova, A., Kong, S.W., Gmuender, H., Sram, R.J., 2017. Altered vulnerability to asthma at various levels of ambient Benzo [a] Pyrene by CTLA4, STAT4 and CYP2E1 polymorphisms. Environ. Pollut. 231, 1134–1144.
Choi, H., Song, W.M., Wang, M., Sram, R.J., Zhang, B., 2019. Benzo [a] pyrene is associated with dysregulated myelo-lymphoid hematopoiesis in asthmatic children. Environ. Int. 128, 218–232.
Dejmek, J., Solansky, I., Benes, I., Lenicek, J., Sram, R.J., 2000. The impact of polycyclic aromatic hydrocarbons and fine particles on pregnancy outcome. Environ. Health Perspect. 108, 1159–1164.
Dobraca, D., Lum, R., Sjödin, A., Calafat, A.M., Laurent, C.A., Kushi, L.H., Windham, G.C., 2018. Urinary biomarkers of polycyclic aromatic hydrocarbons in pre-and peri-

pubertal girls in northern California: predictors of exposure and temporal variability. Environ. Res. 165, 46–54.
Dostal, M., Pastorkova, A., Rychlik, S., Rychlikova, E., Svecova, V., Schallerova, E., Sram, R.J., 2013. Comparison of child morbidity in regions of Ostrava, Czech Republic, with different degrees of pollution: a retrospective cohort study. Environ. Health 12 (1), 74–85.
EFSA (European Food Safety Authority), 2008. Polycyclic aromatic hydrocarbons in food. Scientific opinion of the panel on contaminants in the food chain on a request from the European Commission on polycyclic aromatic hydrocarbons in food. The EFSA Journal 724, 1–114.
Fan, R., Wang, D., Ramage, R., She, J., 2012. Fast and simultaneous determination of urinary 8-hydroxy-2′-deoxyguanosine and ten monohydroxylated polycyclic aromatic hydrocarbons by liquid chromatography/tandem mass spectrometry. Chem. Res. Toxicol. 25 (2), 491–499.
Guo, Y., Senthilkumar, K., Alomirah, H., Moon, H.B., Minh, T.B., Mohd, M.A., Nakata, H., Kannan, K., 2013a. Concentrations and profiles of urinary polycyclic aromatic hydrocarbon metabolites (OH-PAHs) in several Asian countries. Environ. Sci. Technol. 47 (6), 2932–2938.
Guo, Y., Senthilkumar, K., Alomirah, H., Moon, H.B., Minh, T.B., Mohd, M.A., Nakata, H., Kannan, K., 2013b. Concentrations and profiles of urinary polycyclic aromatic hydrocarbon metabolites (OH-PAHs) in several Asian countries. Science & Technology 47, 2932–2938.
Hertz-Picciotto, I., Dostal, M., Yap, P.S., Baker, R.J., Joad, J.P., Lipsett, M., Greenfield, T., Herr, C.E.W., Benes, I., Shumway, R.H., Pinkerton, K.E., Sram, R.J., 2007. Early childhood lower respiratory illness and air pollution. Environ. Health Perspect. 115, 1510–1518.
IARC, 2018. Monographs on the Evaluation of Carcinogenics Risks to Humans: List of Classifications. vols. Volumes 1-122. https://monographs.iarc.fr/list-of-classifications-volumes/.
Kelishadi, R., Sobhani, P., Poursafa, P., Amin, M.M., Ebrahimpour, K., Hovsepian, S., Mansourian, M., Najafi, R., Hashemipour, M., 2018. Is there any association between urinary metabolites of polycyclic aromatic hydrocarbons and thyroid hormone levels in children and adolescents? Environ. Sci. Pollut. Res. 5 (2), 1962–1968.
Kim, K.H., Jahan, S.A., Kabir, E., Brown, R.J., 2013. A review of airborne polycyclic aromatic hydrocarbons (PAHs) and their human health effects. Environ. Int. 60, 71–80.
Lankova, D., Urbancova, K., Sram, R.J., Hajslova, J., Pulkrabova, J., 2016. A novel strategy for the determination of polycyclic aromatic hydrocarbon monohydroxylated metabolites in urine using ultra-high-performance liquid chromatography with tandem mass spectrometry. Anal. Bioanal. Chem. 408, 2515–2525.
Li, Z., Romanoff, L.C., Trinidad, D.A., Pittman, E.N., Hilton, D., Hubbard, K., Carmichael, H., Parker, J., Calafat, A.M., Sjödin, A., 2014. Quantification of 21 metabolites of methylnaphthalenes and polycyclic aromatic hydrocarbons in human urine. Anal. Bioanal. Chem. 406, 3119–3129.
Li, G., Wu, S., Wang, L., Akoh, C.C., 2016. Concentration, dietary exposure and health risk estimation of polycyclic aromatic hydrocarbons (PAUs) in youtiao, a Chinese traditional fried food. Food Control 59, 328–336.
Ma, Y., Harrad, S., 2015. Spatiotemporal analysis and human exposure assessment on polycyclic aromatic hydrocarbons in indoor air, settled house dust, and diet: a review. Environ. Int. 84, 7–16.
Nethery, E., Wheeler, A.J., Fisher, M., Sjödin, A., Li, Z., Romanoff, L.C., Foster, W., Arbuckle, T.E., 2012. Urinary polycyclic aromatic hydrocarbons as a biomarker of exposure to PAHs in air: a pilot study among pregnant women. J. Expo. Sci. Environ. Epidemiol. 22 (1), 70–81.
Onyemauwa, F., Rappaport, S.M., Sobus, J.R., Gajdošová, D., Wu, R.A., Waidyanatha, S., 2009. Using liquid chromatography–tandem mass spectrometry to quantify monohydroxylated metabolites of polycyclic aromatic hydrocarbons in urine. J. Chrom. B 877 (11−12), 1117–1125.
Perera, F.P., Wheelock, K., Wang, Y., Tang, D., Margolis, A.E., Badia, G., Cowell, W., Miller, R.L., Rauh, V., Wang, S., Herbstman, J.B., 2018. Combined effects of prenatal exposure to polycyclic aromatic hydrocarbons and material hardship on child ADHD behavior problems. Environ. Res. 160, 506–513.
Polachova, A., Gramblicka, T., Parizek, O., Sram, R.J., Stupak, M., Hajslova, J., Pulkrabova, J., 2020. Estimation of human exposure to polycyclic aromatic hydrocarbons (PAHs) based on the dietary and outdoor atmospheric monitoring in the Czech Republic. Environ. Res. 182, 108977.
Poursafa, P., Dadvand, P., Amin, M.M., Hajizadeh, Y., Ebrahimpour, K., Mansourian, M., Pourzamani, H., Sunyer, J., Kelishadi, R., 2018. Association of polycyclic aromatic hydrocarbons with cardiometabolic risk factors and obesity in children. Environ. Int. 118, 203–210.
Ramsauer, B., Sterz, K., Hagedorn, H.W., Engl, J., Scherer, G., McEwan, M., Errington, G., Shepperd, J., Cheung, F., 2011. A liquid chromatography/tandem mass spectrometry (LC-MS/MS) method for the determination of phenolic polycyclic aromatic hydrocarbons (OH-PAH) in urine of non-smokers and smokers. Anal. Bioanal. Chem. 399 (2), 877–889.
Sochacka-Tatara, E., Majewska, R., Perera, F.P., Camann, D., Spengler, J., Wheelock, K., Sowa, A., Jacek, R., Mróz, E., Pac, A., 2018. Urinary polycyclic aromatic hydrocarbon metabolites among 3-year-old children from Krakow, Poland. Environ. Res. 164, 212–220.
Sram, R.J., Benes, I., Binkova, B., Dejmek, J., Horstman, D., Kotesovec, F., Otto, D., Perrault, S.D., Rubes, J., Selevan, S.G., Skalik, I., Stevens, R.K., Lewtas, J., 1996. Teplice program–the impact of air pollution on human health. Environ. Health Perspect. 104 (Suppl. 4), 699–714.
Sram RJ, Rossner P, Jr., Rossnerova A, Dostal M, Milcova A, Svecova V, Pulkrabova J, Hajslova J, Veleminsky M, Jr. Impact of air pollution to genome of newborns. Centr. Eur. J. Public Health. 2016, 24 (Suppl.), S40-S44.
Sykorova, B., Kucbel, M., Raclavska, H., Drozdova, J., Raclavsky, K., 2015. Seasonal variations of polycyclic aromatic hydrocarbons (PAHs) in the air of Moravian-Silesian region, Czech Republic. Environment, Energy and Applied Technology. CRC Press, Taylor & Francis Group, pp. 367–372.
Tombolini, F., Pigini, D., Tranfo, G., Paci, E., Carosi, I., Marini, F., Bauleo, L., Ancona, C., Forastiere, F., 2018. Levels of urinary metabolites of four PAHs and cotinine determined in 1016 volunteers living in Central Italy. Environ. Sci. Pollut. Res. 1–8.
Urbancova, K., Lankova, D., Rossner, P., Rossnerova, A., Svecova, V., Tomaniova, M., Veleminsky Jr., M., Sram, R.J., Hajslova, J., Pulkrabova, J., 2017. Evaluation of 11 polycyclic aromatic hydrocarbon metabolites in urine of Czech mothers and newborns. Sci. Total Environ. 577, 212–219.
Veleminsky Jr., M., Manzl, M., Sram, R.J., 2016. The impact of air pollution in the southern Bohemia region on foetuses and newborns. Neuroendocrinol. Lett. 37 (Suppl. 2), 52–57.
Yamamoto, Y., Ishizaki, A., Kataoka, H., 2015. Biomonitoring method for the determination of polycyclic aromatic hydrocarbons in hair by online in-tube solid-phase microextraction coupled with high performance liquid chromatography and fluorescence detection. J. Chromatogr. B 1000, 187–191.

APPENDIX XI

Urbancova K., Lankova D., Rossner P., Rossnerova A., Svecova V.,
Tomaniova M., Veleminsky M., Jr., Sram R. J., Hajslova J., Pulkrabova J.

Evaluation of 11 polycyclic aromatic hydrocarbon metabolites in urine of Czech mothers and newborns

Science of the Total Environment (2017) 577: 212–219.

doi: 10.1016/j.scitotenv.2016.10.165

Science of the Total Environment 577 (2017) 212–219

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Evaluation of 11 polycyclic aromatic hydrocarbon metabolites in urine of Czech mothers and newborns

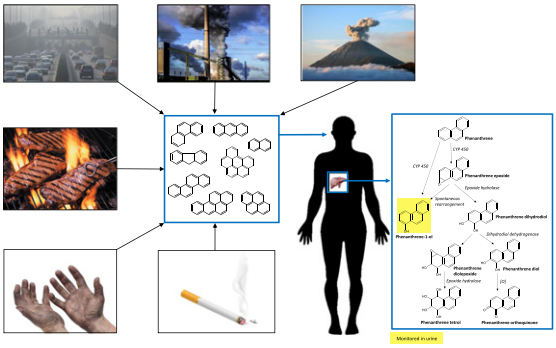
Katerina Urbancova [a], Darina Lankova [a], Pavel Rossner [b], Andrea Rossnerova [b], Vlasta Svecova [b], Monika Tomaniova [a], Milos Veleminsky Jr. [c], Radim J. Sram [b], Jana Hajslova [a], Jana Pulkrabova [a,*]

[a] University of Chemistry and Technology, Prague, Faculty of Food and Biochemical Technology, Department of Food Analysis and Nutrition, Technicka 3, 166 28 Prague, Czech Republic
[b] Institute of Experimental Medicine AS CR, Videnska 1083, 142 20 Prague 4, Czech Republic
[c] Faculty of Health and Social Studies, University of South Bohemia, Ceske Budejovice, Czech Republic

HIGHLIGHTS

- 11 OH-PAHs were measured in 531 urine samples from mothers and their newborns.
- The most frequently detected analyte with the highest concentration was 2-OH-NAP.
- Chrysene-6-ol and benzo[*a*]pyrene-3-ol were not detected in any of analyzed samples.
- ΣOH-PAHs in children's urine was1.6× lower compared to their mothers.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 15 September 2016
Received in revised form 20 October 2016
Accepted 20 October 2016
Available online 29 October 2016

Editor: D. Barcelo

Keywords:
U-HPLC–MS/MS
OH-PAHs

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) represent a large group of ubiquitous contaminants of the environment, including food chain where they are released as by-products of incomplete combustion of an organic matter. Epidemiological studies have shown that exposure to PAHs correlated with increased incidence of cancer. Carcinogenicity is associated mainly with metabolites that are formed during metabolic degradation of these substances in exposed organism. In this study monohydroxylated PAHs (OH-PAHs), the major metabolites excreted into urine, were determined in 531 urine samples collected from mothers and their newborns from two localities of the Czech Republic – heavily air polluted Karvina and control locality of Ceske Budejovice and in two sampling rounds – August–October 2013 (summer, less air polluted season) and January–April 2014 (winter, more air polluted season). From all targeted analytes, naphthalene-2-ol was the most abundant compound present in 100% of the samples and it represented also the analyte with the highest concentration. Median concentration of ΣOH-

Abbreviations: 1-OH-NAP, naphthalene-1-ol; 1-OH-PHEN, phenanthrene-1-ol; 1-OH-PYR, pyrene-1-ol; 2-OH-FLUO, fluorene-2-ol; 2-OH-NAP, naphthalene-2-ol; 2-OH-PHEN, phenanthrene-2-ol; 3-OH-BaP, benzo[*a*]pyrene-3-ol; 3-OH-PHEN, phenanthrene-3-ol; 4-OH-PHEN, phenanthrene-4-ol; 6-OH-CHRY, chrysen-6-ol; 7-OH-BaP, benzo[*a*]pyrene-7-ol; 9-OH-PHEN, phenanthrene-9-ol; BaP, benzo[*a*]pyrene; BMI, body mass index; d-SPE, dispersive solid phase extraction; d_7-1-OH-NAP, $[^2H]_7$-naphtalene-1-ol; d_7-2-OH-NAP, $[^2H]_7$-naphtalene-2-ol; d_8-9-OH-PHEN, $[^2H]_8$-phenanthrene-9-ol; d_9-1-OH-PHEN, $[^2H]_9$-phenanthrene-1-ol; d_9-1-OH-PYR, $[^2H]_9$-pyrene-1-ol; d_9-2-OH-FLUO, $[^2H]_9$-fluorene-2-ol; d_9-2-OH-PHEN, $[^2H]_9$-phenanthrene-2-ol; d_9-3-OH-PHEN, $[^2H]_9$-phenanthrene-3-ol; d_{11}-3-OH-BaP, $[^2H]_{11}$-benzo[*a*]pyrene-3-ol; EFSA, European Food Safety Authority; ESI-, electrospray ionization (negative mode); FLUO, fluorene; HiVol samplers, High-Volume air samplers; JECFA, Joint FAO/WHO Expert Committee on Food Additives; LLE, liquid-liquid extraction; LOQ, limit of quantification; MRM, multiple reaction monitoring; NIST, National Institute of Standards and Technology; OH-PAHs, monohydroxylated PAHs; PAHs, polycyclic aromatic hydrocarbons; PHEN, phenanthrene; PFP, pentafluorophenyl; $PM_{2.5}$, particles smaller than 2.5 μm; PYR, pyrene; S/N, signal to noise ratio; SCF, Scientific Committee on Food; SRM, Standard Reference Material; U-HPLC–MS/MS, ultra-high performance liquid chromatography coupled with tandem mass spectrometry.

* Corresponding author.
E-mail address: Jana.Pulkrabova@vscht.cz (J. Pulkrabova).

http://dx.doi.org/10.1016/j.scitotenv.2016.10.165

Czech Republic
Biomonitoring
Human exposure
3-OH-BaP

PAHs in the urine of children was on average 1.6 times lower compared to the respective mother which correlates with higher intake of PAHs by mothers. ΣOH-PAHs concentrations determined in mothers' urine collected in the summer were comparable in both localities. No significant increase occurred in Ceske Budejovice in winter, while in samples from the Karvina region a statistically significant difference ($\alpha = 0.05$) in the amount of ΣOH-PAHs was observed. The median concentrations of ΣOH-PAHs in mothers' urine samples in the winter were 1.5 times higher than in the summer in the same locality. The amounts of ΣOH-PAHs in newborns' urine from Karvina in the winter season were 1.5 times higher than in the summer collected in the same locality and 3.3 times higher when compared with the less polluted locality of Ceske Budejovice.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) represent an important group of environmental contaminants that may be formed and released during combustion/pyrolysis of organic matter. In addition to a wide range of anthropogenic emission sources (e.g. heavy industry, exhaust fumes) and natural processes (e.g. volcanic activity, forest fires) some culinary practices or industrial processes (e.g. drying, grilling, roasting, frying and smoking) may also be associated with the formation of PAHs. With regards to these multiple sources the occurrence of PAHs in the environment is ubiquitous. They can be found in various compartments such air, soil, water, sediment and also in food supplies (EFSA, 2008). It is worth noting that these pollutants occur here as complex mixtures consisting of PAHs with a different number of aromatic rings (typically 2–6). The incidence of the individual compounds in these mixtures varies largely not only within different localities but also between various seasons depending on the intensity of emissions from heavy industry, traffic or heating frequency in the winter season (Li et al., 2010).

The most common exposure pathway of a human body to PAHs is through the digestion of contaminated foods, which accounts for over 70% of the total PAH exposure for non-smokers. Another important exposure route is the inhalation of airborne PAHs, in the case of smokers tobacco smoke is the major source (Ma and Harrad, 2015; Moustafa et al., 2015). For occupationally exposed individuals the transfer of these substances through the skin may also play an important role (e.g. workers with asphalt or coal, firefighters) (Campo et al., 2010; Oliveira et al., 2016).

Many studies have suggested that PAHs may exhibit a wide range of toxic effects (Alshaarawy et al., 2016; Wang et al., 2016). In the past decade PAHs were evaluated by the International Programme on Chemical Safety (IPCS), the Scientific Committee on Food (SCF) and by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). It has been concluded that 15 PAHs, namely benz[*a*]anthracene, benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, benzo[*k*]fluoranthene, benzo[*ghi*]perylene, benzo[*a*]pyrene, chrysene, cyclopenta[*cd*]pyrene, dibenz[*a,h*]anthracene, dibenzo[*a,e*]pyrene, dibenzo[*a,h*]pyrene, dibenzo[*a,i*]pyrene, dibenzo[*a,l*]pyrene, indeno[1,2,3-*cd*]pyrene and 5-methylchrysene show clear evidence of mutagenicity/genotoxicity in somatic cells in experimental animals in vivo (Boström et al., 2002; IARC, 2010; Onyemauwa et al., 2009; Zhong et al., 2011).

The fate of PAHs in the exposed organism is fairly complex. Carcinogenic and mutagenic PAHs can undergo at least three major metabolic pathways, which form highly reactive electrophilic intermediates capable of binding to cellular macromolecules, in particular proteins and nucleic acids. Following absorption, a rapid biotransformation process starts with phase I metabolism in which these compounds are oxidized by the hepatic cytochrome P450 (CYP 450) monooxygenases to form reactive epoxide intermediates followed by their reduction or hydrolysis yielding hydroxylated derivatives (OH-PAHs). In phase II metabolism, the OH-PAHs are conjugated to glucuronic acid or sulphate to increase water solubility (Li et al., 2006). OH-PAH conjugates with 2–3 benzene rings (low-molecular-weight OH-PAHs) are mostly excreted via urine and those with 4 or more benzene rings are mainly excreted via bile and faeces (high-molecular-weight OH-PAHs) (Campo et al., 2008; Onyemauwa et al., 2009; Ramesh et al., 2004; Zhong et al., 2011).

PAH metabolism in the human body is likely to be influenced by many individual factors such as age, gender, body fat percentage, metabolism of xenobiotics, physical characteristics (body mass index - BMI) and lifestyle (smoking, alcohol consumption, physical activity, location of residence, etc.). These predispositions can lead to the formation of multiple metabolites including epoxides, diolepoxides, monohydroxylated and polyhydroxylated PAHs, dihydrodiols or ortho-quinones of these substances (Campo et al., 2008; Onyemauwa et al., 2009; Yanxin et al., 2011).

PAHs may also interfere with hormones and act as endocrine disruptors (Annamalai and Namasivayam, 2015). Some of these contaminants can behave like estrogens or their antagonists (antiestrogens) and thus disturb estrogen-regulated processes in the body. Moreover, phenanthrene (PHEN) or fluorene (FLUO) have antiandrogen activity (Jedrychowski et al., 2013). After they enter the body, due to their non-polar character PAHs are transported to all lipid-rich tissues. Consequently, repeated and prolonged exposure to PAHs may lead to certain accumulation of these compounds in fat cells, liver and kidney (Hu et al., 2008; Pleil et al., 2010). Recent studies also suggest that nowadays widespread childhood obesity might be associated with exposure of the developing organism to these substances (Jedrychowski et al., 2013; Li et al., 2015; Scinicariello and Buser, 2014).

Due to the above mentioned human health effects of PAHs, a monitoring of an occurrence of metabolites in biological matrices represents an important tool for the assessment of human exposure. Currently the most common biomarkers for the evaluation of overall PAHs exposure are OH-PAHs. Of these biomarkers the most often used is pyrene-1-ol (1-OH-PYR), since its parent compound pyrene (PYR) is one of the principal components in PAHs mixture contaminating air. Because of the molecular symmetry of PYR only one monohydroxylated metabolite (unlike other PAHs) is originated from this parent PAH, so the 1-OH-PYR concentration in urine, to which it is primarily excreted, is relatively high and thus easy to measure. Nevertheless, PYR is not carcinogenic (due to the absence of a 'bay region' in the PYR molecule electrophilic diolepoxides, that belong to the most potent chemical mutagens reported so far, not being formed). The monitoring of its metabolite for the purpose of risk assessment is only based on the assumption that its levels to some extent correlate with benzo[*a*]pyrene (BaP) and other carcinogenic species co-occurring commonly with PYR in PAHs mixtures (Barbeau et al., 2011). However, as noted above, the PAHs compositional pattern in the environment and consequently in organisms varies largely depending on many factors. To obtain more accurate data monitoring more than one OH-PAH marker is needed. The metabolites of BaP, the key representative of carcinogenic PAHs involved in some environmental monitoring studies (Grova et al., 2016; Hecht, 2002; Lutier et al., 2016) might obviously be considered as suitable exposure biomarkers for human studies. Unfortunately, the detection of the most often targeted BaP metabolites benzo[*a*]pyrene-3-ol (3-OH-BaP) and benzo[*a*]pyrene-7-ol (7-OH-BaP) in urine is rather difficult, since they occur here at ultra-trace levels. This is not only due the multiple metabolic pathways that the parent BaP may undergo (yielding various metabolites) but also because the above mentioned OH-PAHs

are relatively non-polar and therefore are mainly excreted in faeces. As analysis of urine is the most common approach employed for a comprehensive assessment of humans' exposure to PAHs, some compromise has to be adopted. It is clearly more convenient to monitor the levels of OH-PAHs with two or three benzene rings, which are major PAHs metabolites measurable in urine. The most commonly targeted species are naphthalene-1-ol (1-OH-NAP), naphthalene-2-ol (2-OH-NAP), fluorene-2-ol (2-OH-FLUO), phenanthrene-9-ol (9-OH-PHEN) and phenanthrene-3-ol (3-OH-PHEN) (Fan et al., 2012; Ramsauer et al., 2011; Xu et al., 2004).

The main aim of this study (which is a part of the national project 'The impact of air pollution on the genome of newborns' (GA CR No. 13-13458S, Ambroz et al., 2016) was to analyse a broader range of OH-PAHs considered as suitable biomarkers of PAHs exposure, namely, 1-, 2-OH-NAP; 2-OH-FLUO; 1-, 2-, 3-, 4-, 9-OH-PHEN; 1-OH-PYR, chrysen-6-ol (6-OH-CHRY) and 3-OH-BaP in the set of 531 urine samples obtained from women and their newborn children living in Ceske Budejovice and Karvina. The Karvina district belongs among the regions with the highest concentrations of BaP in the polluted air in the European Union (Sram et al., 2013).

2. Materials and methods

2.1. Standards

Certified standards of OH-PAHs represented by 1-OH-NAP (1000 μg/mL of methanol) and 2-OH-NAP (1000 μg/mL of methanol) were obtained from Absolute Standards, Inc. (USA). 2-OH-FLUO, 1-OH-PHEN, 2-OH-PHEN and 4-OH-PHEN were supplied by Toronto Research Chemicals, Inc. (USA). 3-OH-PHEN, 9-OH-PHEN, 1-OH-PYR and 3-OH-BaP were purchased from Neochema (Germany). 6-OH-CHRY was obtained from AccuStandard® (USA). Isotopically labelled analogues, specifically $[^2H]_7$-naphtalene-1-ol (d_7-1-OH-NAP), $[^2H]_7$-naphtalene-2-ol (d_7-2-OH-NAP), $[^2H]_9$-fluorene-2-ol (d_9-2-OH-FLUO), $[^2H]_9$-phenanthrene-1-ol (d_9-1-OH-PHEN), $[^2H]_9$-phenanthrene-2-ol (d_9-2-OH-PHEN), $[^2H]_9$-phenanthrene-3-ol (d_9-3-OH-PHEN), $[^2H]_8$-phenanthrene-9-ol (d_8-9-OH-PHEN), $[^2H]_9$-pyrene-1-ol (d_9-1-OH-PYR), $[^2H]_{11}$-benzo[*a*]pyrene-3-ol (d_{11}-3-OH-BaP) were supplied by Toronto Research Chemicals, Inc. Creatinine was delivered by Sigma-Aldrich (USA). The purity of all standards and their isotopically labelled analogues was at least 98%.

Individual OH-PAHs delivered as solids were dissolved with respect to the manufacturers' recommendations. Mixtures of OH-PAHs and their isotopically labelled analogues (d_x-OH-PAHs) were prepared in methanol at concentrations 10, 100 and 1000 ng/mL. Each calibration solution of OH-PAHs corresponding to the calibration curve at levels 0.1, 0.25, 0.5; 1; 2.5; 5; 10; 25; 50 and 100 ng/mL in methanol contained the internal standard at level 10 ng/mL except for d_{11}-3-OH-BaP, for which the concentration was ten times higher (100 ng/mL) because of its lower instrumental sensitivity compared to other d_x-OH-PAHs. All solutions were stored at −20 °C in the freezer.

The Standard Reference Material® 3673 (Organic Contaminants in Non-Smokers' Urine) used for the method evaluation and validation experiments was supplied by the US National Institute of Standards and Technology (NIST, Gaithersburg, Maryland, USA).

2.2. Chemicals, reagents and other materials

Ethyl acetate, picric acid, enzyme β-glucuronidase (type HP-2, glucuronidase activity ≥ 100,000 units/mL, sulfatase activity ≤ 7500 units/mL) and sorbent Supel™ QuE Z-Sep were supplied by Sigma Aldrich. HPLC gradient methanol was delivered by Merck (Germany). Unsterile polytetrafluoroethylene (PTFE, 5.0 μm, Ø 25 mm) filters were purchased from Rotilabo® (Germany). 96-well microtiter plates were obtained from Gama Group (Czech Republic). Polypropylene (PP) centrifuge tube filters (nylon, pore size 0.22 μm) were supplied by Sigma-Aldrich.

2.3. Sample collection

The samples were collected in the Ceske Budejovice Hospital, Department of Obstetrics and Department of Neonatology and in the Karvina Hospital, Department of Obstetrics and Department of Neonatology. The study was approved by the Ethics Committee of both hospitals and the Institute of Experimental Medicine AS CR in Prague. Each mother signed a written consent. The total amount of 531 samples (265 samples from mothers and 266 samples from their newborn children) was obtained. The newborns urine was sampled using a special plastic bag called Adhesive urine bag for children which is made of foil with a hypoallergenic patch. In the center of the patch is a small hole which can be enlarged as required. This bag was directly attached to the newborn baby and the urine sample could be collected.

The sampling was carried out in two rounds, namely in August–September 2013 (summer period) representing the less air-polluted season for PAHs and in January–April 2014 (winter period) as the more contaminated environment for these pollutants due to the more frequent use of heating in the winter season. The sampling was realized in two regions of the Czech Republic – Ceske Budejovice, the control locality with a lower PAHs contaminated environment and Karvina, the locality with high atmospheric pollution due to heavy industry e.g. coke and steel production (levels of $PM_{2.5}$ (particles smaller than 2.5 μm) and levels of individual PAHs in HiVol samplers in the ambient air in Ceske Budejovice and Karvina in August–October 2013 and January–April 2014 are summarized in Table S1). The urine samples were stored in the freezer at −20 °C before analysis.

Each mother filled in a questionnaire regarding their age, BMI, current residency and eating habits. Gestation age, date of child birth, delivery type, birth weight and gender were obtained from the questionnaire filled in by medical personnel. Summary information is shown in Table 1. Levels of individual PAHs in diet are documented in Table S2 in the Supplementary data. Information about dietary intake of PAHs via breast milk is discussed in detail in an article by Pulkrabova et al. (2016).

2.4. Methods description

2.4.1. Determination of urinary creatinine

The creatinine values were used to normalize the urine concentration/dilution in individual samples in order to ensure data comparability. The creatinine concentration was determined using a Jaffe's spectrophotometric method according to our previous study (Lankova et al., 2016). In brief, a coloured complex of creatinine with alkaline picrate was formed and consequently measured at 505 nm using a Spectrophotometer Epoch (BioTek, USA). The data obtained were evaluated by Gen5™ Microplate Data Analysis software.

2.4.2. Sample preparation procedure

A sample preparation procedure based on liquid-liquid extraction (LLE) with clean-up using dispersive solid phase extraction (d-SPE) is described in detail by Lankova et al. (2016). Briefly, deconjugation for the released OH-PAHs was performed by enzyme β-glucuronidase (pH 5, 37 °C, overnight incubation (16 h)) when 10 mL of acetate buffer and 0.02 mL of enzyme to 5 mL urine was added. An amount of 15 mL of ethyl acetate was added to the urine hydrolysate and the tube was vigorously shaken for 1 min and centrifuge for 5 min at 10,000 rpm to facilitate phase separation. The upper organic layer (12 mL) was transferred into a new tube containing 180 mg of Z-Sep and 1.8 g anhydrous $MgSO_4$. The tube was shaken again for 1 min and centrifuged for 5 min at 10,000 rpm. Subsequently, 8 mL of a purified extract were evaporated using a rotary vacuum evaporator near to dryness. The residual solvent was removed under a gentle stream of nitrogen. The residues were dissolved in 250 μL of methanol. The reconstituted extract was filtered

Table 1
The key information about mothers and their newborn children.

		Ceske Budejovice (August–September 2013) *n = 86 mothers, 86 newborns*			Ceske Budejovice (January–April 2014) *n = 76 mothers, 76 newborns*			Karvina (August–September 2013) *n = 60 mothers, 61 newborns*[a]			Karvina (January–April 2014) *n = 43 mothers, 43 newborns*		
		Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max
Mother's age (years)		31	23	41	32	21	44	30	19	42	31	21	42
BMI		23	18	38	23	17	48	23	17	34	23	17	41
Gestation age (weeks)		40	37	42	39	36	41	40	36	48	40	36	42
Child's birth weight (g)		3500	2600	4500	3400	2400	4900	3400	2400	4600	3300	2500	4200
Partner's smoking habits	Unknown (%)	2			0			4			7		
	Yes (%)	15			28			14			27		
	No (%)	83			72			82			66		

[a] Twins.

through a 0.22 μm nylon centrifuge tube filter and transferred into a vial for the U-HPLC–MS/MS analysis.

2.5. Instrumental analysis

The U-HPLC analyses of 11 urinary OH-PAHs were performed using an Acquity Ultra-Performance LC system (Waters, USA) equipped with a 10 μL sample loop. Analytes were separated on a PFP (pentafluorophenyl) Kinetex column, Phenomenex, (USA) (100 mm × 2.1 mm × 1.7 μm) maintained at 40 °C. (A) water and (B) methanol were used as mobile phases at a flow rate of 300 μL/min and with a gradient, specifically 10–40% B over 0.5 min, then 40–100% B over 11 min followed by an isocratic hold at 100% B for 2 min. The total run time for each injection was 13 min. The flow rate began at 0.3 mL/min and the sample volume injected was 5 μL at 10 °C. The U-HPLC system was coupled to a triple quadrupole mass spectrometer Xevo TQ-S (Waters, USA) with electrospray ionization in negative ion mode (ESI-) with capillary voltage −2000 V, ionization and desolvation temperatures were 150 °C and 400 °C, respectively. The instrument was operated in multiple reaction monitoring (MRM) mode (see Tables S3–S4 in the Supplementary data).

2.6. Quality assurance/quality control and validation

The spectrophotometric method based on Jaffe's reaction for the determination of creatinine concentration was validated according to the paper by Lankova et al. (2016) using the NIST SRM 3673 (non-smoker urine) in six replicates. The measured concentration of creatinine was 508 ± 4 mg/kg, RSD 0.8% and limit of quantification (LOQ) 5 mg/kg. This result was in accordance with the reference value on the certificate (505 ± 2 mg/kg).

The validation of the analytical method (LLE followed by clean-up based on d-SPE) for analysis of 11 urinary OH-PAHs is described in detail in our previous study by Lankova et al. (2016). Briefly, the method was evaluated through the analysis of the SRM 3673 in six replicates. LOQs were determined as the lowest calibration standard at which analytes provided signal to noise ratio (S/N) > 10. Concentrations of nine analyzed OH-PAHs (OH-NAP isomers, 2-OH-FLUO, OH-PHEN isomers and 1-OH-PYR) were in good agreement with the certified values, their recoveries ranged between 77 and 113% and repeatability's were <20% and LOQs were in the range of 0.01–0.025 ng/mL. For the 6-OH-CHRY and 3-OH-BaP, which were not certified in the SRM 3673, the performance parameters were determined by the analysis of the artificially contaminated urine blank sample. The validation level was 0.05 ng/mL for 6-OH-CHRY and 1 ng/mL for 3-OH-BaP. The recoveries for 6-OH-CHRY and 3-OH-BaP were 95% (RSD 13%, LOQ 0.01 ng/mL) and 97% (RSD 16%, LOQ 0.9 ng/mL), respectively.

To control background contamination by target analytes the procedural blank (i.e. the same sample procedure only instead of urine the same volume of deionized water was used) was prepared together with each batch of 20 samples (per day). The blank sample contained traces of 1-OH-NAP, 2-OH-NAP, 1-OH-PHEN, 2-OH-PHEN and 3-OH-PHEN (concentrations below 0.03 ng/mL urine). This is common contamination caused by the occurrence of traces of OH-PAHs in the solvents used and in the ambient air. The concentration of contamination in the blank sample was subtracted from all samples prepared on the same day as the blank sample.

3. Results and discussion

As mentioned in the Introduction, selecting the most suitable biomarkers of humans' exposure to carcinogenic PAHs is not an easy task. The relevance of monitored PAH metabolites to the information required for the purpose of health risk assessment has to be considered together with the cost effectiveness and overall feasibility of laboratory analysis targeting compounds occurring in a biological fluid such as urine at very low levels.

3.1. Concentration of OH-PAHs in the urine samples

Using the earlier developed highly sensitive LC-MS/MS method (Lankova et al., 2016) the set of 531 urine samples obtained from mothers and their newborn children was examined for the presence of 11 OH-PAHs. To compensate for the differences in the hydration state of urine donors and thus enable the comparison of biomarker levels, creatinine concentrations were also determined in all samples. Levels of target analytes in urine were normalized using the ratio OH-PAH/creatinine (creatinine concentrations ranged from 300 to 2900 mg/kg, median was 920 mg/kg). The data obtained in this way are summarized in Table 2. From target metabolites 2-OH-NAP was the most abundant compound found in 100% of the samples. 1-OH-PHEN was another frequently detected analyte (97% of samples) followed by 2-OH-PHEN (96% of samples), 2-OH-FLUO (91% of samples), 9-OH-PHEN (85% of samples), 3-OH-PHEN (81% of samples), 4-OH-PHEN (79% of samples), 1-OH-PYR (75% of samples) and 1-OH-NAP (74% of samples). Monohydroxylated metabolites of carcinogenic CHRY and BaP, namely 6-OH-CHRY and 3-OH-BaP were not, like in similar studies (Li et al., 2014; Onyemauwa et al., 2009; Sykorova et al., 2015; Xu et al., 2004), detected in any of the measured samples. As mentioned earlier, urine is not the major excretion route for these rather lipophilic compounds, moreover compared to lower molecular parent PAHs the absolute doses of BaP and CHRY (and other carcinogenic PAHs) to which humans are exposed are relatively lower (Yanxin et al., 2011).

As shown in Table 2, the concentration of all monitored OH-PAHs (expressed as the ΣOH-PAHs) ranged from 650 to 78,000 ng/g creatinine (1.7–92 ng/mL urine). The concentrations of the most abundant 2-OH-NAP varied from 360 up to 41,000 ng/g creatinine (median

Table 2
Results (ng/mL urine, ng/g creatinine) of the analysis of measured urine samples (n = 531).

OH-PAH	% positive samples	ng/mL urine					ng/g creatinine[a]				
		LOQ	Mean	Median	Min	Max	LOQ[b]	Mean	Median	Min	Max
1-OH-NAP	75	0.03	1.1	0.39	0.03	78	30	1000	500	34	30,000
2-OH-NAP	100	0.03	5.9	3.3	0.07	53	30	5500	4300	360	41,000
2-OH-FLUO	91	0.03	0.46	0.23	0.03	10	30	390	280	28	5200
1-OH-PHEN	97	0.01	0.54	0.30	0.01	8.0	10	540	350	21	5700
2-OH-PHEN	96	0.01	0.27	0.16	0.01	3.4	10	280	180	13	2000
3-OH-PHEN	82	0.01	0.10	0.06	0.01	1.4	10	98	71	11	840
4-OH-PHEN	80	0.01	0.36	0.09	0.01	16	10	350	100	12	7700
9-OH-PHEN	85	0.01	0.83	0.20	0.01	19	10	970	300	10	12,000
1-OH-PYR	77	0.03	0.24	0.15	0.03	2.4	30	220	180	27	1200
6-OH-CHRY	0	0.01	<0.01	<0.01	<0.01	<0.01	10	<10	<10	<10	<10
3-OH-BaP	0	0.90	<0.90	<0.90	<0.90	<0.90	900	<900	<900	<900	<900
ΣOH-PAHs	100	–	9.2	5.3	0.17	92	–	8800	7000	650	78,000

[a] The mean, median, minimum and maximum of creatinine was 1.1, 0.92, 0.30 and 2.9 mg/mL urine, respectively.
[b] LOQ was calculated with the median concentration of creatinine (0.92 mg/mL).

4300 ng/g creatinine, 3.3 ng/mL urine). The concentrations of other quantified analytes were about ten times lower. The domination of 2-OH-NAP among other measured OH-PAHs was also found in other similar studies (Fan et al., 2012; Guo et al., 2013; Li et al., 2006; Onyemauwa et al., 2009; Sykorova et al., 2015).

3.2. Comparison of OH-PAH concentrations in mothers' and newborns' urine according to sampling seasons and localities

Fig. 1 shows the profiles of detected OH-PAHs in mothers' and newborns' urine samples collected in the August–October 2013 and January–April 2014 periods in the Ceske Budejovice and Karvina regions. The results obtained are discussed in the following order: *(i)* a comparison of OH-PAH content in samples from mothers and newborn children, *(ii)* concentrations of OH-PAHs in mothers' urine and *(iii)* concentrations of OH-PAHs in urine samples obtained from newborns. To evaluate the differences between concentrations of all detected PAH metabolites in urine of mothers and children from different localities and sampling seasons a statistical *t*-test was performed (data obtained from this test are summarized in the Table S5 in the Supplementary data).

(i) Considering the entire sample set, the median concentration of ΣOH-PAHs in the urine of children was 1.6 times lower (median 5000 ng/g creatinine) compared to their mothers (median 8100 ng/g creatinine). This conclusion correlates with the fact that the exposure of mothers to PAHs via both inhalation and diet is significantly higher compared to their children. Estimated inhalation of air by mothers is 20 m^3/day and by newborns only 3.6 m^3/day and estimated daily intake of food by mothers is on average 1.2 kg of food/day and by newborn children only 510 mL of breast milk per day (EFSA, 2008; U.S. EPA, 2008). For example a daily intake of PYR by all mothers included in this study was approximately 240 ng/day and by their children only 26 ng/day. The median concentrations of 1-OH-PYR measured in urine samples obtained from mothers was then 210 ng/g creatinine and

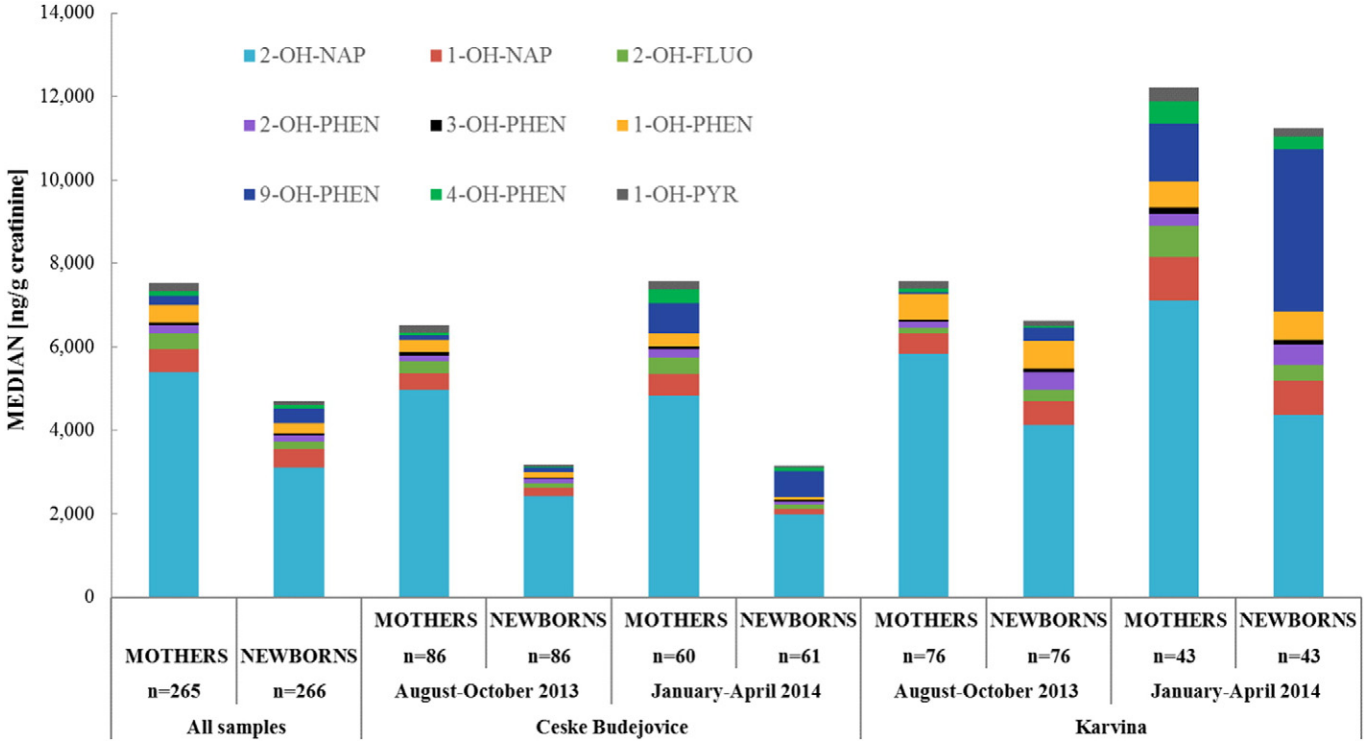


Fig. 1. Concentrations (ng/g creatinine) of detected OH-PAHs in mothers' and newborns' urine samples.

105 ng/g creatinine in urine samples obtained from their children. The lower difference between levels of 1-OH-PYR in urine samples from mothers and their children (compared with relatively high difference in intake of precursor compound) was probably caused by different metabolic pathways in adults and children (Price et al., 2010).

In samples collected in August–October 2013 from mothers living in the Ceske Budejovice region, median concentration of ΣOH-PAHs was 2.3 times higher compared to their newborn children (median 7200 ng/g creatinine and 3100 ng/g creatinine, respectively). The same trend was observed in this locality in mothers' and newborns' urine from the winter sampling round (median 7800 ng/g creatinine and 3300 ng/g creatinine, respectively). Comparing the samples collected in August–October 2013 in Karvina from mothers, no statistically significant difference ($\alpha = 0.05$) was found between the median concentrations of ΣOH-PAHs compared to their children (median 7900 ng/g creatinine and 7200 ng/g creatinine). The same results were observed in January–April 2014 period with median concentrations of ΣOH-PAHs 13,000 ng/g creatinine and 11,000 ng/g creatinine, respectively (Fig. 1).

In urine obtained from all mothers and newborns dominated 2-OH-NAP, the median concentration was 5400 and 3100 ng/g creatinine, respectively. Approximately one order of magnitude lower median concentration of the second dominant compound 1-OH-NAP was found in mothers' (540 ng/g creatinine) and newborns' urine (440 ng/g creatinine). It can be assumed that these results are the consequence of the ubiquitous presence of naphthalene (NAP) in the ambient air (Albuquerque et al., 2016). The profiles of other OH-PAHs in urine from mothers and their children were highly variable. Mainly, the different contribution of individual isomers of OH-PHEN was observed. The major OH-PHEN present in mothers' urine was 1-OH-PHEN (median concentration 420 ng/g creatinine) but in newborns' urine it was 9-OH-PHEN (median concentration 350 ng/g creatinine). These findings are probably caused by different exposure pathways to PHEN (e.g. diet or inhalation) as well as by different metabolic pathways in adults and children or trans-placental transport of these compounds (Price et al., 2010). It is also worth noting that levels of 9-OH-PHEN were higher in both mothers and their newborn children in the winter season (Fig. 1).

Correlations for all measured OH-PAHs between mothers and their newborn children were also investigated. We assumed this relationship between mother and her child based on the premise that the profile of the precursor compounds to which mother and her child are exposed should be similar. However, the concentrations of detected analytes found in newborns did not relate to these compounds found in urine from their mothers. The highest correlation was found for 1-OH-NAP for urine samples collected in Ceske Budejovice in the period from August–October 2013 with the coefficient of determination R^2 only 0.5635. Coefficients of determination of other target compounds were even lower (see Table S6 in the Supplementary data).

(ii) Comparable ΣOH-PAHs concentrations were determined in the mothers' urine collected in the summer in both localities and in the winter period in Ceske Budejovice (median 7200 and 7800 ng/g creatinine). In the case of mothers from the Karvina region, a statistically significant difference ($\alpha = 0.05$) in the ΣOH-PAHs amount was determined between both sampling rounds, when the median concentration in urine collected in the winter was 1.5 times higher than in the summer season (Fig. 1) (median 13,000 and 7900 ng/g creatinine, respectively).

(iii) Regarding the newborns' urine, there were comparable concentrations of ΣOH-PAHs in both seasons (median 3100 ng/g creatinine in the summer season and 3300 ng/g creatinine in the winter season) in Ceske Budejovice. The amount of ΣOH-PAHs in newborns' urine samples from highly industrialized Karvina in the winter season was 1.5 times higher than in the summer season (7200 ng/g creatinine and 11,000 ng/g creatinine) collected in the same locality and 3.3 times higher when compared with the less polluted locality of Ceske Budejovice (statistically significant difference, $\alpha = 0.05$) (Fig. 1).

Significantly higher levels of OH-PAHs in urine from the Karvina region in the winter season are probably caused by the smog situation resulting from local heavy industry and more frequent use of heating than in the summer season as concluded by Sykorova et al. (2015), who studied the seasonal variation of PAHs in the Moravian-Silesian region.

3.3. Comparison of the current study with those performed worldwide

In order to compare the results with similar studies it was necessary to express them in ng/mL urine as this is the most commonly used unit. In the most of recently published scientific papers 2-OH-NAP represents the most abundant metabolite with concentrations ranging from 0.07 up to 50 ng/mL urine (Fan et al., 2012; Guo et al., 2013; Li et al., 2008; Li et al., 2014; Lu et al., 2016; Onyemauwa et al., 2009; Ramsauer et al., 2011; Thai et al., 2016) (Table 3). Our results were comparable with the studies in which different population groups were compared (e.g. general population, children, smokers, steel workers). Concentrations of ΣOH-PAHs in urine collected from Czech mothers (median 8.5 ng/mL) were slightly higher than those reported e.g. by Guo et al. (2013) where urine samples from the general population from India were measured (median 6.4 ng/mL). The amount of ΣOH-PAHs in urine collected from Czech newborns (median 2.9 ng/mL, mean 4.6 mg/mL) was comparable with concentrations reported in the study by Li et al. (2008), where urine samples from US children were measured (mean 5.3 ng/mL). However, the profiles of all targeted analytes differed in each country. These results together with the fact that the PAHs pattern largely varies in the environment and consequently in organisms lead to a conclusion that to obtain more accurate data for biomonitoring purposes more than one OH-PAH marker is needed.

Table 3
Median concentrations of OH-PAHs (ng/mL urine) from various countries.

Analyte	USA, children (Li et al., 2008[a])	Slovakia, steel workers (Onyemauwa et al., 2009[b])	Germany, smokers (Ramsauer et al., 2011[b])	USA, general population (Fan et al., 2012[a])	India, general population (Guo et al., 2013[b])	USA, smokers (Li et al., 2014[a])	China, general population (Lu et al., 2016[b])	Australia, general population (Thai et al., 2016[b])	Czech Republic, mothers (Presented study[b])	Czech Republic, children (Presented study[b])
1-OH-NAP	2.1	7.4	4.3	1.6	1.1	12	3.8	9.2	0.70	0.22
2-OH-NAP	2.5	7.1	8.5	7.7	3.8	20	4.2	4.1	6.0	2.0
2-OH-FLUO	0.32	4.1	1.6	0.87	0.35	2.0	0.40	0.26	0.44	0.11
ΣOH-PHENs	0.38	2.0	0.56	1.8	0.73	0.96	0.40	0.31	1.1	0.52
1-OH-PYR	0.05	0.59	0.2	0.99	0.42	0.48	0.54	0.14	0.22	0.06
6-OH-CHRY	<0.01	<0.001	n.a.	n.a.	<0.01	n.a.	n.a.	n.a.	<0.01	<0.01
3-OH-BaP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	<0.90	<0.90
ΣOH-PAHs	5.3	21	15	13	6.4	35	8.8	14	8.5	2.9

ΣOH-PHENs = 1- + 2- + 3- + 4- + 9-OH-PHEN; n.a. – not analyzed.
[a] Geometric mean (ng/mL urine).
[b] Median (ng/mL urine).

Using the LC-MS based method previously presented by Lankova et al. (2016), we were able to separate and quantify all mostly monitored monohydroxylated isomers of PHEN (1-, 2-, 3-, 4- and 9-OH-PHEN) for the first time. In other similar studies only the sum of either 2- and 3-OH-PHEN or 1- and 9-OH-PHEN was reported (Fan et al., 2012; Onyemauwa et al., 2009; Ramsauer et al., 2011). This is the reason why a detailed comparison of OH-PHEN patterns with other studies was not possible. Since PHEN is the smallest PAH to possess a 'bay region', the ability to measure all isomeric forms of this compound offers promise in studying metabolism across PAH exposed individuals (Hecht et al., 2006; Onyemauwa et al., 2009).

Regarding 1-OH-PYR, which was suggested in many studies as the most suitable exposure biomarker, we found this PYR metabolite in only 77% of the samples, although other PAHs metabolites were present. This clearly documents that a set of PAH metabolites has to be measured to obtain more accurate data for the risk assessment process (Boström et al., 2002; Cahours et al., 2009; Jongeneelen et al., 1990; Oliveira et al., 2016).

4. Conclusions

To our knowledge, this is the most extensive study characterizing the exposure of the Czech population to PAHs based on an analysis of selected biomarkers excreted into urine. Altogether 11 OH-PAHs were measured in 531 urine samples collected from mothers and their newborn children living in two localities in the Czech Republic (the highly air-polluted Karvina and the control locality Ceske Budejovice) during two sampling rounds (August–October 2013 and January–April 2014).

2-OH-NAP was the most abundant of the PAH exposure markers monitored, occurring in 100% of the urine samples. At the same time it was the analyte with the highest concentration (5400 ng/g creatinine for mothers and 3100 ng/g creatinine for newborns, respectively). 6-OH-CHRY and 3-OH-BaP, metabolites of carcinogenic PAHs were not detected in any of the measured samples. Regarding individual OH-PHEN isomers, higher levels of 9-OH-PHEN were observed in urine samples collected from both mothers and their newborn children in the winter season.

The median concentration of ΣOH-PAHs in the childrens' urine was 1.6 times lower compared to their mothers. This conclusion correlates with the fact that the exposure of mothers to PAHs via both inhalation and diet is significantly higher compared to their children. However, a correlation between all detected individual OH-PAHs in urine samples from mothers and their newborn children was not found.

While the content of ΣOH-PAHs in mothers' urine collected in the summer period was comparable in both localities, in the winter period the samples from the Karvina region showed 1.5 times higher amounts of exposure markers. The amounts of ΣOH-PAHs in newborns' urine samples from highly industrialized Karvina in the winter season were 1.5 times higher than in the summer season collected in the same locality and 3.3 times higher when compared with the less polluted locality of Ceske Budejovice. This was probably due to the smog situation resulting from heavy industry and local heating.

Acknowledgments

This study was funded by the Czech Science Foundation Project No. 13-13458S. The 'Operational Programme Prague – Competitiveness' (CZ.2.16/3.1.00/21537 and CZ.2.16/3.1.00/24503) and the 'National Program of Sustainability I' - NPU I (LO1215 and LO1601) are also gratefully acknowledged. The authors deeply appreciate the support of Dr. Radek Susil, vice-director of the Karvina-Raj Hospital and Dr. Bretislav Schon, Director of the Ceske Budejovice Hospital. The study would not have been possible without the close cooperation of all obstetricians, paediatricians, nurses and laboratory workers in both hospitals. The authors would also like to thank Mr. Kip Bauersfeld for language editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.10.165.

References

Albuquerque, M., Coutinho, M., Borrego, C., 2016. Long-term monitoring and seasonal analysis of polycyclic aromatic hydrocarbons (PAHs) measured over a decade in the ambient air of Porto. Portugal. Sci. Total Environ. 543, 439–448.

Alshaarawy, O., Elbaz, H.A., Andrew, M.E., 2016. The association of urinary polycyclic aromatic hydrocarbon biomarkers and cardiovascular disease in the US population. Environ. Int. 89, 174–178.

Ambroz, A., Vlkova, V., Rossner Jr., P., Rossnerova, A., Svecova, V., Milcova, A., Pulkrabova, J., Hajslova, J., Veleminsky Jr., M., Solanskya, I., Srama, R.J., 2016. Impact of air pollution on oxidative DNA damage and lipid peroxidation in mothers and their newborns. Int. J. Hyg. Environ. Health 219, 545–556.

Annamalai, J., Namasivayam, V., 2015. Endocrine disrupting chemicals in the atmosphere: their effects on humans and wildlife. Environ. Int. 76, 78–97.

Barbeau, D., Maître, A., Marques, M., 2011. Highly sensitive routine method for urinary 3-hydroxybenzo [a] pyrene quantitation using liquid chromatography-fluorescence detection and automated off-line solid phase extraction. Analyst 136, 1183–1191.

Boström, C.E., Gerde, P., Hanberg, A., Jernström, B., Johansson, C., Kyrklund, T., Rannug, A., Törnqvist, M., Victorin, K., Westerholm, R., 2002. Cancer risk assessment, indicators, and guidelines for polycyclic aromatic hydrocarbons in the ambient air. Environ. Health Perspect. 110, 451–488.

Cahours, X., Blanchet, M., Rey, M., 2009. Fast and simple method for the determination of urinary 1-hydroxypyrene. J. Sep. Sci. 32, 3403–3410.

Campo, L., Rossella, F., Fustinoni, S., 2008. Development of a gas chromatography/mass spectrometry method to quantify several urinary monohydroxy metabolites of polycyclic aromatic hydrocarbons in occupationally exposed subjects. J. Chromatogr. B 75, 531–540.

Campo, L., Rossella, F., Pavanello, S., Mielzynska, D., Siwinska, E., Kapka, L., Bertazzi, P.A., Fustinoni, S., 2010. Urinary profiles to assess polycyclic aromatic hydrocarbons exposure in coke-oven workers. Toxicol. Lett. 192, 72–78.

EFSA (European Food Safety Authority), 2008. Polycyclic aromatic hydrocarbons in food. Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on Polycyclic Aromatic Hydrocarbons in Food. EFSA J. 724, 1–114.

Fan, R., Wang, D., Ramage, R., She, J., 2012. Fast and simultaneous determination of urinary 8-hydroxy-2′-deoxyguanosine and ten monohydroxylated polycyclic aromatic hydrocarbons by liquid chromatography/tandem mass spectrometry. Chem. Res. Toxicol. 25, 491–499.

Grova, N., Hardy, E.M., Meyer, P., Appenzeller, B.M., 2016. Analysis of tetrahydroxylated benzo[*a*]pyrene isomers in hair as biomarkers of exposure to benzo[*a*]pyrene. Anal. Bioanal. Chem. 408, 1997–2008.

Guo, Y., Senthilkumar, K., Alomirah, H., Moon, H.B., Minh, T.B., Mohd, M.A., Nakata, H., Kannan, K., 2013. Concentrations and profiles of urinary polycyclic aromatic hydrocarbon metabolites (OH-PAHs) in several Asian countries. Sci. Technol. 47, 2932–2938.

Hecht, S.S., 2002. Human urinary carcinogen metabolites: biomarkers for investigating tobacco and cancer. Carcinogenesis 23, 907–922.

Hecht, S.S., Carmella, S.G., Yoder, A., Chen, M., Z-z, L., Le, C., et al., 2006. Comparison of polymorphisms in genes involved in polycyclic aromatic hydrocarbon metabolism with urinary phenanthrene metabolite ratios in smokers. Cancer Epidemiol. Biomark. Prev. 15, 1805–1811.

Hu, Y., Li, G., Xue, X., Zhou, Z., Li, X., Fu, J., Cohen, B., Roy, N., Li, D., Sun, J., Nan, P., Tang, M.S., Qu, Q., 2008. PAH-DNA adducts in a Chinese population: relationship to PAH exposure, smoking and polymorphisms of metabolic and DNA repair genes. Informa healthcare 13, 27–40.

IARC (International Agency for Research on Cancer), 2010. Some non-heterocyclic polycyclic aromatic hydrocarbons and some related exposures. IARC Monogr. Eval. Carcinog. Risk Hum. 92, 1–858.

Jedrychowski, W.A., Perera, F.P., Tang, D., Rauh, V., Majewska, R., Mroz, E., Flak, E., Stigter, L., Spengler, J., Camann, D., Jacek, R., 2013. The relationship between prenatal exposure to airborne polycyclic aromatic hydrocarbons (PAHs) and PAH–DNA adducts in cord blood. J. Expo. Sci. Environ. Epidemiol. 23, 371–377.

Jongeneelen, F.J., van Leeuwen, F.E., Oosterink, S., Anzion, R.B., van der Loop, F., Bos, R.P., van Veen, H.G., 1990. Ambient and biological monitoring of cokeoven workers: determinants of the internal dose of polycyclic aromatic hydrocarbons. Toxicol. Lett. 47, 454–461.

Lankova, D., Urbancova, K., Sram, R.J., Hajslova, J., Pulkrabova, J., 2016. A novel strategy for the determination of polycyclic aromatic hydrocarbon monohydroxylated metabolites in urine using ultra-high-performance liquid chromatography with tandem mass spectrometry. Anal. Bioanal. Chem. 408, 2515–2525.

Li, Z., Romanoff, L.C., Trinidad, D.A., Hussain, N., Jones, R.S., Porter, E.N., Patterson Jr., D.G., Sjödin, A., 2006. Measurement of urinary monohydroxy polycyclic aromatic hydrocarbons using automated liquid-liquid extraction and gas chromatography/isotope dilution high-resolution mass spectrometry. Anal. Chem. 78, 5744–5751.

Li, Z., Sandau, C.D., Romanoff, L.C., Caudill, S.P., Sjodin, A., Needham, L.L., Patterson Jr., D.G., 2008. Concentration and profile of 22 urinary polycyclic aromatic hydrocarbon metabolites in the US population. Environ. Res. 107, 320–331.

Li, J., Shanga, X., Zhaoa, Z., Tanguaya, R.L., Donga, Q., Huanga, C., 2010. Polycyclic aromatic hydrocarbons in water, sediment, soil, and plants of the Aojiang River waterway in Wenzhou, China. J. Hazard. Mater. 173, 75–81.

Li, Z., Romanoff, L.C., Trinidad, D.A., Pittman, E.N., Hilton, D., Hubbard, K., Carmichael, H., Parker, J., Calafat, A.M., Sjödin, A., 2014. Quantification of 21 metabolites of methylnaphthalenes and polycyclic aromatic hydrocarbons in human urine. Anal. Bioanal. Chem. 406, 3119–3129.

Li, J., Fan, R., Lu, S., Zhang, D., Zhou, Y., Lv, Y., 2015. Exposure to polycyclic aromatic hydrocarbons could cause their oxidative DNA damage: a case study for college students in Guangzhou, China. Environ. Sci. Pollut. Res. 22, 1770–1777.

Lu, S.-y., Y-x, L., J-q, Z., Zhang, T., G-h, L., M-z, H., Li, X., J-j, R., Kannan, K., R-l, Q., 2016. Associations between polycyclic aromatic hydrocarbon (PAH) exposure and oxidative stress in people living near e-waste recycling facilities in China. Environ. Int. 94, 161–169.

Lutier, S., Maître, A., Bonneterre, V., Bicout, D.J., Marques, M., Persoons, R., et al., 2016. Urinary elimination kinetics of 3-hydroxybenzo (a) pyrene and 1-hydroxypyrene of workers in a prebake aluminum electrode production plant: evaluation of diuresis correction methods for routine biological monitoring. Environ. Res. 147, 469–479.

Ma, Y., Harrad, S., 2015. Spatiotemporal analysis and human exposure assessment on polycyclic aromatic hydrocarbons in indoor air, settled house dust, and diet: a review. Environ. Int. 84, 7–16.

Moustafa, G.A., Xanthopoulou, E., Riza, E., Linos, A., 2015. Skin disease after occupational dermal exposure to coal tar: a review of the scientific literature. Int. J. Dermatol. 54, 868–879.

Oliveira, M., Slezakova, K., Alves, M.J., Fernandes, A., Teixeira, J.P., Delerue-Matos, C., Carmo Pereira, M., Morais, S., 2016. Firefighters' exposure biomonitoring: Impact of firefighting activities on levels of urinary monohydroxyl metabolites. Int. J. Hyg. Environ. Health (in press).

Onyemauwa, F., Rappaport, S.M., Sobus, J.R., Gajdosová, D., Wua, R., Waidyanatha, S., 2009. Using liquid chromatography–tandem mass spectrometry to quantify monohydroxylated metabolites of polycyclic aromatic hydrocarbons in urine. J. Chromatogr. B 877, 1117–1125.

Pleil, J.D., Stiegel, M.A., Sobus, J.R., Tabucchi, S., Ghio, A.J., Madden, M.C., 2010. Cumulative exposure assessment for trace-level polycyclic aromatic hydrocarbons (PAHs) using human blood and plasma analysis. J. Chromatogr. B 878, 1753–1760.

Price, T.S., Grosser, T., Plomin, R., Jaffee, S.R., 2010. Fetal genotype for the xenobiotic metabolizing enzyme NQO1 influences intrauterine growth among infants whose mothers smoked during pregnancy. Child Dev. 81, 101–114.

Pulkrabova, J., Stupak, M., Svarcova, A., Rossner, P., Rossnerova, A., Ambroz, A., Sram, R.J., Hajslova, J., 2016. Relationship between atmospheric pollution in the residential area and concentrations of polycyclic aromatic hydrocarbons (PAHs) in human breast milk. Sci. Total Environ. 562, 640–647.

Ramesh, A., Walker, S.A., Hood, D.B., Guillén, M.D., Schneider, K., Weyand, E.H., 2004. Bioavailability and risk assessment of orally ingested polycyclic aromatic hydrocarbons. Int. J. Toxicol. 23, 301–333.

Ramsauer, B., Sterz, K., Hagedorn, H.W., Engl, J., Scherer, G., McEwan, M., Errington, G., Shepperd, J., Cheung, F., 2011. A liquid chromatography/tandem mass spectrometry (LC-MS/MS) method for the determination of phenolic polycyclic aromatic hydrocarbons (OH-PAH) in urine of non-smokers and smokers. Anal. Bioanal. Chem. 399, 877–889.

Scinicariello, F., Buser, M.C., 2014. Urinary polycyclic aromatic hydrocarbons and childhood obesity: NHANES (2001–2006). Environ. Health Perspect. 122, 299–303.

Sram, R.J., Dostal, M., Libalova, H., Rossner, P., Rossnerova, A., Svecova, V., Topinka, J., Bartonova, A., 2013. The European hot spot of B[*a*]P and $PM_{2.5}$ exposure—the Ostrava Region, Czech Republic: health research results. ISRN Public Health 2013, 12.

Sykorova, B., Kucbel, M., Raclavska, H., Drozdova, J., Raclavsky, K., 2015. Seasonal variations of polycyclic aromatic hydrocarbons (PAHs) in the air of Moravian-Silesian region, Czech Republic. Proceedings of the Environment, Energy and Applied Technology: Proceedings of the 2014 International Conference on Frontier of Energy and Environment Engineering (ICFEEE 2014), Taiwan, December 6–7, 2014. CRC Press, p. 367.

Thai, P.K., Heffernan, A.L., Toms, L.-M.L., Li, Z., Calafat, A.M., Hobson, P., Broomhall, S., Mueller, J.F., 2016. Monitoring exposure to polycyclic aromatic hydrocarbons in an australian population using pooled urine samples. Environ. Int. 88, 30–35.

U.S. EPA (United States Environmental Protection Agency), 2008. Child-specific exposure factors handbook. EPA/600/R-06/096F.

Wang, S., Bai, Y., Deng, Q., Chen, Z., Dai, J., Li, X., Zhang, W., Zhang, X., He, M., Wu, T., Guo, H., 2016. Polycyclic aromatic hydrocarbons exposure and lung function decline among coke-oven workers: a four-year follow-up study. Environ. Res. 150, 14–22.

Xu, X., Zhang, J., Zhang, L., Liu, W., Weisel, C.P., 2004. Selective detection of monohydroxy metabolites of polycyclic aromatic hydrocarbons in urine using liquid chromatography/triple quadrupole tandem mass spectrometry. Rapid. Commun. Mass Sp. 18, 2299–2308.

Yanxin, Y., Wang, X., Wang, B., Tao, S., Liu, W., Wang, X., Cao, J., Li, B., Lu, X., Wong, M.H., 2011. Polycyclic aromatic hydrocarbons residues in human milk, placenta and umbilical cord blood in Beijing, China. Environ. Sci. Technol. 45, 10235–10242.

Zhong, Y., Carmella, S.G., Hochalter, J.B., Balbo, S., Hecht, S.S., 2011. Analysis of r-7,t-8,c-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene in human urine: a biomarker for directly assessing carcinogenic polycyclic aromatic hydrocarbon exposure plus metabolic activation. Chem. Res. Toxicol. 24, 73–80.